BCG prophylaxis in bladder cancer produces activation of recruited neutrophils

Francesca Vita, MD,[1] Salvatore Siracusano, MD,[2] Rita Abbate, MD,[1] Stefano Ciciliato, MD,[2] Violetta Borelli, MD,[1] Maria Rosa Soranzo, MD,[1] Giuliano Zabucchi, MD[1]

[1]Departments of Physiology, Pathology and Life Sciences, University of Trieste, Trieste, Italy
[2]Department of Urology, University of Trieste, Trieste, Italy

VITA F, SIRACUSANO S, ABBATE R, CICILIATO S, BORELLI V, SORANZO MR, ZABUCCHI G. BCG prophylaxis in bladder cancer produces activation of recruited neutrophils. The Canadian Journal of Urology. 2011;18(1):5517-5523.

Introduction: *Bacillus Calmette-Guérin (BCG) is used to treat high risk superficial bladder cancer, but its anti-tumor effect remains incompletely defined. Recently a role for polymophonuclear (PMN) neutrophils has been suggested. To investigate the role of granulocytes, we monitored the activation state of these cells in the urine of BCG-treated patients.*

Materials and methods: *Ten patients with stage T1, grade 3 (T1G3) transitional cell carcinoma received an 8 week course of BCG after undergoing transurethral resection of the bladder. Cytological and enzymatic analyses of urine samples collected before and 2 hours after the physiological expulsion of BCG were performed. The activation state of urine granulocytes and the presence of activating factors within the urine samples were monitored.*

Results: *BCG immunotherapy stimulated, through soluble factors, the activation of PMN neutrophils, which transmigrated into the bladder, and the degree of activation of the PMN neutrophils was related directly to the number of epithelial cells detached from the urothelial layer.*

Conclusions: *This study suggests that PMN neutrophils can participate in reducing the recurrence of bladder cancer by promoting urothelial cell turnover proportionally to their degree of activation. Our results provide further evidence to support the role of PMN neutrophils in BCG immunotherapy.*

Key Words: bladder cancer, granulocyte, urine, blood, BCG

Introduction

Therapy with Bacillus Calmette-Guérin (BCG) for bladder cancer results in an influx of granulocytes into the urine and an influx of mononuclear cells into the bladder wall.[1] This granulocyte influx is a typical feature of BCG immunotherapy, which cannot be induced by other chemotherapic treatments.

Accepted for publication August 2010

Address correspondence to Dr. Salvatore Siracusano, Department of Urology, Trieste University, Cattinara Hospital, Via Strada di Fiume 447 – 34100 Trieste, Italy

A role for polymophonuclear (PMN) neutrophils has been suggested.[2-4] In a previous study, we hypothesized that in patients with bladder cancer who benefit from BCG therapy, the extent of PMN migration could promote a high turnover of urothelial cells which, together with the chronic granulomatous reaction carried out in the bladder wall, can allow at the same time, the detachment of residual cancer cells.[5] However, in our study we did not find a significant relationship between the number of PMN neutrophils and urothelial cells in the patients' urine samples.

We hypothesized that PMN neutrophils should be activated during their transmigration from blood vessels prior to inducing damage to the urothelial

layer. The activation of these cells allows them to release the cytotoxic content of their granules and to produce free radicals and hydrogen peroxide. Even if the process of recruitment itself requires a degree of activation,[6] the induction of cytotoxic potential in PMN neutrophils cannot be taken for granted. Assuming that our hypothesis is correct, the lack of correlation between the number of detached urothelial cells and invasive PMN neutrophils suggests that other parameters intrinsic to PMN neutrophils should be considered.

In the current study, we investigated the activation state of PMN neutrophils isolated from urine in patients treated with BCG for bladder cancer, and compared this with the resting state of PMN neutrophils purified from blood from the same subjects, in order to determine whether this could be a key process in BCG immunotherapy.

Methods

Patients and urine samples

Ten patients with stage T1, grade 3 (T1G3) transitional cell carcinoma of the bladder who underwent transurethral resection of the bladder at our department, received a 6 week course of BCG therapy (81 mg BCG suspended in 50 mL of physiological solution) consisting of one instillation per week. Urine samples were withdrawn at each BCG-therapy instillation. The BCG suspension was retained in the bladder for 2 hours and then was expelled physiologically. Urine samples were collected before treatment (pre-treatment sample) and 2 hours later, from the physiological expulsions of BCG (post-treatment sample). This 2 hour post-treatment time period was the mean time that patients needed before resuming urination. For each of the 10 patients, we obtained urine samples before and 2 hours after BCG treatments 1, 2, 3, and 5. We did not obtain urine samples from the fourth and sixth BCG treatments.

The urine samples were centrifuged at 250 times gravity (x g) for 7 minutes at room temperature. Supernatants were stored at -20°C. The cells in the pellet were resuspended in phosphate-buffered saline (PBS) and counted in counting chambers; differential counts were carried out on stained smears prepared with a cytospin centrifuge. The number of PMN neutrophils, urothelial cells, and total cells were determined. To investigate the presence of a PMN-stimulating activity in pre-treatment and post-treatment samples, we used pooled samples of urine. These pooled samples were made by mixing equal amounts of all the urine samples for each sampling time, and excluding the pre-treatment urine sample from the first treatment. Pooled samples were centrifuged in an MSE micro centaur (Sanyo, UK) centrifuge for 3 minutes at 13,000 x g.

The study was approved by the hospital's Ethical Committee and informed consent was obtained from all patients involved in the study.

Electron microscopy

PMN neutrophils, 5 x 10^6 cells from urine or blood, were fixed in 1.5% glutaraldehyde and processed as previously described.[7] Ultrathin sections were observed with a transmission electron microscope (EM208; Philips, Eindhoven, The Netherlands). Micrographs were taken with a Morada Camera (Olympus Soft Imaging Solutions [OSIS], Munster, Germany).

Human neutrophils (PMN) and superoxide (O_2-) production

Human PMN neutrophils were isolated from the blood of healthy donors and from the patients' urine as previously described.[5,8] In the urine samples, PMN neutrophils accounted for more than 85% of the total cells, and the remaining cells were urothelial cells and eosinophilic granulocytes. PMN neutrophils, 10^5 in 50 µL, either from urine or from peripheral blood, were added to each well of a 96-well tissue culture plate (Corning, NY) and subsequently superoxide (O_2-) production was measured as previously described;[9] where indicated pooled (pre-treatment and post-treatment) urine samples were added to resting blood PMN neutrophils. Values are expressed as percent of O_2- produced by phorbol,12-myristate,13-acetate (PMA) stimulation taken as 100% and are mean ± SD from four separate experiments.

NBT reduction

Human PMN neutrophils, 0.3 x 10^6/mL, either from urine or from peripheral blood samples, were incubated for 15 minutes at 37°C in phosphate-buffered solution with bovine serum albumin (PBS-BSA) containing 0.5 mg/mL nitroblue tetrazolium (NBT) salt diluted from a dimethyl sulfoxide (DMSO) stock solution (25 mg/mL). PMA 50 ng/mL was added to blood PMN neutrophils as a positive control. After the incubation period, specimens were centrifuged; the pellet was placed onto slides and counterstained with methyl green. The quantification of the NBT signal was based on arbitrary units as follows: 0.5 = rare, weakly positive cells; 1.0 = frequently observed, weakly positive cells; 2 = frequently observed, positive cells; 3 = many highly positive cells.

Biochemical assays

Elastase activity was measured at 37°C in 96-well microtiter plates using methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl~L-valine p-nitroanilide as previously described.[5]

T24 assays

Human transitional carcinoma cell line T24 was obtained from American Type Cell Culture (Rockville, MD) and maintained at 37°C, 5% CO_2 in McCoy's 5A medium (Euroclone) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (complete medium). T24 cells were seeded in 24-well plates with complete medium. Confluent cells were rinsed twice with PBS containing 0.7 mM CaCl2 and 0.7 mM MgCl2 and incubated alone, with resting PMN neutrophils from healthy donors, or with PMN neutrophils and pooled urine at 37°C for 1 hour in 1 mL final volume. After the incubation period, the supernatant (0.1 mL) was diluted with 0.2 mL of PBS 0.1% BSA and used for preparing cytospin specimens. These were stained with Diff-Quik and used for counting detached T24 cells as a measure of PMN neutrophil-induced damage.

Results

Pre- and post-treatment urine samples from 10 patients with bladder cancer who were treated with BCG for 6 weeks were evaluated for number of recruited PMN neutrophils, elastase activity, and number of urothelial cells. Only in one patient was the extent of neutrophil recruitment related to the number of urothelial cells detached during the weeks of treatment. However, in the overall sample of 10 patients, we failed to find any significant relationship between the number of transmigrated neutrophils and urothelial cell detachment (not shown).

Figure 1 shows the ultrastructural appearance of PMN neutrophils recruited into the bladder at the time of the fifth BCG-treatment, for patient #5. As expected, the morphology of the PMN neutrophils found in the urine before the fifth instillation of BCG, Figure 1c-1e, was very similar to the morphology of the PMN neutrophils found in the urine 2 to 3 hours after the fifth instillation of BCG, Figure 1b, with the exception that the former cells were less numerous and contained more apoptotic and necrotic cells. Both pre-treatment and post-treatment urine cell populations, however, had few secretory granules, either specific (weakly electron dense) or azurophilic (highly electron dense) granules, compared with blood PMN neutrophils, Figure 1a, and they contained many of the previously

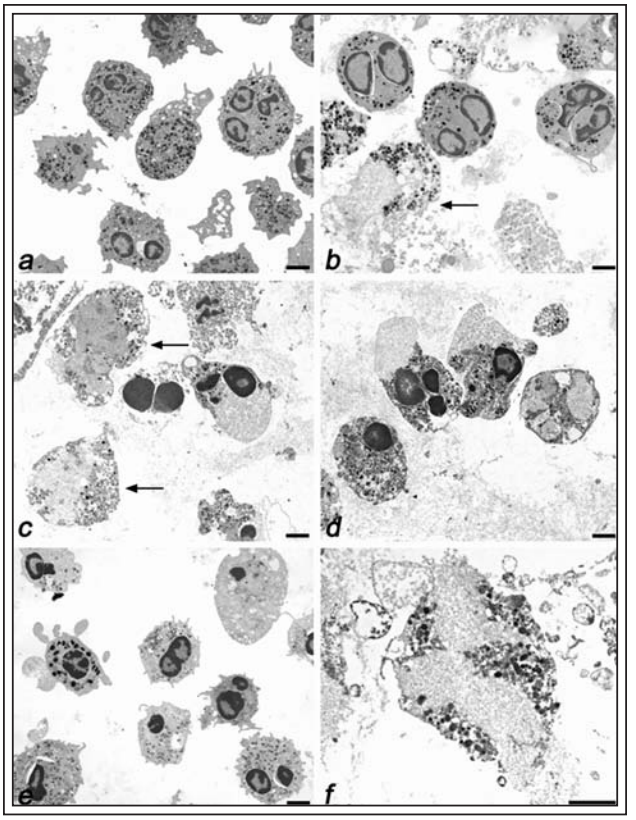

Figure 1. Ultrastructural appearance by transmission electron microscopy of urine polymorphonuclear neutrophils compared with blood PMN neutrophils from one patient (#5) at the fifth Bacillus Calmette-Guérin (BCG) treatment. Resting PMN neutrophils isolated from blood of the patient. **(a)** PMN neutrophils isolated from post-treatment urine **(b)** PMN neutrophils isolated from the urine obtained before the fifth BCG treatment **(c-f)**. Note that the formation of neutrophil extracellular traps (NETs) occurred in both post-treatment urine and pre-treatment urine (arrows). Whole PMN neutrophils in post-treatment sample are degranulated and PMN neutrophils undergoing necrotic (**c and d**) and apoptotic changes are seen more frequently in PMN neutrophils isolated from post-treatment urine (**d and e**). A high magnification of one NET is shown in (**f**). Note the disruption of the nuclear membrane and the DNA entrapping of granules. Bar = 2 micrometers.

described[14] neutrophil extracellular traps (NETs), Figure 1f and arrows in Figure 1b and 1c. Of note, this peculiar cell debris has lost the nuclear membrane, while both apoptotic and necrotic cells retained the nuclear membrane for longer periods.

Furthermore, a NET-like amorphous material associated with detached urothelial cells and PMN

neutrophils undergoing NET-changes was frequently observed, Figure 2. Although Figures 1 and 2 show the morphological appearance of PMN neutrophils isolated from pre-treatment and post-treatment urine samples from patient #5, the ultrastructure findings in the other patients were similar.

PMN neutrophils isolated after treatment, Figure 3a, produced superoxide anion (O_2-) at higher levels than those produced by resting blood PMN neutrophils isolated from the same patients, Figure 3b. The highest production of O_2- from these cells was found during the first and second week of BCG treatment and was comparable to that induced by PMA in blood PMN neutrophils, taken as maximal stimulation. The production of O_2- declined subsequently, but remained at higher levels than in resting cells. A significant production of O_2- was also measured in PMN neutrophils found in pre-treatment urine samples before the next treatment (not shown).

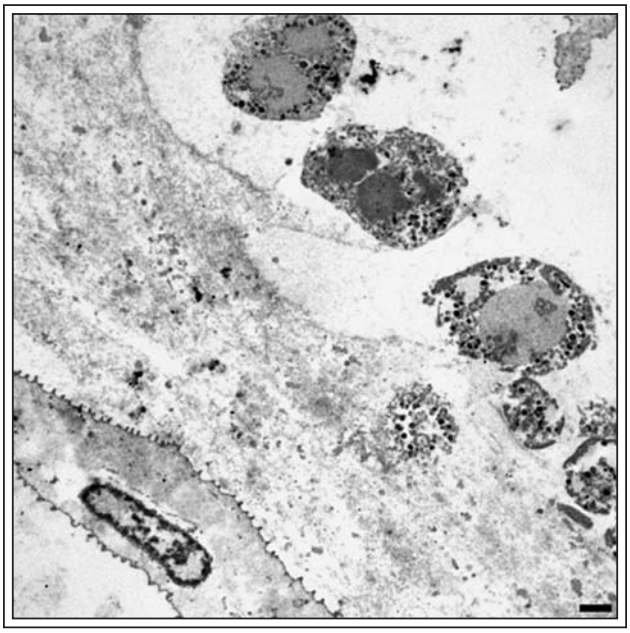

Figure 2 . Ultrastructural appearance by transmission electron microscope of urine PMN neutrophils from one patient at the fifth BCG treatment. Note the association of a urothelial cell (UC) (lower left) with a PMN neutrophil undergoing neutrophil extracellular trap (NET) changes through a NET-like material which also entraps PMN neutrophil granules. This kind of cell cluster was seen frequently in both pre-treatment and post-treatment urine. When PMN neutrophils undergoing NET-formation merge together progressively, to create a biofilm which covers areas of the urothelium surface, the cytotoxic effects of the "weapons" that PMN store in their granules is likely increased. Bar = 2 micrometers.

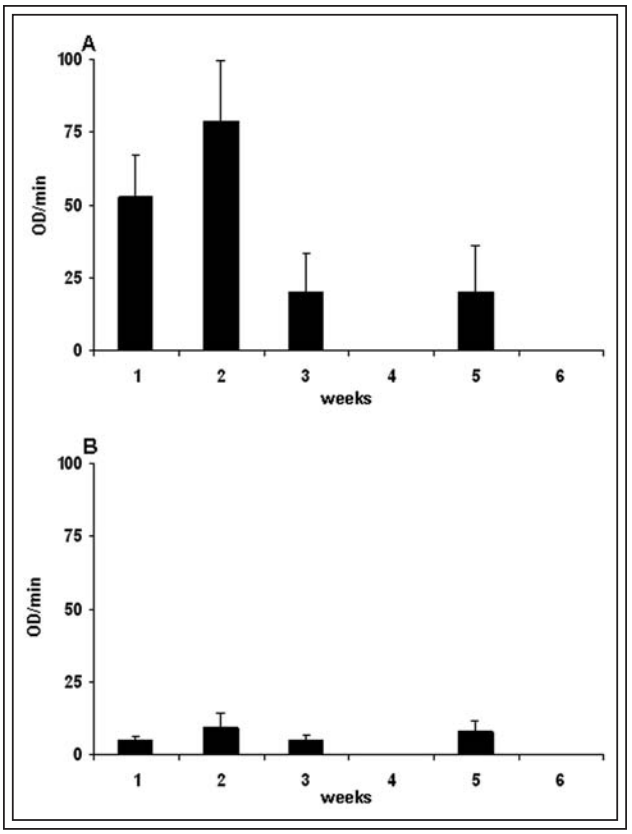

Figure 3. Superoxide (O_2-) production from PMN neutrophils isolated from post-treatment urine (**a**) as compared to that produced by resting blood PMN neutrophils (**b**) from the same patients during BCG immunotherapy. Values in the ordinate axis are expressed as percentages of the maximum value of O_2- production measured in the presence of phorbol,12-myristate,13-acetate (PMA). Statistical analysis carried out with Student's t test (unpaired, one tail) showed that the difference was statistically significant ($p < 0.01$) when the values were compared in each week during the first 3 weeks of treatment. For the fifth week of treatment, the difference was also significant ($p < 0.05$). Post-treatment samples relative to the fourth and sixth week weren't analyzed together with blood PMN neutrophils for all patients. Of note, PMA-induced (O_2-) production was similar in urine and blood PMN neutrophils.

The stimulated metabolic activity of urine PMN neutrophils was confirmed by the morphological analysis of NBT reduction, a sign of PMN neutrophil activation,[15] as shown by the quantification of NBT reduction, Figure 4a. The characteristic appearance of urine and blood PMN neutrophils after a 10 minute incubation with 0.5 mM NBT is shown in Figure 4b. PMN neutrophils isolated from urine, Figure 4b lower panels, show deposits of precipitated reduced NBT,

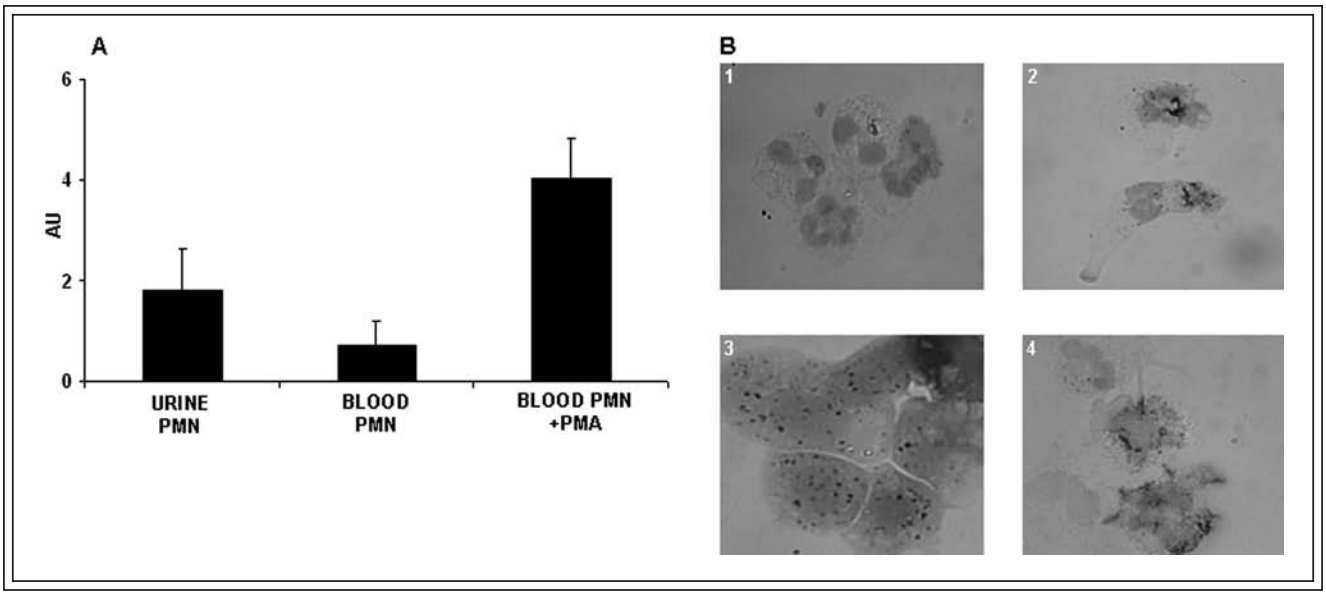

Figure 4. Nitro blue tetrazolium (NBT) reduction by PMN neutrophils isolated from post-treatment and pre-treatment urine as compared to that reduced by blood PMN neutrophils. (**a**) Semiquantitative evaluation of the NBT reduction by PMN neutrophils from post-treatment and pre-treatment urine of all our patients. Values are expressed as arbitrary units (AU). The value of 5 was associated with a very high reduction, and the value of 0.5 was associated with the minimum visible reduction. (**b**) NBT reduction by PMN neutrophils isolated from post-treatment and pre-treatment urine from patient #6 as compared to that reduced by blood PMN neutrophils from the same patient. Upper left panel: resting blood PMN neutrophils; Upper right panel: PMA-stimulated blood PMN neutrophils; Lower left panel: PMN neutrophils from pre-treatment urine; Lower right panel: PMN neutrophils from post-treatment urine. Cells were counter-stained with methyl green; original magnification x 1000.

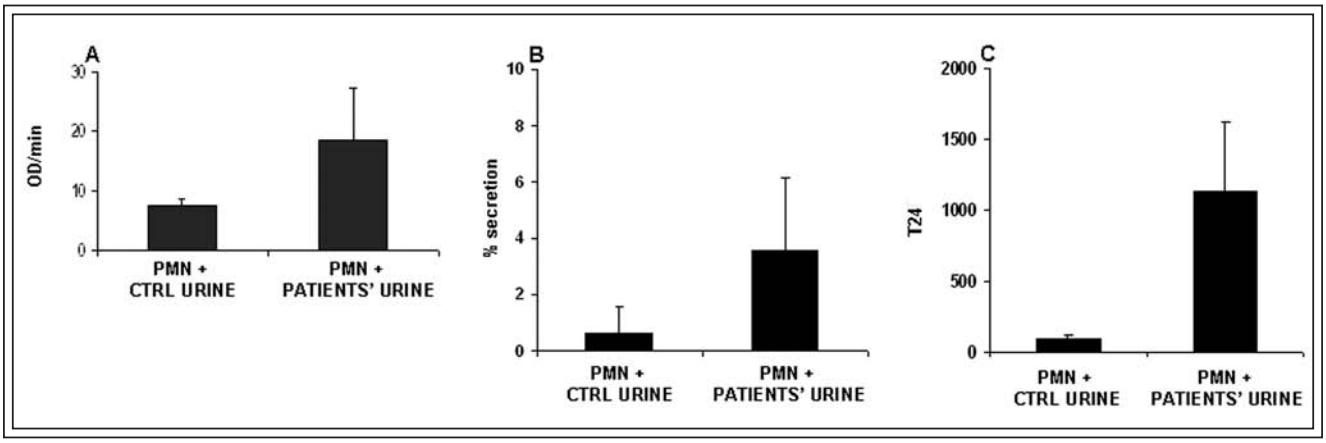

Figure 5. Activation of peripheral blood resting PMN neutrophils by BCG-treated patients' urine. Pooled patient urine, both post-treatment and pre-treatment samples, has greater PMN neutrophil stimulating activity than pooled healthy control urine. (**a**) Superoxide production expressed as percentage of the PMA-induced stimulus taken as 100% (Student's t test unpaired, one tail: $p < 0.05$). (**b**) PMN neutrophil granule secretion evaluated as the percentage of beta-glucuronidase free activity, the total enzyme activity taken as 100% (Student's t test unpaired, one tail: $p < 0.01$). (**c**) Extent of T24 cell detachment from the culture monolayer induced by PMN neutrophils exposed to patients' pooled urine. The total number of T24 cells was calculated by electronically counting the total cell number and by scoring the T24 detached cells on Diff-Quik stained smears. (Student's t test unpaired, one tail: $p < 0.01$).

while no, or very weak NBT reduction was observed in blood PMN neutrophils, Figure 4b upper left panel, unless PMA was added, Figure 4b upper right panel.

Four experiments carried out with different PMN neutrophil preparations revealed that a PMN stimulating activity was present in both pre-treatment and post-treatment pooled urine samples isolated from BCG-treated patients. In fact blood PMN neutrophils exposed to patients' urine, but not urine from healthy controls, were induced to produce a weak but statistically significant amount of O_2^- and release their granular contents, Figure 5a and 5b. Furthermore, after exposure to the urine of BCG-treated patients, PMN neutrophils from healthy donors acquired a strong cytotoxic activity against T24 cells in culture, Figure 5c, while PMN neutrophils exposed to urine samples from healthy subjects did not show this effect. Patients' urine or PMN neutrophils alone had no effect on T24 cells. When the number of urine PMN neutrophils (from both pre-treatment and post-treatment samples) was plotted against the number of detached urothelial cells, no correlation was apparent (not shown). However, if the activation state of these cells was considered by calculating an activation coefficient ψ by multiplying the extent of O_2^- production and the extent of elastase secreted into the urine by the PMN number, a highly significant non-linear correlation was found between activation coefficient ψ and the number of detached urothelial cells ($p < 0.001$; Figure 6).

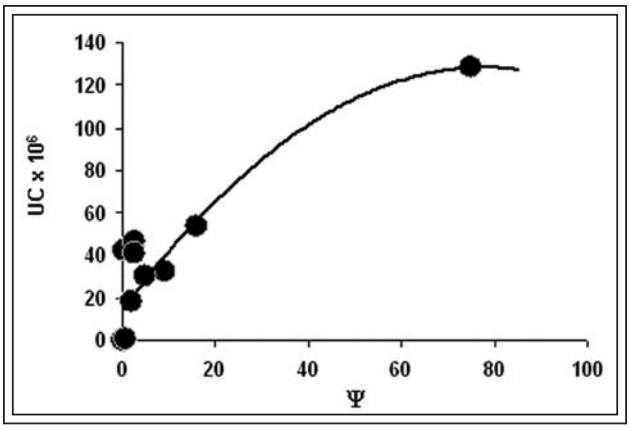

Figure 6. Non linear regression between the number of detached urothelial cells found into patients' urine (both pre-treatment and post-treatment) and the activation state of PMN neutrophils expressed as coefficient ψ. The coefficient was calculated by multiplying the total number of PMN neutrophils found in the urine by the free elastase activity and the amount of O_2^- produced. (See text for more details.) The relationship was highly significant ($R^2 = 0.81$; $p < 0.001$).

Discussion

To the best of our knowledge, this is the first published study showing that PMN neutrophils when recruited into the bladder after BCG immunotherapy are in an activated state 2 hours and even up to 1 week after treatment. This finding appears to confirm previous circumstantial evidence.[2,5,10,11] In those earlier reports, the authors suggested that PMN neutrophil activation state may be related to the presence of elastase or the presence of tumor necrosis factor-related apoptosis-inducing ligand-2 (TRAIL2) in post-treatment urine. However, these processes could also be explained by passive release following cell death. The finding that urine PMN neutrophils are metabolically active excludes the latter possibility as being the main explanation.

Our findings suggest that PMN neutrophils recruited into the bladder after BCG immunotherapy are in an activated state 2 hours and even up to 1 week after treatment. We could measure this state despite cell death, or metabolic exhaustion, which PMN neutrophils probably underwent after migration from blood. These PMN neutrophils reduced NBT and produced superoxide during the 2 first weeks of treatment at levels that were comparable to those produced by PMA in blood PMN neutrophils, suggesting that BCG-induced PMN neutrophil activation can reach high levels. As already reported,[5] the presence of elastase in the urine during BCG-treatment could be ascribed to activation of secretory activity in PMN neutrophils. The ultrastructural appearance of urine PMN neutrophils suggests that a strong reaction occurred in these cells, since they contain fewer granules than blood PMN neutrophils, suggesting again that a specific cell activation process is involved.

However, this cell population, in addition to apoptotic and necrotic cells, also contained many neutrophil extracellular traps (NETs), which have been recently defined and distinguished from both apoptotic and necrotic cells[12] and which can exert cytotoxic activity against host cells.[13] NET formation confirms that PMN neutrophil activation occurs in urine and could continue, even after PMN neutrophil death, to exert persistent cytotoxic activity against urothelium, thus contributing to the increase in its turnover. Accordingly, we observed that NET-like amorphous material is frequently associated with both detached urothelial cells and PMN neutrophils undergoing NET formation. In vivo formation of film, from the fusion of many NETs, could form and coat the urothelium, thus strongly stabilizing and enhancing the cytotoxicity of PMN neutrophil "weapons."

As expected, the urine from patients who were treated with BCG contained some undefined activating factors that stimulated oxygen metabolism and secretory activity of PMN neutrophils isolated from the blood of healthy donors. The activating power of the patients' urine was also confirmed by showing that blood PMN neutrophils can detach many T24 cells from the monolayer when exposed to pre-treatment or post-treatment urine. This activity depends on the release of factors that PMN neutrophils store in their granules, together with elastase, as previously suggested for TRAIL2.[2] Therefore, activation factors capable of activating the recruited PMN neutrophils are still active in urine after BCG treatment.[14,15] We think it is unlikely that the main stimulus for the neutrophils recruited into the bladder is BCG. In our study, BCG-containing urine was voided before the collection of post-treatment urine and we failed to find BCG-associated-neutrophils by Ziel-Nielsen staining (not shown). We think it is more likely that BCG activates the urothelium to produce chemokines, which together with other factors induce neutrophil recruitment. After this step, it is likely that BCG is eliminated almost completely and PMN neutrophils begin to reach the bladder coming from the capillary blood vessels and transmigrating through the urothelium. During this complex migration they are likely activated by a large number of cytokines produced by the urothelium. Soon thereafter the stimulation of the phagocytes can be amplified by cytokines released from macrophages recruited into the bladder wall (as shown in the study by Pages and colleagues),[10] and by those released by neutrophils themselves. While we cannot exclude the possibility that BCG could also be a stimulating factor, it should be active only in the first part of the neutrophil influx. Supporting evidence for this can be derived from our observation that the supernatant of ultra-centrifuged urine can activate neutrophils and it can maintain its activating power even against freshly isolated blood neutrophils from the same patient. Our findings suggest that other stimuli work with BCG in inducing neutrophil activation.

Of note, a statistically significant relationship with the number of detached urothelial cells was found when the number of activated PMN neutrophils was considered. This finding suggests that only activated PMN neutrophils can detach urothelial cells and that migration "per se" is not enough to induce cell damage. □

References

1. Taniguchi K, Koga S, Nishikido M et al. Systemic immune response after intravesical instillation of bacille Calmette-Guérin (BCG) for superficial bladder cancer. *Clin Exp Immunol* 1999;115(1):131-135.
2. Kemp TJ, Ludwig AT, Earel JK et al. Neutrophil stimulation with Mycobacterium bovis bacillus Calmette-Guérin (BCG) results in the release of functional soluble TRAIL/Apo-2L. *Blood* 2005;106(10):3474-3482.
3. Ludwig AT, Moore JM, Luo Y et al. Tumor necrosis factor-related apoptosis-inducing ligand: a novel mechanism for Bacillus Calmette-Guérin-induced antitumor activity. *Cancer Res* 2004;64(10):3386-3390.
4. Suttmann H, Riemensberger J, Bentien G et al. Neutrophil granulocytes are required for effective bacillus Calmette-Guerin immunotherapy of bladder cancer and orchestrate local immune responses. *Cancer Res* 2006;66(16):8251-8257
5. Siracusano S, Vita F, Abbate R et al. The role of granulocytes following intravesical BCG prophylaxis. *Eur Urol* 2007;51(6): 1589-1599.
6. Sengeløv H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. *J Immunol* 1995; 154(8):4157-4165.
7. Lungarella G, Menegazzi R, Gardi C et al. Identification of elastase in human eosinophils: immunolocalization, isolation, and partial characterization. *Arch Biochem Biophys* 1992;292(1):128-135.
8. Brochetta C, Perrotta MG, Jeromin A et al. Identification and subcellular localization of neuronal calcium sensor-1 (NCS-1) in human neutrophils and HL-60 cells. *Inflammation* 2003;27(6): 361-372.
9. Zabucchi G, Berton G, Soranzo MR. Mechanism of the potentiating effect of cytochalasin B on the respiratory burst induced by concanavalin A in leucocytes. *FEBS Lett* 1981;125(2): 165-169.
10. Pagès F, Lebel-Binay S, Vieillefond A et al. Local immunostimulation induced by intravesical administration of autologous interferon-gamma-activated macrophages in patients with superficial bladder cancer. *Clin Exp Immunol* 2002;127(2):303-309.
11. Simons MP, O'Donnell MA, Griffith TS. Role of neutrophils in BCG immunotherapy for bladder cancer. *Urol Oncol* 2008;26(4): 341-345.
12. Fuchs TA, Abed U, Goosman C, Hurwitz R, Schulze I, Wahn V. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007;176(2):231-241
13. Gupta AK, Hasler P, Holzgreve W, Hahn S. Neutrophil NETs: a novel contributor to preeclampsia-associated placental hypoxia? *Semin Immunopathol* 2007;29(2):163-167.
14. Hang L, Haraoka MW, Agace W et al. Macrophage inflammatory protein-2 is required for neutrophil passage across the epithelial barrier of the infected urinary tract. *J Immunol* 1999;162(5): 3037-3044.
15. Miyazaki J, Kawai K, Oikawa T et al. Uroepithelial cells can directly respond to Mycobacterium bovis bacillus Calmette-Guerin through toll-like receptor signaling. *BJU Int* 2006;97(4): 860-864.