Interactions of BCG with urothelial tumor cells in immunotherapy for superficial bladder cancer
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BCG-induced interleukin-6 upregulation and BCG internalization in well and poorly differentiated human bladder cancer cell lines

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Abstract

Intravesical bacillus Calmette-Guérin (BCG) is a successful therapy for superficial bladder cancer. However, the working mechanism of BCG after intravesical instillation is not completely understood. A functional role of urothelial (tumor) cells in the initiation of the BCG-induced immune reaction should be considered. Here, the possibility of a causal relationship between BCG-induced interleukin-6 (IL-6) synthesis and BCG internalization by urothelial tumor cells was examined in a series of human
transitional bladder cancer (TCC) cell lines with different degrees of differentiation. The results showed that the well differentiated TCC cell lines, RT4, SBC-2, and SBC-7, did not possess the capacity to internalize BCG, which was associated with an inability to upregulate IL-6 synthesis when stimulated with BCG. Moreover, these cell lines expressed a low level of constitutive IL-6 synthesis. In contrast, the poorly differentiated TCC cells T-24, TCC-SUP and J-82 were able to internalize BCG. In T24 and J82, but not in TCC-SUP cells, BCG internalization appeared to result in an upregulation of IL-6 synthesis. Constitutive IL-6 synthesis of the high-grade cell lines was found to be cell line-dependent: both TCC-SUP and J82 cells exhibited a high level of constitutive IL-6 synthesis, whereas T24 cells exhibited a low level.

The possible relationship between BCG internalization and IL-6 upregulation was studied in detail with the T24 cell line, which exhibited a low constitutive and high BCG-inducible IL-6 synthesis, using anti-BCG antibodies (αBCG) and Cytochalasin B as internalization inhibitors. Upregulation of IL-6 synthesis was significantly inhibited by αBCG or Cytochalasin B, indicating that internalization is a prerequisite for BCG-induced upregulation of IL-6 synthesis.

In conclusion, upregulation of IL-6 production due to BCG internalization by poorly differentiated bladder carcinoma cells may be part of the mode of action of intravesical BCG therapy.

Introduction

Intravesical BCG therapy has been successfully used for the treatment of superficial bladder cancer [1, 2]. For BCG therapy to be effective, the development of an immune response seems to be important, although the exact mechanism remains to be established [3]. For the initiation of the immune reaction, several steps during the first interaction of BCG with the bladder wall have been suggested to be involved. These initial reactions may include BCG adherence and internalization, cytokine production and BCG antigen presentation by bladder (carcinoma) cells.

The immediate fate of BCG after intravesical instillation is not known. Several lines of evidence suggest internalization of BCG by urothelial cells in the initiation of the BCG-induced immune reaction and/or anti-tumor effect. Attachment and internalization of BCG by bladder tumor cells have been reported [4, 5]. Ingestion of mycobacteria both in vitro and in vivo by other non-professional phagocytic cells has also been reported [6]. The nature of the attachment as well as the number of bacteria attached to the bladder wall and/or urothelial cells are matters of controversy [7-11].
As a reaction to BCG therapy, urothelial cells are able to express several molecules such as MHC class II and ICAM-1 antigens on their cell wall [12, 13]. These molecules are fundamental to many immune functions including antigen presentation and cell-mediated cytotoxicity. BCG-antigen presentation to murine T-lymphocytes by urothelial cells has been reported [13]. In addition, intravesical BCG instillation is associated with an increased level of urinary cytokines [14-16]. The source of some of these cytokines has not been conclusively determined, but epithelial cells, including urothelial cells, do express the capacity to synthesize cytokines [17, 18]. Recently, accumulating data have shown that BCG is capable of upregulating cytokine synthesis such as IL-6 by tumor urothelial cells grown \textit{in vitro} [19, 20]. In patients, IL-6 levels in urine following BCG instillation are reported to be related to the clinical BCG anti-tumor response [20].

In this study the hypothesis that BCG internalization depends on the degree of differentiation of TCC cells and is a prerequisite for upregulation of IL-6 production by bladder tumor cells was tested.

Methods

\textit{Cells and culture conditions.}\n
Stock cultures of a series of cell lines derived from human bladder carcinomas consisting of the well differentiated cell lines RT-4 (grade 1), SBC-2 (grade 1), SBC-7 (grade 1), and the poorly differentiated cell lines T24 (grade 3), TCC-SUP (grade 3) and J82 (grade 3) [21-23], were cultured as monolayers in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 1% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37° C in a humidified 5% CO₂ atmosphere at pH 7.2 until subconfluency. Cultures were routinely screened for mycoplasma.

For passage, cells were trypsinized with 0.05% trypsin and 0.02% EDTA in phosphate buffered saline (PBS). All tissue culture chemicals were obtained from Flow Laboratories (Irvine, Scotland). Tissue culture plastics were from Costar (Cambridge, Mass., USA).

\textit{BCG preparation.}\n
Freeze-dried BCG, Connaught strain (3.2 x 10^8 CFU per vial), was reconstituted in 10 ml prewarmed DMEM. To eliminate large clumps of bacteria, the suspension was centrifuged at 300 g for 3 min and the top 5 ml was used [5]. The concentration of CFU was set by measuring the absorbance at a wavelength of 440 nm, compared to a standard curve relating the CFU count to optical density.
Fluorescence activated cell sorter (FACS) analysis of BCG internalization by bladder cancer cell lines.

Attachment and internalization of BCG were quantitated as described previously [11]. Briefly, cells (3 x 10^5/24-well) were cultured overnight, and BCG (Connaught) labeled with fluorescein isothiocyanate (FITC) was added. Cells were incubated with BCG-FITC for 4 hours, followed by washing, and harvesting, with cell dissociation solution (CDS) obtained from Sigma (St Louis, MO, USA). This procedure resulted in cells containing both attached and internalized BCG-FITC. To exclusively stain the fraction of extracellular or attached BCG, cells were subsequently pre-incubated with polyclonal rabbit anti-BCG antibodies (αBCG) (1:100; Dakopatts, Denmark) followed by incubation with phycoerythrin (PE) conjugated goat anti-rabbit antibodies (1:25; Sigma, St Louis, MO, USA).

Cells (10^4) were measured with a fluorescence-activated cell sorter (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA), to determine the fraction of cells "stained" with FITC (Fl_1^+; intracellular and extracellular BCG) and PE (Fl_2^+; extracellular BCG). The percentage of Fl_1^+/Fl_2^+ cells was taken as the percentage BCG-ingesting cells.

Inhibition studies of BCG phagocytosis.

Inhibition of internalization of BCG was studied using αBCG (1:100-1:25) and rabbit polyclonal anti-FN antibodies (αFN) (1:100-1:25; Dako Corp., Santa Barbara, CA), added 30 min prior to the FACS internalization assay of BCG. To eliminate the effect of possible aspecific binding of the Fc portion of αFN to BCG, inhibition experiments were also performed with goat anti-human FN F(ab)_2 fragments (1:100-1:25; Organon Teknika, West Chester, PA). For inhibition studies with Cytochalasin B (CytB) (Sigma, St Louis, MO, USA), CytB at the indicated concentrations was added to the cells 1 hour before and during the incubation with BCG.

Determination and inhibition of constitutive and BCG-induced upregulation of IL-6 production.

Cells (3 x 10^5 cells) were continuously incubated with BCG at the indicated concentrations. At the indicated time intervals, medium was collected for cytokine determinations. IL-6 was quantified with a human specific, oligoclonal ELISA obtained from Medgenix (Fleurus, Belgium) [24]. Conditions for inhibition of IL-6 production by αBCG, αFN and CytB were similar as those described for the inhibitory studies of BCG internalization.

Statistical analysis.

All experiments were performed at least twice. FACS analysis measurements represent the mean of duplicate cultures; SD was less than
10%. All experiments concerning IL-6 measurements were performed in triplicate. For comparison between groups the Mann-Whitney test or Student's t test for independent samples were used (SPSS 6.1 for Microsoft Windows software).

Results

Internalization of BCG.
The kinetics of BCG internalization in T24 cells has been reported in a previous study [11]. The percentage of cells internalizing BCG appeared to be proportionally related to the time of incubation. As presented in Figure 1,

![Figure 1](image_url)  
*Figure 1.* Internalization of BCG by a series of well and poorly differentiated human bladder cancer. Following incubation of $3 \times 10^5$ cells for 4 hours with FITC-labeled BCG, staining of extracellular BCG was performed with PE-labeled antibodies in harvested cells. Internalization was expressed as the percentage of cells showing internalization of BCG ($Fl_{1+} / Fl_{2-}$ cells) measured in $10^4$ cells using FACS. RT-4, SBC-2 and SBC-7 are well differentiated, grade 1 cell lines, while T24, TCC-SUP and J82 are poorly differentiated, grade 3 cell lines.

the capacity for BCG internalization in a series of human bladder cancer cell lines appeared to be cell-line dependent. The maximum number of cells
internalizing BCG during a period of 24 hours varied from 0-2% for the well differentiated cell lines (RT-4, SBC-2 and SBC-7), to 34-68% for the poorly differentiated cell lines (T-24, TCC-SUP and J-82). These data suggest a relationship between the capacity to internalize BCG and the differentiation grade of TCC cells. Only poorly differentiated tumor cell lines internalized BCG.

**BCG-induced and constitutive IL-6 synthesis.**

To study BCG internalization as a necessary initial step of BCG-induced upregulation of IL-6, initially both the constitutive and BCG-induced upregulation of IL-6 were determined in a series of well and poorly differentiated cell lines. As illustrated in Figure 2 constitutive and BCG-mediated upregulation of IL-6 appeared to be cell line-dependent.

![Figure 2](image)

**Figure 2.** Constitutive and BCG-induced IL-6 production in a series of well and poorly differentiated human bladder cancer cell lines. IL-6 production of $3 \times 10^5$ cells, in the absence of BCG (black) and in the presence of $5 \times 10^6$ CFU/ml BCG (shaded) was determined after a 7 hours incubation. Bars represent mean ± SD.

Determined after a 7 hours incubation period, the well differentiated, non-phagocytic cell lines (SBC-2 and SBC-7) exhibited low (0.4 to 0.5 ng / $3 \times 10^5$ cells) constitutive IL-6 synthesis, which appeared not to be upregulated
in the presence of BCG (0.5 ng / 3 x 10^5 cells). Of the poorly differentiated, BCG internalizing cell lines, the constitutive IL-6 synthesis was found to be high for TCC-SUP (1.1 ± 0.1 ng / 3 x 10^5 cells) and J82 (1.7 ± 0.4 ng / 3 x 10^5 cells) cells, but was low (0.1 ± 0.0 ng / 3 x 10^5 cells) for T24 cells. Stimulation with BCG did not significantly (p = 0.095) upregulate IL-6 synthesis in TCC-SUP cells. In contrast, both the J82 and T24 exhibited a significant (p < 0.005) upregulation of IL-6 production when stimulated with BCG. This BCG-induced upregulation was most pronounced in T24 cells, resulting in an IL-6 production of 2.6 ± 0.1 ng/ml compared to the constitutive synthesis of 0.1 ± 0.0 ng per 3 x 10^5 cells over a period of 7 hours. This upregulation of IL-6 production was BCG concentration-and time-dependent (Figure 3). So, the poorly differentiated TCC cell lines showed, in addition to the capacity to internalize BCG, either a high constitutive (TCC-SUP and J82) and/or a high BCG-inducible production of IL-6 (T24 and J82).

![Figure 3. Kinetics of constitutive (without BCG) and BCG-induced IL-6 production of 3 x 10^5 T24 cells during continuous incubation with BCG at several concentrations: ●: 10^7 CFU BCG; ○: 5 x 10^6 CFU BCG; ■: 2 x 10^6 CFU BCG; □: 2 x 10^5 CFU BCG; ▲: without BCG. Curves were fitted exponentially. Bars represent mean ± SD.](image-url)
Relation between BCG phagocytosis and IL-6 production.

The results outlined above suggest the possibility of a causal relationship between BCG internalization and IL-6 upregulation, which is especially illustrated in T24 cells. In a subsequent series of experiments, the possibility of BCG internalization as a prerequisite to BCG-induced IL-6 production was studied in detail. These experiments were performed with T24 cells, since the characteristics of this cell line, namely the low constitutive IL-6 synthesis associated with a high degree of BCG-induced upregulation of IL-6 synthesis, seemed to be well suited for this purpose. Incubation with BCG, pretreated with αBCG, decreased the percentage of T24 cells associated with BCG to 34% (1:100 αBCG) and 12% (1:25 αBCG) compared to 38% in the absence of αBCG (data not shown). As illustrated in Figure 4, under similar

![Figure 4](image)

**Figure 4.** Effect of addition of αBCG and αFN on IL-6 production. αBCG was added 30 min before and during incubation of 3 x 10^5 T24 cells with 5 x 10^6 CFU/ml BCG. IL-6 in the medium was determined after 7 hours. Bars represent mean ± SD.

conditions a significant (p = 0.028) inhibition of BCG-induced upregulation of IL-6 synthesis occurred in the presence of αBCG at a concentration of 1:25. A condition previously found to reduce the internalization of BCG by T24 cells specifically [11], namely incubation with CytB, was subsequently
tested for its ability to prevent IL-6 upregulation. Under conditions where CytB reduced BCG internalization by T24 cells to 46%, Cyt B inhibited BCG-induced IL-6 production, which was significantly stronger than the inhibition of the constitutive IL-6 synthesis (Table 1).

Table 1. Constitutive and BCG-induced synthesis of IL-6 in T24 bladder tumor cells *in vitro*, in the presence of Cytochalasin B

<table>
<thead>
<tr>
<th>CytB µg/ml</th>
<th>IL-6 production</th>
<th>Mann Whitney test</th>
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<tbody>
<tr>
<td></td>
<td>(in % ± SD) b</td>
<td>p value</td>
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<tr>
<td></td>
<td>Constitutive</td>
<td>BCG - induced</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 8.3 c</td>
<td>100 ± 13.1</td>
</tr>
<tr>
<td>0.25</td>
<td>66.8 ± 7.1</td>
<td>70.0 ± 14.2</td>
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<tr>
<td>2.5</td>
<td>57.3 ± 19.9</td>
<td>30.1 ± 7.0</td>
</tr>
<tr>
<td>10.0</td>
<td>68.5 ± 21.4</td>
<td>35.0 ± 3.9</td>
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a As previously observed [11] internalization of BCG was significantly inhibited by CytB at concentrations of 3.3 µg/ml or higher. At a concentration of 10 µg/ml, an inhibition of internalization of 45% was observed.
b IL-6, expressed as % of synthesis in the absence of Cytochalasin B, was measured in medium containing 3 x 10⁵ T24 cells, incubated for 7 hours with or without 5 x 10⁶ CFU/ml BCG. Cytochalasin B at the indicated concentrations was added 1 hour prior to BCG addition.
c Mean ± SD. The values of 100% correspond to 0.1 and 1.5 ng IL-6/ml for the constitutive and BCG-induced IL-6 synthesis in the absence of Cytochalasin B, respectively.

Kuroda et al. [4] suggest that BCG internalization is associated with fibronectin and can be inhibited by αFN. Using identical antibodies the effect of αFN on BCG-induced upregulation of IL-6 synthesis was studied. The results, presented in Figure 4, show that addition of αFN to T24 cells and subsequent incubation with BCG did not inhibit BCG-induced IL-6 upregulation (2.3 ± 0.1 versus 2.2 ± 0.2 ng/ml per 3 x 10⁵ cells in the absence of αFN; p = 0.272). Since this observation contradicted our expectations, the effect of αFN on BCG internalization was determined. The results indicated that αFN, added at concentrations ranging from 1:100 to 1:25, did not inhibit BCG internalization (data not shown). So, these results
seemed to be in agreement with the absence of an effect of αFN on the induction of IL-6 synthesis by BCG.

Overall, the data suggest that in T24 cells, internalization of BCG is a prerequisite for BCG-induced IL-6 production.

Discussion

Various cytokines can be detected in the urine of patients treated with BCG intravesically [14, 15, 25]. Internalization of BCG by urothelial cells *in vivo* has been reported [5]. This implies that the present study concerning BCG internalization and cytokine production of urothelial cells *in vitro* may be of clinical relevance.

In this study, the phagocytic capacity and high levels of IL-6 production, either constitutive or BCG-induced or both, seemed to be restricted to cell lines with a poor degree of differentiation (T24, TCC-SUP and J82, grade 3). Tumor grade dependency has already been reported with regard to adherence of BCG to bladder tumor cell lines [26], and (BCG-induced) IL-6 production by bladder tumor cell lines [20]. In addition, the present results suggested a relationship between BCG-internalization and IL-6 production. Experiments with Cytochalasin B, a molecule that specifically blocks phagocytosis [11], showed internalization of BCG to be a prerequisite for the BCG-induced IL-6 production in T24 tumor cells. Accepting the hypothesis that BCG internalization and (BCG-induced) cytokine production of (tumor) urothelial cells may play a central role in the mechanism of action of BCG, these findings are in accordance with clinical observations that high grade tumors show a better response to BCG treatment than lower grade tumors [27].

The observed capacity of high-grade TCC tumor cells to produce IL-6, either constitutively and/or during stimulation with BCG, may reflect the clinical situation. Following installations with BCG, various cytokines can be detected in the urine of patients [14,15,25]. These observations suggest that, during intravesical BCG instillation, urothelial (tumor) cells may be a significant source of urinary cytokines, additional to the major cellular sources, such as macrophages and lymphocytes infiltrating the bladder wall. It has been speculated that a BCG-induced upregulation of cytokines in urothelial (tumor) cells of the bladder could be of functional significance *in vivo*, initiating and/or modulating the BCG-induced immune response [24, 25, 28]. It should be noted that upregulation of the cytokine synthesis of urothelial tumor cells by microorganisms seems to be a characteristic most
extensively expressed in the presence of BCG. It has been shown that the upregulation of IL-6 and TNF-α synthesis in T24 cells by BCG is at least 10 times higher compared to the upregulation by *E. Coli* or *S. faecalis* [19].

As indicated for T24 cells, upregulation of IL-6 production appeared to be related to the dose of BCG. An optimal dose *in vitro* was not established with the concentration range used. In patients however, the dose may be limited by the adverse side effects of BCG [29].

In order to inhibit BCG internalization by T24 cells, we used, in addition to Cytochalasin B, polyclonal αBCG and αFN as described by Kuroda et al [4]. In accordance with these investigators, we observed a decreased internalization due to αBCG, suggesting a specific interaction between BCG and the T24 cellular membrane. This condition also reduced the BCG-induced IL-6 upregulation by T24, which further consolidated the findings with Cytochalasin B. In contrast to Kuroda et al. we could not detect an inhibiting effect of αFN on BCG phagocytosis. This discrepancy cannot be explained by the use of different sources of antibodies. Furthermore, differences in BCG strains do not seem likely since thus far all strains have shown to be comparable with regard to clinical efficacy against superficial bladder tumors. Absence of an inhibiting effect of αFN for BCG phagocytosis by bladder carcinoma cell lines has been reported by other investigators too [26,30]. These observations do not favor the previously suggested (opsonizing) role of FN in BCG internalization by bladder tumor cells [4]. However, there may be a difference between the proposed role of FN in mediating BCG binding to the injured bladder wall, initiation of an immune response and the associated antitumor activity in a mouse bladder tumor model [10] and the possible role of FN in the internalization of BCG *in vitro* systems.

In this study, αBCG inhibited *in vitro* internalization of BCG in T24 cells, and IL-6 production. Anti-BCG antibodies (IgG and IgA) have been shown to be present in serum and urine of patients following BCG installations [9] up to 12 months after BCG therapy, possibly interfering with the effectiveness of subsequent BCG installations. Whether the level of anti-BCG antibodies in patients correlates with the antitumor effect or not, and what the implications are for the efficacy of (maintenance) installations with BCG, remains to be established in further studies.

In conclusion, a high constitutive IL-6 production or IL-6 upregulation, initiated by BCG internalization seems to be related to the degree of differentiation of the bladder cancer cell lines. These observations suggest
that constitutive IL-6 production and/or BCG-mediated (after internalization) upregulation of IL-6 production by poorly differentiated bladder carcinoma cells may be part of the mode of action of intravesical BCG therapy. The role of this phenomenon in vivo, however, remains to be established.

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References


