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Increased urinary albumin indicating urothelial leakage following intravesical bacillus Calmette-Guérin therapy for superficial bladder cancer

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Summary. This study on the increase in albumin in the urine of patients with superficial bladder cancer after intravesical bacillus Calmette-Guérin (BCG) treatment was initiated on the basis of two facts. First, extravasation of serum albumin could be expected as a result of the BCG-induced delayed-type hypersensitivity reaction in the bladder wall. Second, appearance of albumin in the urine was a possibility as cytokines also appear in the urine, although probably after being produced suburothelially by infiltrating leukocytes. Albumin and the cytokines interleukin (IL)1β, IL2, IL6, and tumor necrosis factor alpha (TNFα) were determined in urine from 20 patients treated with 6 weekly intravesical BCG instillations, collected prior to each instillation and 2, 4, 6, 8, 12, and 24 h thereafter. The mean concentration of albumin in pre-therapy specimens was 112 ± 118 (range 2–432) μg albumin/ml urine, approximating 14 ± 14 μg/μmol creatinine (creat) ($n = 15$), which was comparable to the mean pre-instillation value of 16 ± 32 μg/μmol creat ($n = 96$). A significant increase in urinary albumin during the 6 weeks of BCG treatment was observed ($P < 0.001$). However, a large variation existed between individual patients and in some patients no reaction was seen. Maximum albumin concentrations were observed after instillations 3–6. A significant correlation between albumin and concentration of the cytokines IL1β, IL2, IL6, and TNFα was found ($P < 0.01$), correlation coefficients (r) being 0.56, 0.56, 0.67, and 0.71 ($n = 418$), respectively. During the first 24 h after instillation cytokines and albumin peaked in the following order: TNFα → IL2 → albumin → IL6 → IL1β. TNF peaked most frequently after 2–4 h and IL1β after 6 h, while IL2, albumin, and IL6 peaked between these time points. In conclusion, the presence of albumin in urine indicates a "leakiness" of the bladder wall after repeated BCG instillations. Since albumin was shown to be stable in urine and the assay is relatively simple and cheap, it may be performed in most hospitals. This will allow large-scale investigations of the correlation between elevation of urinary albumin and (tumor) response on BCG therapy.

Key words: Albumin – Bacillus Calmette-Guérin – Bladder neoplasms – Immunotherapy – Urine

Intravesical treatment with bacillus Calmette-Guérin (BCG) is an effective treatment in patients with carcinoma in situ (Tis) and papillary (Ta/T1) superficial bladder cancer [14, 18]; however a subpopulation of patients remains with tumors refractory to BCG therapy. This demonstrates the need for accurate prognostic indicators with sufficient specificity to identify nonresponders early, so that alternative therapeutic regimens can be initiated before development of muscle-invasive or metastatic disease.

Although the actual mechanism of the BCG-associated antitumor activity is not known, most of the available evidence indicates an immune-mediated mechanism [25]. Therefore factors measuring the immune response to BCG, including purified protein derivate (PPD) skin testing and granuloma formation in bladder biopsy specimens, have been investigated as potential prognostic indicators of a response to BCG therapy. However, the data appear to be conflicting [1, 7, 12, 13, 15, 19, 22]. In a recent report Torrence et al. [29] showed that with extended follow-up the level of statistical significance of the correlation between PPD skin reactivity or presence of granulomas with tumor-free status may become borderline or be lost and that these parameters should not be considered useful as prognostic indicators in individual patients. Evaluation of urinary cytokines (also indicating an immunological response to intravesical BCG) may be advantageous over the PPD skin reaction since they reflect the local immunological reaction. Moreover, cytokines reflect the response of the entire bladder, while demonstration of granulomatous cellular infiltration by bladder biopsy specimens may fail due to focal distri-

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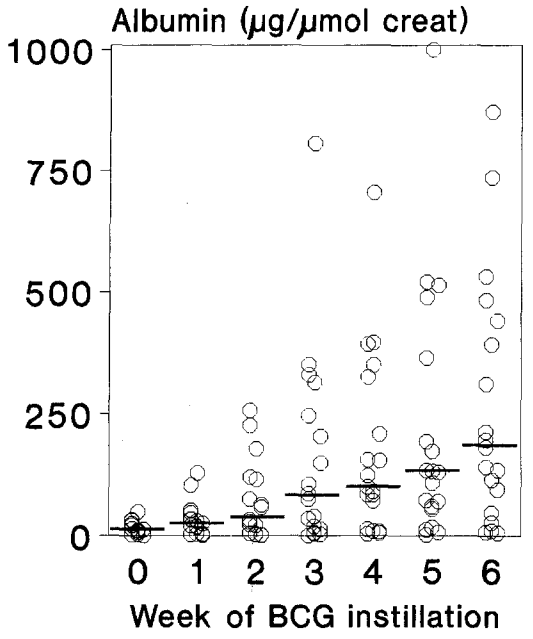

Fig. 1. Urinary albumin concentrations in individual patients during a 6-week course of BCG treatment, showing a significant increase after weeks 2 ($P < 0.05$, $n = 17$), 3 ($P < 0.01$, $n = 18$), and 4, 5, and 6 ($P < 0.001$, $n = 19$), compared with values prior to therapy (0; $n = 15$). Measurements were performed in serially collected urine specimens (2, 4, 6, 8, 12, and 24 h) after BCG instillations. The highest concentration measured after each weekly (*1–6*) BCG instillation is shown for each patient. *Horizontal lines,* Median

bution [17, 24]. The induction of urinary IL2 and TNF in the urine has been suggested to be of prognostic significance [27].

In the case of a delayed-type hypersensitivity (DTH) reaction an increased content of serum albumin in the extravascular tissue appears which originated from the circulation and probably gained access to the extravascular spaces via gaps between endothelial cells. In mice this event has been suggested to be necessary for the initiation of DTH-like reactions [16, 30]. Local vascular permeability is thought to facilitate leukocyte entry into the extravascular tissue. The repeated intravesical administration of BCG in the bladder may be considered as an immunological antigen challenge to the sensitized patient resulting in a DTH-like reaction in the bladder wall. The reaction of antigen-specific T lymphocytes has been illustrated by the induction of IL2 and activation markers on T cells [4, 9, 26]. Consequently the appearance of albumin in the extravascular tissue within the bladder wall after BCG instillation was expected. Since after repeated instillations cytokines appear in the urine [3, 6, 23, 26, 27] – although they are probably produced within the bladder mucosa by the infiltrating leukocytes [6] – we initiated investigations into the presence of albumin in the urine after intravesical instillation of BCG. The kinetics of albumin appearance in relation to cytokines in the urine was studied in order to gain insight into the process of local cytokine production and the apparent "leakage" into the urine after intravesical BCG instillation. Furthermore the stability of urinary albumin was tested and the correlation between urinary albumin and urinary cytokines was evaluated. Since albumin determination in urine is a routine assay in most hospitals, this may allow large-scale investigations into the predictive value of this parameter.

Materials and methods

Patients and treatment

Urine was obtained from 20 patients with primary (except solitary TaG1) or recurrent superficial bladder carcinoma [3 TaG1, 7 TaG2, 2 TaG3, 1 T1G2, 4 T1G3 (2 associated with TisG3), 3 TisG3 only] treated with 6 weekly intravesical BCG instillations in 50 ml 0.9% saline after complete transurethral resection of papillary tumor(s).

Detection of albumin and cytokines in urine

Urine was basically collected according to the following scheme: prior to BCG instillation and 2,4, 6, 8, 12, and 24 h thereafter, during 6 weekly instillations. The total numbers of samples obtained for the respective time points were 96, 38, 104, 71, 31, 35, and 43. Urine was immediately frozen to −20°C. It was then thawed and centrifuged at 4°C and stored in aliquots at −20°C until analysis.

To measure albumin in urine specimens a nephelometric microalbumin assay was performed using N-antiserum from rabbit to human albumin (Behringwerke, Marburg, Germany) and a Behring nephelometer 100. With N-protein (Behringwerke) as albumin standard, the albumin concentration in urine specimens was determined by measuring each specimen in two different dilutions which provided an albumin concentration within the measurable range. Using this method the albumin level in urine from normal individuals was found to be 2–40 mg/l per 24 h, which is in accordance with levels reported by others [28].

The cytokines IL1β, IL2, IL6, and TNFα in urine were determined as reported previously [27] with commercially available, highly specific and reproducible ELISAs using an oligoclonal system (Medgenix, Fleurus, Belgium). Using this method pre-instillation values of 3.7 ± 6.2 pg/µmol creatinine (creat) IL1, 0.0 ± 0.1 U/µmol creat IL2, 5.9 ± 12.8 pg/µmol creat IL6, and 0.2 ± 0.8 pg/µmol creat TNF ($n = 134$) were established. Albumin and cytokine data were standardized to urine creatinine.

Statistical analysis

Correlation coefficients were calculated by the Pearson product-moment correlation method. Differences in urinary albumin levels prior to and after BCG instillations were analyzed using the Wilcoxon two-sample test for the unpaired case.

Results

The mean concentration of albumin in pre-therapy specimens was 112 ± 118 (range 2–432) µg albumin/ml urine, approximating 14 ± 14 µg/µmol creat ($n = 15$), which was comparable to the mean pre-instillation value of 16 ± 32 µg/µmol creat ($n = 96$). The kinetics of albumin increase during the 6 weeks of BCG treatment are shown in Fig. 1. Generally an increase during the 6 weeks was observed as represented by the median values in Fig. 1. However, a large variation existed between individual

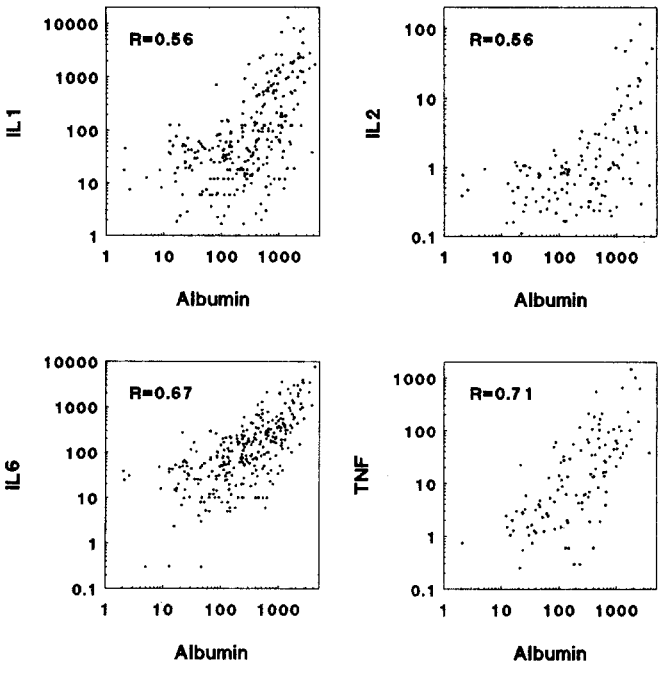

Fig. 2. Correlation between urinary albumin and cytokines after intravesical BCG instillations. Albumin and cytokine (IL1β, IL2, IL6, TNFα) concentrations, determined in serially collected urine specimens (prior to, and 2, 4, 6, 8, 12, and 24 h) after 6 weekly BCG instillations, were significantly correlated ($P<0.01$, $n=418$). Values of the correlation coefficient r are shown in corresponding parts of the figure. Some of the data are not depicted since the samples were negative for either albumin or cytokines. Albumin is expressed as μg/μmol creatinine (creat); IL1β, IL6, and TNFα as pg/μmol creat; IL2 as U/μmol creat

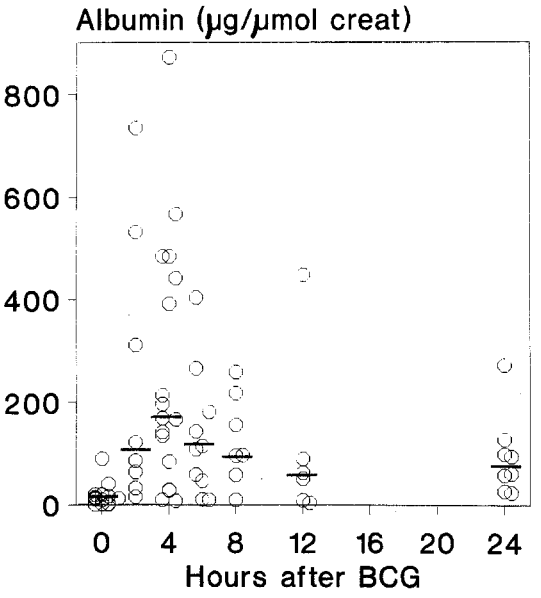

Fig. 3. Urinary albumin concentrations in individual patients during the first 24 h after BCG instillation 6, showing a significant increase after 2 h ($P<0.001$, $n=8$), 4 h ($P<0.001$, $n=17$), 6 h ($P<0.01$, $n=10$), 8 h ($P<0.01$, $n=7$), and 24 h ($P<0.001$, $n=8$), compared with values prior to the sixth instillation (0; $n=16$). *Horizontal Lines,* Median

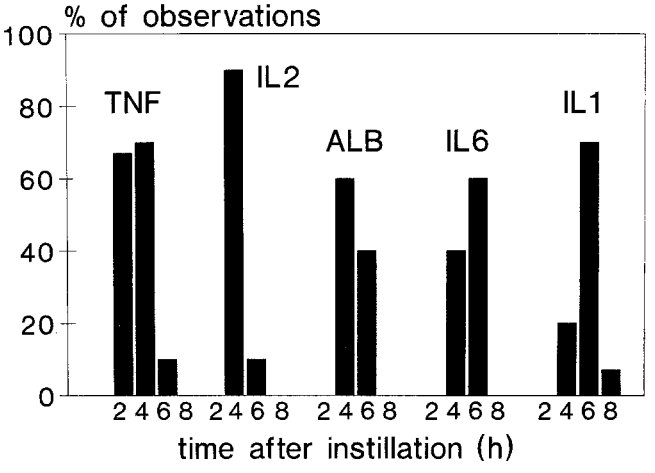

Fig. 4. Time of occurrence of cytokine and albumin peak levels in urine after repeated BCG instillations. For 10 patients (selected for showing a clear increase in urinary albumin as well as all cytokines and for availability of samples for most time points of collection during the first 8 h after BCG instillations 5 and 6), the time of occurrence of the cytokine/albumin peak was determined. The number of peaks per time point was expressed as a percentage of the total number of samples evaluated at that time point. It is seen that cytokines and albumin peaked in the order TNFα → IL2 → albumin → IL6 → IL1β. TNF peaked most frequently after 2–4 h and IL1 after 6 h, while IL2, albumin, and IL6 most frequently peaked between these time points

patients and in some patients no reaction was seen. Depending on the patient, maximum concentrations were observed after instillations 3–6, which generally correspond with kinetics of urinary cytokines [3, 6, 23, 26, 27].

In the urine of patients with clearly elevated albumin levels at least three of the urinary cytokines IL1β, IL2, IL6, or TNFα were also elevated, while in the urine of patients not showing an albumin increase cytokine levels were low or absent, suggesting a correlation. Indeed a significant correlation between urinary albumin concentration and concentration of urinary cytokines IL1, IL2, IL6, and TNF was found ($P<0.01$): correlation coefficients (r) were 0.56, 0.56, 0.67, and 0.71 ($n=418$), respectively (Fig. 2).

As shown in Fig. 1, elevation of urinary albumin occurred after repeated BCG instillations. Figure 3 shows that during the first 24 h after instillation the highest albumin levels are generally found in specimens collected 4 h after start of the BCG instillation. After 24 h the albumin concentration was still significantly increased.

For urine samples obtained after instillatons 5 or 6 that were positive for albumin as well as for IL1β, IL2, IL6, and TNFα the time of occurrence of peak levels during the first 24 h after the start of BCG instillation was determined. Cytokines and albumin peaked in the following order: TNFα → IL2 → albumin → IL6 → IL1β. TNF peaked most frequently after 2–4 h and IL1 after 6 h, while IL2, albumin, and IL6 most frequently peaked between these time points (Fig. 4).

In contrast to cytokines in the urine (data not shown), albumin was stable in the urine during 24 h of incubation at 4, 20, and 37°C (Table 1).

Table 1. Stability of albumin in urine

Time (h)	Temperature (°C)		
	4	20	37
0.5	102	n.d.	100
2	102	98	102
6	101	99	102
12	101	100	99
24	97	100	98

Mean albumin concentration of four albumin-positive urine samples after incubation at 4, 20 or 37 °C during 0.5, 2, 6, 12, or 24 h. Data are expressed as a percentage of the pre-incubation value. *n.d.*, Not determined

Discussion

In this study data are presented for the first time on the elevation of albumin levels in urine from bladder cancer patients, indicating urothelial leakage as a result of intravesical BCG treatment. The kidney serves to conserve albumin and other high molecular weight proteins within the plasma and the normal concentration of serum albumin in urine is therefore low, i.e. 30 μg/ml [28]. The mean pre-therapy value of 112 ± 118 (range 2–432) μg/ml ($n = 15$) we found in the patients may be dependent on the extent of the tumor and/or transurethral resection, although no clear association could be detected.

Prior to its appearance in the urine, extravasation of albumin into the extravascular tissue of the bladder mucosa was presumed to occur as a result of the DTH-like reaction caused by repeated BCG administration [16, 30]. Although in this case probably restricted to the bladder, the phenomenon may essentially be comparable to the capillary leak syndrome associated with endotoxemia or recombinant IL2 cancer therapy [2, 8, 21]. The pathophysiologic mechanisms may include histamine-associated effects, prostaglandin-mediated changes, direct cytokine effects on the endothelium, and cell-mediated effects on endothelial cells, either through direct lysis or via mechanisms mediated by oxygen free radicals [21]. In this study TNF was the first cytokine to appear in the urine during the 24 h after BCG instillation (Fig. 4), suggesting an inducing role of TNF in capillary leakage resulting in extravasation of albumin, and in urothelial leakage resulting in the appearance of cytokines and albumin in the urine. This is in agreement with the key role of TNF in endotoxemia-associated capillary leak syndrome [2, 11]. Moreover, Engelhardt et al. [10] also found TNF production to precede the release of other cytokines in cancer patients after intravenous administration of endotoxin. TNF has been reported to affect epithelial tight junctions resulting in an increase in transepithelial permeability [20]; hence in this system TNF might possibly increase urothelial permeability, explaining why cytokines are detectable in the urine although probably produced within the bladder mucosa by the infiltrating leukocytes [6]. In this way cells throughout the bladder mucosa may come into contact with cytokines. The importance of this phenomenon for the mode of action and efficacy of BCG therapy is not clear.

The BCG treatment schedule is mainly empirical. An early identifiable factor for ascertaining whether an immune response is initiated by the initial 6 weekly instillations most commonly used in clinical practice is thus urgently needed. The PPD skin reaction and granuloma formation in the bladder wall have been of little value to date [29]. Large-scale investigations on the predictive significance of urinary cytokines (de Boer et al., unpublished data) have been hampered due to restrictions on urine collection related to the instability of cytokines in urine, the diversity in cytokine assays and their cost, and the transient occurrence of urinary cytokines. Since urinary albumin is stable in urine (Table 1) and the assay can be routinely performed in most hospitals, the predictive potency of this marker could be investigated in large groups of patients. This study suggests albumin measurement in urine collected from 2 to 6 h after the start of BCG instillation (Fig. 4). Additionally it could be investigated whether there is any value in giving patients who do not show albumin elevation after 6 weeks an adjusted treatment (i.e. a prolonged schedule) immediately, in order to obtain an immunological reaction and subsequent antitumor response. Although the correlation between the elevation of urinary albumin and clinical response remains to be investigated, the data on this small group of patients may possibly suggest an association: of the 9 patients with albumin levels above the median after week 6 (see Fig. 1) 2 (22%) had early recurrence of disease (≤ 6 months) and 7 (78%) did not, while of the 9 patients with albumin below the median 4 (44%) had early recurrence and 5 (56%) did not.

Although BCG therapy may be optimized by monitoring albumin in the urine as suggested above, a subpopulation of clinically nonresponding patients may remain despite showing an immune response to intravesical BCG. This may partly be the result of intrinsic factors of the tumor. In order to discriminate these patients the antitumor reaction itself needs to be measured. For this, elucidation of the antitumor mechanism of intravesical BCG therapy in superficial bladder cancer is required.

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