The Immunomodulating Effect of Interferon-γ Intravesical Instillations in Preventing Bladder Cancer Recurrence

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ABSTRACT

Purpose: The purpose is to investigate the prophylactic effect of intravesically instilled recombinant IFN-γ against recurrence of superficial transitional cell carcinoma of the bladder and to evaluate its effect in local immune response, presumably mediating its therapeutic efficacy.

Experimental Design: We prospectively randomized in two groups 123 patients with initially diagnosed superficial transitional cell carcinoma and stage Ta, T1, grade 2 tumors, who underwent transurethral tumor resection (TUR). In group A, 60 patients received IFN-γ (1.5 × 107 IU/instillation), whereas 63 patients, consisting of the control group B, received mitomycin C (40 mg/instillation). The annual administration schedule consisted of eight weekly followed by four biweekly and then by every 6 months instillations for both regimens. We also analyzed the immunophenotype of the intratumoral and intramural leukocytes by immunohistochemical and flow-cytometric techniques. To this purpose, tumor samples were obtained at TUR and random biopsies at TUR and during cystoscopy at 6 and 12 months, and bladder washings were collected before TUR and at prescheduled time points.

Results: In group A, 44 of 60 (73.4%) patients, and in group B, 36 of 63 (57.2%) patients, were tumor free during the median follow-up period of 26.5 months (range, 3–49 months). IFN-γ was well tolerated. Six months after starting treatment, follicular cystitis was detected in patients responding to IFN-γ. After IFN-γ instillations, statistically significant increases in T cells, T-helper cells, T-cytotoxic cells, natural killer cells, and total leukocytes, as well as in the number of B cells expressing intercellular adhesion molecule-1 and total leukocytes expressing HLA-DR, were observed by flow cytometry in tissue specimens and bladder washings.

Conclusions: Recombinant IFN-γ appears to be effective against stage Ta, T1, grade 2 bladder tumors’ recurrence. Recruitment and activation of intramural leukocytes seem to be involved in the mechanism of IFN-γ action.

INTRODUCTION

sTCC,5 stages Ta, T1 constitute the majority (80%) of human bladder cancers. TUR of all visible tumors is currently the standard treatment for patients with tumors limited to the bladder mucosa and lamina propria. Despite resection, 50–70% of these patients will present recurrences (mainly within the first year) and up to 20% with progression to muscle invasive disease. The prevention or delay of tumor recurrence and disease progression, as well as the eradication of any nondetected disease, requires complementary therapy. Numerous chemo- or immunotherapeutic agents have been suggested as suitable adjuvants to TUR of bladder tumors (1). Although intravesical instillation of BCG is the treatment of choice for sizeable, multifocal, high-grade (G3), or recurrent tumors and CIS, this treatment is not advocated by many for stage Ta, T1 and grades 1 and 2 (G1, G2) tumors (2). In this last group of low-risk patients, the serious side effects caused by BCG do not counterbalance its therapeutic efficacy. This is particularly true in patients who do not present extensive T1 tumors or those without multiple T1 tumors. On the other hand, various other chemo- or immunotherapeutic agents used in the treatment of small, solitary or multifocal, low-grade (G1, G2) and stage (Ta, T1) tumors have not shown significant efficacy in long-term follow-up (3).

It has been shown that sTCC patients who respond to BCG treatment have a nonspecific cellular and humoral immune response toward tumor cells. Apart from the BCG-induced detectable alterations in leukocytes’ subpopulations infiltrating the bladder wall (intramural leukocytes), the bacillus affects the local and systemic expression of molecules such as cytokines, ILs, and adhesion molecules (4). The basis of the BCG tumoricidal effect is immunological and both IFN-γ and TNF-α are the main mediators. On the basis of the fact, this study was de-

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5 The abbreviations used are: sTCC, superficial transitional cell carcinoma; TUR, transurethral resection; BCG, Bacillus Calmette-Guérin; CIS, carcinoma in situ; TNF-α, tumor necrosis factor α; MMC, mitomycin C; mAb, monoclonal antibody; CD, clusters of differentiation; NK, natural killer; IL, interleukin; IL-2R, IL-2 receptor; ICAM-1, intercellular adhesion molecule-1; AUC, area under the curve; LAK, lymphokine-activated killer.
signed to examine the efficacy of IFN-γ intravesical instillation in the treatment of patients with primary Ta or T1 and grade 2 bladder tumors after TUR as a means of recurrence prevention. Several studies have investigated the antineoplastic action of IFN-γ, proving the immunomodulatory and tumor-suppressive potential of this agent (5).

However, few studies have supported the concept of the in vivo immunostimulating and antiproliferative effect of the agent in bladder cancer and even fewer its effectiveness against tumor recurrence (6–9).

In vitro, a direct cytotoxic (in grades 1 and 2) or cytostatic (in grade 3) effect of IFN-γ has been shown in urothelial cancer cells (10, 11).

The objectives of the current study are: (a) to determine the efficacy and the tolerance of intravesically instilled recombinant human IFN-γ in stage Ta, T1, grade 2 bladder tumor recurrence of initially diagnosed sTCC patients; and (b) to examine a series of immunological parameters in an attempt to elucidate the mechanism of IFN-γ action.

MATERIALS AND METHODS

Patients. A total of 123 eligible patients, treated in the participating departments from January 1997 to February 2001, was enrolled in this study, which was approved by the Ethical Committee of the Hospital. All patients underwent routine clinical examination, including chest radiography, complete blood count, liver function tests, urine culture, serum creatinine measurements, total protein electrophoresis, urography, cystoscopy, and ultrasonography. Each patient with sTCC underwent TUR of all visible tumors by the same group of urologists. A tumor’s tissue specimens obtained during TUR were histopathologically examined by two pathologists. The tumors were graded according to the WHO recommendations and staged according to the tumor-node-metastasis system. Inclusion criterion for enrollment in the study was the histopathological determination of stage Ta or T1 and grade 2 tumors of initially diagnosed sTCC patients with no more than two tumor’s foci and with initial specimens sufficient to document the absence of muscle invasion. Patients with a previous history of cancer and immunodeficiency or coexistence of CIS or high-grade (G3) TCC, as well as suspicion of upper tract TCC, were excluded from the study. All patients were informed about the applied protocol and their written consent was obtained.

These patients were randomized in two groups. Group A consisted of 60 patients who received IFN-γ (1.5 × 10^7 IU/instillation) instillations after TUR, whereas group B consisted of 63 who received MMC (40 mg/instillation).

Treatment of Patients with IFN-γ or MMC and Follow-Up Protocol. The current study was an open-label, prospective, randomized, comparative, multicenter clinical trial. Two groups, A and B, consisting of 60 and 63 patients, respectively, were followed-up. Group A patients were treated by intravesical instillations of recombinant human IFN-γ 1b, (Imukin; Boehringer Ingelheim Pharma K.G., Biberach, Germany) after TUR, whereas group B patients received intravesical instillations of MMC (Mitomycin-C Kyowa; Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). The first instillation of both regimens was administered 2 weeks after the operation. IFN-γ 1b was administered in an annual scheme consisting of 20 instillations of 1.5 × 10^7 IU (or 0.5 mg)/instillation. The scheme comprised of eight weekly instillations followed by four biweekly and then by eight monthly instillations (three cycles). Before each instillation, the agent was diluted in 50 ml of normal saline and was intravesically retained for 2.5 h after instillation. MMC was administered to group B patients per the aforementioned scheme of 20 instillations of 40 mg each under the same procedure. The MMC instillation scheme was adopted from the current knowledge at the starting time of our study (12).

Hematological, renal, and hepatic functions, urine cytology, and bladder cystoscopy were performed every 3 months for the first year and every 6 months thereafter for patient monitoring. Confirmation of recurrence-free status was based on the absence of any visible tumor or suspicious bladder lesion during cystoscopy and on a negative urine cytology result. In case of tumor’s recurrence, the intravesical treatment was stopped for both regimens.

Histopathological and Immunohistochemical Examination. During the initial TUR, tissue specimens were taken from the resected tumors, and random “cold cup” biopsies were obtained from normally appearing urothelium. The sites of random biopsies included the areas around the ureteric orifices, the dome of the bladder, the posterior wall, and the prostatic urethra. Random cold cup biopsies from the bladder wall urothelium were also obtained during cystoscopy for disease reevaluation at 6 and 12 months. All of the above tissue samples were routinely fixed and separately embedded in paraffin to be histopathologically and immunohistochemically analyzed. The immunohistochemical analysis was performed using the three-stage immunoperoxidase technique. The tested antibody panel comprised of L26 (B cells), OPD4 (T cells), CD4+ (T-helper cells), and CD8+ (T-cytotoxic cells; Dako reagents). The density of the tumor (intratumoral) and bladder wall (intramural)-infiltrating leukocytes was scored on a three-point scale: absent or weak; moderate; and intense.

Flow Cytometric Analysis. During TUR, a small piece of tumor was separately stored fresh at 4°C in RPMI culture medium. Bladder-washing samples were collected before TUR and at preselected time points during the administration period of the agent. From 30 of 60 group A patients receiving IFN-γ, the bladder washings were obtained five times during the 1-year administration schedule. These time points were at 0, 3, 9, 17, and 47 weeks. Point 0 was defined as the time of TUR. The next bladder-washing samples were collected before each instillation (second and last weekly, last biweekly, and last monthly). To obtain these washing samples, normal saline (100 ml) was intravesically instilled and followed by repeated washing of the bladder under pressure. Additionally, bladder-washing samples were collected from 20 of 63 group B patients receiving MMC. These samples were similarly obtained at three time points (0, 17, and 47 weeks).

Double-immunophenotypic analysis was performed by flow cytometry using the fresh tumor’s tissue specimens and the bladder-washing samples. Cell separation from the tumor’s tissue was done as described previously (13). Cells obtained after centrifugation from tumor and bladder-washing samples were properly analyzed following standard procedure (14). mAbs
conjugated with FITC or phycoerythrin were directed against certain CD. The following mAbs (Becton Dickinson, Lyon, France) were used: anti-CD45 (total leukocytes’ number); anti-CD3 (total T cells); anti-CD4 (T-helper cells); anti-CD8 (T-cytotoxic cells); anti-CD16 (NK cells); anti-CD19 (B cells); anti-CD25 (IL-2R); anti-CD54 (ICAM-1); and anti-HLA-DR. The IL-2R was detected on both T-cell subpopulations, and cytotoxic, whereas ICAM-1 was detected on B cells and HLA-DR on total leukocytes. The whole procedure was carried out using a FACS Scan flow cytometer at a density of $1 \times 10^5$ cells/sample. Flow cytometric analysis results are reported as a percentage of selectively gated total events corrected for non-specific binding by isotype-matched controls (14).

**Statistical Analysis.** Analysis for this study followed the intention-to-treat principle. The baseline comparability of the treatment groups was explored with respect to demographic and other patients’ data. The patients’ age was compared with Student’s $t$ test. Tumor’s stage, size, and multifocality were evaluated using the Pearson’s $\chi^2$ test with Yates correction. Estimations of recurrence-free function were derived from the Kaplan-Meier curves. Differences between the two groups of patients with respect to time of recurrence were evaluated by the Mantel-Haenszel test. Because data were not equally distributed, median values were used to present alterations on flow cytometrically tested parameters of fresh tissue specimens obtained at TUR and bladder-washing samples taken before TUR. Bladder-washing results during treatment were presented using the standardized AUC values (trapezoidal rule). For each patient and a specific parameter (X), the standardized AUC value was calculated as follows: the values (percentage of positive cells) of this parameter ($X_1, X_2, X_3, X_4$) were measured on four bladder-washing samples taken, as mentioned above. The values define a curve. The area between this curve and the horizontal axis of time (AUC) was calculated according to the trapezoidal rule (AUC value). The AUC value was divided by the duration of treatment of the patient (in months), and the quotient represented the standardized AUC value of this parameter. In bladder washings, the standardized AUC values were compared with baseline values using the Wilcoxon signed rank-sum test (baseline value was taken before TUR). All $P$s were not adjusted and derived from two-sided tests. The level of significance was fixed at $\alpha = 5\%$. $P \leq 0.05$ was considered to indicate statistical significance.

Statistical analysis was carried out with the software product SAS (SAS Institute, Cary, NC) version 8.1 (15).

**RESULTS**

**Efficacy of IFN-$\gamma$ in Preventing Recurrences.** Patients’ data and tumor characteristics are shown in Table 1. There were no statistically significant differences between the two groups with respect to the patients’ age and sex, as well as stage, size, and multifocality of tumors. Moreover, all of the resected tumors were of the same grade 2.

Table 2 presents the differences between the two groups concerning the number of patients with recurrent tumors or progressive disease. Forty-four of 60 (73.4%) patients in group A remained tumor free, compared with 36 of 63 (57.2%) of group B during the median follow-up period. This median follow-up period, including agent administration time, for group A patients was 26.5 months (range, 4–49) versus 24 months (range, 3–49) for group B. Within the first postoperative year, only 6 of 60 (10%) group A patients presented recurrence. The recurrences were stage T1, grade G2 tumors, with one being CIS. During the same postoperative period in group B, 15 of 63 (23.8%) patients presented recurrence. Two of them (initially T1) had invasive disease (pT2), whereas one of them presented CIS. After the first year of follow-up, a total of 10 of 60 (16.6%) patients showed recurrences in group A, two of which represented disease progression (1 CIS and 1 pT2 from pT1). Twelve of 63 (19%) group B patients recurred at the same period; four of them (initially T1) presented invasive disease (pT2). All of the patients who recurred were initially either pTa or pTa, and none had CIS evidence on any earlier random biopsy. However, the majority of those patients had multiple tumors at diagnosis and initial TUR. Estimation of the patients’ recurrence-free status for both administered agents, with respect to recurrence time, was made using Kaplan-Meier curves and is shown in Fig. 1. Statistically significant differences between the two groups were derived from the comparison of the patients’ recurrence-free status using the Mantel-Haenszel test. According to this, the $P$ within the first year was found to be 0.040 and for the total study period, 0.051.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patients’ data and tumor’s characteristics (group A receiving IFN-$\gamma$, group B receiving MMC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A ($n = 60$)</td>
</tr>
<tr>
<td>Median age (yrs)</td>
<td>68 [34, 86]</td>
</tr>
<tr>
<td>Sex (male-female)</td>
<td>48–12 (80%)</td>
</tr>
<tr>
<td>$T_1 G_2$ Multifocal</td>
<td>12 (20%)</td>
</tr>
<tr>
<td>Solitary</td>
<td>28 (46.7%)</td>
</tr>
<tr>
<td>$T_1 G_2$ Multifocal</td>
<td>7 (11.7%)</td>
</tr>
<tr>
<td>Solitary</td>
<td>13 (21.6%)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt;1 mc$^3$</td>
</tr>
<tr>
<td>&gt;1 mc$^3$</td>
<td>36 (60%)</td>
</tr>
</tbody>
</table>

$^a$ Pearson $\chi^2$ test with Yates correction.  
$^b$ Student’s $t$ test.
Tolerability of IFN-γ Intravesical Instillations. Intravesically instillated recombinant IFN-γ 1b was well tolerated with no serious side effects. Two of group A patients suffered cystitis-like symptoms lasting for 24 h after administration. No patient refused treatment because of any local or systemic toxicity caused by the agent. In seven patients from group B, mild chemical cystitis-like symptoms were observed; however, treatment was not interrupted.

Immunohistochemical Examination. To characterize intramural and intratumoral leukocytes, immunohistochemical examination was performed on a tumor's tissue obtained at TUR and tissue samples from the bladder wall, taken both during TUR and at disease reevaluations. The density of intratumoral leukocytes was rather low at the time of TUR in all patients, whereas in the bladder urothelium, no expression of intramural leukocytes was observed.

At the time of disease reevaluation on the sixth month after starting treatment, follicular cystitis was microscopically observed in the majority of group A patients in the tissue specimens from apparently normal bladder urothelium obtained by cold cup biopsies. Spread leukocytes and numerous lymphoid follicles with germinal centers appeared in the submucosa. It is noteworthy that numerous follicles formed by B and T cells were only observed in group A patients, who had no recurrence. Furthermore, all patients with recurrence within the first year of IFN-γ administration had absence of follicles and weak infiltration with leukocytes of the bladder wall in cold cup bladder biopsies obtained at the time of the second tumor's resection. Of the 10 patients in group A, who recurred after the first postoperative year, 2 had weak and 8 absence of leukocyte infiltration of the bladder wall. In the bladder of group B patients, there was no expression of immune cells infiltrating the bladder wall at the time of disease reevaluation.

Development of the local immune reaction against IFN-γ is given in Figs. 2–5. The presence of B cells (Fig. 2), T cells (Fig. 3), T-helper cells (Fig. 4), and T-cytotoxic cells (Fig. 5) was examined in tumor (A) and random biopsies (B) during TUR and in random biopsies (C and D) taken 6 months later.

Flow Cytometric Analysis. Flow-cytometric analysis was performed using fresh tumor specimens obtained during TUR and bladder-washing samples collected before TUR and during treatment. Leukocytes' subpopulations, namely B cells, T cells, T-helper, T-cytotoxic cells, NK cells, and total leukocytes, were determined. Immune cells expressing specific molecules on their surface such as HLA-DR on total leukocytes, IL-2R on T-helper and T-cytotoxic cells and ICAM-1 on B cells, were also determined.

Table 3 lists the median values and range of the immune parameters determined in tumor samples. The presentation of T-helper, as well as T-cytotoxic cells expressing IL-2R, seems to be lower than the other tested parameters. In the same table, the median values and range of parameters measured in the washing samples obtained before TUR, as well as median values and range of AUC standardized values measured at four time points during IFN-γ administration period, are presented. The percentage of the positive cells was the basis of the descriptive statistics used. Statistically significant differences were derived from almost all leukocytes' subpopulations, including those that express specific molecules when their median values were compared before TUR and during IFN-γ administration time (AUC standardized values). IL-2R expression and T-cytotoxic cells were the only parameters, which gave statistically nonsignificant differences between the preoperative and postoperative values (P = 0.169 and P = 0.064, respectively). The above-mentioned differences in the leukocytes' subpopulations were all statistically nonsignificant in group B patients.

**DISCUSSION**

The unpredictable biological behavior of bladder tumors makes prognosis of the disease outcome very difficult and provides a powerful rationale for complementary to TUR treat-
ment. Treating undetectable tumor, preventing recurrence of tumor, or disease progression and prolonging survival are the specific objectives of postoperative, intravesical drug instillation. Although various chemo- or immunotherapeutic agents have been tested as such complementary treatments, clear evidence for beneficial effect has been provided only in the case of BCG treatment against recurrent and high-grade tumors, as well as CIS (1, 2).

However, by analyzing the immunological basis of the BCG tumoricidal effect, important conclusions about bladder physiology have been reached. Bladder wall infiltrating immune cells communicating via certain cytokines trigger the effective antitumoral activity induced by BCG (16). It must be highlighted that IL-2 and IFN-γ are the most frequently involved cytokines in several key steps of BCG action (2). IFN-γ, as well as IFN-α, are endogenously produced cytokines that exhibit growth, immunomodulatory, and antiproliferative activity (5).

Several studies exist on the action of IFN-γ in the bladder. This cytokine is produced by macrophages, T cells, NK cells, and LAK cells, which are associated with a T-cell mediated response to BCG instillation. It is noteworthy that maximal levels of IL-2 and IFN-γ (T-helper 1 cell response) in the urine or serum were noted after the fourth BCG instillation and simultaneous LAK activity, induced by BCG in the peripheral

\[ \text{Fig. 2} \] At the time of TUR, scattered single B cells are visible on tumor (A) while there is no expression on bladder wall (B). On the sixth month of IFN-γ treatment, the distribution of B cells on bladder wall was prominent in lymphoid follicles (designated by arrows in C) as well as diffusely (D). Specific immunocytochemical staining was made using L26 (CD20 for B cells).

\[ \text{Fig. 3} \] At the time of TUR, scattered single T cells are visible on tumor (A) while there is no expression on bladder wall (B). On the sixth month of IFN-γ treatment, the distribution of T cells on bladder wall was prominent in lymphoid follicles (designated by arrows in C) as well as diffusely (D). Specific immunocytochemical staining was made using OPD4 (CD45RO for T cells).
blood mononuclear cells, was significantly increased (17). This BCG-mediated activation of LAK cells was found to be inhibited after IFN-\(\gamma\) neutralization by monoclonal antibodies. Furthermore, IFN-\(\gamma\) and IL-2 mRNAs were present in the peripheral blood lymphocytes of 70% of bladder cancer patients who achieved remission. On the other hand, these molecules were absent in 96% of patients nonresponding to BCG (18). After BCG instillation, IFN-\(\gamma\) and TNF-\(\alpha\) mRNAs were also detected in the bladder wall of syngeneic mice, growing murine bladder tumors. Even when BCG is administered in combination with IFN-\(\alpha\), IFN-\(\gamma\) production is accelerated, suggesting that IFN-\(\alpha\) is a potent BCG enhancer, which directs the BCG-induced response toward T-helper 1 cell response (IL-2, IFN-\(\gamma\), and TNF-\(\alpha\) production; Refs. 19, 20). Moreover, it is the very absence of IFN-\(\gamma\) in the initially diagnosed bladder cancer patients, which is strongly indicative of its role in the immune regulation of these patients.

According to the above findings, IFN-\(\gamma\) is substantially involved in the immunoresponse against bladder tumor cells, induced by the most effective immunotherapeutics. Apart from this immune cell-mediated tumoricidal effect, the direct effect of recombinant IFN-\(\gamma\) was documented on the growth of three human bladder cancer cell lines (10, 11). It was found to be cytotoxic and cytostatic on grade 1 or 2 cells and cytostatic on

**Fig. 4** At the time of TUR, scattered single T-helper cells are visible on tumor (A) while there is no expression on bladder wall (B). On the sixth month of IFN-\(\gamma\) treatment, the distribution of T-helper cells on bladder wall was prominent in lymphoid follicles (designated by arrows in C) as well as diffusely (D). Specific immunocytochemical staining was against CD4+ (T-helper cells).

**Fig. 5** At the time of TUR, scattered single T-cytotoxic cells are visible on tumor (A) while there is no expression on bladder wall (B). On the sixth month of IFN-\(\gamma\) treatment, the distribution of T-cytotoxic cells on bladder wall was prominent in lymphoid follicles (designated by arrows in C) as well as diffusely (D). Specific immunocytochemical staining was against CD8+ (T-cytotoxic cells).
grade 3 cells, showing that high grade bladder cancer cells were less sensitive to recombinant IFN-γ. In breast cancer and melanoma cells, this effect was mediated by IFN-γ receptors (IRF-1 and IRF-2). In the bladder, HLA-DR antigen expression, not appearing in normal urothelial cells, was observed in urothelial cancer cell lines in response to IFN-γ. The HLA-DR antigen, which is responsible for the recognition and specific interaction of effector cells, was induced by IFN-γ in the urine after BCG treatment and neutralized by polyclonal antibodies to IFN-γ (21). Furthermore, ICAM-1, which is required for the contact and nonspecific interaction of effector cells, was induced directly by IFN-γ in urothelial cancer cell lines, suggesting a direct mode of IFN-γ action against tumor cells (22). Finally, IFN-γ seems to be involved in the angiogenic process because secretion of Inducible Protein-10 by human sTCC follows BCG or IFN-γ stimulation.

Despite evidence supporting the concept of IFN-γ effect against bladder cancer, the clinical experience with IFN-γ instillation in sTCC patients remains very limited. Two studies, both in abstract form, were recorded some years ago on the use of the recombinant form of intravesically instilled IFN-γ against bladder tumor recurrence (23, 24). Recently, recombinant IFN-γ was instilled in bladder cancer patients and its antiproliferative action, as well as alterations in immune cells infiltrating marker tumor were examined (6, 7). However, the only clinical data concerning tumor recurrence in treated patients were recently reported in a small patient population with a big variety of inclusion criteria (9). Currently, a Phase I/II clinical trial is in process on the safety and efficacy of autologous IFN-γ-activated macrophages, intravesically administered in sTCC patients (8). In the field of immunogenetic therapy, retroviruses carrying the IFN-γ gene transduced murine bladder tumor-2 cells in vivo.

Considering the potential of IFN-γ and given the insufficiency of currently used chemotherapeutics as well as the unsuitability of BCG against low-grade and stage bladder tumors, we considered the pure form of IFN-γ as a promising candidate for the treatment of these patients. In this study, we intravesically instilled the human recombinant IFN-γ in initially diagnosed sTCC patients with Ta and/or T1 tumors, of grade 2, as a means of prevention of recurrence. We excluded patients with grade 3 or recurrent tumors or CIS. Patients with grade 1 tumors were not enrolled because watchful waiting is the current follow-up practice for them. Initiation of patients’ treatment started 15 days after TUR to avoid complications in case of remaining intravesical trauma or infection. We chose MMC in a comparison arm because of its adequate effectiveness in sTCC of the bladder, which has been well documented (12, 25). Recent studies observed that optimal concentration of MMC in high-risk patients is 2 mg/ml in six weekly instillations (25). When our study started, the usual concentration for this agent was 0.4–0.8 mg/ml, and we elected to use the maximum dose (12). On the other hand, by administering IFN-γ, we aimed to enhance patients’ immune response and to eliminate any side effect. Thus, we selected to instillate low, repeated doses of IFN-γ (6). Even in well-known effective drugs such as BCG with a rather high rate of complications, the current trend is to lower the dose to diminish toxicity (26). Additionally, in the case of immunotherapy, it has been shown that although the administered instillations were less than the maximum tolerated, the procedure with initial induction courses and maintenance therapy afterward has given optimal responses (16). After these observations, we administered both agents in the two groups of the study patients in the same annual schedule.

The recurrence-free status during the follow-up period after MMC treatment was 57.2%. This percentage was in accordance with major studies treating patients with similar characteristics (25). The recurrence-free status for IFN-γ patients was 73.4% for the same follow-up period and up to 90% during the year of instillations. These encouraging results along with the optimal tolerability of the instilled IFN-γ, counterbalance and justify its relative high cost. Our data showed a statistically significant difference in favor of IFN-γ within the first follow-up year (P = 0.040) between the two groups. However, previous studies without our strict selection criteria produce nonpromising results (23, 24). This beneficial influence of IFN-γ within the administration time may have been because of the combination of initial shock tactic (weekly intervals) followed by maintenance therapy (monthly intervals). As a consequence, early treatment with IFN-γ could help avoid the necessity of a more toxic treatment during the course of the disease. After the first year, for the rest of the study period the difference between the

### Table 3 Local immune response to IFN-γ

<table>
<thead>
<tr>
<th>mAbs (cell subpopulations)</th>
<th>Tumor samples at TUR</th>
<th>Bladder-washing samples Before TUR</th>
<th>AUC standard value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median value (min-max)</td>
<td>Median value (min-max)</td>
<td>P</td>
</tr>
<tr>
<td>CD3+ (T cells)</td>
<td>1.00 (0.0–30.0)</td>
<td>1.00 (0.0–8.5)</td>
<td>0.031</td>
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<tr>
<td>CD4+ (T helper)</td>
<td>0.00 (0.0–3.0)</td>
<td>0.60 (0.0–4.5)</td>
<td>0.025</td>
</tr>
<tr>
<td>CD8+ (T cytotoxic)</td>
<td>1.00 (0.0–29.0)</td>
<td>1.00 (0.0–8.5)</td>
<td>0.064</td>
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<tr>
<td>CD16+ (NK cells)</td>
<td>1.00 (0.0–15.0)</td>
<td>1.00 (0.0–14.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD19+ (B cells)</td>
<td>3.00 (0.0–6.0)</td>
<td>2.70 (0.0–10.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>CD45+ (total leukocyte)</td>
<td>3.75 (0.0–50.0)</td>
<td>5.00 (0.0–56.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>CD25+ (IL-2R) on CD8+, CD4+</td>
<td>0.00 (0.0–70.0)</td>
<td>0.00 (0.0–5.0)</td>
<td>0.169</td>
</tr>
<tr>
<td>CD54+ (ICAM-1) on CD19+</td>
<td>0.75 (0.0–5.0)</td>
<td>1.35 (0.0–11.0)</td>
<td>0.009</td>
</tr>
<tr>
<td>HLA-DR on CD45+</td>
<td>2.50 (0.0–35.0)</td>
<td>4.00 (0.0–25.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*% positive cells.

Wilcoxon signed rank-sum test.
two arms of the study was still in favor of IFN-γ, showing a borderline statistical significance (P = 0.051). This observation may suggest the need for repeated stimulation of the immune system (booster effect) as has been implemented in the case of BCG treatment. According to our results, recombinant IFN-γ exerts a substantially protective effect against tumor recurrence and disease progression, potentially influencing the overall survival of those patients.

In parallel with patient follow-up during IFN-γ or MMC treatment, the possible effect of instillations on local immune reaction was immunohistochemically monitored. In recurrence-free patients and 6 months after starting IFN-γ treatment, a massive immune reaction of the entire bladder wall ensued. Follicular cystitis with the intense presence of T and B cells organized in follicles was observed in patients responding to IFN-γ. On the contrary, weak infiltration and absence of follicles were intramurally observed in patients with recurrent tumors. In recurrence-free patients, persistent immune reaction was observed until the end of the 1-year treatment with IFN-γ. In patients treated with MMC, the absence of follicular cystitis might be a clear indication that no local immune reaction is implicated in the mode of action of this agent. Reportedly, T-helper cells and macrophages increased significantly after intravesical instillations of recombinant IFN-γ in the marker tumors, whereas HLA-DR antigens were either up-regulated or remained stable and T-cytotoxic cells decreased (6). A marked infiltration with mononuclear cells, expressing activation markers and accumulated follicle granulomatous structures, has also been shown immunohistochemically in bladder cancer patients responding to BCG. Intramurally recruited leukocytes after BCG treatment were assumed as clear evidence that humoral and cellular mechanisms are involved in its mode of action (16). Similarly, recruitment of immunocompetent cells, observed in our patients responding to IFN-γ, might serve as a qualitative depiction of IFN-γ local mode of action.

On the other hand, a reliable model for the quantitative evaluation of local immunity is the leukocytes derived from bladder-washing samples. An accurate phenotypic and functional analysis of intramurally detectable leukocytes’ populations and subpopulations can be achieved by flow cytometry (27). In this study, phenotypic analysis was performed, both in tumor samples taken at the initial TUR and in bladder-washing samples obtained at predetermined time points. The first washing sample, before TUR, was considered as the patient baseline immune profile. The next samples were before second and last weekly, last biweekly, and last monthly instillations respectively and were taken to control the persisting stimulation of patient immune reaction and the suitability of the selected intervals between instillations. In the MMC group, washing samples were obtained at fewer time points, assuming that after the instillation of an alkylation agent, alterations in local immune reaction are unlikely to occur.

In our study, the tested leukocytes’ populations and subpopulations presented low concentrations in tumor samples confirming our immunohistochemical results. Regarding bladder-washing samples collected before TUR, all of the mentioned leukocytes’ populations were also detected. In a previous study, in untreated bladder cancer patients, the large majority of bladder-washing samples contained a pure population of T lymphocytes, whereas B and NK cells were absent in a number of individuals. However, in BCG-treated patients, higher frequency of lymphocytes derived from the bladder wall was observed (2, 16). Similarly, in our study, statistically significant differences were found between median values determined in leukocytes’ subpopulations, which derived from bladder-washing samples before TUR and during treatment (AUC-standardized values). Patient variability, as well as the difficulty of selection of the proper time point for the measurement of an immune parameter, makes studies dealing with immunoresponse evaluation difficult. On the basis of the observation that the typical dose response curve with biological response modifiers such as BCG or IFNs is bell-shaped, we obtained the washing samples at the end of each induction cycle (exactly before the next instillation) to check the durability of immunoresponse after IFN-γ instillation. By this conservative approach (at the time when immune response is speculated to be diminished), we found statistically significant differences in almost all leukocytes’ subpopulations tested before TUR and during treatment. These data support the persistence of immune reaction after each IFN-γ instillation and the possible suitability of the administration schedule.

After extensive investigation, the induction of local immunological response has been strongly confirmed, following BCG treatment in bladder cancer. As a potent immune stimulator, BCG has established its ability to substantially elicit both humoral and cellular immune responses, showing effective antitumor immune reaction. In the current study, for the first time to our knowledge, both qualitative and quantitative observations registered an active immune reaction, which occurred in the bladder, after IFN-γ instillations. Additionally, our clinical data support the concept of the beneficial influence of human recombinant IFN-γ instillation in selected bladder cancer patients. These data could be considered as a critical indication that the locally observed alterations are a specific component of the immune reaction against tumor cells induced by the agent. Should our data be confirmed in additional studies, human recombinant IFN-γ may be suggested as an attractive option for adjuvant intravesical treatment of superficial bladder cancer.

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Prevention of Bladder Cancer Recurrence by IFN-γ

The Immunomodulating Effect of Interferon-γ Intravesical Instillations in Preventing Bladder Cancer Recurrence

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