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Leukocytes in the urine after intravesical BCG treatment for superficial bladder cancer

A flow cytofluorometric analysis

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Summary. Cellular immunologic reactions occurring in the bladder after intravesical treatment with bacillus Calmette-Guérin (BCG) were investigated by flow cytofluorometric analysis of leukocytes present in the urine. Urine specimens from 11 superficial bladder cancer patients were collected before and 5, 24, 48 and 72 h after repeated BCG instillations. Monoclonal antibodies specific for granulocytes, monocytes/macrophages, and T- and B-lymphocytes were used to characterize and quantify leukocyte subpopulations. The total number of cells in urine was found to be 2- to 485-fold increased 24 h after BCG administration. The predominant cell type present was the polymorphonuclear granulocyte, probably representing a defense mechanism against mycobacteria. The main mononuclear leukocytes in urine specimens were monocytes/macrophages and T-lymphocytes, indicating an ongoing immune response in the bladder wall. Although percentages of lymphocytes were low, T- and B-cells could be identified using a selective cell measurement procedure. In conclusion, a clear increase in the numbers of granulocytes, monocytes/macrophages and T-lymphocytes in urine after intravesical BCG administration was demonstrated, indicating local activation of the immune system.

Key words: Intravesical BCG therapy – Bladder cancer – Urine sediment – Flow cytofluorometry – Cellular immunological reaction

Since the report of Morales et al. [15] in 1976, intravesical treatment with bacillus Calmette-Guérin (BCG) has been proved to be an effective therapy for superficial bladder cancer [5, 10]. However, due to the complexity of the immunological effects that BCG can exert, the mechanism of the antitumor activity of BCG is still unclear. Depending on the route of administration, BCG is known to stimulate various parts of the immune system both at the humoral and at the cellular level. The cell mediated immunity seems to be important for the activity of BCG as an immunotherapeutic agent against cancer [4, 7, 17].

In the bladder, repeated administration of BCG can induce inflammatory reactions. Histologically these reactions have been described as mononuclear cell infiltrates with granulomatous characteristics [8, 15, 20]. Recent immunohistochemical studies of this BCG-induced reactions have shown that the major infiltrating cell type is the T-lymphocyte. Macrophages and B-lymphocytes have been found in lower amounts. Induction of major histocompatibility complex class II (HLA-DR) antigen expression on urothelial cells as a result of intravesical BCG administration has also been reported [13, 16, 19]. Another evidence of a local immunological effect is the presence of interleukin 2 in the urine after intravesical administration of BCG [6, 9].

In the present study the immunological effects of BCG administration in the bladder were investigated by flow cytofluorometric analysis of leukocyte subpopulations present in the urine after repeated BCG instillations. Probably the leukocytes in urine are a reflection of the cellular immunological reactions taking place in the bladder wall of superficial bladder cancer patients during treatment with BCG.

Materials and methods

Patients and treatment

The presence of leukocytes in the urine was studied in 11 patients with superficial bladder carcinoma (stage pTa, pT1 and/or carcinoma in situ) who were intravesically treated with BCG after transurethral resection of papillary tumor(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. Some patients were treated with more than six intravesical BCG instillations, either in a second series of 6 weekly administrations or with instillations every 2 weeks or once a month.

Collection and preparation of cells from urine

Voided urine specimens were collected before and approximately 5, 24, 48 and 72 h after intravesical BCG administration. These specimens were collected after the fifth or more BCG instillation.

Table 1. Specificity of monoclonal antibodies for identification of leukocytes

mAb	Anti-CD	Cell type	FITC-/PE-conjugated
Anti-Leu-4	CD3	T lymphocytes	FITC
Anti-Leu-12	CD19	B lymphocytes	PE
Anti-Leu-M3[a]	CD14	Monocytes/macrophages	PE
FK32	CD15	Granulocytes	-[b]

[a] Simultest Anti-Leukocyte FITC + Anti-Leu-M3 PE, Becton Dickinson
[b] FITC-conjugated $F(ab')_2$ fragments from rabbit IgG anti-mouse was used as second antibody
CD, Cluster designation

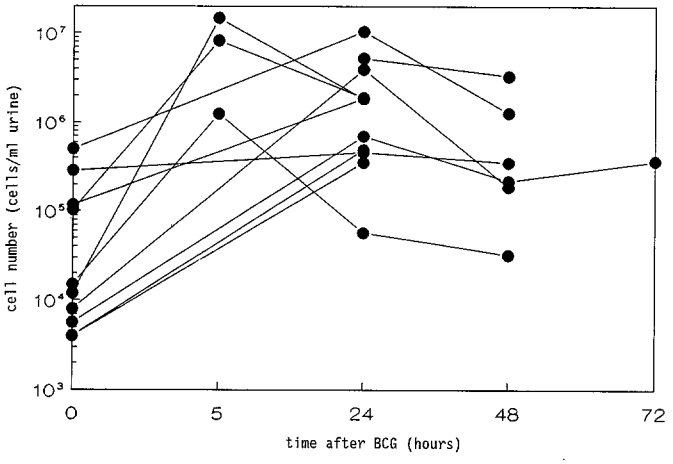

Fig. 1. Increase in the total number of viable cells in patients' urine after repeated BCG instillations

The voided urine was centrifuged (10 min, 300 g) and the cellular sediment was suspended in RPMI 1640 tissue culture medium (Gibco Europe b.v., Breda, The Netherlands), supplemented with 10% fetal calf serum (FCS; Gibco), penicillin (100 IU/ml), and streptomycin (100 µg/ml), hereafter referred to as complete RPMI. To obtain high cell viability all urine specimens were immediately cooled and processed at 4°C or on melting ice.

After washing once in complete RPMI, viable nucleated non-squamous cells in urinary sediments were counted by trypan blue (0.5%) exclusion, and the cell suspensions were adjusted to 1×10^7 cells/ml in RPMI with 2% FCS for immunofluorescence staining or cytospin preparation.

Immunofluorescence staining

The specificity of monoclonal antibodies (mAbs) used for identification of different subpopulations of leukocytes from urine samples is shown in Table 1. MAb FK32 was kindly provided by Dr. F. Koning (University of Leiden, Leiden, The Netherlands). All other mAbs were obtained from Becton Dickinson (Etten-Leur, The Netherlands).

Incubation of 5×10^5 cells in 50 µl RPMI with 2% FCS and 50 µl mAb was performed in 96-well Microtest III assay plates (Becton Dickinson). The mAbs were diluted in Dulbecco's phosphate buffered saline (Gibco) with 0.01% NaN_3 and 2% FCS, hereafter referred to as DPBS+. Final dilutions of mAbs used were 1:100 (FK32) or 1:5. After 30 min incubation at 4°C the cells were washed three times (centrifugation 300 g) with 200 µl DPBS+. A second incubation with FITC-conjugated $F(ab')^2$ fragments from rabbit anti-mouse IgG (Organon Teknika, West Chester, Pa, USA), diluted 1:40 in DPBS+, was performed with cells that had been incubated with mAb FK32, followed by washing three times with DPBS+. Finally all cells were fixed in 100 µl paraformaldehyde solution (0.25% in DPBS+) and stored in the dark at 4°C. As negative controls, mAbs of irrelevant specificity (anti-KLH) but appropriate isotypes (Becton Dickinson), or FITC-conjugated rabbit anti-mouse mAb only, were used in all experiments.

Flow cytofluorometry and analysis

Twelve thousand cells were measured with a fluorescence-activated cell sorter (FACScan, Becton Dickinson Immunocytometry System, Mountain View, Calif. USA). Storage and analysis of the data was performed with FACScan software on a Hewlett Packard 9920S computer. For each cell four parameters were measured simultaneously; forward scatter (FSC), indicating cell-volume; side scatter (SSC), indicating cell-structure; green fluorescence (FITC); and red fluorescence (PE). Selection of cells for fluorescence analysis was performed within the scattergram (FSC vs SSC). The percentage of cells of a specific leukocyte subset was determined by analysis of fluorescence histograms.

Cytospin preparations and staining

Approximatley 2×10^5 cells in 100 µl RPMI were cytocentrifuged per slide. The slides were air dried, fixed for 5 min in methanol and subsequently stained with May-Grünwald Giemsa staining (Fluka, Buch, Switzerland).

Quantitative assessment of the cells was performed as a control for flow cytofluorometric data. Percentages of each cell type were determined by counting of cells in five microscopic fields (×400), resulting in a mean total cell count of 293 (range 146–500) per sample.

Results

Number of cells in the urine and viability

The amount of viable cells in urine specimens collected from 11 patients 24 h after the fifth or more intravesical instillation of BCG was 2–485 times higher than in pretreated specimens. The increase in the number of cells within 24 h after BCG instillation was followed by a gradual decrease (Fig. 1). There was substantial variation between individual patiens in the number of cells obtained from the urine specimens, both before and after BCG instillation.

The viability of cells in the urine samples is shown in Table 2. The cell viability in samples collected before BCG instillation was lower (65%) than in samples collected after BCG instillation (at 24 h 87%).

Identification of leukocyte subpopulations by flow cytofluorometry

The presence of granulocytes, monocytes/macrophages, and T- and B-lymphocytes was investigated in the urine of eight patients. In the scattergram (FCS vs SSC) of the cells

Table 2. Viability of cells in urine after repeated intravesical BCG instillations in superficial bladder cancer patients

Time before/after BCG[a]	Viability of total cell population[b]
0	65 ± 27 (11)[c]
5	98 ± 2 (3)
24	87 ± 21 (11)
48	89 ± 15 (7)
72	73 ± 33 (2)

[a] Hours before (time = 0) and after instillation no. 5 or higher
[b] Viable nucleated cells were counted by trypan blue (0.5%) exclusion
[c] Mean percentage viable cells ±SD (*n*)

from urine four different cell clusters could be distinguished (Fig. 2a). However, in the fluorescence histogram of the total cell population only two cell types could be identified: granulocytes and monocytes/macrophages (Fig. 3) The fluorescence of the cells in the four clusters was therefore investigated separately. The cells in the clusters were selected for analysis by setting four gates, as shown in Fig. 2a. Because the number of cells in gates 2, 3, and 4 was not sufficient for analysis, selective cell measurement was performed. With this method we selected for measurement of the cells outside gate 1 only, resulting in an increased number of cells within gates 2, 3, and 4 (Fig. 2b). Fluorescence histograms of the cells in the four gates are shown in Fig. 4. The cells in both gate 1 and gate 2 were predominantly granulocytes. Monocytes/macrophages were the major cell type present in gate 3. The cells in gate 4 mainly consisted of T-lymphocytes, together with a few B-lymphocytes. Thus, in addition to granulocytes and monocytes/macrophages, a relatively small number of lymphocytes was also present in the urine specimens of BCG-treated patients. The cell located in the lower left corner in the scattergram (Fig. 2a) did not react with any mAb. It is likely that these dots represent dead cells, erythrocytes, and cell debris; these cells were excluded from analysis.

The relative quantities of leukocyte subpopulations in urine determined with flow cytofluorometry is presented

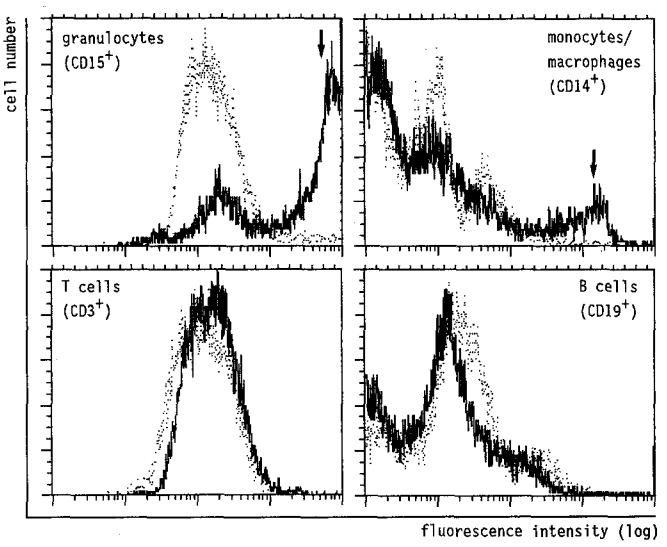

Fig. 3. Fluorescence histograms of the total cell population in urine, showing the presence of granulocytes and monocytes/macrophages. Data for cells obtained from urine collected 24 h after sixth BCG instillation. —— = Specific mAb; ····· = control mAb; *arrow* indicates presence of specific leukocyte subtype

in Table 3, which shows data relating to the sediments in the urine collected from three representative patients before and 24 h after BCG instillation. Granulocytes were the predominant cell type; monocytes/macrophages and T-lymphocytes were present in much lower amounts, and B-lymphocytes rarely. The number of cells of the various subpopulations of leukocytes per milliliter urine increased after BCG instillation (data from the same three patients are shown in Table 4).

Cell types identified by examination of cytospin preparations

Examination of May-Grünwald-Giemsa-stained cytospin preparations, performed as a control for flow cytofluorometry, confirmed that the main cell type in the urinary sediments was the granulocyte. The presence of monocytes/macrophages and lymphocytes was also confirmed.

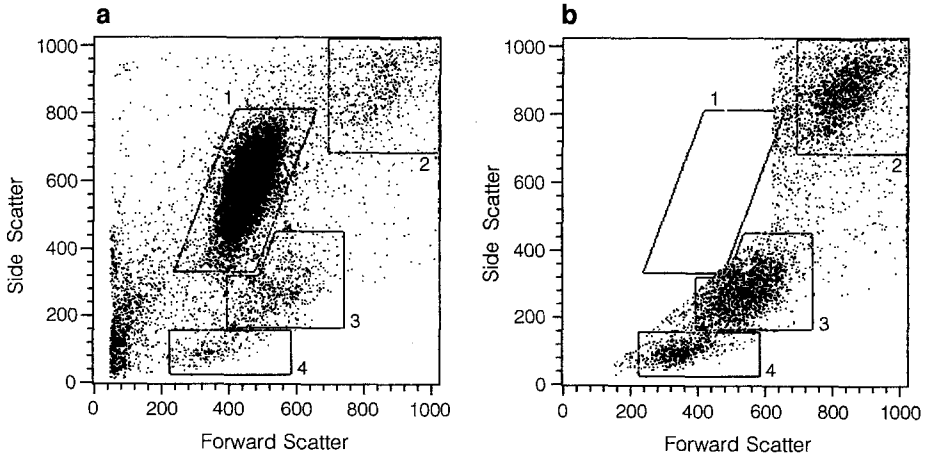

Fig. 2a, b. Scattergram (FSC vs SSC) of cells from urine collected 24 h after sixth BCG instillation. **a** Four cell clusters can be distinguished, surrounded by a gate for fluorescence analysis (the dots in the *lower left* corner represent dead cells, erythrocytes and cell debris). **b** By selective cell measurement the number of cells within gates 2, 3, and 4 can be increased for proper fluorescence analysis

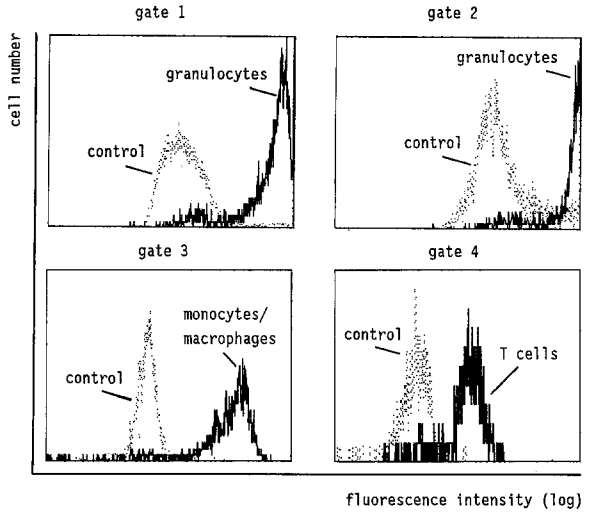

Fig. 4. Fluorescence histograms of the major cell type present in the four cell clusters, showing the intensity of fluorescence in the cells after labeling with specific mAb as opposed to after labeling with control mAb. Gates 1, 2, 3, and 4 were set in the scattergram (FSC vs SSC) as shown in Fig. 2. Data for cells obtained from urine collected 24 h after sixth BCG instillation

Additionally, eosinophils and urothelial cells, not identified with flow cytofluorometry, were observed. Eosinophils were incidentally present. The number of urothelial cells counted in cytospin preparations of pretreated specimens from four patients investigated was 6%, 19%, 32%, and 48% respectively. In 24-h specimens the number of urothelial cells was always low (0%–5%). An example of a cytospin preparation is shown in Fig. 5.

The relative quantities of the various cells identified in the cytospin preparations from urine specimens from the three patients listed in Table 3 are shown in Table 5. The percentages of granulocytes were in general higher here then the percentages determined using flow cytofluorometry, the mean percentage of monocytes/macrophages was comparable, while the number of lymphocytes counted in cytospin preparations was too low to confirm the flow cytofluorometrically based percentages.

Discussion

With regard to the mechanism of action of BCG in prevention of papillary recurrences and cure of carcinoma

Table 3. Relative quantities of leukocyte subpopulations present in urine after intravesical BCG administration

Patient	Instillation number	Time before/ after BCG	Granulocytes[a] (%)	Monocytes/ macrophages[a] (%)	Lymphocytes[b]	
					T (%)	B (%)
A	6	0	54.2	3.7	n.d.	n.d.
		24	65.0	9.6	1.8	0.1
B	12	0	80.8	0.4	0.5	0.1
		24	58.2	1.8	0.5	0.2
C	19	0	26.2	16.2	3.4	0.0
		24	80.0	5.2	0.6	0.0

[a] Determined by flow cytofluorometric analysis of the total cell population
[b] Determined by flow cytofluorometric analysis of the cells in the lymphocyte gate
Numbers of cells expressed as percentages of total cell population.
n.d. not detectable (amount of cells in the lymphocyte gate not sufficient for analysis)

Table 4. Increase in the total number of cells leukocyte subpopulations per mililiter urine after intravesical BCG administration

Patient	Instillation number	Time before/ after BCG	Granulocytes[a]	Monocytes/ macrophages[a]	Lymphocytes[b]	
					T	B
A	6	0	8.1×10^3	5.6×10^2	n.d.	n.d.
		24	3.7×10^4	5.4×10^3	4.4×10^3	3.4×10^1
B	12	0	4.0×10^5	2.1×10^3	2.4×10^3	3.2×10^2
		24	6.1×10^6	1.9×10^5	5.1×10^4	1.7×10^4
C	19	0	1.0×10^3	6.1×10^2	1.4×10^2	1.2×10^0
		24	3.9×10^5	2.6×10^4	3.0×10^3	5.4×10^1

[a] Determined by flow cytofluorometric analysis of the total cell population
[b] Determined by flow cytofluorometric analysis of the cells in the lymphocyte gate
n.d. not detectable (amount of cells in the lymphocyte gate not sufficient for analysis)

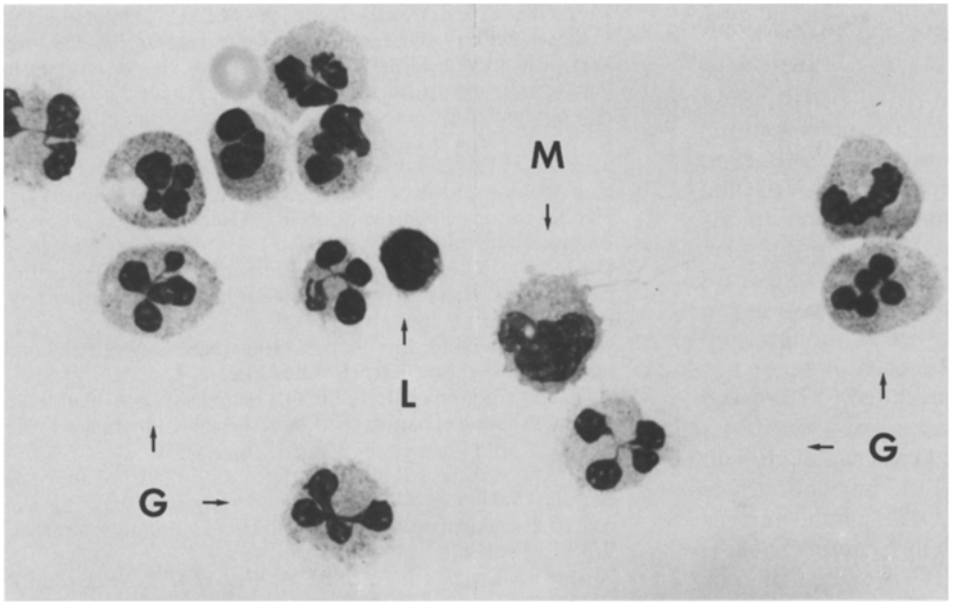

Fig. 5. Cytospin preparation of the cells in urine collected 24 h after sixth BCG instillation. In addition to the large number of polymorphonuclear granulocytes (*G*), monocytes/macrophages (*M*) and lymphocytes (*L*) are present. May-Grünwald-Giemsa staining; magnification ×1,000

Table 5. Cell types identified in cytospin preparations of urine after intravesical BCG administration

Patient	Instillation number	Time before/ after BCG	Granulocytes (%)	Monocytes/ macrophages (%)	Lymphocytes (%)	Eosinophils (%)	Urothelial cells (%)
A	6	0	77.1	2.0	0.0	1.5	19.0
		24	90.6	7.7	0.2	0.0	1.4
B	12	0	90.2	0.9	2.2	0.6	6.0
		24	94.3	3.3	0.5	0.0	0.0
C	19	0	54.2	12.5	0.6	0.6	32.1
		24	86.0	6.5	1.5	0.6	5.3

Numbers of cells expressed as percentages of the total number of cells counted

in situ of the bladder, the ultimate effector mechanism is not yet known [7, 20]. Both cells and/or cytokines may be responsible for the killing of tumor cells. Cells of the immune system with a cytotoxic potential against tumor cells are cytotoxic T cells, NK cells, macrophages, and granulocytes. From immunohistochemical studies on bladder biopsies from patients it is known that cytotoxic/suppressor T cells and macrophages are present in the BCG-induced cell infiltrate. However, the majority of infiltrating leukocytes were T-lymphocytes of the helper/inducer subset. In these biopsies few NK cells were detected. No substantial numbers of granulocytes were reported [16, 19]. Weiner et al. [21] reported that T helper/inducer cells were the main subset of T-lymphocytes present in bladder irrigation specimens of patients after BCG therapy. At present, the actual function of the leukocytes present in the bladder wall after BCG administration is not clear.

Investigating the cellular immunological reactions exerted by intravesical BCG administration by means of FACS analysis of cells in urine, we predominantly found granulocytes. Large quantities of these cells were observed especially shortly after the BCG administration, probably representing a defense mechanism against the mycobacteria [11]. Polymorphonuclear neutrophils are known to be the first cells to appear in tuberculous exudates [14]. Antony et al. [1] have reported that polymorphonuclear neutrophils may play an important role in the initiation of inflammatory responses to BCG. Granulocytes are potentially cytotoxic for tumor cells in vitro, but their role in the antitumor reaction in bladder cancer is not known [2].

The percentages of granulocytes as determined by flow cytofluorometry (Table 3) were lower than the percentages determined by microscopic counting in cytospin preparations (Table 5). This may be explained by loss of surface antigens as a result of the presence of granulocytes in urine. However, it has been demonstrated that characterization of mononuclear leukocytes from urine with mAbs against surface antigens is possible [12, 18, 21].

Two populations of granulocytes could be discriminated with FACS analysis (Fig. 2a, gates 1 and 2). The cells in gate 2 may represent clumps of granulocytes, since they exhibit higher fluorescence than the granulocytes in gate 1 after labeling with the mAb FK32 (Fig. 4). Additionally,

the volume (FSC) of these cells is increased (Fig. 2a). Monocytes/macrophages were also detected in gate 2 in some specimens. Clumps of leukocytes were also observed in cytospin preparations (Fig. 5).

Besides granulocytes, monocytes/macrophages and T-lymphocytes were the main mononuclear leukocytes present in urine specimens after repeated BCG instillations, indicating an ongoing immune reaction in the bladder. Although the percentages of lymphocytes were low, T- and B-subsets could be analyzed using a selective cell measurement procedure (Fig. 2b). Phenotype analysis of other lymphocyte subsets, e.g., helper/inducer and suppressor/cytotoxic T cells and NK cells, is needed to investigate the role of T-lymphocytes in the BCG-induced antitumor activity [7, 17].

Variable percentages of urothelial cells can be present in urine specimens collected prior to BCG instillation. The extensive increase in the number of cells of the various leukocyte subpopulations after BCG instillation (Table 4) explains the decrease in percentages of urothelial cells (Table 5).

We do not know if the leukocytes in the urine are actually a reflection of the leukocytes present in the bladder wall at the same time. The predominant presence of macrophages instead of monocytes, observed in cytospin preaparations, suggests that the cells originated in the bladder wall. Moreover, few erythrocytes were present. The number of macrophages in urine specimens, however, was higher than the number of T-lymphocytes after BCG administration. This is in contradiction with the reports on bladder wall biopsies in which T-cells predominated [16, 19]. The difference in the time of collection may be an explanation of this.

In conclusion, a clear increase was demonstrated in the number of granulocytes monocytes/macrophages, and T-lymphocytes in patients' urine after repeated intravesical administration of BCG, indicating local activation of the immune system. The functional properties of these leukocytes are currently under investigation.

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