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# 7 Effects of isoniazid on the proliferation and cytokine production of bladder cancer cells *in vitro* induced by bacille Calmette-Guérin

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## Abstract

**Objective** To determine the effects of isoniazid (isonicotinic acid hydrazide), used to reduce the serious side-effects of immunotherapy of superficial bladder cancer with bacille Calmette-Guérin (BCG), on the proliferation and constitutive BCG-induced synthesis of interleukins 6 (IL6) and 8 (IL8) in human bladder cancer cells cultured *in vitro*.

**Materials and methods** Three poorly differentiated human cell lines, T24, TCC-SUP and BT-B, were used to study the effect of isoniazid on cell proliferation. Cells were inoculated in tissue culture plates and various

concentrations of isoniazid added to the medium. Cell density was then monitored for up to 6 days using a colorimetric assay. To determine the effects of isoniazid on constitutive and BCG-induced cytokine synthesis, cells were cultured in medium containing no additions, BCG, isoniazid or BCG with isoniazid, at various concentrations. Samples of medium were collected regularly for 6 h and the cytokine content (IL6 and IL8) determined using enzyme-linked immunosorbent assays.

*Results* Continuous incubation of proliferating T24, TCC-SUP and BT-B cells with isoniazid at concentrations of 0-100 µg/mL did not affect the rate of proliferation. Unlike TCC-SUP and BT-B cells, T24 cells released more IL6 and IL8 during incubation with BCG. At 6 h after the addition of BCG, the cumulative mean (SD) IL6 and IL8 production of T24 cells was 2.6 (0.1) and 2.3 (0.4) ng per  $3 \times 10^5$  cells, compared with a constitutive level of 0.1 (0.0) and 1.3 (0.2) ng, respectively. There was no significant effect of isoniazid (1-100 µg/mL) on either the constitutive or BCG-induced synthesis of IL6 and IL8 in T24 cells.

*Conclusion* Assuming an essential role of (tumor) epithelial cells in the local immune response induced by BCG, these *in vitro* results suggest that the administration of isoniazid does not interfere with this part of the mechanism by which BCG operates.

## Introduction

Intravesical therapy with BCG against superficial bladder cancer recurrences and carcinoma *in situ* has been shown to be highly effective in many clinical trials [1,2]. Although being an attenuated strain of *Mycobacterium bovis* without virulence, about 5% of patients present with moderate to severe local or systemic adverse effects after treatment with BCG, necessitating treatment with the tuberculostatic agent isoniazid [3]. The prophylactic administration of isoniazid, initiated before BCG instillation has been considered to diminish these side effects and currently the prophylactic use of isoniazid is being investigated in a clinical trial [4]. However, the prophylactic application of isoniazid assumes that it does not adversely affect the BCG-induced activity against the tumor. Data on potentially interfering effects of isoniazid on the successive steps involved in the process of BCG-induced antitumor activity are scarce or lacking. Conflicting results about the effect of isoniazid on the BCG-associated immune reaction have been reported: animal studies indicated either an impairment of BCG-induced reactions or absence of an isoniazid-associated effect [5-8]. No impairment by isoniazid of the local immunological stimulation after BCG

instillation has been reported in man, suggesting that isoniazid does not affect the antitumor efficacy of BCG [9]. BCG-mediated antitumor activity is probably associated with local immunological phenomena [10], but the detailed mode of action is not clearly understood [11]. When used prophylactically, isoniazid may interfere at all levels within the complex host response to BCG, in addition to its direct antitubercular properties on BCG viability [12]. The present study addresses the direct and indirect effects of isoniazid associated with BCG on urothelial tumor cell lines, modeling the possible interference of isoniazid with the initial interaction between BCG and urothelial cells. Presuming that urothelial cells contribute significantly as participants in the locally induced immune response [11,13], the influence of isoniazid on cell proliferation and gene expression was determined in several human bladder tumor cell lines.

## Material and methods

Three poorly differentiated human cell lines, T24, TCC-SUP and BT-B, were used; detailed information (e.g. origin, stage and grade) about the T24 and TCC-SUP cell lines was reported previously [14,15]. The BT-B cell line (kindly provided by Dr A. Böhle, Department of Urology, Medical University Lübeck, Lübeck, Germany) was directly established *in vitro* from a poorly differentiated (grade 3) surgical specimen (personal communication, Dr A. Böhle and [16]). Stock cultures of the cells were maintained as monolayers in RPMI-1640 medium with 1 % glutamine, 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 % CO<sub>2</sub> atmosphere at pH 7.4 until subconfluency. For passage and experiments, cells were trypsinized with 0.05 % trypsin and 0.02 % EDTA in PBS (8 g of NaCl, 0.2 g of KCl, 1.16 g of Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O and 0.2 g of KH<sub>2</sub>PO<sub>4</sub> per liter at pH 7.4). Cultures were screened routinely for mycoplasma. All tissue culture chemicals were obtained from Flow Laboratories (Irvine, Scotland). Tissue culture plastics were from Costar (Cambridge, MA, USA). A vial lyophilized BCG, strain Connaught (Connaught Laboratories, North York, Canada) containing  $1.9 \times 10^8$  c.f.u. of BCG per vial was reconstituted in prewarmed culture medium to the appropriate concentration for experiments. To determine the effect of isoniazid on bladder tumor cell proliferation,  $2.5 \times 10^3$  cells were inoculated in 96-well flat-bottom tissue culture plates containing 100 µL medium. After 24 h the cells were refreshed with RPMI-1640 (100 µL) without or with various concentrations (1-100 µg/mL) of isoniazid (Sigma, St.Louis, MI, USA). Cell density was monitored for up to

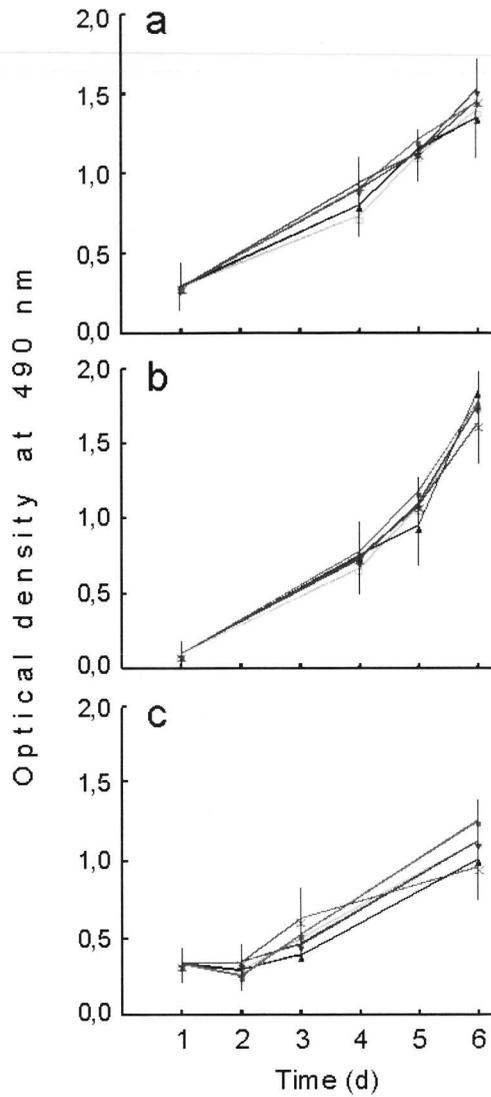
6 days using a colorimetric assay measuring the formation of a formazan dye, soluble in aqueous solutions, from XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate) [17]. Briefly, 50  $\mu$ L XTT solution, containing 1 mg XTT per 1 mL RPMI-1640 and 0.383 mg N-methyl dibenzopyrazine methyl sulphate (Sigma) per 1 mL PBS, was added to the cultures. After incubation for 3 h at 37° C, the optical density was read at 490 nm and 650 nm as a reference wavelength using a microplate reader (Thermomax, Molecular Devices, Sopachem, Lunteren, The Netherlands). The spectrophotometer was blanked on the first column of control wells containing medium (without cells) and XTT solution; isoniazid did not interfere with the assay. In some experiments, data obtained with the XTT assay were verified by cell counting with a Coulter counter and/or a haemocytometer.

To determine the effects of isoniazid on constitutive and BCG-induced cytokine synthesis, cells ( $3 \times 10^5$ ) were cultured for 24 h in 24-well plates containing 1 mL medium/well. Subsequently, the medium was replaced with medium containing (i) no additions, (ii) BCG ( $1$  or  $5 \times 10^6$  c.f.u.), (iii) isoniazid (100  $\mu$ g/mL) or (iv) BCG and isoniazid at these concentrations. Medium was collected at regular intervals for 6 h, centrifuged and stored at -20° C before cytokine content was determined. IL6 was quantified as described previously, using a human specific, oligoclonal ELISA (Medgenix, Fleurus, Belgium) [13]. IL8 was determined with an ELISA developed by CLB (Amsterdam, The Netherlands) [18]; the detection limits of the two assays were 3 pg/mL IL6 and 5 pg/mL IL8.

To test for differences between groups, a series of one-way ANOVAs with equal or unequal size was used. When significant differences ( $P < 0.05$ ) were found by analysis of variance, Duncan's multiple-range test [19], or for unequal numbers of replications Duncan's multiple-range test adjusted by Kramer, was applied ( $P = 0.05$ ) [20]. For differences between groups, paired comparisons were assessed using Student's t-test.

## Results

Addition of isoniazid at concentrations of 1-100  $\mu$ g/mL at 24 h after inoculation appeared to have no effect on the proliferation of T24, TCC-SUP and BT-A cells (Fig. 1). Initially, the constitutive and BCG-induced



**Figure 1.** Proliferation of the bladder carcinoma cell lines: **a.** BT-B, **b.** TCC-SUP and **c.** T24, in the presence of various concentrations of isoniazid. At 24 h after inoculation the cell cultures were refreshed with medium containing 0 (x crosses), 1 (■ closed squares), 10 (▼ down triangles), 50 (▲ up triangles) or 100 (□ open squares) µg/ml isoniazid. The number of cells was determined using the XTT assay and expressed as the optical density at 490 nm.

upregulation of IL6 and IL8 of the three bladder cancer cell lines was determined. BCG was applied as an inducer, as a BCG-associated upregulation of the synthesis of various cytokines has been reported in the T24 bladder cancer cell line *in vitro* [13]. The present results indicated that BCG upregulated the synthesis of both IL6 and IL8 in T24 cells, but not in TCC-SUP or BT-A cells (Table 1). Moreover, T24 cells showed a profound

**Table 1.** Cumulative production of IL-6 and IL-8 in the medium of BT-B, TCC-SUP and T24 cells during 6 h incubation in the absence and presence of BCG

	Mean (SD) concentration (ng / 3 x 10 <sup>5</sup> cells)	
	IL-6	IL-8
BT-B		
- BCG	0.3 (0.0)	<< <sup>1</sup>
+ BCG	0.4 (0.0)	<<
TCC-SUP		
- BCG	1.1 (0.1)	<<
+ BCG	1.0 (0.1)	<<
T24		
- BCG	0.1 (0.0)	1.3 (0.2)
+ BCG	2.6 (0.1) <sup>2</sup>	2.3 (0.4) <sup>2</sup>

<sup>1</sup>: << indicates an IL-8 concentration of <10 pg/mL.

<sup>2</sup>: p < 0.01 compared to cytokine accumulation in the absence of BCG.

constitutive synthesis of IL8 compared with BT-B and TCC-SUP cells. Because both IL6 and IL8 synthesis were upregulated by BCG in T24 cells, the effect of 1-100 µg/mL isoniazid on the constitutive and BCG-induced synthesis of IL6 and IL8 was determined in this cell line. However, a control experiment was performed to determine whether the penicillin and streptomycin in the culture medium interfered with the capacity of BCG to induce cytokine synthesis in T24 cells. The BCG-associated upregulation of IL6 and IL8 in the presence and absence of penicillin and streptomycin was similar, suggesting that there was no interference with the BCG-induced upregulation of the cytokines within the duration of the experiments. The presence of isoniazid did not interfere with the observed continuous, linear increase of either constitutive or BCG-induced production of IL6 and IL8 (Table 2). In addition, there was no effect of isoniazid on the relatively high constitutive synthesis of IL6 in TCC-SUP cells (results not shown).

**Table 2.** Cumulative production of constitutive and BCG-induced IL6 and IL8 in the medium of T24 cells during 6 h in the absence and presence of isoniazid

Isoniazid ( $\mu\text{g/ml}$ )	Mean (SD) concentration (ng / ml)	
	IL 6	IL 8
- BCG		
0	0.3 (0.0)	1.1 (0.0)
1	0.3 (0.1)	1.3 (0.3)
10	0.3 (0.2)	0.9 (0.2)
100	0.3 (0.1)	0.9 (0.0)
+ BCG		
0	2.3 (0.4)	2.2 (0.9)
1	2.1 (0.2)	2.0 (0.4)
10	2.2 (0.0)	1.7 (0.0)
100	2.3 (0.2)	1.7 (0.2)

## Discussion

Several lines of evidence indicate that (specialized) epithelial cells, such as mature pulmonary epithelial cells, may contribute to the host defense system through interactions with inflammatory cells [21]. It has been suggested that uroepithelial cells are part of a mucosal cytokine network, depending on microbial stimulation of epithelial cells, as is the case in urinary tract infections [22]. During intravesical therapy of superficial bladder cancer with BCG, urothelial (tumor) cells are exposed to BCG mycobacteria. *In vitro*, this situation has been shown to upregulate cytokine synthesis by human bladder tumor cells [13,18]; some of these *in vitro* observations seem to be analogous to observations *in vivo* [11,18,23] supporting the notion that urothelial cells may contribute to the modulation of the BCG-induced immune reaction that is probably important for antitumor activity.

Isoniazid is one of the most widely used antituberculosis drugs, although its precise target of action on mycobacteria is unknown [24,25]. In addition, adverse effects on eukaryotic cells, such as chromosomal aberrations, have been noted by some but not by others [24,26]. The latter report suggests a possible direct effect of isoniazid on urothelial cells during BCG therapy of superficial bladder cancer. The present results indicate no effect, stimulatory or inhibitory, of isoniazid on cell proliferation and cytokine (IL6 and IL8) synthesis, either constitutive or BCG-induced, of bladder cancer cell lines *in*

*vitro*. These results were obtained at isoniazid concentrations comparable to those found in the urine (38.0, SD 60.9, mg/ml urine) of patients treated with isoniazid (300 mg) orally the day before, 2 h before and the day after BCG instillation [9]. This protocol of isoniazid administration is currently under investigation in man (EORTC protocol 30911) [4].

In conclusion, the present results suggest that the administration of isoniazid does not interfere with the postulated, modulating role of cytokine-producing urothelial cells after intravesical BCG installations. However, these results do not exclude possible interference of isoniazid with additional steps of the BCG-induced cascade.

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