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Immunostimulation in the Urinary Bladder by Local Application of *Nocardia rubra* Cell-Wall Skeletons (Rubratin) and Bacillus Calmette-Guérin as Therapy for Superficial Bladder Cancer: A Comparative Study

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Twelve patients with superficial bladder cancer were treated with intravesical instillations of Rubratin (ASTA Pharma AG, Frankfurt, Germany), a cell-wall preparation of *Nocardia rubra*. The objective was to compare the immunostimulating effect of Rubratin with that of bacillus Calmette-Guérin (BCG). Local immunostimulation was determined by cytokine induction in serially collected urine samples during the first 24 h after each instillation, leukocyte influx into the urine, and phenotypic analysis of the lymphocyte fraction. Levels of Rubratin-induced interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α were significantly elevated compared with pretherapy levels. Rubratin induced leukocyte influx into the urine. T-cell activation (IL-2 receptor and human leukocyte antigen-DR expression) can be induced, and CD4:CD8 cell ratios can be increased. All parameters indicated that Rubratin-induced immunostimulation was less than that associated with BCG. In conclusion, although local Rubratin-induced immunostimulation occurs in a limited number of patients, the amount of immunocompetent cells attracted to the bladder seems to be less than that associated with BCG therapy, thus resulting in lower levels of cytokine production (which may reflect less clinical efficacy).

Recurrent superficial bladder cancer provides unique possibilities to investigate the immunotherapeutic potential of new agents. We studied the immunostimulating and immunotherapeutic potential of a commercially available cell-wall skeleton preparation of *Nocardia rubra* (Rubratin, ASTA Pharma AG, Frankfurt, Germany), which is a bacterial species taxonomically related to BCG. Intravesical immunotherapy with the biological response modifier BCG is an effective treatment for carcinoma in situ (Tis) and superficial (Ta, T1) papillary transitional-cell carcinoma of the urinary bladder [1, 2]. Most patients tolerate local BCG treatment well, and complications are usually minor; however, serious and even fatal reactions resulting from systemic infection with the viable microorganism can occur [3], indicating a need for new therapeutics.

The immunostimulating and immunotherapeutic activity of Rubratin has been reported in a number of experimental tumors in mice, rats, and guinea pigs [4–8]. Clinical studies from Japan and Germany have confirmed these experimental results [7, 9,

10]. In prospective, randomized, phase III trials, a benefit of Rubratin, with respect to remission or survival time, was shown for a variety of nonurologic tumors of different origin [11–16]. Rubratin was also used in monotherapy, in adjuvant therapy, and in combination with chemotherapy for patients with lung, colorectal, and gastric carcinoma, malignant glioma, and acute leukemia. In these studies, the preparation was given intradermally and by the intratumoral and intrapleural route. However, nothing is known regarding the immunotherapeutic activity of Rubratin if it is applied intravesically in superficial bladder cancer.

In this study, patients with superficial bladder cancer (pTa, pT1) were treated intravesically with Rubratin, according to a treatment schedule generally used for BCG. The objective was to compare the immunostimulating activity of Rubratin with that of BCG, with regard to the potential use of Rubratin in the treatment of superficial bladder carcinoma. Local immunostimulation was determined by measurement of cytokine (IL-1 β , IL-2, IL-6, and TNF- α) induction in the urine and leukocyte influx into the urine and by phenotypic analysis of the lymphocyte fraction of these leukocytes, as described elsewhere [17–19] for BCG therapy.

Materials and Methods

Patients and treatment protocol. This study included 12 patients (9 males and 3 females) with primary or recurrent histologically proven superficial papillary transitional-cell carcinoma of the urinary bladder (5 patients with TaG1, 6 with TaG2, and 1 with

This study was approved by the local ethical committee, and informed consent was obtained from the patients.

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T1G2; all were designated as having "low risk" for progression, according to the criteria of Kurth et al. [20]). Patients with carcinoma in situ (TisG3) were excluded. To study the immunologic effect as well as the antitumor efficacy of Rubratin, a marker lesion (0.5–1 cm) was left in the bladder after transurethral resection of all other visible tumors in the initial 4 patients. These 4 patients were treated with 6 weekly intravesical instillations of Rubratin (dose, 1.5 mg). Because no response by the marker lesion was observed in these patients, the other 8 patients were treated prophylactically according to a revised protocol; in these patients, all visible tumors were resected by transurethral resection. In these 8 patients, the immunologic effect of 6 weekly intravesical instillations of Rubratin (dose for 5 patients, 3.0 mg/week; dose for 3 patients, 4.5 mg/week) was studied, and they were followed up for recurrence of disease by 3 monthly examinations with cystoscopy and cytological analysis. Patients were monitored for local (cystitis and hematuria) and systemic (fever and allergic reactions) adverse events.

A group of 11 consecutive patients who had superficial transitional-cell carcinoma and were treated, within the same period, with 6 weekly intravesical instillations of BCG (~5–10 × 10⁸ cfu or 27 mg of dry weight; Connaught Laboratories, North York, Ontario, Canada) were control subjects for comparison of immunostimulation.

Rubratin. Rubratin is a lyophilisate that, on reconstitution, forms an injectable oil-in-water emulsion [8]. The contents of an injection vial (0.5 mg) were reconstituted in 1 mL of 0.9% saline. For intravesical instillation, the required number of vials for the 1.5-, 3.0-, or 4.5-mg dose was diluted to an end volume of 50 mL in saline.

Determination of cytokines in urine. Urine samples (obtained during spontaneous voiding) were collected before instillation and at 2–4, 4–6, 6–12, and 12–24 h thereafter during each of the 6 instillations. Samples were immediately frozen to –20°C. Afterward, specimens were thawed, centrifuged (300g) to remove cells and debris, and stored in aliquots at –20°C until analysis. Levels of IL-1 β , IL-2, IL-6, and TNF- α in urine were determined, as reported elsewhere [19], with the use of commercially available, highly specific, reproducible ELISAs using an oligoclonal system (Medgenix, Fleurus, Belgium). By use of this method, baseline urinary cytokine levels (3.7 \pm 6.2 pg of IL-1 β / μ mol creatinine, 0.0 \pm 0.1 U of IL-2/ μ mol creatinine, 5.9 \pm 12.8 pg of IL-6/ μ mol creatinine, and 0.2 \pm 0.8 pg of TNF- α / μ mol creatinine) were established in 134 preinstillation samples from 25 patients with superficial bladder cancer. Data on cytokines were standardized to urinary creatinine levels to correct for hydration in voided urine.

Fluorescence-activated cell sorter (FACS) analysis of lymphocytes from urine. In fresh urine samples obtained 2–4 h after the first, third, and sixth instillations, the total number of viable leukocytes was determined using the trypan blue exclusion. Leukocytes were washed (centrifugation for 5 min at 300g) twice in PBS (pH 7.2) with 0.2% albumin (Boehringer Mannheim, Mannheim, Germany), 0.02% K-EDTA (Merck, Darmstadt, Germany), and 0.01% NaN₃ (Merck). Cells (5 × 10⁵) were labeled with 100 μ L of monoclonal antibody (mAb) in PBS with the described additions. CD45 mAb to leukocytes (1:10), CD3 mAb to T cells (1:10), CD4 mAb to Th/i (1:10), CD8 mAb to Ts/c (1:10), CD25 mAb to IL-2 receptor (1:10), CD14 mAb to monocytes/macrophages (1:10), CD66 mAb

granulocytes (1:500), and mAb to human leukocyte antigen-DR (HLA-DR; 1:500) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam), and CD20 mAb to B cells (1:5) and CD56 mAb to natural killer/Tc cells (1:10) were from Becton Dickinson (BD Biosciences, Woerden, Netherlands). A fluorescein-conjugated F(ab')₂ fragment from rabbit antibody to mouse Ig (Dako, Glostrup, Denmark) was used as a second antibody (100 μ L; 1:40 dilution in PBS with the described additions). Labeled cells were fixed in 300 μ L of 0.5% paraformaldehyde in PBS. FACS analysis (FACSscan, BD Biosciences, Woerden, Netherlands) was performed the following day, as described elsewhere [18].

Statistical methods. Differences between Rubratin- or BCG-treated patients and control subjects were analyzed using the 2-tailed Wilcoxon's rank-sum test for the unpaired case.

Results

Cytokines in urine. In serial urine samples obtained during the first 24 h after each of the 6 weekly instillations of Rubratin, all cytokines (i.e., IL-1 β , IL-2, IL-6, and TNF- α) could be detected. The highest cytokine concentrations were generally observed in urine samples obtained 2–4 h or 4–6 h after instillation, in accordance with cytokine induction by BCG [18]. No differences between the 1.5-, 3.0-, and 4.5-mg doses of Rubratin were observed with regard to the levels of cytokine induction (results not shown); therefore, the data were combined. In figure 1, cytokine levels during the 6 weeks of intravesical treatment with Rubratin are presented in comparison with those seen during BCG therapy. Pretherapy cytokine levels were not different for the Rubratin- and BCG-treated patients ($P = .621$). For IL-1 β (figure 1A), an increase over the pretherapy level was already observed after the first instillation of Rubratin ($P = .025$); this level increased moderately up to the sixth instillation of Rubratin ($P = .015$). No significant differences in levels of Rubratin- and BCG-induced IL-1 β (week 6, $P = .442$) were seen, although maximum urinary IL-1 β concentrations were higher (see upper range) during BCG treatment.

For urinary levels of Rubratin-induced IL-2 (figure 1B), a trend for an increase over the pretherapy level was found (lowest level [$P = .070$] at week 4; highest level [$P = .162$] at week 2). In contrast, IL-2 levels clearly increased during a 6-week treatment course with BCG: at week 6, significantly lower amounts of IL-2 were induced by Rubratin compared with BCG ($P = .002$). For IL-6 (figure 1C), Rubratin-induced levels were higher than pretherapy levels after the first instillation ($P = .002$); however, these levels declined thereafter to a non-significant difference at week 6 ($P = .926$). At week 6, the difference between BCG- and Rubratin-induced amounts of IL-6 was highly significant ($P = .0001$). Urinary levels of Rubratin-induced TNF- α (figure 1D) were significantly increased at week 3 only ($P = .017$), compared with pretherapy levels. At weeks 5 and 6, levels of Rubratin-induced TNF- α were lower

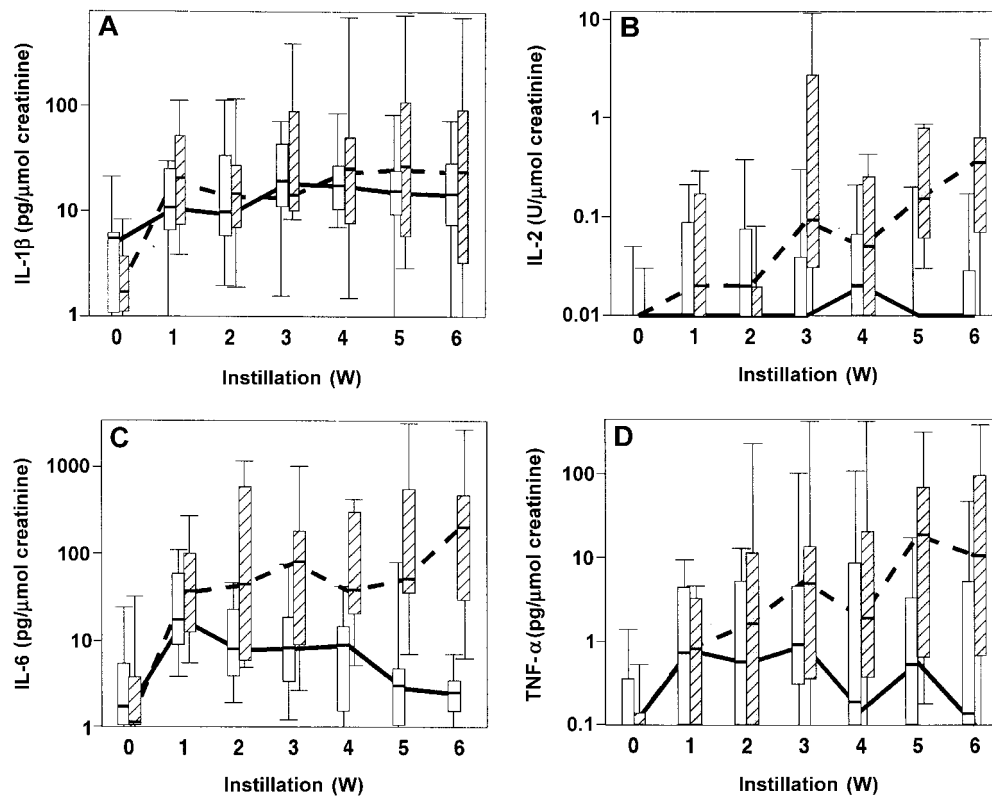


Figure 1. Urinary levels of IL-1 β (A), IL-2 (B), IL-6 (C), and TNF- α (D) during a 6-week course of intravesical therapy with Rubratin (ASTA Pharma AG, Frankfurt, Germany) or BCG for superficial bladder cancer. Urine samples were collected before therapy (week 0) and serially during the first 24 h after each of 6 weekly instillations (weeks 1–6). For individual patients, the highest cytokine concentration observed after each week was evaluated. White boxes denote cytokine concentrations in 12 Rubratin-treated patients; shaded diagonally striped boxes, cytokine concentrations in 11 BCG-treated patients. The lower and upper quartiles are indicated by the corresponding vertical ends of the box. The median value is indicated by the central horizontal line in the box. Median values are connected by an unbroken line for Rubratin and by a broken line for BCG. Minimum and maximum cytokine concentrations (range) are indicated by the T bars

than levels of BCG-induced TNF- α ($P = .017$ and $P = .022$, respectively).

Leukocyte counts in urine. Leukocyte counts were determined for 8 patients treated with the highest 2 doses of Rubratin. Rubratin was found to induce leukocyte influx into the urine after intravesical instillation: for all samples investigated (which were obtained 2–4 h after the first, third, and sixth instillations), the leukocyte count after instillation was higher than that noted before instillation. No differences were observed between the 3.0- and 4.5-mg doses of Rubratin, with regard to the numbers of induced leukocytes (results not shown); therefore, the data were combined. In figure 2, the leukocyte counts in urine samples obtained from 8 patients during 6 weeks of intravesical treatment with Rubratin are compared with those in urine samples from 8 BCG-treated patients. Pretherapy leukocyte counts were not different for the Rubratin- and BCG-treated patients ($P = .518$). After weeks 1, 3, and 6, counts of Rubratin-induced leukocytes in urine samples obtained 2–4 h after instillation were greater than pretherapy counts ($P = .027$, $P = .002$, and $P = .006$, respectively). Uri-

nary leukocyte counts after Rubratin therapy increased from the first to the third instillation but not from the third to the sixth instillation. This finding was clearly in contrast to that for BCG therapy, in which a gradual elevation was seen during the 6-week treatment course. Leukocyte counts in urine samples were significantly lower after the sixth instillation of Rubratin than after that of BCG ($P = .005$).

FACS analysis of leukocytes. FACS analysis was performed on leukocytes in urine samples obtained 2–4 h after the first, third, and sixth instillations in 7 patients treated with the 2 highest doses of Rubratin (3.0 and 4.5 mg). Small percentage of monocytes/macrophages ($3.3 \pm 2.1\%$) and lymphocytes ($0.4 \pm 0.3\%$) were detectable in addition to most granulocytes, which is similar to the situation after BCG instillation [18]. No differences within these percentages were observed for the 2 different doses of Rubratin; therefore, the data were combined. Because the composition of the lymphocyte population in urine after BCG therapy seems to be representative of the BCG-induced chronic lymphocytic infiltrate in the bladder wall, the phenotype of urinary lymphocytes after Rubratin instillations

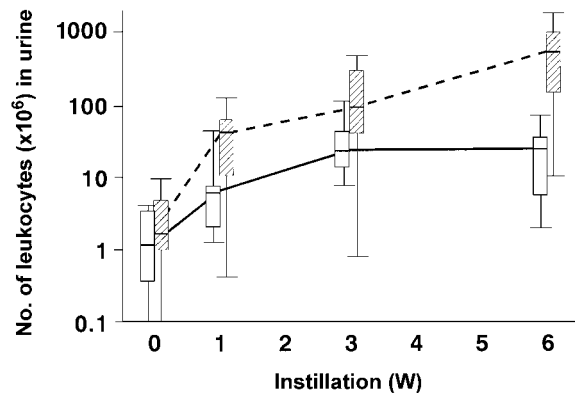


Figure 2. Leukocyte counts in urine during a 6-week course of intravesical therapy with Rubratin (ASTA Pharma AG, Frankfurt, Germany) or BCG for superficial bladder cancer. Urine samples were obtained before therapy (week 0) and 2–4 h after the first, third, and sixth instillations. White boxes denote leukocyte counts in 8 Rubratin-treated patients; shaded diagonally striped boxes, leukocyte counts in 8 BCG-treated patients. Lower and upper quartiles are indicated by the corresponding vertical ends of the box. The median value is indicated by the central horizontal line in the box. Median values are connected by an unbroken line for Rubratin and by a broken line for BCG. Minimum and maximum numbers of leukocytes (range) are indicated by the T bars.

was determined. Because of the small number of lymphocytes, accurate FACS analysis was not possible after the first instillation. After the sixth instillation, analysis was possible for 5 of the 7 patients: cells in the lymphocyte gate consisted mostly of CD3⁺ T cells ($59 \pm 17\%$), with lower percentages of CD20⁺ B cells ($14 \pm 20\%$) and small percentages of CD56⁺ natural killer/Tc cells ($2 \pm 3\%$). For 2 of these 5 patients, relatively high percentages of lymphocytes expressing IL-2 receptors (CD25⁺) and HLA-DR were observed, indicating T-cell activation. For the same 2 patients, an increased CD4:CD8 cell ratio was found. In figure 3, these data are shown in comparison with levels of IL-2 receptor ($P = .143$) and HLA-DR ($P = .222$) expression and CD4:CD8 cell ratios ($P = .256$) after 6 BCG instillations and suggests a trend for a lower level of T-cell activation and a lower CD4:CD8 cell ratio after Rubratin treatment than after BCG therapy.

Tumor response. A response was not observed in the marker lesions (at week 10) in 4 Rubratin-treated patients. Of the additional 8 Rubratin-treated patients who received prophylaxis, 4 had early recurrence of disease (mean time to recurrence, 3.8 months). For the 4 patients without recurrence, the mean time of follow-up was 10.5 months.

Discussion

This study assessing the immunostimulating capacity of intravesically applied Rubratin provided a unique opportunity for comparison with the level of immunostimulation induced

by intravesical treatment with BCG; both Rubratin and BCG are similar types of biological response modifiers (i.e., bacterial preparations). It is shown that local immunostimulation can be accomplished by intravesical application of the *N. rubra* cell-wall skeleton preparation Rubratin. Significantly elevated amounts of IL-1 β , IL-6, TNF- α , and leukocytes were observed in urine samples, whereas a trend for an increase was found for IL-2. FACS analysis of lymphocytes obtained from urine samples suggested that T-cell activation may be induced (IL-2 receptor and HLA-DR expression) and CD4:CD8 cell ratios can be increased after Rubratin treatment. However, immunostimulation by Rubratin was considerably less than that by BCG, which is the classical biological response modifier used as treatment of carcinoma in situ (Tis) and prophylaxis for superficial (Ta/T1) bladder tumors.

Our observations of local immunostimulation resulting from intravesically applied Rubratin agree with the findings of Jurincic et al. [21], who reported an increased number of macrophages, monocytes, granulocytes, and CD4⁺ T cells in mucosa and submucosa of the bladder; these findings were determined by immunohistochemical analysis. With regard to urinary cytokine induction by Rubratin, Jurincic et al. [21] studied only IL-1 β . They found an increase in the IL-1 β level, which was also observed in this study.

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 bacterial cell walls only. For BCG, viability of the preparation is probably important for therapeutic efficacy [22], and alternative immunomodulators have not yet shown comparable antitumor efficacy [3, 23–25]. Experimental data obtained from the guinea pig model described by Van der Meijden et al. [26] suggested the need for viable organisms: heat-killed BCG had less immunostimulating capacity than did viable BCG when applied intravesically (unpublished data, E. C. de Boer). In agreement with these observations, assuming that the BCG-associated immune reaction is required for antitumor efficacy, Kelley et al. [22] reported on the importance of the viability of the BCG preparation for clinical efficacy against superficial bladder cancer. The low level of immunostimulation observed in the present study after intravesical instillations of Rubratin may be comparable. However, Chin et al. [27] recently reported experimental results indicating that a mycobacterial cell-wall preparation obtained from *Mycobacterium phlei* invokes an inflammatory response in the mouse similar to that of a live BCG organism and retains antitumor action.

Regarding the intravesical administration of bacteria other than BCG, it is not clear whether the type of bacterial species (either viable or nonviable) is an influence on the level of immunostimulation. Bacteria commonly associated with urinary tract infections can induce elevated levels of urinary cytokines [28], although the levels are not as high as those associated with BCG [29]. In addition to being produced by infiltrating cells

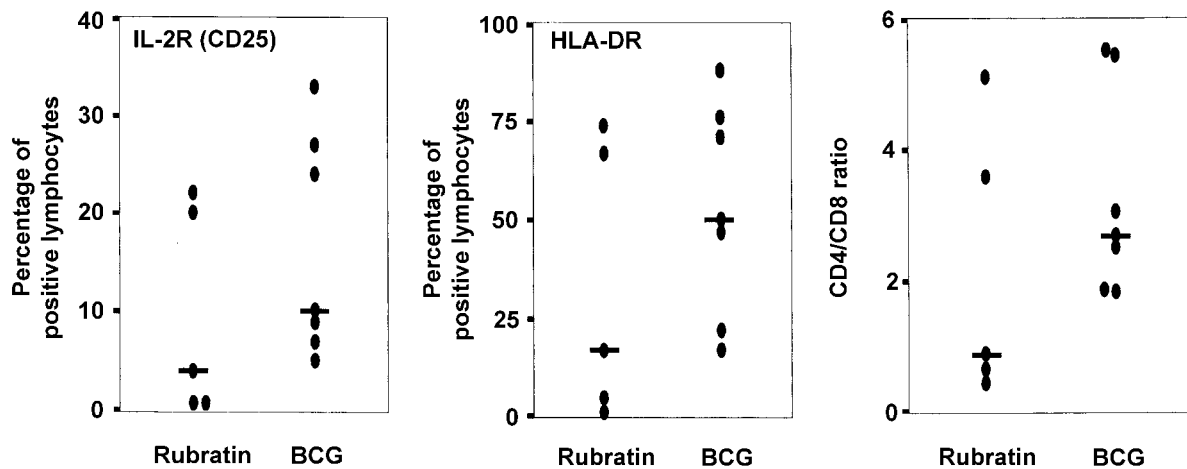


Figure 3. Flow cytometric analysis of IL-2 receptor (IL-2R) and histocompatibility complex class II (human leukocyte antigen-DR [HLA-DR]) expression and CD4:CD8 cell ratios in lymphocytes in urine samples obtained 2–4 h after 6 instillations of Rubratin (ASTA Pharma AG, Frankfurt, Germany) or BCG as therapy for superficial bladder cancer. Data for individual patients (5 Rubratin-treated patients and 7 BCG-treated patients) are shown. Unbroken lines represent median values.

of the immune system, urothelial cells may be a source of urinary cytokines such as IL-1 β , IL-6, and TNF- α [30, 31]. Recently, it was reported by our group that interaction of urothelial cells with BCG resulted in enhanced secretion of IL-6 and TNF- α by the urothelial cells, which was >10-fold higher than that with Rubratin or other whole, viable, urinary tract infection-inducing bacteria [32].

The data agree with results obtained in this study and are thus suggestive of a role for this phenomenon within local immunostimulation following bladder instillation.

Although there is increasing evidence regarding the importance of local T-cell infiltration and immune activation for efficacy [33–36], the exact mode of action of BCG as an antitumor modality in bladder cancer is not known, and a firm link between an immune-mediated mode of action and a clinical (tumor) response is still lacking. Our recent results from investigations of induction of urinary cytokines by means of intravesical therapy with BCG indicate an association between absence of cytokine induction and early recurrence of disease [37]. In agreement with these observations, the low levels of Rubratin-induced cytokines in this study seem to be associated with the absence of appreciable antitumor efficacy of intravesical instillations of Rubratin, although no firm conclusions regarding the antitumor efficacy of Rubratin can be drawn from this study because of the small number of patients.

Intravesical BCG treatment for superficial bladder cancer is well tolerated by >95% of patients, although serious complications, such as systemic BCG sepsis, can occur. The most frequently encountered side effect of the treatment is local bladder irritation (which occurs in >90% of patients); this adverse effect usually persists for ~2 days and begins after repeated instillations. It is probably a result of locally induced inflam-

matory and immunologic processes. That no local or systemic side effects were observed in the patients treated with Rubratin in the present study is in agreement with the limited immunologic effects reported here.

The results of phenotypic analysis for a limited number of Rubratin-treated patients suggested that T-cell activation was induced in some (2 of 5) patients. However, in contrast with observations for BCG-treated patients [38], no correlation between phenotypic T-cell activation and the amount of urinary cytokines was observed for the Rubratin-treated patients. This finding may be explained by the considerably lower amount of urinary leukocytes induced by Rubratin compared with BCG (figure 2). In conclusion, although local immunostimulation by intravesical administration of Rubratin may be induced in a limited number of patients, the amount of immunocompetent cells attracted to the bladder is probably not as high as that seen with intravesical treatment with BCG, resulting in lower amounts of cytokine production (which may be reflected in less clinical efficacy). Further study using intravesical instillation of Rubratin (even with doses of >4.5 mg) in superficial transitional cell carcinoma of the bladder is limited because of the presented immunologic data, clinical data, and cost-effectiveness of this treatment. For future studies with similar immune modulators, cytokine induction may be used in addition to clinical evaluation in the testing of new agents for treatment of superficial bladder cancer.

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