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Correlation between clinical response and urinary interleukin levels using different doses and intravesical administration schedules of interferon-alpha-2b combined with epirubicin: a pilot study

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Summary. A total of 62 patients at high risk for recurrence of superficial bladder cancer were selected for a study designed to compare the prophylactic efficacy of different doses and schedules of sequential intravesical instillations of epirubicin and interferon-alpha-2b and to evaluate which sequence could enhance the release of cytokines in the urine. Our investigations showed a significant increase in urinary concentrations of interleukins in patients who received the sequential intravesical administration of epirubicin and interferon-alpha-2b. Higher urinary concentrations of interleukins and a lower recurrence rate were detected in patients who received interferon-alpha-2b 24 h after epirubicin instillation.

Key words: Intravesical chemotherapy – Intravesical immunotherapy – Interferon-alpha-2b – Epirubicin – Interleukins (urinary concentration of)

No significant progress has been achieved in recent years in the field of topical treatment of superficial bladder tumours. Despite the sagacious use of the currently available chemotherapeutic agents, the recurrence rate after transurethral resection has been reduced but not abolished. Progression to higher stages and death from invasive bladder cancer continue to occur [1].

The combination of different anticancer drugs in systemic chemotherapy of advanced bladder cancer is more effective than the use of single agents. Likewise, it is hoped that the efficacy of intravesical therapy can be enhanced by combining various agents lacking cross-resistance [2]. Doxorubicin and epirubicin have been proven to be effective against superficial bladder cancer when used intravesically and are well established in clinical use [3].

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With regard to the agents used for intravesical immunotherapy, an increase in urinary concentrations of lymphokines can be observed after intravesical administration of bacille Calmette-Guérin (BCG) [4]. BCG may represent only an aspecific stimulus on the immune responding cells that could be mediated by different factors, such as interferons and interleukins. The intravesical use of interferon (IFN)-alpha in the treatment of papillary superficial bladder tumours has not been shown to be superior to conventional agents [5]. Its action against carcinoma in situ is dose-related. A randomized study comparing 10 vs 100 million units per instillation showed a better response when the higher dose was used [6]. However, such a high dose is very expensive (roughly U.S. $1365/instillation).

The antitumoural action of IFN-alpha given intravesically may be explained by three different concurrent mechanisms:

A. Direct cytotoxic action. Although well demonstrated in vitro, this mechanism does not seem to be of clinical relevance in vivo following intravesical administration. When IFN-alpha was used as a single agent, even at high doses, no objective response was observed against papillary vesical lesions [7].

B. Induction of progressive differentiation of the neoplastic cell [8]. This interesting property of IFN-alpha might play a role in the prophylaxis of recurrent bladder tumours by acting against premalignant lesions. However, such action has not been demonstrated in vivo.

C. Immunomodulation. It has been proven that IFN-alpha given at the tumour site has the ability to stimulate a local aspecific immune response through an activation of the natural killer (NK) cells and a promotion of the activity of the T-lymphocytes [9]. There is evidence that IFN-alpha is more effective in vivo than IFN-gamma in promoting an immune response [10].

Immunomodulation with IFN is possible only if an adequate lymphocyte population is present at the tumour

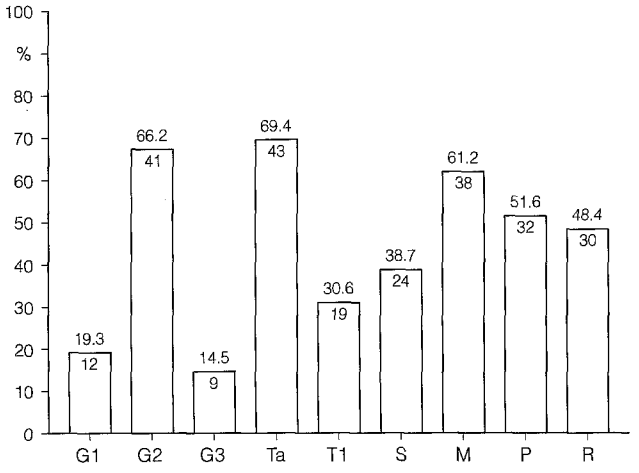

Fig. 1. Characteristics of the 62 evaluable patients at entry. Mean number of tumours, 2.7 (range 1–9); mean recurrence rate, 1.4/year; *S*, single tumours; *M*, multilple tumours; *P*, primary disease; *R*, recurrent disease

Table 1. Distribution of the main prognostic factors into the four groups

		Groups			
		I [%]	II [%]	III [%]	IV [%]
Tumour category	Ta	66.6	81.2	55.5	76.9
	T1	33.3	18.8	44.5	23.1
Tumour grade	G1	13.3	25.0	5.5	23.1
	G2	80.0	68.7	61.2	69.2
	G3	6.7	6.3	33.3	7.6
Multiplicity	Single	40.0	37.5	44.4	23.0
	Multiple	60.0	62.5	55.6	77.0
Recurrence	Primary	53.3	37.5	61.1	46.1
	Recurrent	46.7	62.5	38.9	53.9

site. The role of the anticancer drug epirubicin would be to promote necrosis, inflammation and infiltration by lymphocytes within and around the tumour. The sequential use of epirubicin followed by IFN could activate a local immune response through an activation of the T-lymphocytes at the tumour site and the production of interleukins to obtain an effective reduction of local recurrence. Preliminary clinical experience with the intravesical combination of IFN and epirubicin (EPI) has shown encouraging results [11–13].

As the immune regulation of T-cell subsets is mediated by a complex network of interleukin (IL) production that modifies specific and non-specific T-cell responses, the present study was designed to compare different doses and schedules of sequential intravesical instillations of EPI and IFN-alpha-2b to show which sequence could enhance the release of cytokines in the urine (IL-1-alpha, IL-2, IL-4), considered as a sign of efficient immunostimulation and possible antitumour activity. An increase in the urinary release of IL-1-alpha, which is mainly produced by macrophages and monocytes, could be considered as a sign of aspecific inflammation. A progressive increase in urinary levels of IL-2 and IL-4, which are mainly produced by activated T-lymphocytes, would be a sign of local activation of an immune response [14].

Table 2. Schedule of urine storage for interleukin determination

Urine was stored at the following times:

a. At entry
b: At the time of the 1st, 4th and 8th instillation

At the time of the 1st, 4th and 8th instillation, urine was taken as follows:

Group 3:

a. Before the instillation
b. At 2 h after the start of IFN-alpha instillation
c. At 4 h after the start of IFN-alpha instillation

Group 4:

a. Before the instillation
b. At 2 h after the start of EPI instillation
c. At 2 h after the start of IFN-alpha instillation
d. At 4 h after the start of IFN-alpha instillation

Patients and methods

A total of 62 patients were entered into the study. Their characteristics at entry are reported in Fig. 1. All patients were at high risk for recurrence since their tumours were recurrent in 50% of cases, were multiple in over 70% of cases and were of relatively high grade (G2 or G3) in most cases. At least one of these unfavourable prognostic factors was present in all patients. The mean number of lesions was 2.7 (range, 1–9) and the mean recurrence rate at entry was 1.4/year.

Patients were distributed into four groups, comparable for the main prognostic factors (Table 1), according to different modalities of treatment:

Group I, EPI at 30 mg/30 cc + IFN at 5×10^6 IU/30 cc after 1 h (15 patients);
Group II, EPI at 30 mg/30 cc + IFN at 5×10^6 IU/30 cc after 24 h (16 patients);
Group III, EPI at 50 mg/50 cc + IFN at 10×10^6 IU/30 cc after 1 h (18 patients); and
Group IV, EPI at 50 mg/50 cc + IFN at 10×10^6 IU/30 cc after 24 h (13 patients).

Both drugs were diluted in normal saline solution, EPI at a concentraton of 1 mg/ml and recombinant IFN-alpha-2b (Schering-Plough) in 30 ml, and were maintained in the bladder for 1 h. Starting 15 days after transurethral resection, the drugs were given weekly for the 1st month. For the following 5 months, EPI was given monthly and IFN was given every 15 days. In this study no effort was made to evaluate the stability of IFN in urine.

Determination of the urinary concentrations of IL-1, IL-2 and IL-4 was performed in 12 patients belonging to groups III and IV (6 patients from each group). The patients considered for the study had normal renal function and were maintained on the same hydration regimen. Urine was stored as shown in Table 2. All urine samples (30 ml) were centrifuged at 2000 rpm for 15 min, concentrated 10-fold using Millipore filters and stored at −70°C within 2 h of their collection.

Table 3. Urinary IL-1 concentrations determined in groups III and IV

	1st week	4th week	8th week
Before instillation at entry:			
Group III	112 ± 37	198 ± 43	165 ± 41
Group IV	109 ± 42	179 ± 54	199 ± 61
2 h after EPI:			
Group III	ND	ND	ND
Group IV	189 ± 39	212 ± 39	221 ± 58
2 h after IFN:			
Group III	187 ± 67	184 ± 49	152 ± 71
Group IV	121 ± 39	197 ± 63	201 ± 87
4 h after IFN:			
Group III	223 ± 48	198 ± 43	165 ± 41
Group IV	211 ± 72	220 ± 45	240 ± 89

Data represent mean values ± SD expressed in pg/ml (minimal detection limit, 15 pg/ml). *IFN*, Interferon-alpha-2b; *ND*, not dermined

Table 4. Urinary IL-2 concentrations determined in groups III and IV

	1st week	4th week	8th week
Before instillation at entry:			
Group III	0.2 ± 0.1	1.0 ± 0.2	1.2 ± 0.3
Group IV	0.3 ± 0.1	0.7 ± 0.3	1.1 ± 0.4
2 h after EPI:			
Group III	ND	ND	ND
Group IV	0.4 ± 0.2	0.6 ± 0.2	0.5 ± 0.1
2 h after IFN:			
Group III	0.3 ± 0.1	1.1 ± 0.5	2.6 ± 1.1
Group IV	1.2 ± 0.6[a]	2.5 ± 0.7[a]	4.2 ± 0.6[a]
4 h after IFN:			
Group III	1.0 ± 0.4	1.6 ± 0.9	2.1 ± 0.7
Group IV	3.5 ± 0.9	4.1 ± 1.1[a]	3.9 ± 1.4[a]

Data represent mean values ± SD expressed in U/ml (minimal detection limit, 0.1 U/ml). *IFN*, Interferon-alpha-2b; *ND*, not dermined
[a] $P < 0.01$

Table 5. Urinary IL-4 concentrations determined in groups III and IV

	1st week	4th week	8th week
Before instillation at entry:			
Group III	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1
Group IV	0.1 ± 0.02	0.2 ± 0.1	0.3 ± 0.2
2 h after EPI:			
Group III	ND	ND	ND
Group IV	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.05
2 h after IFN:			
Group III	0.2 ± 0.1	0.1 ± 0.05	0.4 ± 0.1
Group IV	0.7 ± 0.2[a]	0.5 ± 0.09[a]	0.8 ± 0.2[a]
4 h after IFN:			
Group III	0.5 ± 0.2	0.6 ± 0.3	2.1 ± 0.7
Group IV	0.6 ± 0.3	1.5 ± 0.4[a]	1.1 ± 0.6

Data represent mean values ± SD expressed in ng/ml (minimal detection limit, 0.045 ng/ml). *IFN*, Interferon-alpha-2b; *ND*, not determined
[a] $P < 0.01$

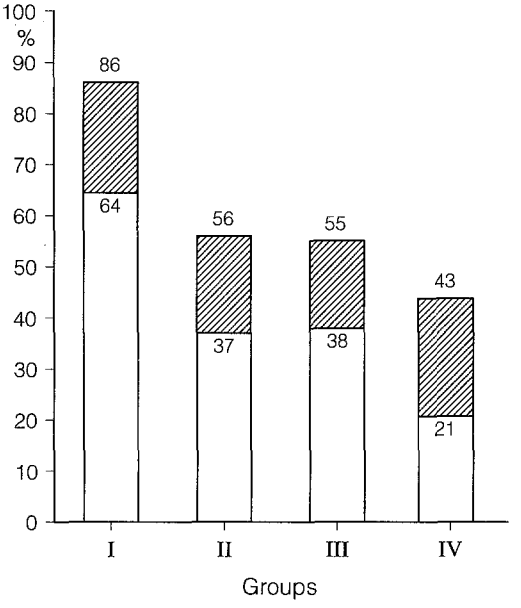

Fig. 2. Percentage of recurrence in relation to treatment; □ 9 months; ▨ 24 months

IL-1-alpha, IL-2 and IL-4 determinations were performed by immuno-enzymatic assay. Urine samples and standards were incubated for 18 h at 25°C on 96-well plates coated with IL-specific monoclonal antibodies (Genzyme CO.). Thereafter, washed plates were incubated with anti-IL-1, -IL-2, and -IL-4-specific antisera for 18 h at 25°C and then with enzyme-conjugated antibodies for 4 h at 37°C. The substrate reaction was measured and related to the absorbances of the standard curves.

Although our study was a preliminary one involving a small number of patients, the results were evaluated by Student's *t*-test to show any possible statistical difference.

Results

The urinary concentration of IL-1 (Table 3) was not significantly modified by IFN instillation in either arm of the study. In contrast, IFN-alpha instillation induced a progressive increase in IL-2 and IL-4 urinary concentrations (Tables 4 and 5).

The values reported in the tables refer to the concentrations of ILs detected in the original urine samples (urine specimens were thereafter concentrated 10-fold). Urine

Table 6. Recurrence rate according to dose and schedule

Group I	86%
Group II	56%
Group III	55%
Group IV	43%
Low dose	70%
High dose	50%
Short interval	68%
Long interval	50%

Mean follow-up period, 24 months

concentrations of IL-1, IL-2 and IL-4 are reported in original concentrations per milliliter of urine (and not in relation to urinary creatinine concentrations, since the possibility of a direct action of these lymphokines against tumour cells cannot be excluded. IL-2 and IL-4 urinary levels were significantly ($P<0.01$) higher in patients receiving IFN-alpha instillation 24 h after EPI than in those in whom a 1-h interval between the two instillations was adopted.

The clinical results have been extensively reported elsewhere [12]. They can be summarized as follows. At a mean follow-up of 24 months (range 16–30 months), 37 patients (59.6%) showed tumour recurrence. The mean recurrence rate was 0.48/year in the overall series and 0.55/year in patients who had experienced previous recurrence. The recurrence rates were 86% in group I, 56% in group II, 55% in group III and 43% in group IV (Fig. 2). The recurrence rates at a mean follow-up of 24 months are shown in Table 6. Tolerability was excellent, and in no case was the treatment discontinued. Mild to moderate chemical cystitis was observed in 12 patients (19.3%).

Discussion

The aim of our research was to study the urinary concentrations of IL-1-alpha, IL-2 and IL-4 after the sequential intravesical instillation of EPI and IFN-alpha-2b. Immunohistochemical investigations suggest that the presence of a marked lymphocytic infiltration, mainly involving NK cells, correlates with a good prognosis for vesical tumours [15]. It has been demonstrated by several studies that the intravesical instillation of antineoplastic drugs induces aspecific phlogistic changes in the bladder wall along with a relevant increase in monocytes, macrophages and lymphocytes [16, 17].

The elevated basal levels of IL-1-alpha and their slight increase after epirubicin instillation may be explained by the phlogistic aspecific alterations induced by the recent transurethral resection (TUR) and by the antineoplastic drug. In constrast, EPI alone was not capable of inducing any increase in the urinary concentrations of IL-2 and IL-4 (see the values recorded at 2 h for patients of group IV; the values obtained at 4 h are not reported herein since they were similar). On the other hand, although it is unlikely, we cannot exclude that EPI itself might influence urinary levels of IL-2 and IL-4 after a delay of 24 h.

The progressive increase in IL-2 and IL-4 concentrations to levels of up to 10–20 times the basal values after the sequential instillation of IFN-alpha-2b could be considered a sign of local immune activation since the lymphokines are mainly produced by activated T-lymphocytes. Elevated local concentrations of IL-2 and IL-4 could promote a progressive enhancement of the immune response mediated by T-cells (IL-2) and B-cells (IL-4) [18] and might themselves exert an antiproliferative action [19]. A recent investigation demonstrated an increase in the T-cell population in the bladder wall of patients affected by superficial bladder tumours following the sequential intravesical administration of EPI and IFN-alpha-2b [20].

We also wanted to investigate whether any difference was evident in urinary IL concentrations in relation to the interval between the instillation of EPI and that of IFN, since in our pilot clinical trial the patients receiving IFN 24 h after EPI (group IV) showed a trend towards recurrence rate lower than that found in the patients receiving IFN instillation 1 h after that of EPI (group III). Although a progressive increase in levels of IL-2 and IL-4 was also evident in group III patients, the urinary levels were lower (50%) than those reached in group IV. Thus, there seems to be a correlation between our clinical results and the laboratory data obtained in the present study. The difference in the urinary levels of ILs detected in the two groups of patients could be explained by two concurrent mechanisms: (1) a latency time is necessary for the elimination of the antiproliferative action and the inhibition of protein synthesis induced by EPI on the immune cell population present at the tumour site and (2) a latency period is also necessary for the recruitment of an adequate lymphocytic population.

Conclusions

Our investigations showed a significant increase in the urinary concentrations of ILs in patients who received the sequential intravesical administration of EPI and IFN-alpha-2b. Higher values were detected in patients who received IFN-alpha 24 h after EPI instillation. This treatment could realize a lymphokine-activated tumour inhibition (LATI) system. In fact, as suggested by Forni et al. [21], the immunocompetent cells infiltrating the neoplastic area could be stimulated by exogenous lymphokines to mount an efficient specific response. In this view, the decrease in the recurrence rate after TUR reported herein for the intravesical sequential combination of EPI and IFN-alpha-2b could be explained by the induction of a more efficient and specific antitumour immune response.

Our research seems to support the general concept of a possible synergism between a conventional antiproliferative drug and a biological response modifier in activating an antitumoral local response. We therefore suggest that such an approach should be subjected to further investigation for use in future clinical studies comparing the

combined intravesical therapy versus the single antiproliferative drug.

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