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BCG treatment and the importance of an inflammatory response*

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Summary. A prospective study was performed on patients with superficial bladder tumour treated with bacillus Calmette-Guérin (BCG). The kinetics of interleukin-6 (IL-6) titres were monitored in urine collected at regular intervals for 24 h during 14 BCG treatments, each consisting of six weekly intravesical instillations. IL-6 titres were quantified with an ELISA system and compared with a bioassay (biologically active IL-6) system. After instillation, urinary IL-6 titres transiently increased, reaching maximum levels between 2 and 6 h after instillation. IL-6 titres appeared to be significantly correlated with an increase of total cells retrieved by bladder washout 3 h after instillation. The kinetics of the weekly maximum biologically active IL-6 titres indicate that three types of BCG-induced response occur: an "early" response starting at the first instillation; a "late" response after the third instillation; or no IL-6 response. The "early" response appeared to be associated, but not strictly correlated, with an IL-2 response. The results suggest that the effectiveness of BCG treatment is determined by two processes, an inflammatory one, followed by a delayed type of hypersensitivity response.

Key words: Superficial bladder cancer – Bacillus Calmette-Guérin – Cytokines

Intravesical instillation of bacillus Calmette-Guérin (BCG) is an established and effective form of adjuvant therapy for cases of superficial bladder carcinoma [7, 10]. However, the mechanisms of action of BCG, resulting in inhibition of tumour growth, are not well understood [8]. Recent studies have stressed the importance of local immunological mechanisms at the mucosal surface of the bladder, emphasizing the delayed-type sensitivity (DTH) reaction [8]. An association between anti-tumour activity and a T-cell-dependent mode of action has been concluded from histological studies [2] and from the absence of response in tumour-bearing athymic mice [9]. Knowledge concerning the appearance of cytokines in the course of BCG therapy is sparse and limited to interleukin (IL)-1, IL-2 and tumour necrosis factor (TNF) [3, 5]. In addition, the significance of a BCG-associated inflammatory reaction can at present not be excluded. No data are available on BCG-induced IL-6 production, despite the pleiotropic character of this interleukin [4, 6]. Considering the characteristics of IL-6, including its major role in the inflammatory reaction, the aim of this study was to monitor the kinetics of urinary IL-6 secretion as part of BCG-induced immune modulation in patients with superficial bladder tumour and to evaluate IL-6 secretion in relation to BCG-induced IL-2.

Materials and methods

Patients and BCG treatment protocol

Fourteen patients with superficial bladder cancer (TCC), category Tis, Ta/T1 were treated with various BCG strains, containing at least 5×10^8 colony-forming units (CFU) per BCG instillation, which was retained in the bladder for 2 h. A total of six BCG instillations were performed at weekly intervals. Instillation was initiated after biopsy (TisG3) or complete resection of all visible tumours (Ta/T1) and histological confirmation of the diagnosis.

Collection and processing of urine specimens and bladder washouts

Urine samples were obtained prior to catheterization by voiding and by catheter 1, 2 and 3 h after BCG instillation. Subsequently, voided urine was collected during 3–6, 6–12 and 12–24 h intervals.

Urine samples were centrifuged at 1000 *g* for 10 min, stored at −20°C and tested for cytokines within 2 months of collection. Bladder washouts (BWOs) of 100 ml saline were performed immediately before, and 3 and 24 h after the instillation. BWOs were processed immediately. Cells were collected by centrifugation, resuspended in 1–2 ml phosphate-buffered saline containing 2 mg

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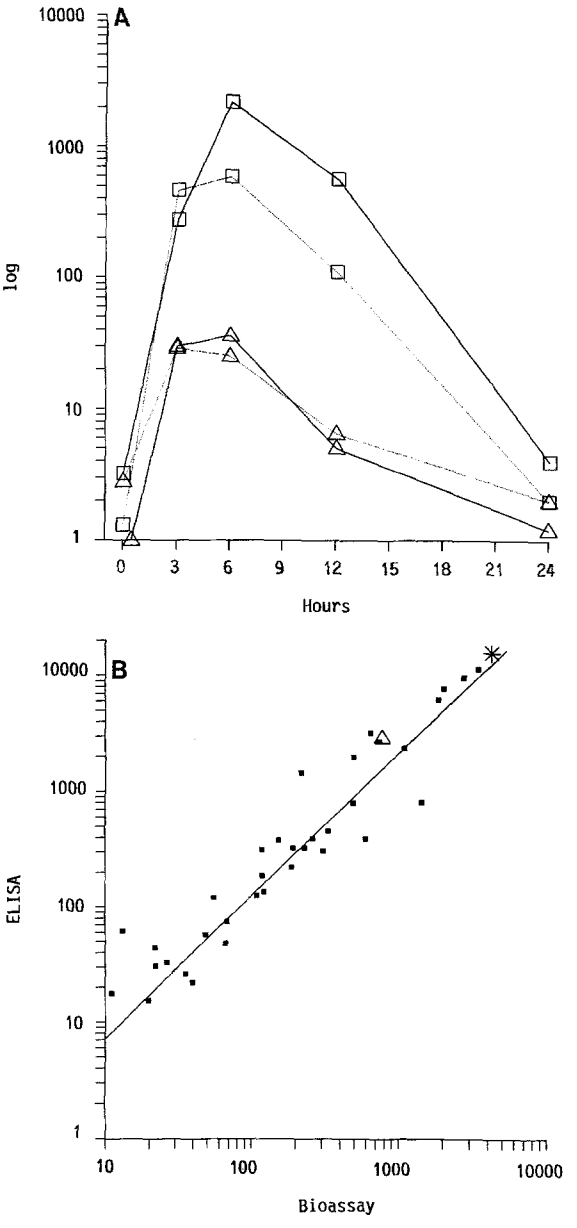

Fig. 1. A Comparison obetween the kinetics of interleukin-6 (IL-6) production in two patients (□, △) prior to and after installation 6, as measured by bioassay (– – –) or ELISA (———). B Double log relation between IL-6 concentrations determined by bioassay and ELISA: log [ELISA] = 0.13 × 1.17 log [bioassay]. The data consist of patient material (■), an IL-6 standard, HGF (*) [11] and IL-6 produced by human endothelial cells (HECS) in vitro (△)

bovine serum albumin and 0.2 mg EDTA per millilitre. Appropriate dilutions were prepared, stained with a Türks solution and counted under a microscope.

Analysis of urinary cytokines

For the IL-6 bioassay, urine specimens were filtered using Centricon-10 (10,000 mol. wt. cut-off; Amicon, Danvers, Mass.) in order to remove low-molecular-weight inhibitors of the IL-6 assay. After filtration, the samples were reconstituted to the original volume with phosphate-buffered saline. The bioassay was performed with a variant (subclone 9.9) of the original IL-6-dependent hybridoma cell line B 13.29 [1, 11]. In addition to the bioassay, IL-6 was determined by ELISA (Medgenix, Fleurus, Belgium). IL-2 was measured by radioimmunoassay (Amersham, Houten, The Netherlands). The immunoassay were directly performed on centrifuged urine samples. In a total of 294 urine specimens the cytokines were measured in duplicate or triplicate, including standard titration curves.

Statistical analysis

Correlation coefficients were calculated by the Pearson product-moment correlation method. Comparison of the different groups was achieved using Student's *t*-test; a P value of less than 0.05 was considered to be significant.

Results

Kinetics of IL-6

Initially, the 24-h kinetics of BCG-induced elevation of urinary IL-6 in two patients were studied extensively by both ELISA and bioassay after each intravesical BCG instillation. As shown in Fig. 1A, a transient increase of IL-6 secretion was observed, reaching a maximum between 2 and 6 h after BCG instillation. These kinetics appeared to be independent of the assay system used. Titres returned to normal within 24 h. It is significant to note that in the course of this study with increasing IL-6 concentrations the values obtained by ELISA were higher than those using the bioassay. By determining urinary IL-6 levels of various patients and standard, purified IL-6 preparations with both assays, a double log relation ($r = 0.952$; $P<0.01$) was found, indicating that this result was independent of the patient (Fig. 1B). Addition of standard IL-6 preparations to the urine specimens resulted in a recovery of at least 96%, which shows that there were no factors interfering with either assay system and no loss of IL-6 during the filtration step used in the bioassay.

In a further series of experiments, the 24-h kinetics of IL-6 in seven patients, each receiving 6 weekly instillations were determined. Expressed as frequencies, the number of IL-6 peak values were 33%, 64% and 0% at 2–3, 3–6 and 6–12 h after BCG instillation, respectively. The results of 42 instillations demonstrate that the maximum elevation of IL-6 occurred within 2–6 h after instillation. In the same urine samples figures were 62%, 28% and 10% for IL-2, showing that the majority of IL-2 peak values were found in urine collected between 2 and 3 h after instillation.

Subsequently, the biologically active IL-6 titres prior to therapy and maximum IL-6 titres after each of the six instillations in 14 patients were determined by ELISA and subsequently converted using the correlation presented in Fig. 1B (Fig. 2). The mean IL-6 titre prior to therapy was found to be 1.9 ± 3.1 pg/μmol creatinine ($n = 14$). Analyzing the kinetics of the weekly maximum IL-6 titres indicates that three types of BCG-induced responses occur: (1) an "early" response (7 patients), of which, (compared to the pre-therapy value) the kinetics of the weekly maximum IL-6 titre could be characterized by an increase during each instillation, starting from the first

Table 1. Average (mean ± SD) of maximum interleukin-6 (IL-6) titre after instillations 1–6 in 13 patients exhibiting an "early" ($n = 7$) or "late" ($n = 6$) IL-6 response

Number of instillation	1	2	3	4	5	6
IL-6 response:						
Early	32 ± 27	78 ± 45	197 ± 101	129 ± 87	206 ± 252	241 ± 316
Late	4 ± 8	10 ± 17	6 ± 6	6 ± 2	16 ± 16	70 ± 90
Significance	$P<0.05$	$P<0.01$	$P<0.001$	$P<0.02$	NS	NS

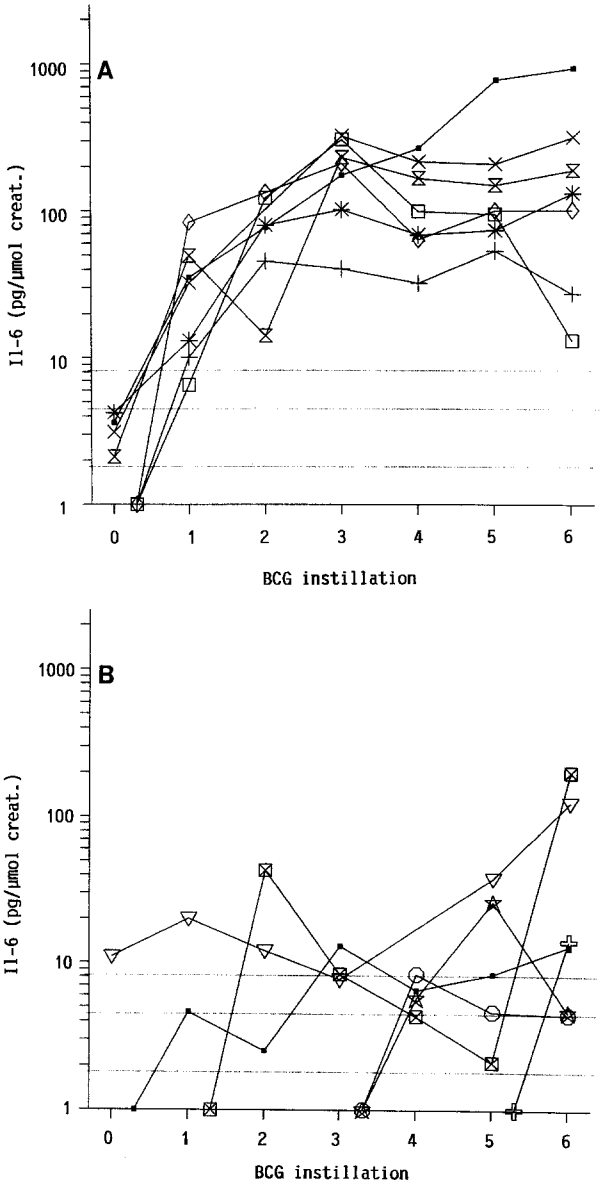

Fig. 2A, B. Kinetics of the maximum titre of IL-6 during the course of BCG treatment in patients exhibiting an "early" **(A)** or "late" **(B)** response. The dotted lines indicate the mean, mean + 1 SD and mean + 2 SD. Also indicated are the pre-therapy levels (0)

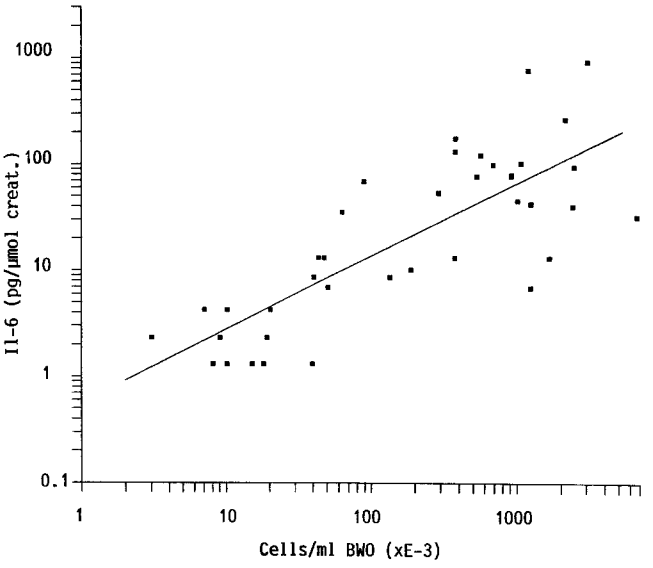

Fig. 3. Correlation between maximum IL-6 titre after BCG instillation and total cell number, obtained by bladder washout 3 h after instillation

instillation (Fig. 2A); (2) a "late" response (6 patients), characterized by a (generally) lower enhancement of the IL-6 titre initiated at a later phase of therapy (Fig. 2B); and (3) no IL-6 response (1 patient), i.e. not exceeding the pre-therapy titre (results not shown). Table 1 shows the mean maximum IL-6 titre obtained after each instillation in patients exhibiting an "early" or "late" response, quantifying the difference in response.

Kinetics of cell release

Cell release, obtained by BWO, was analysed prior to and 3 h after each BCG instillation. Disregarding the occasionally found erythrocytes, the majority of this cellular material consisted of granulocytes (>95%), but lymphocytes (CD 3+) and urothelial cells were also detected. The weekly kinetics of total release paralleled the observed weekly maximum IL-6 titres, reflected by a significant correlation ($r = 0.808$; $P<0.01$; $n = 41$) obtained by plotting the corresponding maximum values of IL-6 and cell amounts after each instillation in seven patients (Fig. 3).

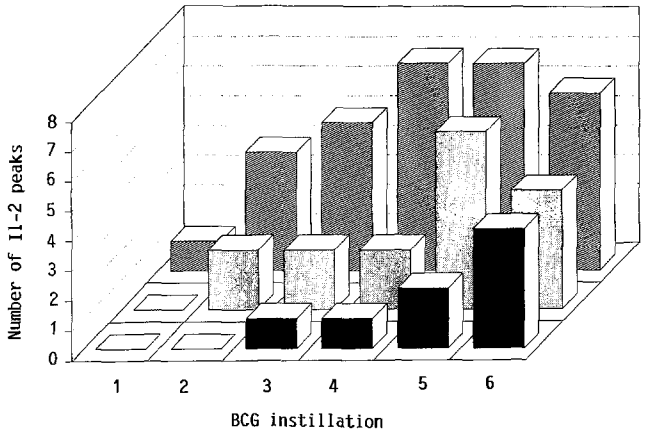

Fig. 4. Number of maximum IL-2 titres during the indicated instillations exceeding the pre-therapy mean (▨), mean + 1 SD (▩) and mean + 2 SD (■) in 14 patients. The pre-therapy mean ± SD was found to be 415 ± 376 pg/μmol creatinine

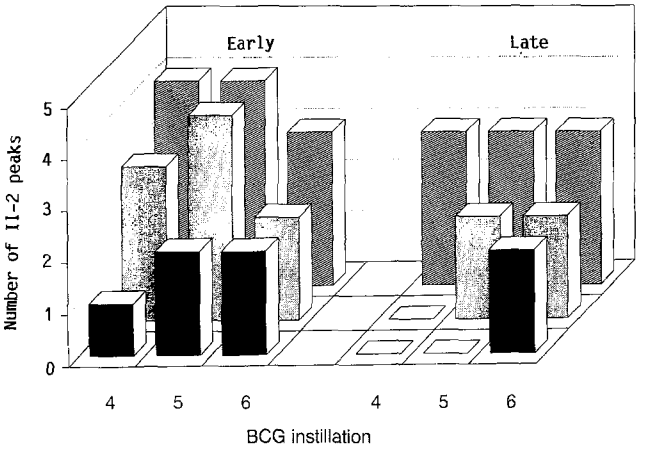

Fig. 5. Number of maximum IL-2 titres during the indicated instillations exceeding the pre-therapy mean (▨), mean + 1 SD (▩) and mean + 2 SD (■) in 14 patients, presented according to "early" (7 patients) and "late" (6 patients) type of IL-6 response. The patient with no IL-6 response was not included in this analysis

Kinetics of IL-2

The maximum urinary IL-2 titres were determined after measuring the 24-h kinetics of 84 instillations (6 in each of 14 patients). The mean pre-therapy value was 415 ± 376 pg/μmol creatinine. Figure 4 shows the number of maximum IL-2 levels, increasing the mean, mean + 1 SD and mean + 2 SD of the pre-therapy value, respectively. The results show an enhancement of IL-2 in urine collected during the later phases of therapy (instillations 4–6).

IL-6 response and induction of IL-2

Urinary secretion of IL-2 was evaluated in patients exhibiting an "early" and "late" IL-6 response. Analysing instillations 4–6, the number of maximum IL-2 titres secreted within 6 h after instillation were scored as either elevated or non-elevated using as "cut-off" levels the pre-therapy mean, mean + 1 SD and mean ± 2 SD values (Fig. 5). Taking into consideration increased "cut-off" values, the date indicate that an "early" IL-6 response appeared to be associatd with an IL-2 response following instillations 4–6. In patients with a "late" IL-6 response, the BCG-associated IL-2 response appeared to occur during a later phase of therapy (instillation 6).

Discussion

The present observations demonstrate for the first time the occurrence of a BCG-induced IL-6 response in patients with bladder carcinoma. The response could be described by several characteristics, distinguishing the IL-6 response from that of IL-2 [5]. First, the transient induction of IL-6 occurred either at a very early stage of BCG treatment (instillation 1; "early" response), reoccurring during each subsequent instillation or during a later phase of therapy (instilations 3–6; "late" response). Secondly, the absolute maximum IL-6 titre after instillation correlates with a cellular response, mainly reflected by the number of neutrophils.

Among the large variety of cells, including T-lymphocytes, endothelial cells and fibroblasts, monocytes/macrophages seem to be the major source of IL-6 production within the human mononuclear cell population [1]. Human IL-6 exhibits multiple actions and seems to be highly indicative of inflammation [1, 4, 6].

Although still controversial, most authors currently accept a local response of the immune system as the most likely primary mechanism, explaining the anti-tumour effect of intravesical BCG instillations in patients with superficial bladder carcinoma (see [8] for review). Recently, Böhle and associates [3] reported increased levels of IL-1, IL-2 and TNF after the sixth instillation of BCG in patients with superficial bladder carcinoma, providing additional evidence in support of the concept of a T-cell-dependent mechanism of action. Furthermore, the present results confirm those reported by Haaff and coworkers [5], indicating maximum elevation of urinary IL-2 after instillations 4–6.

However, at present no conclusive data are available to exclude other, additional mechanisms, including direct (toxic) effects of BCG on tumour cells and/or inflammation without antigen recognition, as important effectors.

Considering urinary IL-6 secretion paralleled by an increased release of granulocytes to be a conclusive inflammatory response, these parameters were considered worthwhile for study of the effects of an inflammatory response on the secretion of additional urinary cytokines. The present report indicates that secretion of IL-2 is associated, but not strictly correlated, with the occurrence of a preceding inflammatory response.

Although only a limited number of patients were studied, it is tempting to consider BCG-induced antitumour activity the result of two cooperative processes, i.e. (1) an initial, continuous inflammatory reaction and (2) a T-cell-mediated response, reflected by an increase in the urinary IL-2 titre, at a later stage of therapy [5].

Whether IL-6 is involved in B-cell proliferation/maturation, possibly associated with BCG-induced anti-tumour activity, remains to be established [6]. With respect to the practical implications, monitoring the absolute levels and kinetics of IL-6 seems to provide a sensitive, non-invasive parameter indicative of the occurrence of an inflammatory response. It seems worthwhile to explore the value of this parameter as part of a systematic investigation of the BCG-induced immunological response, as this may provide information regarding dose and/or number of instillations, adapted to individual patients. In general, immunomonitoring, emphasizing the kinetics of a local (BCG-specific) production of cytokines and mobilization of immunocompetent cells during the course of BCG treatment, should be considered as an attempt to address the working mechanism of BCG or BRMs in general. Such an approach may provide a rationale for this therapy and the clinical (prognostic) parameters to monitor.

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