Urol Res (1997)25:19–24

ORIGINAL PAPER

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Local expression of cytokines in rat bladder carcinoma tissue after intravesical treatment with *Nocardia rubra* cell wall skeleton and bacille-Calmette-Guerin

Received: 22 May 1996 / Accepted: 21 August 1996

Abstract This study aimed to investigate local immunotherapy with *Nocardia rubra* cell wall skeleton (N-CWS) and bacille-Calmette-Guérin (BCG) in chemically induced rat bladder carcinoma. After being fed with 0.025% *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine for 26 weeks, female Wistar rats were treated once a week by intravesical instillation of 100 μg N-CWS or 5×10^6 Colony-Forming Units (CFU) BCG. Tissue specimens were obtained 4 h after the ninth instillation and analyzed by reverse transcription polymerase chain reaction (RT-PCR) for mRNA expression of rat cytokines. The analysis showed high expression of interleukin (IL)-1α, tumor necrosis factor (TNF)-α, IL-2, and interferon (IFN)-τ in BCG (7/7, 7/7, 7/7, 5/7) and N-CWS (9/9, 9/9, 8/9, 8/9) treated tumors in comparison to low expression in controls (3/9, 0/9, 3/9, 3/9). Interestingly, intravesical instillation of N-CWS tended to be less effective at preventing local invasion of the tumors. It is speculated that this difference may result from a more strongly induced expression of T-helper cell-derived lymphokines (IL-2, IFN-τ) by BCG.

Key words Bladder neoplasms · BCG · Nocardiaceae · Cell wall skeleton · Immunotherapy · Nitrosamines

Introduction

Intravesical instillation of bacillus Calmette-Guérin (BCG) after transurethral resection of bladder tumors and in situ cancer is considered to be highly effective in the treatment of recurrent superficial bladder carcinoma [19, 22]. However, the mode of action is still not completely understood.

The antitumor effects of BCG seem to be related to local immunological mechanisms [7]. The transplantable mouse transitional cell carcinoma model of the bladder, MBT-2, has been used as an animal model for intravesical BCG therapy. The efficacy of intravesical BCG therapy against MBT-2 has been shown to correlate with augmentation of natural killer cell activity and systemic sensitization to BCG [31]. Ratliff et al. [28] demonstrated that athymic nude mice, which lack the ability to develop T-lymphocyte response, also lack the ability to resist MBT-2 growth in response to intravesical BCG therapy. The adoptive transfer of spleen cells containing BCG-sensitized T lymphocytes restored BCG-mediated antitumor activity. This formally demonstrated the requirement for T lymphocytes in the antitumor response.

Nocardia rubra cell wall skeleton (N-CWS) is suggested as an immunostimulatory drug in different experimental tumor models. In combination with chemotherapy and/or by intratumoral, intradermal, intrapleural application, N-CWS led to increased survival of patients with advanced cancer (lung [36, 37], gastric [17, 23], leukemia [24], prostate, pancreas, thyroid [4]). Some evidence exists that N-CWS is capable of inhibiting growth of malignant tumors in syngeneic mice more than BCG [4]. Moreover, the clinical application of N-CWS has been shown to cause less adverse effects [3]. Therefore N-CWS might be suggested as an alternative drug for treating patients with bladder carcinoma.

To characterize the potency of the two drugs in preventing the development of bladder carcinoma and furthermore to investigate the local immune response in bladder carcinoma after topical immunotherapy, bladder biopsies from female Wistar rats with chemically induced bladder carcinoma were examined after a 9-week course of intravesical treatment with BCG or N-CWS. Since cytokines are suggested to be critical for

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immunostimulation after intravesical therapy with BCG [6, 8, 9, 13, 30], the expression of cytokines was analyzed at the tissue level.

Materials and methods

Animal experiment

Adult, female Wistar rats were continuously supplied with 0.025% (v/v) *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) (German Cancer Research Center, Heidelberg, Germany) in drinking water for 26 weeks. Control animals (control TO) were sacrificed to investigate the incidence of bladder carcinoma ($n = 12$). Other animals were treated once a week for the subsequent 9 weeks by intravesical instillation (0.4 ml; 30 min) of 5×10^6 CFU BCG-Pasteur (Merieux, Paris, France) ($n = 8$) or 100 µg N-CWS ($n = 9$); controls (control t1) received no instillation during that period ($n = 16$). N-CWS was provided as a lypohilized preparation in vials of 0.5 g (Asta Medica, Frankfurt, Germany). Its final concentration was achieved by reconstitution with physiological saline. Bladder (tumor) tissue was obtained from both controls and treated animals 4 h after the ninth instillation of drugs. They were cut into equal halves and snap frozen in liquid nitrogen. Animal experiments were carried out in accordance with the principles of laboratory animal care and the German law on the protection of animals. Representative tissue specimens were fixed in formalin, embedded in paraffin, sectioned at 5 µm, stained with hematoxylin-eosin, and classified according to WHO criteria.

RNA isolation

Tissue samples were kept frozen at –80°C until processing. Total RNA was isolated from frozen tissue samples by a guanidinium thiocyanate/phenol/chloroform extraction procedure using the RNAzol kit (Cinna/Biotecx, Houston, Tx, USA). Extracted RNA was analyzed for integrity by agarose gel electrophoresis.

Message amplification by reverse transcription PCR (RT-PCR)

Message amplification was performed using RT-PCR as described previously [12]. Briefly, total RNA (1 µg) was reverse transcribed in a final volume of 20 µl reaction mixture containing: 50 mM TRIS-HCl (pH 8,3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 50 µM each of dATP, dCTP, dGTP, and dTTP (Pharmacia, Piscataway, NJ, USA), 500 ng random hexamer primers (Pharmacia), 12 units RNA guard (Pharmacia), and 200 units Moloney murine-leukemia-virus reverse transcriptase (PRL, Gaithersburg, MD, USA). The mixture was incubated at 37°C for 1 h, heated to 95°C for 10 min, and stored at –20°C. cDNA was denatured by heating to 95°C for 5 min; 1 µl was then added to a 24-µl reaction mixture containing 2.5 µl 10 × PCR reaction buffer [0.5 M KCl, 0.1 M TRIS (pH 8,0), 15 mM $MgCl_2$, 0.1% gelatin, 9.6 µM each dATP, dCTP, dGTP, and dTTP (Pharmacia)], 75 ng of each priming oligomer, 1.0 unit *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA), and H_2O. A negative control consisting of a 25-µl aliquot without the addition of cDNA was included in each amplification. Amplification was performed using a DNA thermal cycler (Perkin-Elmer Cetus) for 35 cycles. A cycle profile consisted of 1 min at 94°C for denaturation, 1 min at 60°C for primer annealing, and 1 min at 72°C for primer extension. After amplification, polymerase chain reaction (PCR) products were electrophoresed in 1.5% agarose (IBI, New Haven, CT, USA) and stained with ethidium bromide; size markers from $\phi \times 174$ DNA digested with *Hae* III endonuclease (Gibco BRL, Gaithersburg, MD, USA) were included. Amplified cDNA fragments were visualized using a UV transilluminator. Specific bands were identified by their anticipated molecular weight and by comparison with amplified cDNA from control templates (Clontech, Palo Alto, CA, USA).

Oligonucleotide primers

Oligonucleotide primers for rat ß-actin, IL-1α, IL-2, IL-6, IFN-τ, and TNF-α were purchased from Clontech (Palo Alto) (Table 1). All primer pairs used were designed to cDNA sequences that in genomic DNA cross an intron-exon boundary.

Table 1 Nucleotide sequences of the primer pairs used in this study

Gene	Primer	Amplified PCR fragment size (bp)
β-Actin	Sense: 5′-TTGTAACCAACTGGGACGATATGG-3′ Antisense: 5′-GATCTTGATCTTCATGGTGCTAGG-3′	764
IL-1α	Sense: 5′-CTAAGAACTACTTCACATCCGCAGC-3′ Antisense: 5′-CTGGAATAAAACCCACTGAGGTAGG-3′	623
IL-2	Sense: 5′-CAAAGGAAACACAGCAGCACCTGG-3′ Antisense: 5′-TCCTCAGAAATTCCACCACAGTTGC-3′	342
IL-6	Sense: 5′-CAAGAGACTTCCAGCCAGTTGC-3′ Antisense: 5′-TTGCCGAGTAGACCTCATAGTGACC-3′	614
IFN-γ	Sense: 5′-ATCTGGAGGAACTGGCAAAAGGACG-3′ Antisense: 5′-CCTTAGGCTAGATTCTGGTGACAGC-3′	288
TNF-α	Sense: 5′-TACTGAACTTCGGGGTGATTGGCTC-3′ Antisense: 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	295

Results

Tumor incidence and histology

After 26 weeks of BBN intake, 1/12 animals had developed a superficial carcinoma (8%; TO) while 9 weeks later 14/16 tumors appeared (87.5%) in the control group (t1); 5/14 (36%) were found to be invasive (> pTa) (Table 2). Most of the carcinomas were of a urothelial and squamous cell carcinoma cell type (Fig. 1).

Neither N-CWS nor BCG treatment prevented tumor development (i.e., 100% in each test group). After BCG treatment, one out of eight tumors (12%) was found to be invasive since four out of nine (44%) invasive cancers developed during N-CWS therapy (Table 2).

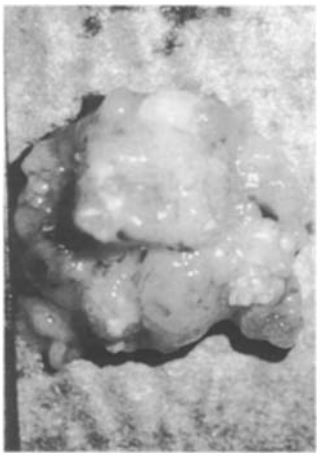

Fig. 1 View inside an opened rat bladder with carcinoma

Local transcription of inflammatogenic cytokines (Fig. 2, Table 3)

RT-PCR was performed to assess the expression of cytokine genes in tumor samples from controls (control t1; $n = 9$) and BCG- ($n = 7$) and N-CWS- ($n = 9$) treated animals (Table 3). ß-Actin transcripts were used as a control and detected in all the samples under investigation ($n = 25$). With regard to proinflammatory cytokine gene expression, transcripts for rat interleukin (IL)-1α, tumor necrosis factor (TNF)-α, and IL-6 were analyzed. After repeated intravesical instillation of BCG or N-CWS, a message for IL-1α and TNF-α was found in virtually all tumor samples (Fig. 2, Table 3). Notably, topical immunotherapy had only a minor effect on IL-6 transcription in tumor tissue, but TNF-α and IL-6 were exclusively expressed in BCG- or N-CWS-treated tumors compared to controls.

Occasionally, IL-1α transcription could be observed in tumor specimens from untreated three of nine animals (Table 3).

Local transcription of T-cell-derived lymphokines (Fig. 3, Table 3)

Lymphokines which are typically expressed by activated helper T lymphocytes, such as IL-2 and IFN-τ, were found to be transcribed in three of the nine untreated tumors (Table 3). Following topical administration of BCG or N-CWS, however, IFN-τ transcripts were detected in five of seven and eight of nine tumor samples, respectively. IL-2 transcripts were found in virtually all tumor specimens (Table 3). Notably, IL-2 transcription in tumor tissue appeared to be more strongly induced by BCG than by N-CWS.

Discussion

Cytokines are soluble signal molecules secreted by a variety of cell types; many of them are critical for regulating the growth and functional activities of immunocompetent cells. Cytokine expression can be induced by various nonspecific immunostimulators including BCG or purified bacterial components, such as N-CWS. BCG [7] and N-CWS [3] have been thoroughly tested for their antitumor activity in animal models and in patients.

BCG has come to be recognized as the most effective treatment currently available for the treatment and secondary prevention of superficial bladder cancer [18]. The weight of current evidence suggests that immunological mechanisms are involved and that T cells play an important role in the BCG-induced antitumor response [7, 28]. Detailed histological studies of human bladder carcinomas treated with topical BCG have been reported [5, 26]. The local response is characterized by mucosal ulceration and submucosal granulomas con-

Table 2 Classification of bladder (carcinoma) specimens according to WHO criteria (T;G) (*S/G* urothelial carcinoma with squamous and glandular differentiation)

Test group	Histology						
	Normal	pTa	>pTa	G1	G2	G3	S/G
BBN, 26 weeks ($n = 12$)	11	1	0	0	1	0	0
BBN, 35 weeks ($n = 16$)	2	9	5	8	5	1	6
N-CWS ($n = 9$)	0	5	4	5	4	0	5
BCG ($n = 8$)	0	7	1	6	2	0	6

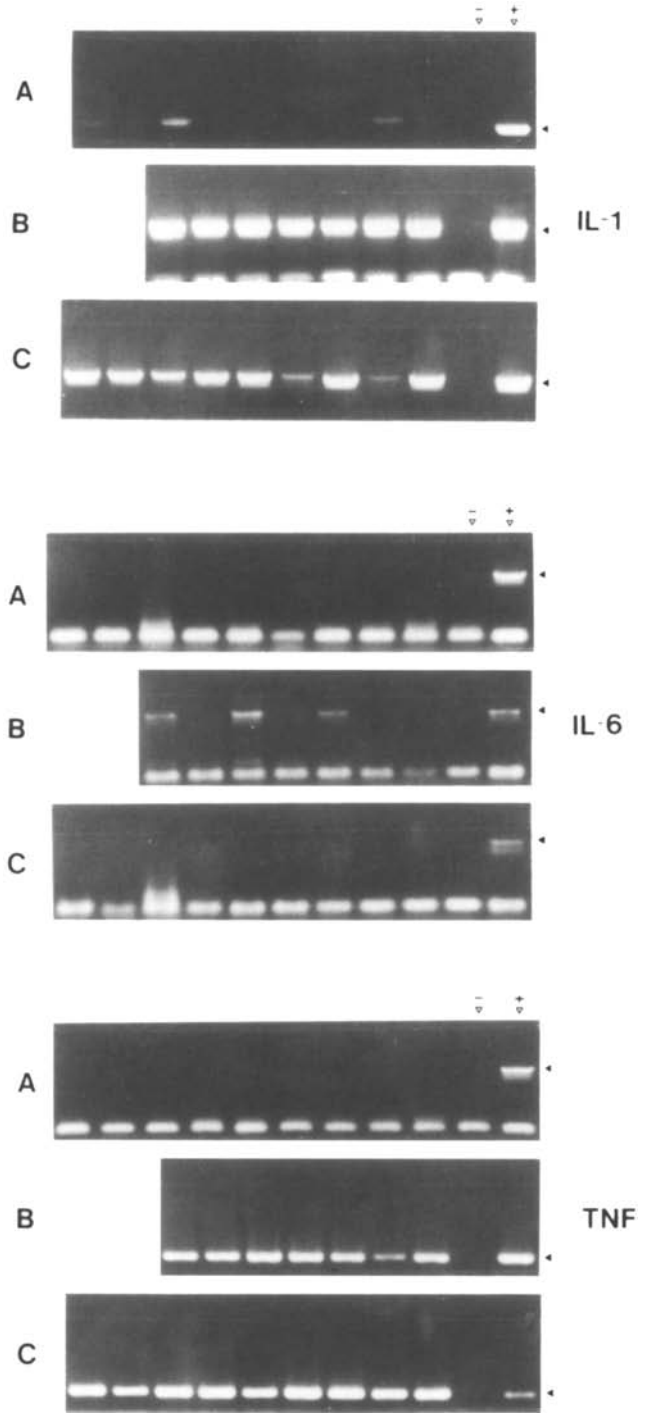

Fig. 2 Transcription of rat IL-1α, IL-6, and TNF-α. RT-PCR products were run on agarose gels in the presence of ethidium bromide, and photographed under ultraviolet transillumination. A negative (–) and a positive (+) control were run on the right margin of the gel. Samples from controls (*A*), BCG-(*B*), and N-CWS-treated (*C*) animals. Molecular weight markers were run in parallel (not shown)

taining activated macrophages and lymphocytes. Several studies have demonstrated the preponderance of activated helper T lymphocytes in the granulomas and the bladder tumors [5, 25, 26].

Table 3 Cytokine gene expression in rat bladder tissue samples

Treatment	Gene	Tumor tissue, Positive samples / all samples
None	β-Actin	25/25
None	IL-1-α	3/9
BCG		7/7
N-CWS		9/9
None	IL-2	3/9
BCG		7[a]/7
N-CWS		8/9
None	IL-6	0/9
BCG		3/7
N-CWS		4/9
None	IFN-γ	3/9
BCG		5/7
N-CWS		8/9
None	TNF-α	0/9
BCG		7/7
N-CWS		9/9

[a]Strong expression in all samples

N-CWS has also been shown to activate macrophages [14, 15, 20, 32, 33], NK cells [29, 35], and T cells [16, 34]. Moreover, previous studies demonstrated induction of IL-1, IFN-α, IFN-ß, and IFN-τ [3], caused by N-CWS.

In this study we used a rat bladder carcinoma model to investigate the effect of intravesical instillations of BCG or N-CWS on the cytokine expression in tumor tissue. To analyze the local cytokine transcription we employed RT-PCR, which is characterized by a much higher sensitivity than Northern blotting or conventional in situ hybridization techniques.

The rapid induction of cytokine transcription in rat bladder carcinoma after BCG therapy, described in the present study, is in agreement with cytokine levels measured in the urine of patients with superficial bladder cancer receiving topical BCG. Peak levels of IL-1α, IL-1ß, IL-2, IL-6, and to a lesser extent TNF-α and IFN-τ have been shown [6, 8, 9, 13, 30] during the first hours after BCG instillation. Moreover, there is clear evidence that BCG treatment induces MHC class II antigen expression on bladder tumor cells [26] and that there is a direct correlation between the amount of IL-2 released into the urine following BCG therapy and the tumor response [11]. Therefore the expression of IL-2 was considered to indicate the activation of T cells (T-helper/inducer > T-suppressor/cytotoxic) in the bladder wall after stimulation with BCG [5, 8, 19]. In addition it has been shown that the synergism of IL-1, IL-2, and IFN-τ can enhance the NK-cell-mediated cytotoxicity [10], although NK cells are not supposed to be a major contributor to the antitumor activity of BCG [27]. Moreover, the presence of IL-1, IL-6, and TNF-α has been suggested to reflect activation of macrophages [1, 2, 5, 8].

In our study, transcription of classical inflammatogenic cytokines, like IL-1α and TNF-α, and lymphokines, such as IL-2 and IFN-τ, was found in virtually all

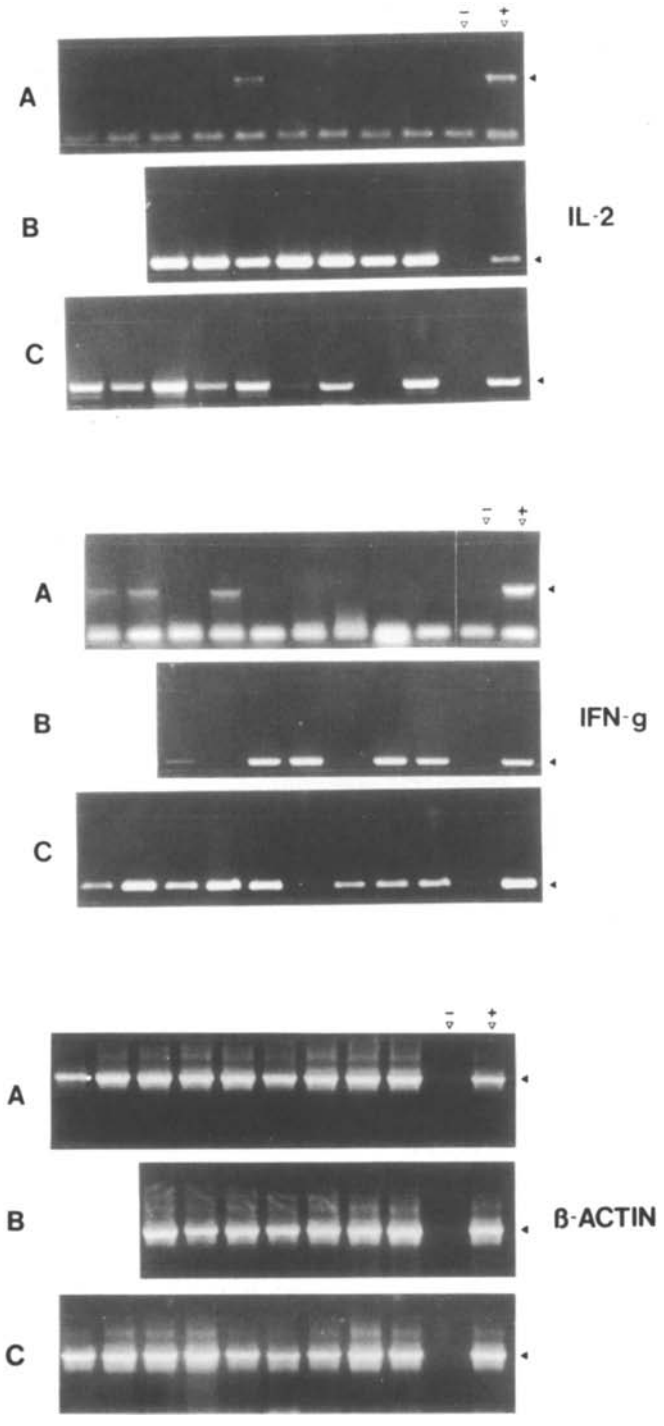

Fig. 3 Transcription of IL-2, IFN-τ, and ß-actin genes in rat bladder carcinoma specimens. For details see legend to Fig. 2

BCG- or N-CWS-treated tumors. These cytokines are probably part of a complex immunological cascade, induced by the local presence of BCG or N-CWS. IL-1 and TNF-α can be expressed by a variety of cell types including T cells, macrophages, and normal or malignant epithelial cells. In contrast, expression of IL-2 and IFN-τ has been exclusively attributed to activated T-helper lymphocytes and NK cells. With regard to the possible antitumor mechanisms, the role of the induced inflammatogenic cytokines and T-helper-cell-derived lymphokines might be the attraction and activation of cytotoxic T cells, lymphokine-activated killer (LAK) cells, or macrophages. Of note, IL-2 in our series was most strongly enhanced following BCG administration. Therefore the present data are consistent with the hypothesis that intravesical BCG, and to a lesser extent N-CWS, causes a shift to a type 1 T-helper cell response with the subsequent development of a productive antitumor response [21].

Besides immunomodulating properties, the cytokine levels in tumor tissue and urine might be sufficicently high to cause direct cytotoxic and/or cytostatic effects on tumor cells. But, interestingly, intravesical instillations of N-CWS tended to be less effective at preventing local invasion of bladder carcinoma than BCG, although these histopathological data did not reach statistical significance. Nevertheless, the impression that N-CWS is less effective in treating bladder carcinoma was independently confirmed in a study on a few patients with residual marker lesions after transurethral resection of bladder carcinoma, who were treated by intravesical instillations of N-CWS (K.H. Kurth, personal communication).

In conclusion, both drugs, BCG and N-CWS, are capable of stimulating the inflammatogenic cytokines IL-1α, IL-6, and TNF-α in a comparable fashion. An increased efficacy of BCG in inhibiting invasion of rat bladder carcinoma is suggested, which may result from its distinct potency for inducing a profound local expression of immunoregulatory lymphokines (IL-2, IFN-τ), activating T-lymphocytes, and stimulating a local type 1 T-helper cell response.

Acknowledgements We would like to thank N.H. Bander for the opportunity to carry out part of this work at the New York Hospital-Cornell Medical Center, and the Memorial Sloan Kettering Cancer Center, New York. In addition, we thank Beatrice Totten and Markus Goertz for their excellent assistance with running the animal experiments. This study has in part been supported by Asta Medica, Frankfurt, Germany.

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