

Cytokine production by the human bladder carcinoma cell line T24 in the presence of bacillus Calmette-Guerin (BCG)

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Summary. The study was initiated as an in vitro approach to the situation existing during intravesical bacillus Calmette-Guerin (BCG) instillation in patients with superficial bladder cancer. Cytokine secretion of a human bladder carcinoma cell line T24 treated with BCG was investigated. A 24-h treatment of T24 cells with BCG resulted in a tenfold higher secretion of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α) when compared with T24 cells treated with *Escherichia coli*, *Streptococcus faecalis* or a cell wall preparation of *Nocardia rubra* (N-CWS). No secretion of IL-1 β and IL-2 was detected. Pre-exposing T24 cells to BCG for various periods of time indicated that a minimum exposure time of 0.5–1 h was required to upregulate IL-6 and TNF α production. Extending the BCG pre-exposure time to 2 and 3 h further increased the rate of cytokine production. No significant difference was found, however, between the rate of secretion initiated after a 2-h or 3-h pre-exposure period. The amounts of these cytokines secreted in the presence of BCG-conditioned medium did not differ significantly from the constitutively secreted amounts, excluding an effect of products possibly secreted by BCG on the upregulation of IL-6 and TNF α . In addition, upregulation of cytokine production appeared to be dependent on the concentration of BCG. The results suggest that cytokines may be produced by urothelial tumor cells after intravesical instillation in patients with superficial bladder cancer, which may play a role in the mode of action of BCG.

Key words: Bladder carcinoma cell line – BCG immunotherapy – Cytokines

Intravesical instillation of bacillus Calmette-Guerin (BCG) has been recognized as an effective treatment in patients with superficial bladder cancer [10, 15]. Howev-

er, the absence of a clinical response of a subpopulation of patients is not understood, explaining the interest in basic research concerning BCG-associated antitumor activity and the search for prognostic markers.

Although a great deal of information on the mode of action of BCG as an antitumor modality is still lacking, the majority of the available evidence indicates an immune-mediated reaction, associated with local production of cytokines [2–4, 9, 18, 19, 23]. Knowledge of the mechanism of interaction of BCG with the bladder wall that initiates the immunological reaction is inadequate [12, 22]. Data on a possible direct interaction between BCG and normal urothelium and/or tumor cells are scarce [1, 26].

It is generally assumed that after BCG instillation the major cellular sources of the cytokines detected in urine are macrophages and lymphocytes infiltrating the bladder wall [4]. However, cytokine determinations in urine reflect the response of the *entire* bladder and other cellular sources cannot be excluded. The release of interleukin-1 and -6 (IL-1, IL-6) and tumor necrosis factor alpha (TNF α) has been reported for a number of other cell types, such as polymorphonuclear granulocytes, fibroblasts, and endothelial and epithelial cells [6, 13, 25], and in vitro studies with bladder tumor cell lines have shown that these cells are capable of cytokine gene expression. The bladder tumor cell line 5637 synthesizes mRNAs encoding various cytokines under steady-state conditions [11]. The constitutive synthesis of B cell growth factor (BCGF), at present known as interleukin-6 (IL-6), has been observed for T24 cells [20]. In view of these observations, an investigation was initiated to study the possible effects of BCG on bladder tumor cells.

This report describes the effects on the production of various cytokines by T24, a human bladder carcinoma grown in vitro, of treatment with BCG and various other bacteria commonly known to induce cystitis. BCG appeared to upregulate the production of IL-6 and TNF α by T24 cells. The consequences of these observations are discussed in relation to the BCG-associated inflammatory and immune reactions.

Materials and methods

Cells and culture conditions

Stock cultures of the human bladder carcinoma cell line T24 [5] were cultured as monolayers in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37°C in a humidified 10% CO₂ atmosphere at pH 7.4 until subconfluency. Cultures were screened for mycoplasma. For passage and experiments, cells were trypsinized with 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS) consisting of 8 g NaCl, 0.2 g KCl, 1.16 g Na₂HPO₄ × 2H₂O and 0.2 g KH₂PO₄ per liter at pH 7.4. All tissue culture chemicals were obtained from Flow Laboratories (Irvine, UK). Tissue culture plastics were from Costar (Cambridge, Mass.).

Bacterial preparations

Escherichia coli and *Streptococcus faecalis* were grown according to standard microbiological procedures, washed and diluted to appropriate concentrations with DMEM. Freeze-dried BCG, strain Connaught, was reconstituted in 1.0 ml diluent consisting of 0.85% NaCl, 0.025% Tween, 0.06% NaH₂PO₄ and 0.25% Na₂HPO₄ and diluted to appropriate concentrations with DMEM. Five hundred micrograms Rubratin (N-CWS), a lyophilized cell wall skeleton preparation of *Nocardia rubra* with reported immunomodulating activity [7], was reconstituted in 1.0 ml saline and diluted with DMEM. For determination of the effects of the various bacteria and N-CWS on cytokine secretion, the bacteria or N-CWS were added to T24 cultures for various periods of time. Subsequently, the medium was collected, centrifuged and stored at -20°C. The amounts of the various cytokines were determined within 2 months of collection.

Study design

A total of 3 × 10⁵ T24 cells (2 cm²; 1 ml medium) were treated according to the following protocols:

1. Cells were incubated *continuously* for 24 h with BCG as well as with several other bacteria at various concentrations. At the end of the 24-h period medium was collected for cytokine determinations.
2. Cells were *pre-exposed* to BCG (2 × 10⁶ cfu) for time intervals ranging from 0.25 to 3 h, washed 3 times with DMEM and fresh culture medium added. Subsequently samples of medium were taken regularly over a period of 8–12 h for cytokine determinations.

Determination of IL-6, IL-1β, IL-2 and TNFα

IL-1β, IL-2, IL-6 and TNFα were quantified with a human-specific, oligoclonal enzyme-linked immunosorbent assay (ELISA) obtained from Medgenix (Fleurus, Belgium). We recently demonstrated a significant relation between the Medgenix ELISA and the bioassay utilizing a variant (subclone 9.9) of the original IL-6 dependent hybridoma cell line B13.29 as described by van Oers et al. [17, 23].

Determination of total protein and the rate of protein synthesis

Proliferation of T24 cells during the experiments was determined by measuring total cellular protein [16, 21]. Labeling and measurement of the rate of protein synthesis were performed as described by Schamhart et al. [24].

Statistical analysis

To test for differences between groups, series of one-way analyses of variance of equal or unequal size were used. When significant ($P < 0.05$) differences were found by analysis of variance, Duncan's multiple range test [8] or, in the case of an unequal number of replications, Duncan's multiple range test as adjusted by Kramer, was performed ($P = 0.05$) [14].

Results

Bacteria-induced cytokine secretion by T24 cells

Initially, secretion of IL-6 and TNFα by T24 cells was determined after a 24-h exposure to BCG Connaught, N-CWS, *E. coli* and *S. faecalis* at various concentrations (Table 1). The results showed a strong enhancement of IL-6 and TNFα secretion in the presence of BCG compared with the amounts produced constitutively or in the presence of the other bacteria investigated. The highest BCG-induced IL-6 and TNFα secretion was about 10 times higher than that induced by *E. coli*, *S. faecalis* or N-CWS. In addition, the induction of IL-6 and TNFα appeared to be concentration dependent. No proliferation or lysis of T24 cells was observed during the time course of the experiments.

Under these conditions no bacteria-induced IL-1β or IL-2 was detected.

Production of IL-6 and TNFα after BCG pre-exposure for various periods of time

In a next series of experiments the effects of pre-exposure of T24 cells to BCG (2 × 10⁶ cfu) for 0, 0.25, 0.5, 1, 2, and 3 h on IL-6 and TNFα production were studied (Fig. 1). Subsequent monitoring over a period of at least 8 h after pre-exposure indicated that a minimum pre-exposure period of 0.5–1 h was required to upregulate cytokine production to a significant ($P < 0.05$) degree. Increasing the pre-exposure time to 2 or 3 h resulted in an increase of the initial rate of IL-6 and TNFα production by a factor 2.9 ± 0.7 and 4.8 ± 2.0 respectively compared with a 1-h pre-exposure period. No significant ($P < 0.05$)

Table 1. IL-6 and TNFα secretion of T24 cells after 24 h of incubation with different bacteria and bacterial cell walls^a

Incubated with		IL-6 (ng)	TNF (pg)
Organism or bacterial cell wall	Quantity		
None		1.0 ± 0.6 (6) ^b	9.5 ± 3.0 (4)
BCG Connaught	2 × 10 ⁵ cfu	2.1 ± 0.8 (4)	23.2 ± 3.6 (3) ^c
	2 × 10 ⁶ cfu	37.8 ± 1.6 (4) ^c	136.0 ± 6.3 (3) ^c
	2 × 10 ⁷ cfu	22.9 ± 1.0 (3) ^c	94.7 ± 7.0 (3) ^c
<i>Escherichia coli</i>	1 × 10 ⁵ cfu	0.4 ± 0.1 (3)	9.9 ± 2.8 (3)
	1 × 10 ⁷ cfu	2.0 ± 0.3 (3) ^c	9.8 ± 0.1 (3)
	1 × 10 ⁹ cfu	4.1 ± 0.0 (3) ^c	7.6 ± 11.4 (3)
<i>Streptococcus faecalis</i>	1 × 10 ⁵ cfu	0.5 ± 0.0 (3)	5.7 ± 0.6 (3)
	1 × 10 ⁷ cfu	0.4 ± 0.0 (3)	8.8 ± 0.1 (3)
	1 × 10 ⁹ cfu	1.8 ± 0.5 (3)	6.3 ± 1.5 (3)
N-CWS	0.6 µg	0.4	—
	6.0 µg	0.7	—
	60.0 µg	3.3	—

IL-6, Interleukin-6; TNF, tumor necrosis factor; —, not determined
^a 3 × 10⁵ T24 cells were incubated for 24 h in the presence of the indicated number of bacteria or bacterial cell walls (N-CWS)

^b Mean ± SD (number of replications)

^c $P < 0.05$ compared with constitutive secretion ("None")

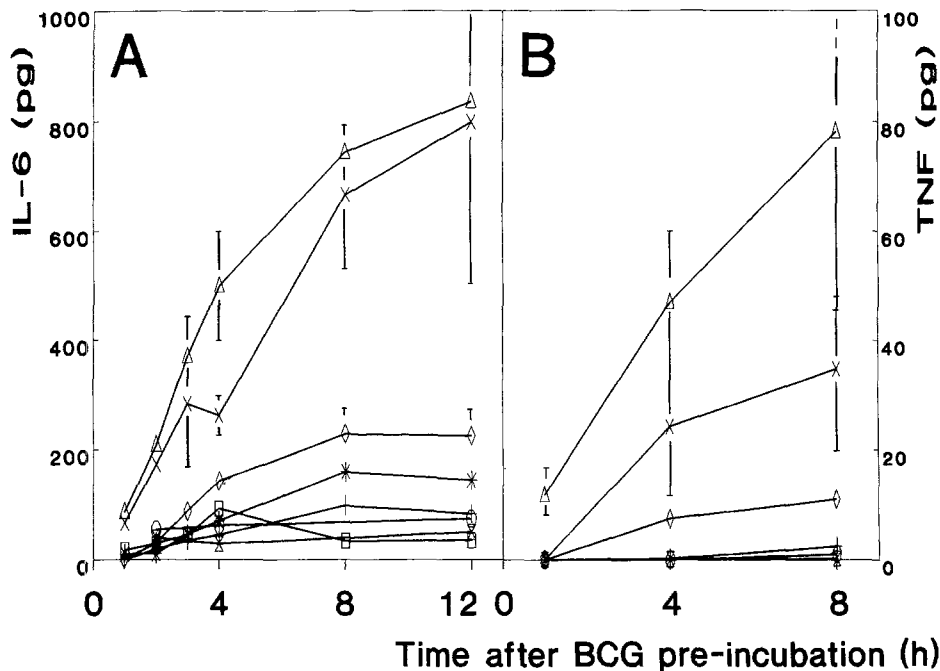


Fig. 1A, B. Secretion of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF α) by T24 cells after pre-exposure to BCG. T24 cells (3×10^5) in 1 ml Dulbecco's modified Eagle's medium were exposed to BCG (2×10^6 cfu) for 0 (+ - +), 0.25 (□ - □), 0.5 (* - *), 1 (◇ - ◇), 2 (x - x) or 3 (Δ - Δ) h. After removal of BCG by washing, fresh medium (1 ml) was added and IL-6 (A) and TNF α (B) were determined during the indicated time intervals. Cytokine secretion after "exposure for 0 h" represents the constitutively secreted amounts of IL-6 or TNF α . The effects of BCG-conditioned medium on IL-6 and TNF α were determined after incubating T24 with 4-h (○ - ○) or 24-h (x - x) BCG-conditioned medium. Error bars (\pm SD) are indicated.

differences between 2-h and 3-h pre-exposures to BCG were found in the kinetics of IL-6 or TNF α production.

Determination of the total rate of protein synthesis of T24 cells pre-exposed to BCG for 3 h showed that BCG did not affect the total rate of protein synthesis compared with that in untreated T24 cells (results not shown). These results establish that the upregulation of IL-6 and TNF α production by BCG did not result from an increase in the rate of total protein synthesis.

Bacteria-secreted products and T24 cytokine secretion

In order to investigate whether the observed cytokine secretion resulted from products secreted by the bacteria, T24 cells were incubated with BCG-conditioned (4 and 24 h) medium and the secretion of IL-6 and TNF α monitored over a period of 12 and 8 h, respectively (Fig. 1). No significant ($P > 0.05$) differences in cytokine secretion were found compared with their constitutive production. In contrast, incubating T24 cells with *E. coli*-conditioned medium for 24 h resulted in an IL-6 production (4.1 ± 0.1 pg) identical to that during incubation with 1×10^9 cfu *E. coli* (4.1 ± 0.0 pg), indicating the involvement of products secreted by *E. coli* in the (slight) upregulation of IL-6.

Discussion

This study has demonstrated that, in contrast to various other bacteria, bacillus Calmette-Guerin upregulates the production of IL-6 and TNF α , but not of IL- β or IL-2, by the human bladder carcinoma cell line T24. The upregulation appeared to depend on both the concentration of BCG and the pre-exposure time to BCG. It should be noted that extrapolation of the data on cytokine production after pre-exposing T24 cells for 2 or 3 h to BCG results in a theoretical amount of approximately 2 ng IL-6

and 250 pg TNF α over a period of 24 h (Fig. 1), whereas after continuous incubation for 24 h with BCG total amounts of 37 ng IL-6 and 136 pg TNF α were observed (Table 1). Although not the immediate subject of this paper, the discrepancy between these figures, in particular of IL-6 production, may most probably be explained by a progressive increase in the rate of IL-6 synthesis during continuous incubation with BCG.

The pre-exposure period to BCG of 2–3 h required to obtain appreciable secretion of IL-6 and TNF α seems to agree with the reported time-dependent internalization of BCG by the bladder tumor cell lines T24 and MBT-2, which show a maximum at 3 h [1]. However, the current data do not exclude BCG-induced upregulation of IL-6 and TNF α production by other, as yet unidentified mechanism(s) not related to internalization of BCG. Of these alternative mechanisms, upregulation of IL-6 by product(s) secreted by BCG into the medium has been excluded in the present study. Additional studies, including inhibition of the phagocytic process and the use of non-phagocytosing bladder (tumor) cell lines, are in progress to determine whether or not internalization is a prerequisite for upregulation of cytokine production.

The absence of an effect of BCG on the rate of total protein synthesis and cell lysis and the absence of proliferation of T24 cells during the course of the experiments suggest a specific upregulation of de novo IL-6 and TNF α synthesis.

As currently understood, BCG-mediated antitumor activity in humans results from stimulation of local immunological reactions [2, 4, 6, 9, 18, 19, 23, 26] initiated by the attachment of BCG to the bladder. However, no conclusive data are available on this latter event [12, 19, 22]. Becich et al. [1] have suggested a potential active role of transitional epithelial cells (after BCG internalization) in the antitumor process. The present data, showing both a concentration dependency and the requirement for an

exposure period of at least 2 h to obtain significant cytokine secretion by T24 cells, correlate with clinical observations and animal studies on BCG concentration dependency and the general clinical practice of retaining BCG in the bladder for 2 h. Furthermore, the specificity of BCG, compared with that of several other bacteria, in upregulating cytokine production by T24 cells seems to agree with clinical observations showing an increase in urinary cytokines after BCG instillation [3, 23] and no significant increase in urinary cytokines in patients with urinary tract infections [4, 9, unpublished observations].

It is generally assumed that urinary cytokines found after intravesical BCG instillation are produced by leukocytes. From the present data it is tempting to speculate that cytokines may also be produced by tumor cells and/or normal urothelial cells as a result of their interaction with BCG. This phenomenon may be of additional importance in the BCG-associated immune response. However, further studies, including experiments using additional bladder cell lines with a different degree of differentiation, are needed to verify this hypothesis.

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