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Effects of isoniazid (INH) on the BCG-induced local immune response after intravesical BCG therapy for superficial bladder cancer

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Abstract Because recent investigations showed that the use of isoniazid (INH) severely impaired the local immune reaction to intravesical bacillus Calmette-Guérin (BCG) in the bladder of guinea pigs, in this study the effect of INH in man has been investigated. Patients were treated with BCG with or without oral INH. The concentration of free INH in most urine samples of patients treated with BCG/INH was much higher (mean 38.0 ± 60.9 μg INH/ml) than the minimal inhibitory concentration (MIC; 0.1 μg INH/ml), suggesting at least a bacteriostatic potential of the INH present. However, in vitro studies showed that these urinary concentrations of INH did not kill BCG organisms effectively, even at a concentration of 150 μg/ml for 24 h. After the fifth and sixth BCG instillations a significant increase in the concentration of cytokines (IL2, IL6, IL8 and TNFa), IgG and IgA antibodies to BCG and the number of leukocytes in urine was observed. The leukocytes mainly consisted of granulocytes, besides monocytes/macrophages and, in lower amounts, T- and B-lymphocytes and natural killer (NK) cells. The absolute number of granulocytes and the concentration of IgG antibodies after BCG instillation were significantly suppressed by INH, whereas INH appeared to have no effect on the urinary cytokine and IgA antibody concentrations or the total number and phenotype of the leukocytes present. In conclusion, the results of this study indicate that INH does not impair the local immunological stimulation after BCG instillation in man as severely as was observed in the guinea pig and it may be expected that INH does not impair the antitumor efficacy of BCG.

Key words BCG immunotherapy · Isoniazid · Superficial bladder cancer

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Intravesical bacillus Calmette-Guérin (BCG) therapy is one of the most effective therapies for both prophylaxis and treatment of superficial bladder cancer [17]. BCG belongs to the mycobacteria and is an attenuated strain of the bovine tubercle bacillus. The mode of action of BCG is probably based on local stimulation of the immune system [11, 23], but the actual mechanisms by which BCG mediates antitumor activity are not clearly understood.

Although BCG has lost its virulence, complications of intravesical BCG have been reported [16, 28]. Side effects after intravesical instillation of BCG can be local (cystitis) or systemic, such as granulomatous hepatitis, pneumonitis and even lethal sepsis [15]. To diminish the local irritating bladder symptoms and to prevent systemic side effects some urologists prescribe the bacteriostatic drug isoniazid (INH) prophylactically. It has been assumed that INH will not influence the antitumor effect in man. However, the bacteriostatic potential of INH may reduce the effective dose of viable BCG organisms and thus reduce the antitumor activity [13]. Recently the effect of INH treatment on the immune response after repeated intravesical BCG instillation has been studied in guinea pigs [5]. In that study the administration of INH severely impaired

the immunological stimulatory effects of BCG. The induction of a mononuclear cell infiltrate in the bladder wall was reduced. Enlargement of the regional lymph nodes and an increase of MHC class II expression on the lymph node cells, normally observed after intravesical BCG administration, were inhibited by INH. Systemic immunity to mycobacteria was also diminished.

The influence of INH on the antitumor efficacy is now investigated in man (EORTC protocol 30911: comparative study of intravesical instillation of epirubicin, BCG or BCG+INH in intermediate and high risk PTa-PT 1 papillary carcinoma of the urinary bladder). In previous studies we have investigated the local immunological effects after treatment of superficial bladder cancer patients with BCG by examining the urine for the presence of leukocytes and cytokines [2, 3, 4].

In the present study we have investigated the effect of INH on the local immune response induced by intravesical BCG administration in patients with superficial bladder carcinoma. In the urine of patients treated with BCG or BCG/INH the number of cells, the leukocyte subpopulations, and amounts of IL2, IL4, IL6, IL8, TNFα and of class IgG and IgA antibodies to BCG were compared. The concentration of INH in the urine and the effect of these concentrations on the viability of BCG were also determined.

Materials and methods

Patient treatment

Urine specimens were obtained from 22 patients with superficial bladder carcinoma (stage PTa, PT 1 and/or carcinoma in situ) who were treated with BCG (Tice and RIVM) after transurethral resection of papillary tumor(s). BCG, approximately 5×10^8 culturable particles, was administered in 50 ml 0.9% saline once a week for 6 consecutive weeks. Twelve of the 22 patients investigated also received 300 mg INH orally the day before the BCG instillation, 2 h before instillation and on the day after instillation (instillation 1–6). After treatment with six instillations of BCG patients were followed up by cytoscopy every 3 months.

Collection and preparation of cells from urine

Because previous investigations showed maximal urinary concentrations of cells and cytokines after repeated (> 4) instillations [2, 3, 4], in this study urine specimens were examined during the fifth and sixth BCG instillations. Samples were collected before instillation and 2–6 h (pooled specimens) and 24 h thereafter. The specimens were centrifuged (5 min, 300 *g*) and the supernatants were immediately frozen to −20°C and stored at −70°C. Supernatants were afterwards thawed and used for measuring cytokines, antibodies and INH.

The cellular sediment was suspended in RPMI 1640 tissue culture medium (Gibco Europe, Breda, The Netherlands), supplemented with 10% fetal calf serum (FCS; Gibco), penicillin (100 IU/ml) and streptomycin (100 μg/ml), referred to as complete RPMI. To obtain high cell viability all urine specimens were immediately cooled and processed at 4°C or on melting ice. After washing twice in complete RPMI, viable nucleated nonsquamous cells in urinary sediments were counted by trypan blue (0.5%) exclusion. The cells were used for immunofluorescence staining.

Immunofluorescence staining

Cells (5×10^5) were labelled with 100 μl mAb in Dulbecco's phosphate-buffered saline (Gibco) with 0.01% NaN and 2% FCS, referred to below as $DPBS^+$, in 96-well Microtest III assay plates (Becton Dickinson, Etten-Leur, The Netherlands). Monoclonal antibody (MAb) FK32 (anti-CD15; granulocytes) was kindly provided by Dr. F. Koning (University of Leiden, Leiden, The Netherlands). Other mAbs were obtained from Becton Dickinson: anti-HLE 1 (anti-CD45, leukocytes), anti-LeuM 3 (anti-CD14, monocytes/macrophages), anti-Leu12 (anti-CD19, B-lymphocytes), anti-Leu 4 (anti-CD3, T-lymphocytes), anti-Leu 3 (anti-CD4, helper/inducer T-lymphocytes), anti-Leu 2 (anti-CD8, suppressor/cytotoxic T-lymphocytes), anti-Leu 11c (anti-CD16, NK cells), anti-Leu 19 (anti-CD56, NK cells), anti-IL2R (anti-CD25, IL2-receptor), anti-HLA-DR (Ia non-polymorphic), anti-TCR-1 α/β (T-cell receptor-α/β), anti-TCR-γ/δ-1 (T-cell receptor-γ/δ). Fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments from rabbit anti-(mouse Ig) (Organon Teknika, West Chester, Pa.) were used as second Ab to detect binding of FK32. All other mAbs were conjugated to either FITC or phycoerythrin (PE). As negative controls, mAb of irrelevant specificity (anti-KLH) but corresponding isotypes (Becton Dickinson) or FITC-conjugated rabbit anti-(mouse Ig) mAb only, were used. Labelled cells were finally fixed in 200 μl paraformaldehyde solution (0.25% in $DPBS^+$).

Flow cytofluorometry and analysis

Samples of 1×10^4 cells were measured with a fluorescence-activated cell sorter (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). During measurement the forward scatter threshold was set on channel 52 for background exclusion. Quantification of granulocytes, monocytes/macrophages, and T- and B-lymphocytes was based on cells with forward scatter > 200. The fluorescence of the granulocytes and the monocytes/macrophages was determined in the fluorescence histogram of the total cell population. Analysis of lymphocyte subsets was made possible by a selective cell measurement procedure to increase the number of cells within the lymphocyte gate [2]. The number of cells within the lymphocyte gate reacting with a specific mAb is expressed as a percentage of the $CD45^+$ cells (leukocytes) within the gate. However, in 11 of the 15 urine sediments of patients treated with BCG and in 10 of the 20 sediments of patients treated with BCG/INH, the leukocytes present just before the BCG instillation could not be analyzed because of low cell numbers.

Detection of IL2, IL4, IL6, IL8, TNFα and antibodies specific to BCG

IL4, IL6 and TNFα were determined using specific enzyme-linked immunosorbent assays (ELISA) from Medgenix (Fleurus, Belgium). For detection of IL2 a specific bioassay with the IL2-dependent murine T-cell line CTLL-16 was used as previously described [12]. A human recombinant IL2 preparation was used as a standard (Proleukin; EuroCetus Benelux B.V., Amsterdam, The Netherlands). IL8 was measured with an ELISA according to a procedure described previously [9]. The detection limits of the various assays were 0.1 IU IL2, 2 pg IL4, 3 pg IL6, 5 pg IL8, and 3 pg TNFα per ml urine. The results were standardized to urine creatinine levels (pg/μmol creatinine). Urine creatinine was determined photometrically (500 nm) with a Cobas-Bio centrifugal analysator (Hoffmann-La Roche, Basle, Switzerland), using alkaline pikrate as reagent. The mean creatinine concentration for all samples was 9.76 ± 5.18 ($n = 252$). Class IgG and IgA antibodies to BCG were determined with an ELISA [14]. For this purpose flat bottomed ELISA microplates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with 100 μl carbonate buffer containing 10 μg/ml purified protein derivative (PPD) of *Mycobacterium tuberculosis*.

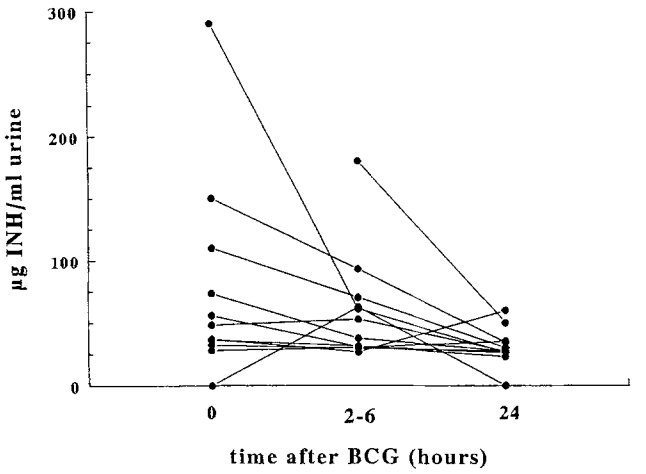

Fig. 1 Isoniazid concentration in urine before (0 h), 2–6 h after and 24 h after fifth bacillus Calmette-Guérin/isoniazid (BCG/INH) instillation

Table 1 Effect of isoniazid (INH) on bacillus Calmette-Guérin (BCG) organisms exposure (24 h)

Concentration of INH (µg/ml)	No. ($\times 10^5$) of colony-forming units of BCG
0	6.0
50	7.5
150	7.0

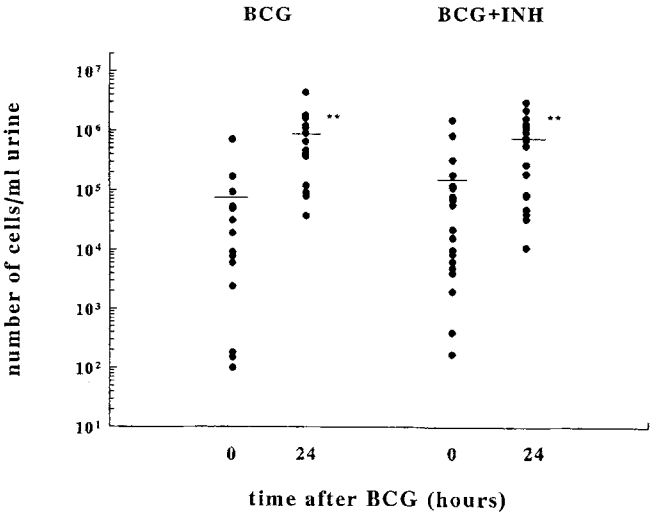

Fig. 2 Total number of leukocytes before (0 h) and 24 h after fifth and sixth BCG and BCG/INH instillation (determined by trypan blue exclusion). -, Mean number of cells. The number of cells is significantly increased 24 h after BCG instillation,** $P < 0.01$ (two-sample sign test)

Isoniazid (INH) determination

For determination of INH, urine was collected just before, 2–6 h after (pooled specimens), and 24 h after the BCG instillation. After centrifugation (5 min, 300 *g*) the supernatant was stored at −70°C until analysis. Analysis of INH was based on acetylation of INH with pentafluorobenzoylchloride in an aqueous solution, resulting in the formation of 2-(4-pyridyl)-5-pentafluorophenyl-1,3,4-oxadiazole. Quantification was performed using benzoic hydrazide (BAH) as internal standard. Both derivatives have favorable electron capture properties and can be analyzed with high sensitivity with negative ion chemical ionization GC/MS, monitoring their molecular ions $[M]^-$.

Effect of urinary INH concentrations on BCG viability

The direct cidal effect of INH to BCG was determined in a quantitative suspension test. Approximately 1.2×10^7 colony forming units (cfu)/ml PBS were exposed to 0, 50 or 150 µg/ml INH. After 24 h INH was inactivated with 0.5% pyruvate. Hereafter mycobacteria were cultured on Middlebrook 7H 10 medium. The number of cfu was counted after 4 weeks of growth at 35°C in 5% CO_2.

Statistical analysis

Significance of differences between the total cell number, number of cells in various cell subsets, cytokine and antibody concentrations before and after treatment within each group of patients (BCG or BCG/INH) was determined with two-sample sign test for equal medians [26]. Differences between the percentages of cells (determined by flow cytometric analysis), the number of cells and the amount of cytokines and antibodies in BCG-treated and BCG/INH treated patients were statistically determined with Mann-Whitney two-sample (non-matched) test [26].

Results

Urinary INH concentration and effect on BCG viability

INH was administered orally the day before, 2 h before and the day after BCG instillation. The concentration of INH in urine was measured just before, 2–6 h after (pooled urine specimens), and 24 h after the fifth BCG instillation (Fig. 1). Although INH concentrations showed large variations between individual patients, nearly all concentrations were far higher (mean: 38.0 ± 60.9 µg INH/ml) than the minimal inhibitory concentration (MIC; 0.1 µg/ml) determined for BCG in vitro by the agar dilution technique (after 21 days of incubation with INH, the number of cfu of BCG was counted) [14]. Only 1 patient showed no detectable urinary INH concentrations just before and 24 h after BCG instillation. As a negative control, INH was also measured in urine of 2 patients treated with BCG only. In these samples no INH could be detected (data not shown). To study the effect of high INH concentrations on the viability of BCG fresh mycobacteria were incubated with 0, 50 and 150 µg/ml INH, comparable to INH concentrations found in the urine after the fifth BCG instillation (Fig. 1). Four weeks after addition of INH during 24 h to a BCG culture, none of the INH concentrations tested appeared to be bactericidal to the mycobacteria (Table 1).

Effect of INH on urinary cell numbers

Before the fifth and sixth BCG instillation, the number of viable cells in urine from patients treated with BCG or

Table 2 Leukocyte subpopulations and subsets of lymphocytes in urine before (0 h) and after (24 h) fifth and sixth BCG instillation. Percentages given are mean ± SD of (*n*) patients. **P* < 0.05 (Mann-Whitney) for difference between 0 h and 24 h

Cell type	Cell composition (%) after			
	BCG		BCG/INH	
	0 h	24 h	0 h	24 h
Granulocytes[a]	68 ± 10 (4)	63 ± 18 (14)	71 ± 13 (10)	65 ± 14 (17)
Monocytes/macrophages[a]	1 ± 0 (4)	3 ± 2* (14)	2 ± 2 (10)	6 ± 4* (17)
T lymphocytes[b]	1 ± 1 (4)	1 ± 1 (14)	1 ± 1 (10)	1 ± 0 (17)
B lymphocytes[b]	0 ± 0[d] (4)	0 ± 0[d] (14)	0 ± 0[d] (10)	0 ± 0[d] (17)
NK cells[b]	0 ± 0[d] (4)	0 ± 0[d] (14)	0 ± 0[d] (10)	0 ± 0[d] (17)
T helper/inducer[b,c]	64 ± 13 (3)	38 ± 16* (14)	44 ± 19 (8)	39 ± 15 (17)
T suppressor/cytotoxic[b,c]	16 ± 2 (3)	18 ± 8 (14)	19 ± 9 (8)	19 ± 11 (17)
IL2 receptor[b,c]	37 ± 32 (3)	16 ± 11 (14)	20 ± 12 (7)	21 ± 17 (17)
HLA-DR[b,c]	67 ± 30 (3)	50 ± 21 (14)	47 ± 22 (8)	55 ± 15 (17)
α/β T cell receptor[b,c]	81 ± 13 (3)	58 ± 26 (14)	56 ± 21 (8)	61 ± 21 (17)
γ/δ T cell receptor[b,c]	17 ± 13 (3)	9 ± 9 (14)	5 ± 4 (8)	7 ± 8 (17)
CD4/CD8 ratio	4.0 ± 1.3 (3)	2.5 ± 1.9 (14)	3.3 ± 3.2 (8)	2.5 ± 1.2 (17)

[a] Determined by flow cytometric analysis of the total cell population with forward scatter > 200
[b] Determined by flow cytometric analysis of the cells in the lymphocyte gate
[c] Positive cells expressed as percentage of the $CD45^+$ cells
[d] Percentages 0–0.5%

BCG/INH ranged from 1.0×10^2 to 7.1×10^5/ml and from 1.7×10^2 to 1.5×10^6/ml, respectively. In both groups of patients (BCG and BCG/INH) the total number of cells in the urine was significantly ($P < 0.01$) higher 24 h after BCG instillation than before instillation (Fig. 2).

Effect of INH on leukocyte subpopulations

The relative quantities of leukocyte subpopulations of urine sediments before and after the fifth and sixth BCG instillation, determined by flow cytofluorometry, are presented in Table 2. Granulocytes ($CD15^+$) were predominant (about 67%), in addition to which about 3% of monocytes/macrophages ($CD14^+$), and about 1% T-lymphocytes ($CD3^+$) were found. B-lymphocytes ($CD19^+$) and NK cells ($CD56^+$) were present in very small amounts or even absent (between 0% and 0.5%).

Table 2 shows that after both BCG and BCG/INH treatment the percentage of monocytes/macrophages had increased significantly by 24 h after BCG instillation compared with pre-instillation values ($P < 0.05$). The percentage of T helper/inducer cells ($CD4^+$) was significantly decreased 24 h after BCG treatment, but not after BCG/INH treatment. This decrease was probably caused by high pre-instillation values, in which case it is not relevant. The two groups of patients (BCG and BCG/INH) did not differ significantly in the percentage of leukocytes either before or 24 h after BCG instillation.

For both treatments (BCG and BCG/INH) the mean percentage of T suppressor/cytotoxic ($CD8^+$) was lower than the percentage of $CD4^+$-cells both before and 24 h after BCG instillation (Table 2). Before and after BCG or BCG/INH treatment most of the lymphocytes expressed α/β T-cell receptor (TCR) and a considerable percentage of T-cells showed HLA-DR and IL2-receptor (CD25) expression. No indications were found for different percentages of α/β TCR, HLA-DR or IL2-receptor expression between samples of patients treated with BCG and patients that received BCG/INH. However, when the absolute numbers of leukocyte subpopulations are calculated, INH induced no significant increase of the absolute number of granulocytes 24 h after BCG instillation, whereas the absolute numbers of monocytes/macrophages, T-cells and B-cells were significantly ($P < 0.05$) increased 24 h after both treatments (BCG and BCG/INH) (Fig. 3).

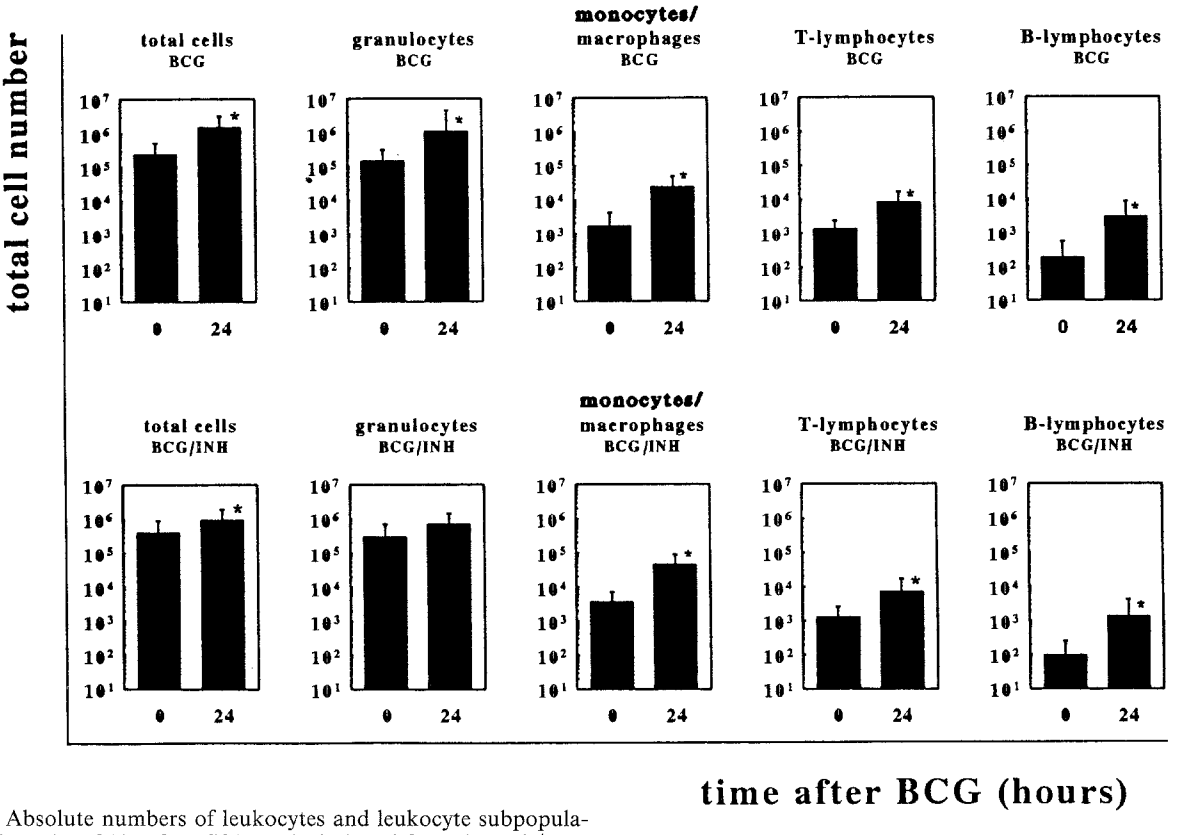

Fig. 3 Absolute numbers of leukocytes and leukocyte subpopulations in urine 24 h after fifth and sixth BCG and BCG/INH instillation (determined by flow cytometric analysis). * $P < 0.05$ (Wilcoxon matched-pairs signed-rank test)

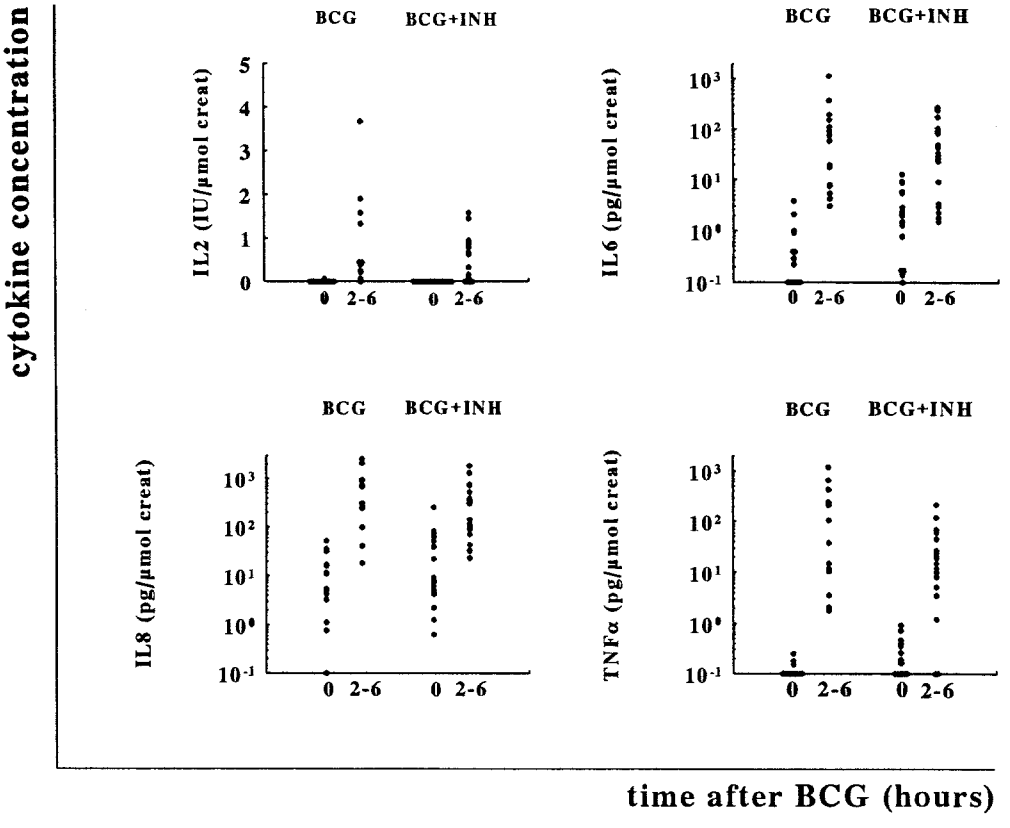

Fig. 4 Concentration of cytokines in urine before and after fifth and sixth BCG and BCG/INH instillation [determined by ELISA (IL6, IL8, TNFα) or CTLL 16-bioassay (IL2)]. Differences between concentrations of these four cytokines 2–6 h after and before (0 h) instillation are significant ($P < 0.01$; Mann Whitney)

Table 3 Increase in concentrations of IL2, IL6, IL8 and TNFα after fifth and sixth BCG instillations, with and without INH. Values shown are means ± SD for (*n*) specimens of concentrations 24 h after instillation minus concentration before. Differences not significant

Cytokine	Treatment	Increase
IL2	BCG	0.79 ± 1.07 (13)
	BCG/INH	0.49 ± 0.51 (17)
IL6	BCG	146 ± 283 (15)
	BCG/INH	61 ± 78 (19)
IL8	BCG	799 ± 883 (15)
	BCG/INH	345 ± 471 (19)
TNFα	BCG	224 ± 327 (15)
	BCG/INH	35 ± 53 (19)

Table 4 Presence of BCG-specific IgG and IgA antibodies in urine before (0 h) and after (2–6 and 24 h) the fifth and sixth BCG instillations [mean ± SD for *n* samples; 1U = [(OD(450 nm)-background OD(450 nm))/mmol creatinine] × 1000; $^{**}P < 0.01$ (two-sample sign test)]

Treatment	Time (h)	Concentration (U) of antibodies	
		IgG	IgA
BCG	0	11.20 ± 14.95 (15)	7.63 ± 7.26 (15)
	2–6	87.74 ± 81.27 (15)**	27.82 ± 35.99 (15)**
	24	21.08 ± 25.29 (15)	4.68 ± 5.29 (15)
BCG/INH	0	28.24 ± 31.32 (20)	6.91 ± 9.35 (20)
	2–6	38.02 ± 41.63 (20)	9.96 ± 11.50 (20)**
	24	10.92 ± 23.38 (20)	2.46 ± 2.90 (20)

Effect of INH on urinary concentrations of IL2, IL4, IL6, IL8 and TNFα

Figure 4 shows the levels of IL2, IL6, IL8 and TNFα in urine before and 2–6 h after (obtained from pooled urine samples) the fifth and sixth BCG instillations. In most pre-instillation samples the concentrations of IL2 and TNFα were low or not detectable. In contrast, the levels of IL6 and IL8 in urine were relatively high just before BCG instillation, ranging from zero to 12.8 pg IL6/μmol creatinine and zero to 254 pg IL8/μmol creatinine.

The concentrations of urinary IL2, IL6, IL8 and TNFα showed a significant increases during 2–6 h after BCG instillation in both groups of patients ($P < 0.01$). However, there were considerable variations between individual patients in the concentrations during 2–6 h after BCG or BCG/INH treatment. In none of the urine samples tested were detectable IL4 concentrations both before and 2–6 h after BCG or BCG/INH found (data not shown). The increase in urinary cytokine concentrations after the fifth and sixth BCG instillations can be seen from Table 3, showing no significant differences between BCG and BCG/INH treatment.

Effect of INH on urinary concentrations of anti-BCG IgG and IgA antibodies

The presence of urinary IgG and IgA antibodies to BCG is presented in Table 4. Anti-BCG IgG and IgA were present before the fifth and sixth instillations of both treatments (BCG and BCG/INH). The concentration of urinary IgA was significantly increased ($P < 0.01$) 2–6 h after BCG and BCG/INH treatment. Also IgG was significantly increased ($P < 0.01$) 2–6 h after BCG treatment, whereas after BCG/INH treatment no significant increase could be found. By 24 h after both BCG and BCG/INH treatment the concentrations of IgG and IgA decreased.

Discussion

In the present study we investigated the effect of oral INH administration on local cellular immunological stimulation after 5 and 6 intravesical BCG instillations in man. The great majority of individuals can be characterized as either slow or rapid inactivators of INH [19]. The large variations observed in INH concentrations between individual patients may indicate the presence of slow and fast inactivators of INH in this study (Fig. 1). The concentration of free INH in most urine samples was far higher than the minimal inhibitory concentration (MIC) as determined for BCG in vitro (0.1 μg/ml) [18], suggesting a bacteriostatic potential of the intravesical INH. The MIC was determined by the agar dilution technique, in which the number of cfu of BCG was counted after 21 days of incubation with INH. As shown in Fig. 1, INH is present in urine only for about 24 h. The consequences of these temporary high urinary INH concentrations were studied in vitro (Table 1), showing no short term bactericidal activity of INH to BCG.

Both BCG and BCG/INH administration induced a significant increase in the total number of leukocytes in the urine 24 h after BCG instillation (Fig. 2). However, when the cell number after the sixth instillation is determined separately from the fifth instillation, the increase after 24 h was significantly inhibited by INH after the sixth instillation, but not after the fifth instillation (data not shown).

The absolute and relative amounts of leukocytes and leukocyte subsets after intravesical BCG therapy are in agreement with observations of De Boer et al. [2]. Although the percentage of granulocytes, B-cells and T-cells 24 h after BCG instillation showed no significant increase (Table 2), the absolute numbers of the leukocyte subsets were significantly increased after BCG instillation (Fig. 3). These findings are comparable with results reported by De Boer et al. [3]. In contrast to the relatively low T-cell number in the urine after both treatments (BCG and BCG/INH), T-lymphocytes are the main cell type present in bladder wall infiltrates in patients after intravesical BCG administration [6, 20].This is possibly the result of differences in time of sample collection after BCG treat-

ment. Because T-lymphocytes have been shown to play an important role in the antitumor activity of BCG [10, 21, 22], subsets of lymphocytes in urine after intravesical BCG administration have been characterized. Both before and 24 h after BCG instillation, T-cells showed expression of HLA-DR and IL2-receptors, indicating activation of T-lymphocytes. The presence of the activation markers HLA-DR and IL2 receptor is in accordance with their presence in bladder wall biopsies [6, 20]. This suggests that the lymphocytes in the urine are probably a reflection of the events that take place in the bladder wall after BCG administration.

After both BCG and BCG/INH treatment, the levels of IL2, IL6, IL8, and TNFα showed a significant increase during 2–6 h after BCG administration. These data are in agreement with results of other studies on cytokines in urine after BCG treatment [1, 4, 7, 12, 21, 24]. However, when cytokine concentrations of the fifth and sixth instillations are determined separately, the increase of IL8 and TNFα was significantly reduced by INH after the fifth instillation (data not shown). After the sixth instillation no significant differences between BCG and BCG/INH treatment could be observed (data not shown), suggesting probably a transient reduction of the cytokine induction by INH. The absence of IL4 in urine after both treatments (BCG and BCG/INH) probably indicates the absence of the Th 2 subset of $CD4^+$ T-cells. It has been shown that mycobacteria can selectively induce human T-cells with a Th1-like cytokine secretion profile [8]. The release of BCG-specific antibodies of both IgA and IgG classes after intravesical BCG administration was also found by Van Der Sloot et al. [27]. Probably these antibodies lead to lysis of mycobacteria by opsonization and subsequent increased phagocytosis by granulocytes [29].

In conclusion, the antitumor activity of BCG has been reported to be dependent on the dose of viable mycobacteria [13, 25]. In our study, INH (tested with concentrations comparable to those found in urine after the fifth instillation) did not kill BCG organisms. Furthermore, INH did not impair the local immunological effects after BCG instillation as has been reported in guinea pigs [5]. Although INH may inhibit the proliferation of BCG because the urinary concentrations found were much higher than the MIC, viable BCG organisms are still able to induce the observed local immune response and antitumor activity. If INH does not influence the immunological action of BCG, it may be expected that INH does not impair the antitumor efficacy of BCG. If this assumption is true and if INH would inhibit the potential side effects of BCG, then the use of prophylactic INH would improve therapy of intravesical BCG in patients with superficial bladder cancer. Both issues are now being investigated in man.

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References

1. Bettex-Galland M, Studer UE, Walz A, Dewald B, Baggiolini M (1991) Neutrophil-activating peptide-1/interleukin-8 detection in human urine during acute bladder inflammation caused by transurethral resection of superficial bladder cancer and bacillus Calmette-Guérin administration. Eur Urol 19:171
2. Boer EC de, Jong WH de, Meijden APM van der, Steerenberg PA, Witjes JA, Vegt PDJ, Debruyne FMJ, Ruitenberg EJ (1991) Leukocytes in the urine after intravesical BCG treatment for superficial bladder cancer: a flow cytofluorometric analysis. Urol Res 19:45
3. Boer EC de, Jong WH de, Meijden APM van der, Steerenberg PA, Witjes JA, Vegt PDJ, Debruyne FMJ, Ruitenberg EJ (1991) Presence of activated lymphocytes in the urine of patients with superficial bladder cancer after intravesical immunotherapy with bacillus Calmette-Guérin. Cancer Immunol Immunother 33:411
4. Boer EC de, Jong WH de, Steerenberg PA, Aarden LA, Tetteroo E, Groot ER de, Meijden APM van der, Vegt PDJ, Debruyne FMJ, Ruitenberg EJ (1992) Induction of urinary IL1, IL2, IL6, and TNF during intravesical immunotherapy with BCG in superficial bladder cancer. Cancer Immunol Immunother 34:306
5. Boer EC de, Steerenberg PA, Meijden APM van der, Klingeren B van, Jong WH de, Elgersma A, Debruyne FMJ, Ruitenberg EJ (1992) Impaired immune response by isoniazid treatment during intravesical BCG administration in the guinea pig. J Urol 148:1577
6. Böhle A, Gerdes J, Ulmer AJ, Hofstetter AG, Flad HD (1990) Local BCG therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall. J Urol 144:53
7. Böhle A, Nowc C, Ulmer AJ, Musehold J, Gerdes J, Hofstetter AG, Flad HD (1990) Detection of urinary TNF, IL1, and IL2 after local BCG immunotherapy for bladder carcinoma. Cytokines 2:175
8. Haanen JBAG, Waal Malefijt R de, Res PCM, Kraakman EM, Ottenhoff THM, Vries RRP de, Spits H (1991) Selection of a human T helper type 1-like T cell subset by mycobacteria. J Exp Med 174:583
9. Hack CE, Hart M, Strack van Schijndel RJM, Eerenberg AJM, Nuijens JH, Thijs LG, Aarden LA (1992) Interleukin-8 in sepsis: relation to shock and inflammatory mediators. Infect Immun 60:2835
10. Hanna MG Jr, Snodgrass MJ, Zbar B, Rapp HJ (1973) Histopathology of tumor regression after intralesional injection of Mycobacterium bovis:IV Development of immunity to tumor cells and BCG. J Natl Cancer Inst 51:1897
11. Jong WH de, Steerenberg PA, Ruitenberg EJ (1987) Bacillus Calmette-Guérin (BCG) and its use for cancer immunotherapy. Res Monogr Immunol 11:283
12. Jong WH de, Boer EC de, Meijden APM van der, Vegt PDJ, Steerenberg PA, Debruyne FMJ, Ruitenberg EJ (1990) Presence of interleukin-2 in urine after intravesical treatment of superficial bladder cancer patients with bacillus Calmette-Guérin. Cancer Immunol Immunother 31:182
13. Kelley DR, Ratliff TL, Catalona WJ, Shapiro A, Lage JM, Bauer WC, Haaff EO, Dressner SM (1985) Intravesical BCG therapy for superficial bladder cancer: effect of BCG viability on treatment results. J Urol Res 143:48
14. Klein WR, Steerenberg PA, Poelma F, Wiel E van de, Rutten VPM, Misdorp W, Jong WH de, Ruitenberg EJ (1986) Immune reactivity in cattle with ocular squamous cell carcinoma after intralesional BCG immunotherapy. Cancer Immunol Immunother 22:87

15. Lamm DL (1992) Complications of bacillus Calmette-Guérin immunotherapy. Urol Clin North Am 19:565
16. Lamm DL, Meijden APM van der, Morales A, Brosman SA, Catalona WJ, Herr HW, Soloway MS, Steg A, Debruyne FM (1992) Incidence and treatment of complications of bacillus Calmette-Guérin intravesical therapy in superficial bladder cancer. J Urol 147:596
17. Meijden APM van der (1991) Non specific immunotherapy with BCG in superficial bladder cancer: an overview. In Vivo 5:599
18. Meijden APM van der, Klingeren B van, Steerenberg PA, Boer EC de, Jong WH de, Debruyne FMJ (1991) The possible influence of antibiotic therapy on results of bacillus Calmette-Guérin intravesical therapy for superficial bladder cancer. J Urol 146:444
19. Peters JH (1960) Studies on the metabolism of isoniazid. Amer Rev Resp Dis 81:485
20. Prescott S, James K, Busuttil A, Hargreave TB, Chisholm GD, Smyth JF (1989) HLA-DR expression by high grade superficial bladder cancer treated with BCG. Br J Urol 63:264
21. Ratliff TL (1992) Role of the immune response in BCG for bladder cancer. Eur Urol 21 [Suppl 2]:17
22. Ratliff TL, Haaff EO, Catalona WJ (1986) Detection of interleukin 2 in the urine of patients with superficial bladder tumors after treatment with intravesical BCG. Clin Immunol Immunopathol 40:375
23. Ratliff TL, Gillen DP, Catalona WJ (1987) Requirement of a thymus-dependent immune response for BCG-mediated antitumor activity. J Urol 137:155
24. Schamhart DHJ, Kurth K (1990) Detection of urinary IL-6 during intravesical BCG therapy (abstract 531). J Urol 143:321A
25. Shapiro A, Ratliff TL, Oakley DM, Catalona WJ (1983) Reduction of bladder tumor growth in mice treated with bacillus Calmette-Guérin intravesical and its correlation with bacillus Calmette-Guérin viability and natural killer activity. Cancer Res 43:1611
26. Siegel S, Castellan NJ (1988) Nonparametric statistics. McGraw-Hill, New York
27. Sloot E van der, Kuster S, Böhle A, Braun J, Wood WG (1992) Towards an understanding of the mode of action of bacillus Calmette-Guérin-therapy in bladder cancer treatment, especially with regard to the role of fibronectin. Eur J Clin Chem Clin Biochem 30:503
28. Steg A, Adjiman S, Debré B (1992) BCG therapy in superficial bladder tumours – complications and precautions. Eur Urol 21 [Suppl 2]:35
29. Teppema JS, Boer EC de, Steerenberg PA, Meijden APM van der (1992) Morphological aspects of the interaction of bacillus Calmette-Guérin with urothelial bladder cells in vivo and in vitro: relevance for antitumor activity. Urol Res 20:219