Urol Res (1997) 25:117–120

ORIGINAL PAPER

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Immunostimulation in the urinary bladder by local application of *Nocardia rubra* cell wall skeleton preparation (Rubratin) for superficial bladder cancer immunotherapy – a phase I/II study

Received: 15 March 1986 / Accepted: 20 September 1996

Abstract *Objectives*: Twelve patients with superficial papillary transitional cell carcinoma of the bladder (pTa, pT1) were treated with six consecutive weekly intravesical instillations of Rubratin (in a dose of 1.5, 3.0, or 4.5 mg), a cell wall skeleton preparation of *Nocardia rubra* (NCW). The main objective of this study was to look for local immunomodulating effects of NCW and in the first four patients the effect on a marker lesion was also investigated.
Methods: Local immunostimulation in all 12 patients was determined by (1) measurement of cytokine induction [interleukin 1β (IL1β), IL2, IL6, and tumor necrosis factor alpha (TNFα)], (2) leukocyte influx into the urine, and (3) phenotypic analysis of the lymphocyte fraction of these leukocytes.
Results: Significantly elevated levels of Rubratin-induced IL1β ($P < 0.001$), IL2 ($P < 0.001$), IL6 ($P < 0.01$), and TNFα ($P < 0.001$) were found compared to control pretherapy levels. Rubratin also induced leukocyte influx into the urine. Fluorescence-activated cell sorter (FACS) analysis of the urinary leukocytes indicated T-cell activation (IL2 receptor and HLA-DR expression), while in two out of five patients the CD4/CD8 ratios were increased. Urinary cytokine induction by Rubratin was comparable with cytokine induction observed in non-responding bacillus Calmette-Guérin (BCG) patients (recurrent tumor within 6 months), but less compared with responding BCG patients (no recurrent tumor within 6 months). Clinical results showed no response on the marker lesion and in five out of eight patients early recurrence was found after complete transurethral resection (TUR) of the bladder tumors. This biological response modifier caused no local or systemic side effects at the doses used.
Conclusion: Although local immunostimulation by intravesical Rubratin administration can be induced, the amount of immunocompetent cells attracted to the bladder is not as high as observed in BCG-responding patients, resulting in lower amounts of cytokines produced. This could also explain the lack of clinical efficacy.

Key words Rubratin · Superficial bladder cancer · Cytokines · Immunotherapy

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Introduction

For prophylaxis and treatment of superficial bladder cancer (Ta,T1,Tis), bacillus Calmette Guérin (BCG) immunotherapy is generally considered to be the treatment of choice, especially in patients with a poor prognosis [9]. However, using intravesical BCG, the risk of local and occasionally systemic side effects with even fatal reactions in rare cases is higher than with chemotherapeutic instillations [10]. Thus, there is interest in the search for safer biological response modifiers (BRMs).

The clinical efficacy of new immunotherapeutic agents can be tested through the ablative effect on a marker lesion [11]. Based on observations that BCG-induced immunological events appear to be predictive of early clinical response in superficial bladder cancer, investigations directed to immunological events could also be a logical initial approach for the evaluation of new BRMs [18]. Several effects associated with BCG immunotherapy can be monitored, e.g., the conversion of the PPD skin test, granuloma formation in the bladder wall, production of cytokines and the presence of subtypes of leukocytes in the urine as a reflection of immunological events in the bladder wall [3, 17].

The purpose of the present study was to investigate changes in urinary cytokine production after intravesical instillation of the immunomodulating agent Rubratin. Rubratin is a commercially available cell wall skeleton preparation of *Nocardia rubra*, a bacterial species taxonomically related to bacillus Calmette-Guérin (BCG). In a number of experimental tumors in mice/rats and guinea pigs, immunostimulating and immunotherapeutic

activity of Rubratin has been reported [5, 12, 14, 20, 21]. Clinical studies in Japan and Germany have confirmed these experimental results [1, 4, 21]. In prospective randomized phase III trials a benefit of Rubratin with respect to remission and/or time to survival was shown for a variety of nonurological tumors of different origin [7, 15, 16, 22, 24]. The immunotherapeutic activity of *Nocardia rubra* if applied intravesically in patients with superficial bladder cancer has not yet been examined.

Material and methods

In this study 12 patients (9 male, 3 female) were included with primary or recurrent histologically proven superficial papillary transitional cell carcinoma (TCC) of the urinary bladder (5 pTaG1, 6pTaG2, 1 pT1G2); all were designated "low risk" (= primary G1, recurrence rate < 1/year, size < 3.0 cm *or* primary G2, recurrence rate < 1/year, size < 1.5 cm) for progression according to Kurth et al. [8]. Patients with concomitant carcinoma in situ were excluded from this study. Intravesical instillations with Rubratin (ASTA Pharma AG, Frankfurt, Germany) were started within 2 weeks after transurethral resection (TUR) and the drug was retained in the bladder for 2 h. Patients received no antibiotics during the instillation period. Escalating doses of Rubratin were instilled according to a treatment schedule generally used for BCG (weekly for 6 weeks). The objective was to investigate whether Rubratin had any immunostimulating activity and in the first four patients the response of Rubratin on a marker lesion was also tested. According to pre-defined stopping rules the compound should be given only adjuvantly after complete resection of all tumors, if no reaction is observed in the first four patients with a marker lesion, with the purpose of monitoring only toxicity and the possible immunostimulatory effects of this BRM as expressed by altered cytokine levels and/or leukocyte appearance in the dose applied to elucidate potential dose-related effects. Following Rubratin instillations, local immunostimulation was determined by the measurement of urinary cytokine induction [interleukin (IL)1β, IL2, IL6, and tumor necrosis factor alpha (TNFα)], leukocyte influx into the urine and phenotypic analysis of the lymphocyte fraction of these lymphocytes, as performed previously for BCG therapy [2, 3, 19].

After TUR a marker lesion (0.5–1 cm) was left in the bladder in the first four patients to establish the antitumor efficacy of Rubratin 1.5 mg. All visible tumors were resected by TUR in the following eight patients and Rubratin given in six weekly intravesical instillations at a dose of 3.0 mg ($n = 5$) or 4.5 mg ($n = 3$). Patients were followed for recurrence of disease by 3-monthly cystoscopy combined with cytology. The study design was approved by the local Ethics Committee and written informed consent was obtained from each patient.

Rubratin

Rubratin is a lyophylisate which upon reconstitution forms an injectable oil-in-water emulsion [20]. The contents of an injection vial (0.5 mg) were reconstituted in 1 ml 0.9% saline. For intravesical instillation the required number of vials for the 1.5-, 3.0-, or 4.5-mg dose were diluted to an end volume of 50 ml in saline.

Cytokine determination in urine

Spontaneous voided urine samples were collected during the six instillations, prior to instillation, and 2–4 h, 4–6 h and 12–24 h thereafter. Samples were immediately frozen to −20°C. Afterwards specimens were thawed and centrifuged (300 *g*) to remove cells and debris and stored in aliquots until analysis. IL1β, IL2, IL6, and TNFα in urine were determined with an enzyme-linked immunosorbent assay (ELISA) using an oligoclonal system (Medgenix, Fleurus, Belgium). Cytokine data were standardized to urine creatinine.

FACS analysis of lymphocytes from urine

From fresh 2–4 h urine samples obtained after instillations 1, 3, and 6, the total number of viable leukocytes was determined. Leukocytes were washed (centrifugation 5 min, 300 *g*) twice in phosphate-buffered saline (PBS) (pH 7.2) with 0.2% albumin (Boehringer Mannheim, Germany), 0.02% K-ethylenediaminetetraacetate (EDTA) (Merck, Darmstadt, Germany) and 0.01% NaN_3 (Merck). Cells (5×10^5) were labeled with 100 µl monoclonal antibody (mAb) in PBS with described additions. Monoclonal antibodies anti: -CD45, leukocytes (1:10); -CD3, T cells (1:10); -CD4, Th/i (1:10); -CD8, Ts/c (1:10); -CD25, IL2 receptor (1:10); -CD14, monocytes/macrophages (1:10); -CD66, granulocytes (1:500); and -HLA-DR (1:500) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands), and anti: -CD20, B cells (1:5) and -CD56, NK/Tc cells (1:10) were obtained from Becton Dickinson (Etten Leur, The Netherlands). Fluorescein-conjugated F(ab′)2 fragment from rabbit anti-(mouse Ig)(DAKO, Glostrup, Denmark) was used as second antibody (100 µl, 1:40 in PBS with described additions). Labeled cells were fixed in 300 µl paraformaldehyde solution (0.5% in PBS). Fluorescence-activated cell sorter (FACS) analysis (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) was performed the following day as described previously [3].

Data gained from patients with superficial bladder cancer and intravesically treated with BCG were used for comparison of the immunological effects after instillation of the two BRMs.

Statistics

Differences between Rubratin-induced cytokine concentrations and leukocyte numbers, and pre-therapy levels were analyzed using the Wilcoxon two-sample test for the unpaired case.

Results

Cytokines in urine by Rubratin

In serially collected urine samples during the first 24 h after each of the six weekly instillations of Rubratin, IL1β, IL2, IL6, and TNFα could be detected. Highest cytokine concentrations were generally observed in the 2–4 h or 4–6 h urine sample. Because no differences in cytokine induction between the various doses of Rubratin were observed, all data were pooled. Significantly elevated levels of Rubratin-induced IL1β ($P = 0.0001$), IL2 ($P = 0.0007$), IL6 ($P = 0.0007$) and TNFα ($P = 0.0012$) were found compared to pre-therapy cytokine levels (Table 1). IL6 induction was found after the first instillation and IL1β, IL2, and TNFα were found after the second or subsequent instillations.

The urinary cytokine levels were compared with those of nonresponding and responding BCG patients. Responding BCG patients showed no tumor recurrence within 6 months after BCG instillations, while nonresponding patients had a recurrent tumor within 6 months [18]. Urinary cytokine levels in nonresponding and responding BCG-treated patients are shown in Table 3. Comparison of these levels with the Rubratin-induced cytokine levels as shown in Table 1 reveals that the highest urinary cytokine levels after six Rubratin instillations were comparable to those of the nonresponding BCG patients, but were clearly less comparable to those of the BCG-responding patients.

Table 1 Comparison of pretreatment urinary cytokine levels with peak levels plus ranges after intravesical instillation of Rubratin ($n = 12$)

	Pretreatment	Rubratin
IL1β (pg/μmol creatinine)	5.1 (0.4–21.3)	37.5 (17.5–113.3)
IL2 (U/μmol creatinine)	0.0 (0.0–0.1)	0.1 (0.0–0.4)
IL6 (pg/μmol creatinine)	1.7 (0.0–24.0)	22.2 (6.7–110.6)
TNF (pg/μmol creatinine)	0.0 (0.0–1.4)	5.9 (0.0–109.3)

Table 2 Leukocyte influx after Rubratin instillation measured in seven patients and FACS analysis results, measured in five patients plus ranges

	Pretreatment	Rubratin
Total cells $\times 10^3$	12.6 (1.0–40.9)	24.8 (1.2–70.9)
IL2 $^+$-R lymphocytes (%)		4 (0–22)
HLA-DR $^+$lymphocytes (%)		17 (1–74)
CD4/CD8 ratio		0.88 (0.46–5.1)

Leukocyte numbers in urine

As shown in Table 2, Rubratin induced leukocyte influx into the urine after intravesical instillation ($P = 0.0019$). During the 6 weeks of treatment numbers of Rubratin-induced leukocytes in 2–4 h urine samples increased from the first to the third instillation, but not from the third to the sixth instillation. No dose-response effect of Rubratin was observed.

FACS analysis of leukocytes

In a subgroup of patients ($n = 7$), instilled with 3.0 and 4.5 mg Rubratin, FACS analysis of the leukocytes was performed. Small percentages of monocytes/macrophages (3.3 ± 2.1%) and lymphocytes (0.4 ± 0.3%) were detectable in addition to a majority of granulocytes (> 95%). Due to the small number of lymphocytes after the first instillation, accurate FACS analysis was not possible. After the sixth instillation, analysis was possible for five out of seven patients: cells in the lymphocyte gate consisted of $CD3^+$ T cells in the majority (59 ± 17%), with lower percentages of $CD20^+$ B cells (14 ± 20%) and small percentages of $CD56^+$ NK/Tc cells (2 ± 3%) present. For two of these five patients, relatively high percentages of lymphocytes expressing IL-2 receptors ($CD25^+$) and HLA-DR were observed, indicating T-cell activation. In the same two patients an increased CD4/CD8 ratio was found (data not shown).

Tumor response

No ablative effect of Rubratin was observed in the first four patients with a marker lesion at follow-up cystoscopy at month 3. In patients treated by complete TUR, of all visible lesions early recurrent tumor was found in five (mean time to recurrence 4.0 months). Mean time of follow-up of the three recurrence-free patients was 36 months. This BRM did not induce local and/or systemic side effects or adverse reactions.

Discussion

This study shows that local immunostimulation can be accomplished by intravesical application of the *Nocardia rubra* cell wall skeleton preparation (Rubratin). Elevated amounts of the interleukins IL1β, IL2, IL6, TNFα, and leukocytes were found in the urine compared to pre-instillation levels. FACS analysis of the lymphocytes obtained from urine indicated that the T-cell activation may be induced (IL2 receptor and HLA-DR expression) and CD4/CD8 ratios can be increased in some patients after Rubratin treatment. However, compared to responding BCG patients, the urinary cytokine levels induced by various dosages of Rubratin were clearly lower. Recent investigations by our group concerning the induction of urinary cytokines (especially IL2 and IL6) with intravesical BCG therapy indicate an association between absence of cytokine induction and "early" (≤ 6 months) tumor recurrence [18]. In agreement with these observations, in this study low levels of Rubratin-induced cytokines were associated with absence of appreciable antitumor or prophylactic efficacy after intravesical instillations of Rubratin.

A major cause of the reduced immunostimulating capacity of Rubratin compared with BCG may be that the BCG preparation contains viable bacteria whereas Rubratin contains cell walls only [6, 13]. However, this suggestion cannot be verified since there are no clinical and/or experimental data on cytokine induction of intravesically applied nonviable BCG or other bacterial preparations.

In contrast to the frequently reported local complaints after BCG instillation, no local or systemic side effects were observed after Rubratin instillation, which could be in agreement with the minimal immunomodulatory effect of Rubratin.

Phenotypic analysis in a limited number of Rubratin-treated patients indicated that in some patients (two out

Table 3 Urinary cytokine levels after BCG instillation measured in patients with superficial bladder tumors, subdivided into responding patients (recurrent tumor after ≥ 6 months) and nonresponding patients (recurrence < 6 months)

Urinary cytokines	Nonresponding BCG patients	Responding BCG patients
IL1β (pg/μmol creatinine)	9.9 (1.4–139.8) $n = 5$	64.6 (23.5–712.1) $n = 5$
IL2 (U/μmol creatinine)	0.1 (0.0–0.8) $n = 6$	1.6 (0.3–11.6) $n = 8$
IL6 (pg/μmol creatinine)	35.5 (3.0–420.4) $n = 10$	286 (16.1–3802) $n = 13$
TNF (pg/μmol creatinine)	9.3 (1.0–67.0) $n = 8$	78.1 (4.0–561.0) $n = 10$

of five) T-cell activation was induced. However, in contrast to observations made in BCG-treated patients (unpublished data), for the Rubratin-treated patients no correlation between phenotypic T-cell activation and the amount of urinary cytokines was observed. This may be explained by the considerably lower amount of urinary leukocytes induced by Rubratin compared to BCG (data not shown).

In conclusion, local immunostimulation by intravesical Rubratin administration could be induced in this small number of patients; however, the amount of immunocompetent cells attracted to the bladder is low as is the level of cytokines measured. Clinical effectiveness could not be demonstrated. Continuation of this study using higher doses of Rubratin was not considered useful for both clinical and cost-effectiveness reasons. This study provides further evidence that monitoring cytokine release during intravesical treatment with BRMs can be useful for the investigation of local immunostimulatory effects.

Acknowledgements We thank H.A. Simoons (Andreas Ziekenhuis, department of Urology, Amsterdam, The Netherlands) for providing urine specimens and Dr. R.J.W.M. Vet (Laboratory for Haematology, University of Amsterdam, The Netherlands) for cooperation with FACS analyses. This study was financially supported by ASTA Pharma AG, Frankfurt, Germany and the BUWO Foundation, Amsterdam, The Netherlands.

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