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Induction of urinary interleukin-1 (IL-1), IL-2, IL-6, and tumour necrosis factor during intravesical immunotherapy with bacillus Calmette-Guérin in superficial bladder cancer

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Summary. To study the local immunological effects of intravesical bacillus Calmette-Guérin (BCG) therapy in superficial bladder cancer patients, the production of interleukin-1 (IL-1), IL-2, IL-6, tumour necrosis factor α (TNFα), and interferon γ (IFNγ) was investigated in the urine. Urine specimens were collected during the six weekly BCG instillations, before instillation, and 2, 4, 6, 8, and 24 h thereafter. Results were standardized to urine creatinine. In general, the concentration of IL-1 increased markedly during the first three BCG instillations, reaching a plateau from instillations 3 to 6. IL-2 was not detected after the first BCG instillation, but from the second instillation onwards the mean IL-2 concentration increased rapidly. With respect to IL-6, patients had relatively high levels in the urine after the first BCG instillation. A relatively moderate increase of the IL-6 concentration was observed during the following weeks. Like IL-2, TNFα was only detected after repeated BCG instillations. Generally the highest TNF levels were found after BCG instillation 5. The presence of IFNγ could not be demonstrated. With respect to the occurrence of the cytokines during the first 24 h after the BCG instillation, TNF, IL-2, and IL-6 were detectable 2 h after the instillation. In contrast, IL-1 seemed to appear later, i.e. from 4 h onwards. TNF decreased most rapidly; it was nearly absent in 6-h samples. Generally IL-2 was not detectable in the 8-h samples, whereas IL-1 and IL-6 were present up to 8 h after instillation of BCG. The presence of TNF was found less frequently than the presence of IL-1, IL-2, and IL-6. Neutralization experiments indicated that most of the IL-1 present in the urine after BCG treatment was IL-1α. In conclusion, activation of BCG-specific T cells was indicated by the detection of IL-2. The presence of IL-1, IL-6, and TNFα might suggest activation of macrophages by intravesically administered BCG, although production by other cell types cannot be excluded. It is suggested that these cytokines, in combination with the leucocytes that are known to be recruited to the bladder in reaction to the BCG treatment, may play an important role in the antitumour activity of BCG against bladder cancer. For monitoring purposes, collection of urine might be performed during the first 6 h after BCG instillations 4–6. A correlation between the presence of cytokines in the urine and the clinical response has yet to be evaluated.

Key words: BCG vaccine – Immunotherapy – Bladder neoplasms – Interleukin-1 – Interleukin-2 – Interleukin-6 – Tumour necrosis factor

Introduction

Superficial transitional cell carcinoma of the bladder urothelium has a high recurrence rate of 50%–70% after transurethral tumour resection. It is well established that bacillus Calmette-Guérin (BCG), an attenuated *Mycobacterium bovis* strain commonly used for vaccination against tuberculosis, is one of the most effective intravesically applied agents to reduce the recurrence rate [25, 40]. Moreover, BCG has been found highly effective for cure of carcinoma in situ of the bladder urothelium.

The mode of action of BCG is probably based on a local stimulation of the immune system, and has been reported to be T-cell-dependent [19, 29]. However, the actual antitumour effector mechanism is still unclear. To gain further insight we have been studying the immunological reactions induced by intravesical BCG administration. In the urine of patients a cellular reaction, consisting of a marked increase of the number of leucocytes, has been observed. These leucocytes consist of granulocytes, which are abundantly present, and mononuclear cells. The mononuclear cells have been flow-cytometrically identified as being largely

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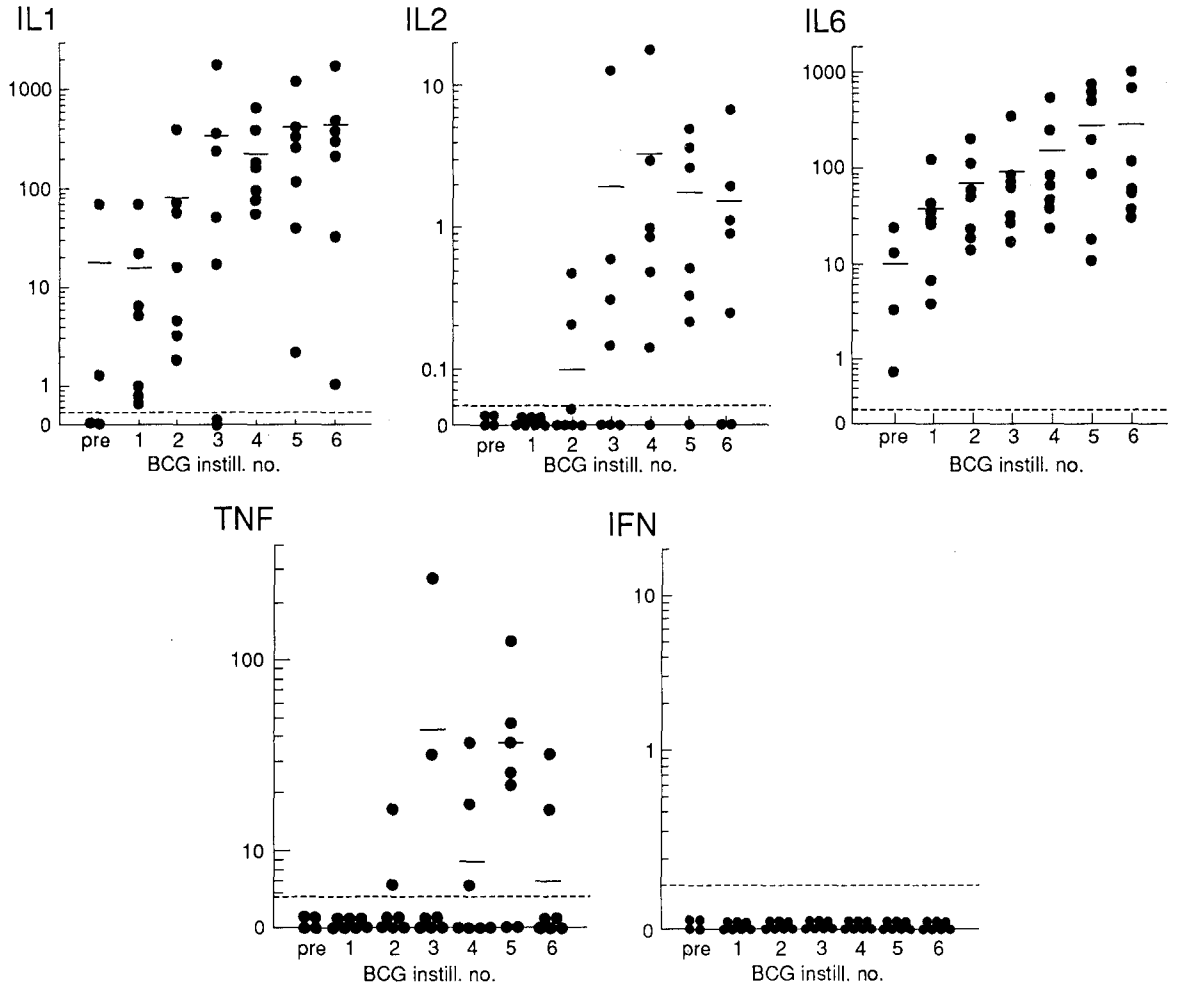

Fig. 1. Highest cytokine levels in urine during six consecutive bacillus Calmette-Guérin (*BCG*) instillations once a week. For each patient ($n = 7$) the highest cytokine concentration determined per instillation is presented (●). x *axis*, BCG instillation number; *pre*, pretherapy (i.e. before the first BCG instillation). y *axis*, cytokine concentration: interleukin(*IL*)-1, -2, -6, and interferon γ (*IFN*) U/μmol creatinine; tumour necrosis factor α (*TNF*) pg/μmol creatinine. —, Mean; – –, minimal detection limit

monocytes/macrophages ($CD14^+$) and T lymphocytes ($CD3^+$) [6, 7]. Furthermore we have found T lymphocytes to be the cell type predominantly present in BCG-induced bladder wall infiltrates in the guinea pig [9]. This correlates with data reported on bladder wall biopsies from patients after BCG therapy [1, 12]. Demonstration of the expression of interleukin-2 receptors and HLA-DR antigens has also suggested activation of T cells by intravesical therapy with BCG [1, 7, 9, 12].

In the present study we have investigated the presence of cytokines in the urine of patients after BCG treatment. This may give us more insight in whether locally present leucocytes are activated. Interleukin-2 (Il-2) and interferon γ (IFNγ) were measured, which are mainly produced by activated T lymphocytes [37, 46]. Furthermore the presence of interleukin-1 (IL-1), interleukin-6 (IL-6), and tumour necrosis factor α (TNFα) was investigated. These cytokines are mainly produced by activated monocytes/macrophages [10, 23, 34]. The induction of cytokines was serially measured within the first 24 h after BCG instillation, during a complete treatment course of intravesical BCG instillations, once a week for 6 consecutive weeks. Measuring the immunological reaction in the bladder by the production of cytokines may provide us with a prognostic tool to predict clinical response in future.

Materials and methods

Patient treatment. Urine specimens were obtained from seven patients with superficial bladder carcinoma (stage pTa, pT1 and/or carcinoma in situ) who were intravesically treated with bacillus Calmette-Guérin (BCG) after transurethral resection of papillary tumour(s). BCG-RIVM or BCG-TICE, approximately 5×10^8 culturable particles, was administered in 50 ml 0.9% saline once a week for 6 consecutive weeks [41].

Urine specimens. Urine specimens were collected during the six weekly BCG instillations, before instillation and 2, 4, 6, 8, and 24 h thereafter. The specimens were immediately frozen to –20° C. Afterwards specimens were thawed and centrifuged (300 *g*) to remove cells and debris. Specimens were subsequently extensively dialysed (Spectrapor 1 dialysis membrane, molecular mass cut-off 6–8 kDa; Spectrum, Los Angeles, Calif.) to remove constituents that are inhibitory in the cytokine bioassays, and sterilized (Acrodisc 0.2-μm filter unit, Gelman Sciences, Ann Arbor, USA) [8]. Thereafter, samples were stored (–20° C) until determination of cytokines.

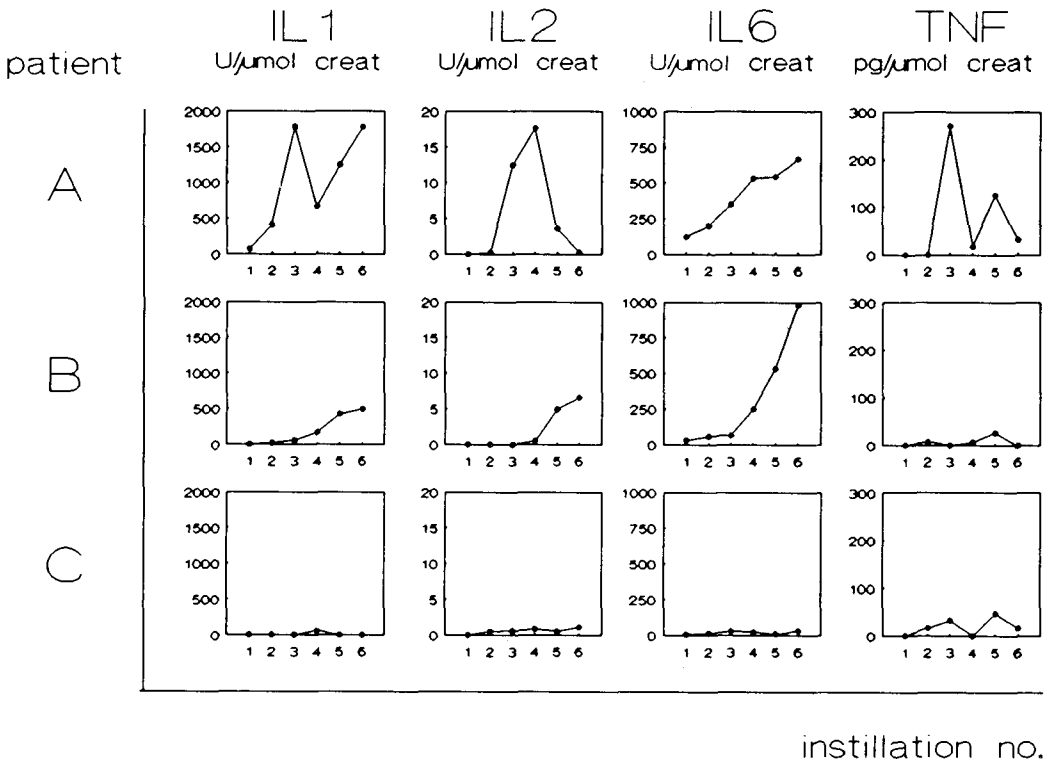

Fig. 2. Cytokine levels in urine of three individual patients during six consecutive BCG instillations once a week. For each patient the highest cytokine concentration determined per instillation is presented

Detection of IL-1, IL-2, IL-6, and IFNγ. IL-1 was measured with the T cell line D10(N4)M as described by Helle et al. [20]. This assay is specific for IL-1 and can detect both IL-1α and IL-1β. Recombinant human IL-1β (rhIL-1β; Hoffmann-LaRoche, Nutley, N. J.) was used as a standard. The nature of the IL-1 in some urine samples was investigated by neutralization tests. During the D10 assay the biological IL-1 activity in urine samples, diluted to approximately 10 pg/ml IL-1, was inhibited with goat anti-IL-1α antibodies, a kind gift of Dr. I. Otterness (Pfizer, Groton, Conn., USA), and/or with anti-IL-1β [39], kindly provided by Dr. J. Van Damme (Leuven, Belgium). The residual IL-1 activity after inhibition was expressed as a percentage of the non-inhibited IL-1 concentration.

For detection of IL-2 a specific bioassay with the IL-2-dependent murine T cell line CTLL-16 was used as previously described [8]. A recombinant human IL-2 preparation (rhIL-2) was used as a standard (Sanofi, Toulouse, France). IL-2 in urine specimens was expressed in units/ml urine; 1 unit was defined as the amount of IL-2 resulting in 50% proliferation of CTLL-16 cells using the standard rhIL-2.

Detection of IL-6 was performed by the use of the hybridoma growth factor assay B9 [20], with rhIL-6 as standard [3]. TNFα was determined using a TNFα-specific enzyme-linked immunosorbent assay, (ELISA), described by Van Kooten et al [43].

The assay used for detection of IFNγ was a commercially available ELISA (Holland Biotechnology, Leiden, The Netherlands).

The detection limits of the various assays were 6 pg IL-1, 0.5 U IL-2, 2 pg IL-6, 50 pg TNFα, and 1 U IFNγ/ml urine. The results were standardized to urine creatinine (U/μmol or pg/μmol creatinine) so that sample data were comparable, regardless of the urine volume. Urine creatinine was photometrically (500 nm) determined with a Cobas-Bio centrifugal analyser (Hoffmann-La Roche Ltd., Basle, Switzerland), using alkaline picrate as a reagent. The mean creatinine concentration for all samples was 7.9±4.1 μmol/ml urine (n = 207).

Results

Occurrence of IL-1, IL-2, IL-6, and TNF during BCG instillations 1–6

Figure 1 shows the highest concentrations of IL-1, IL-2, IL-6, TNFα, and IFNγ in the urine of superficial bladder cancer patients during a 6-week BCG treatment course. In pretreatment specimens, i.e. samples collected before the first BCG instillation, considerable levels were occasionally observed for IL-1 and IL-6. Generally the cytokine concentrations in pretreatment samples were low or zero, which was also observed for the other pre-instillation samples (for instillations 2–6, data not shown). During the first three BCG instillations the concentration of IL-1 increased markedly, reaching a plateau from instillation 3 to 6. IL-2 was not detected after the first BCG instillation, but from the second instillation and onwards the mean IL-2 concentration increased rapidly. With respect to IL-6, patients had relatively high levels in the urine already after the first BCG instillation. A relatively moderate increase of the IL-6 concentration was observed during the following weeks. Like IL-2, TNFα was only detected after repeated BCG instillations. The total number of samples positive for TNF was low. Generally the highest TNF levels were found after BCG instillation 5. In the urine of all of the seven patients investigated, positive concentrations of IL-1, IL-2, and IL-6 were measured at least once during the 6 weeks, however for urinary TNF two of the seven patients remained negative during the complete 6-week instillation course (data not shown). The presence of IFNγ could not be demonstrated in any of the specimens during

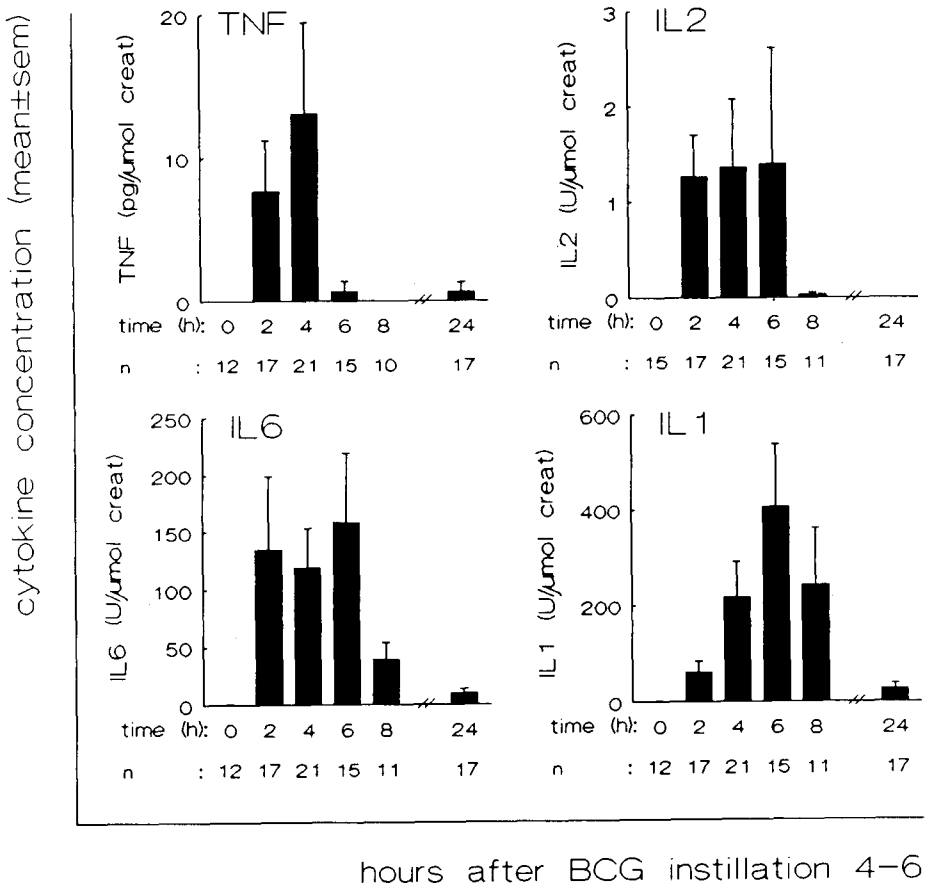

Fig. 3. Mean concentration of cytokines in urine during the first 24 h after intravesical BCG instillation. Data presented for instillations 4–6. *n* = number of samples investigated

any of the 6 weeks of BCG treatment. Overall, Fig. 1 shows that a considerable difference between individual patients was present with respect to the highest cytokine levels after each BCG instillation. As an example, the course of highest cytokine levels during six weekly BCG instillations is shown for three individual patients in Fig. 2. In the urine of patient A, relatively high cytokine levels were measured; cytokine concentrations were more or less intermediate for patient B, whereas for patient C relatively low cytokine concentrations were found.

We also determined after which of the six BCG instillations the highest level of IL-1, IL-2, IL-6, and TNFα (cytokine peaks) occurred in the urine. For most of the patients the cytokine peak occurred after instillations 4, 5 and 6 (data not shown).

Occurrence of IL-1, IL-2, IL-6, and TNF during the first 24 h after the BCG instillation

In Fig. 3 the concentrations of IL-1, IL-2, IL-6, and TNFα in urine samples collected at regular intervals during the first 24 h after the BCG instillation are presented. For this, data of instillations 4–6 are shown, as the highest cytokine concentrations occurred during these instillations (see above). TNF, IL-2, and IL-6 were clearly detectable within 2 h after the instillation. In contrast, IL-1 seemed to occur later, i.e. from 4 h onwards. TNF decreased most rapidly; it was nearly absent in 6 h samples. Generally, IL-2 was no longer detectable in 8-h samples, whereas IL-1 and IL-6 were present up to 8 h after instillation of BCG.

In addition, we evaluated at which hour the highest cytokine concentrations (cytokine peaks) after BCG instillation occurred. For IL-2 it was noticed that concentration peaks were scarcely present at $t = 6$ h (data not shown), although at this time point the mean IL-2 concentration was still considerable (cf. Fig. 3). Likewise, peaks of IL-1 were relatively infrequent in 8-h specimens (data not shown), although the mean concentration was still considerable at this time (cf. Fig. 3). Overall it is concluded that, for the four cytokines together, the highest concentrations occurred within the first 6 h after BCG instillations 4–6. Thus, to obtain an indication on the amount of IL-1, IL-2, IL-6, and TNFα produced, urine should be collected during these first 6 h.

The bioassay used to measure IL-1 in the urine did not discriminate between IL-1α and IL-1β. By neutralization of the biological IL-1 activity with anti-IL-1α or anti-IL-1β antibodies the nature of the IL-1 in the urine after BCG treatment was investigated. For this, from each of the seven patients investigated the specimen with the highest IL-1 activity during the six BCG instillations was investigated. Figure 4 shows that inhibition of the biological IL-1 activity was mainly exerted by the anti-IL-1α mAb in each patient sample, as the residual IL-1 activity was 10%–32% of the non-inhibited activity. In contrast, after inhibition with anti-IL-1β mAb most (72%–98%) of the IL-1 activity remained detectable. Inhibition with a

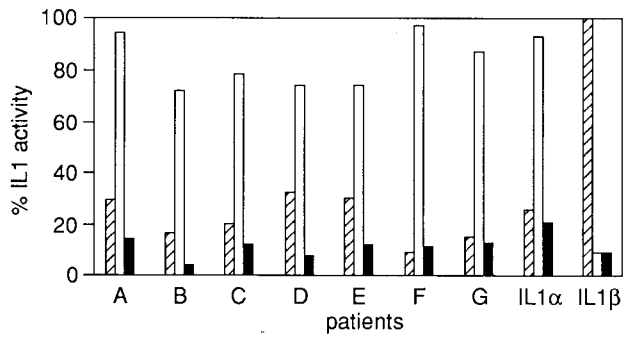

Fig. 4. Neutralization of IL-1 activity present in urine after intravesical BCG treatment. The IL-1 activity left after neutralization with anti-IL-1α (hatched bars), anti-IL-1β (blank bars), or anti-IL-1α + anti-IL-1β (black bars) antibodies was expressed as percentage of the IL-1 activity in each non-neutralized sample

combination of anti-IL-1α and anti-IL-1β resulted in 4%–14% IL-1 activity left. With rhIL-1α and rhIL-1β controls, inhibition with anti-IL-1α and anti-IL-1β resulted in 26% and 10% residual IL-1 activity, respectively, being measured. The observations indicate that most of the IL-1 induced by the BCG treatment is IL-1α.

Cytokine concentrations were also determined in urine specimens from two patients intravesically treated with mitomycin C, a chemotherapeutic agent. None of these specimens, obtained both before and at several times after the sixth mitomycin C instillation, was positive (data not shown).

Discussion

In this paper we have shown the induction of urinary IL-1, IL-2, IL-6, and TNFα, indicating immunological stimulation after intravesical treatment with BCG. In pre-instillation urine samples, IL-2 and TNFα were not detected; IL-1 and IL-6 were either absent or found in low levels. These data agree with data reported by other investigators, showing the occasional presence of IL-1, IL-2, IL-6, and TNF in urine of bladder cancer patients (non-BCG-treated), patients with cystitis, febrile patients, or healthy persons, the concentrations being relatively low [2, 22, 28, 33, 44].

During the course of six weekly BCG instillations, IL-1 and IL-6 were already present in the urine after the first BCG instillation. As monocytes/macrophages can be directly stimulated by BCG or mycobacterial antigens resulting in the release of IL-1 and IL-6 [32, 45], these cytokines may be produced by macrophages. The presence of macrophages in the normal human bladder has been described [11, 17]. However, upon such direct stimulation production of TNFα has also been reported [38], which we could detect only after repeated BCG instillations. In general, monocytes/macrophages are reported to be the major producers of IL-1, IL-6, and TNFα [10, 23, 34]. Recently Van Kooten et al. have demonstrated that T cells, when sufficiently stimulated, are also effective producers of IL-1, IL-6, and TNFα [43]. Moreover, the release of IL-1, IL-6, and TNFα has been reported for a number of other cell types, such as endothelial cells, fibroblasts, epithelial cells or polymorphonuclear granulocytes [10, 23, 34]. Thus, the possibility of some production by cell types other than monocytes/macrophages cannot be excluded. The type of IL-1 found in the urine after BCG therapy was mainly IL-1α. The detection of IL-1α rather than IL-1β as the main type of IL-1 produced is in agreement with our in vitro studies on IL-1 production by human monocytes and T cells [43] (and unpublished results, L. A. Aarden).

During the initial BCG instillation(s) immunological sensitization to BCG probably occurs. The detection of IL-2 in the urine after repeated BCG instillations indicates the induction of a T-cell-mediated immune reaction [46], which might be comparable to the cutaneous delayed-type hypersensitivity (DTH) reaction to tuberculin. For guinea pigs Coe and Feldman have demonstrated that the bladder is an organ in which a DTH reaction might well be induced [5]. A number of cytokines are known to be induced during the DTH reaction [18], which agrees with our data. The kinetics of IL-2 appearance in the urine confirms previous observations made by Ratliff et al. and by our own group [8, 28]. Although antigenically activated T cells commonly produce IFNγ, we were not able to detect this cytokine in the urine. IFNγ was possibly not stable in non-dialysed urine [27].

Several investigators have demonstrated the induction of mononuclear cell infiltrates in the bladder wall, sometimes with granulomatous characteristics, by repeated intravesical BCG administration [2, 9, 12]. The general increase we observed for the concentration of cytokines during the 6-week instillation course may reflect an increase in the number of leucocytes infiltrating the bladder wall. It is not known whether the cytokine secretion of the individual cells increases after subsequent BCG instillations. Moreover, the permeability of the urothelium may influence the detection of cytokines in the urine after BCG instillation. TNF effects on epithelial tight junctions, resulting in an increase of transepithelial permeability, have been reported [26]. Indeed our unpublished data on the presence of albumin in the urine of the patients show an increase of the albumin concentration, after each BCG instillation as well as during the 6-week BCG course. This might indicate an increased permeability of endothelial cells and of the urothelium, which coincides with the cytokine increase in the urine during BCG treatment.

We have demonstrated the presence of IL-1, IL-2, IL-6, and TNF mostly during the first 8 h, while peak concentrations mainly occurred during the first 6 h after BCG instillation. These data on the time during which such substances are present are comparable to the results of other studies on cytokines in the urine of bladder cancer patients after BCG treatment [2, 8, 28, 33]. Furthermore, induction of cytokines within several hours after stimulation has been reported for serum IL-6 in cancer patients after i. v. administration of a muramyltripeptide derivative [16]. For serum IL-1, IL-6, and TNF in baboons during lethal bacteraemia and in cancer patients after i. v. administration of endotoxin this rapid production was also found [14, 15].

The cytokines found are probably part of an immunological cascade, induced by the local presence of BCG in the bladder. The rationale of BCG treatment is to activate the immune system, eventually producing an immunologi-

cal reaction against locally present bladder tumour cells and tumour cell degradation [30]. With regard to the theoretically possible antitumour effector cells, the role of the induced IL-2 might be the activation of cytotoxic T cells or induction of lymphokine-activated killer cells [21, 46]. Other mechanisms, like macrophage- or granulocyte-mediated cytotoxicity to tumour cells, may also be part of the mode of action of BCG [24, 42]. Augmentation of these possible effector mechanisms by IL-1, IL-6, and TNFα has been reported [10, 23, 34]. Besides immunomodulating properties, the cytokines themselves may have direct cytotoxic/cytostatic effects on tumour cells [4, 13, 31, 35].

The treatment of patients with superficial bladder cancer would be significantly improved by the availability of a prognostic indicator to monitor whether a sufficient number of BCG instillations has been given to a patient, and to predict the response on therapy. Several parameters, including purified protein derivative skin reactivity and the presence of granulomatous reactions in the bladder wall, have been evaluated for use as a prognostic tool. However, no sufficient correlation with the clinical response has been found [36]. Correlation of the IL-2 concentration in urine with clinical outcome has also been suggested [28]. Currently we are evaluating, for a larger number of patients, the correlation of clinical response to therapy with the concentration of IL-1, IL-2, IL-6, and TNFα in the urine. On the basis of data presented in this paper, for monitoring purposes we suggest the collection of urine during the first 6 h after BCG instillations 4–6.

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