In Vitro and In Vivo Effects of CpG-Oligodeoxynucleotides (CpG-ODN) on Murine Transitional Cell Carcinoma and on the Native Mucine Urinary Bladder Wall

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Abstract. Background: Intravesical BCG instillation is established and efficient in the prophylaxis of recurrent transitional cell carcinoma. A Th-1 biased immune response is postulated. Recent work has proven the efficacy of synthetic CpG-Oligodeoxynucleotides (ODN) as inducers and adjuvants for a strong Th1-response and there is evidence for a direct and/or adjuvant anti-neoplastic effect. The purpose of this study was to examine the local effects of CpG-ODN on the murine bladder wall after intravesical instillation and the effects on cytokine expression in an orthotopic murine bladder cancer model. Materials and Methods: Histopathology, immunohistochemistry and fluorescence microscopy were performed after different instillation schedules of stimulatory, non-stimulatory biotinylized and FITC-labelled CpG-ODN into the murine bladder. MB-49 murine bladder cancer cells were tested for TLR-9 expression to exclude a potential direct responsiveness to CpG-ODN. Furthermore induction of apoptosis was tested by annexin V staining and FACS analysis of CpG-ODN stimulated tumor cells. In an orthotopic C57/Bl6 murine bladder cancer model, the expressions of IL-12, IFNγ, IL-10 and TGF-β were evaluated after repeated CpG-ODN treatment. Results: Single and repeated instillation of CpG-ODN induced subepithelial and urothelial lymphocytic infiltrations with consecutive apoptoses. PBS and non-stimulative ODN induced no visible reaction. Bladder submucosa stained positive for biotin. Controls showed no endogenic biotin staining. FITC-labelled ODN adhered to the bladder mucosa and penetration of the mucosal barrier was not detected. MB-49 TCC cells did not express TLR-9 and CpG-ODN did not induce apoptosis in these cells. Repeated intravesical instillations of CpG-ODN in orthotopic murine tumor bearing urinary bladders resulted in significant up-regulation of both Th-1 and Th-2 cytokines. Conclusion: CpG-ODNs have promising anti-neoplastic potential. They exert a pronounced immunological response both in the native murine urinary bladder and in murine TCC. The mechanisms of action appear to be mediated immunologically, There was no direct effect of CpG-ODN on the tumor cells in this model.

More than 50 years ago, DNA was recognized as the coding molecule of genetic information. Initially, the DNA molecule was considered to be immunologically inert, causing immune responses only in rare autoimmune diseases. Today, it is known that certain sequence motifs are sensed by cells of the innate immune system. These sequences are common in prokaryotic DNA, whereas in mammalians they are less frequent. Bacterial DNA has the potency to initiate and bias immune responses, since it preferentially induces a T helper cell-1 (Th-1) type reaction (1).

In the 1970s and 1980s a DNA-rich fraction of Mycobacterium bovis was identified as an active compound in terms of antitumoral potency in animal models, inducing interferon secretion from spleen cells and natural killer (NK)-cell activation (2, 3). A 45-mer palindromic DNA-sequence with a central CG-motif was identified that showed to be essential for the effects of the preparations (4). Furthermore, in antisense oligodeoxynucleotide (ODN) research, certain sequences had unexpected immunological side-effects such as B-lymphocyte stimulation, major
histocompatibility complex (MHC)-up-regulation and splenomegaly (5, 6). Reversal or methylation of the CG-motif completely abrogated the mentioned effects (5). It was found, that the central immunostimulatory motif is necessary to induce a response in murine immune cells. The 5’ and 3’ flanking regions have modulating character, however, the mechanisms behind these effects are not yet understood. Possible immunostimulating effects by CpG-ODN are achieved via a toll-like receptor 9 mediated pathway (7) and comprise the induction of cytokine production by immune cells; direct B-cell activation; activation of dendritic cells to promote a Th-1-reaction; T-helper cell-independent induction of cytokine T-cell activity, anti-neoplastic effects promote a Th-1-reaction, T-helper cell-independent induction of cytokine production by immune cells; direct B-cell activation, activation of dendritic cells to promote a Th-1-reaction, T-helper cell-independent induction of cytokine T-cell activity, anti-neoplastic effects in vivo when applied locally into/near the tumor (8-10), and adjuvant potency in monoclonal antibody-therapy in several malignancies (11, 12). The data on the role of TLR-9 agonists in cancer therapy are excellently reviewed by Krieg (13) and Tsan (14).

Transitional cell carcinoma (TCC) of the urinary bladder is a common malignancy of the urogenital tract. In the US and in Western Europe it is second only to prostate cancer in urogenital cancer statistics (15, 16). Superficial bladder cancer (pTa, pT1, carcinoma in situ) will recur in 70% of cases and 20-30% of patients will experience progression to a muscle-invasive stage, requiring more invasive therapy like radical cystectomy or radiochemotherapy (17). Intravesical instillation of cytotoxic or immunologically active compounds offers a chance to reduce recurrence rates significantly after transurethral resection (18, 19). In low-risk tumors, cytotoxic agents such as mitomycin c or doxorubicin are favored because of their moderate side-effects (18). In intermediate and high-risk superficial TCC, especially pTa/pT1G3 and CIS, viable Bacille Calmette Guérin (BCG) has shown to be superior in randomized trials in terms of recurrence and it seems to reduce progression rates (20). These clinical data show that TCC is amenable to a local, immunotherapeutic concept.

The mechanisms of action of BCG-therapy in TCC are complex and far from being fully understood, but the following aspects seem to be essential (21, 22); induction of a T-helper cell-dependent, delayed-type hypersensitivity reaction (23, 24); shift of the CD4+:CD8+ ratio in favour of CD4+ cells (25); secretion of a cytokine pattern (IL-2, IFNγ, TNFα) implying a Th-1 type response (26, 27); and, ability of BCG to induce differentiation of mononuclear cells into lymphokine-activated killer cells and cytotoxic effector cells (28).

The parallels of the experimentally proven effects of CpG-ODN and of the anti-neoplastic potency of BCG in TCC formed the rationale for the evaluation of the antineoplastic effect of CpG-ODN in hetero- and orthotopic murine transitional cell carcinoma (8, 9, 29). After demonstrating activity of the agent in the murine model, research is now being focused on the evaluation of possible mechanisms of action. The present work was intended to differentiate whether the CpG-effects on TCC of the bladder are mediated immunologically or whether there might be an additive direct cytotoxic effect on the tumor cells. Furthermore, initial studies on the cytokine expression pattern of orthotopic murine bladder cancer under experimental, topical treatment with CpG-ODN have been performed.

Materials and Methods

Oligodeoxynucleotides. Lyophilized, completely phosphorothioated modified ODN was purchased from TIB-MolBiol, Berlin, Germany. CpG, sequence: 5’-TCCATGAAGTTCTGATGCT-3’ (1668), GpC, sequence: 5’-TCCATGAAGCTTCTGATGCT-3’ (1668), 5’-FITC (Fluorescein-isothiocyanate), 5’-Biotin-labelled and unmodified phosphodiester (PO) ODN 1668 were purchased from MWG-Biotech, Munich, Germany.

Lyophilized ODNs were dissolved in distilled H2O to a 250 μM stock solution and sterile filtered. For intravesical instillation, 50 μL aliquots were prepared containing 10 nM of the respective ODN. The ODNs were then stored at −20°C.

Cell culture (MB-49). MB-49 cells were maintained under standardized conditions at 37°C in DMEM+Glutamax, supplemented with 10% FCS and 1% penicillin/streptomycin in the presence of 5% CO2.

Animal models. Animal experiments were approved by the responsible animal experiment committee (N˚ II 25.3-19 c 20-15 (1) MR 20/20-Nr.03/2001).

C3H/He-N mouse model: Female C3H/He-N mice (20-25g) were purchased from Charles River, Sulzdelfeld, Germany and were kept under SPF conditions in groups of 4 animals and put on a standard diet and fresh water ad lib. No antibiotics were given. For intravesical instillation, mice were anesthetized by intraperitoneal injection of 100 mg/kg body weight Ketamin-HCl (Ketanest®; Parke-Davis/Goedecke, Karlsruhe, Germany) and 10 mg/kg body weight Xylazin (Rompun® 2%; Bayer, Leverkusen, Germany). The anesthetized mice were catheterized in supine position after local disinfection of the urethral meatus and the introitus vaginae. The 24 G sheath of a baby i.v. cannula (Insysy W; Becton Dickinson, Franklin Lakes, NJ, USA) was used for catheterization. Meatus and catheter position were secured by tape. After a period of 2 h the catheter was removed and the mice voided spontaneously. Anesthesia lasted for 2.5 to 3 h. Details of the ODN-instillation-schedule are displayed in Table I. Briefly, group 1 received a single instillation, group 2 three consecutive instillations of stimulative CpG-ODN, animals were sacrificed 7 days after the last dose. Group 3 received 3 consecutive doses of non-stimulative GpC-ODN and group 4 served as control with 3 doses of PBS according to the same schedule as groups 2 and 3.

Because of potential retroviral contamination of the murine MBT-2 TCC cell line (27) and uncertain effects on the results of immunotherapeutic research with these cells, the syngeneic murine MB 49 TCC model in the C57Bl/6 mouse was used instead for all further experiments involving tumor cells.
Table I. Instillation schedule in C3H/He-N mouse model for the evaluation of CpG-ODN effects in the native urinary bladder (n=5 in each group).

<table>
<thead>
<tr>
<th>Day</th>
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<th>Group 3</th>
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<td>Day 0</td>
<td>CpG</td>
<td>CpG</td>
<td>GpC</td>
<td>PBS</td>
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<tr>
<td>Day 1</td>
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Table II. Instillation schedule in C57/Bl-6/MB49 syngeneic orthotopic mouse model for the evaluation of CpG-ODN effects in transitional cell carcinoma (n=5 in each group).

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<th>Day</th>
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<th>Group 2</th>
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<td>Day 1</td>
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<td>Day 9</td>
<td>PBS</td>
<td>CpG</td>
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<td>Day 10</td>
<td>PBS</td>
<td>CpG</td>
<td>ø</td>
<td>ø</td>
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<tr>
<td>Day 11</td>
<td>PBS</td>
<td>CpG</td>
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C57/Bl-6 orthotopic TCC mouse model: Female C57/Bl-6 mice (20-30 g) were purchased from Charles River and kept under the same conditions as described above. Anesthesia and catheterization were performed in the same way. Before tumor cell instillation, the bladders were pre-conditioned by instillation of 50 μL poly-cationic poly-L-lysine (MW 50,000-75,000; Sigma, Munich, Germany) to enhance cell-adhesion. For tumor implantation, cell suspensions of 10⁶ MB-49 cells/50 μL PBS were prepared. Dwelling time was 1-2 h. ODN instillation was performed as described above. Details of the ODN and tumor-cell instillation schedule are displayed in Table II. Briefly, group 1 served as control with tumor-cell instillation on day 0 and 5 consecutive doses of PBS. Group 2 was treated according to the same schedule by 5 consecutive doses of CpG-ODN, starting on day 1. Group 3 received only 3 CpG-ODN instillations, starting on day 3 and group 4 were completely untreated and tumor-free animals.

Conventional histology (CH3/He-N model): After completion of the instillation schedule mice were sacrificed, the urinary bladders were removed and immediately fixed in a 10% buffered formalin-solution, thereafter dehydrated in graded alcohols and paraffin-embedded. Three micrometer sections were prepared and stained with hematoxylin-eosin.

Immunohistochemistry and fluorescence microscopy (CH3/He-N model): Three mice received a single instillation of 50 μL S’ biotinylated ODN 1668. Immediately after 1 h dwelling time, the mice were sacrificed, bladders were removed and paraffin-embedded sections were prepared as described above. The slides were stained immunohistochemically by the avidin-biotin-peroxidase method. Three native bladders were also stained to control for endogenous biotin staining.

Three mice received a single instillation of 5’ FITC-labelled ODN 1668. Immediately after 1 h of dwelling time, the mice were sacrificed, bladders were removed and frozen sections were prepared. Fluorescence microscopy and photography of the slides were performed at an excitation wavelength of 494 nm.

TLR expression in MB-49 TCC cells.

RNA preparation and cDNA synthesis: Total RNA from 1×10⁶ cells was isolated by using HighPure™ RNA-kit (Roche, Mannheim, Germany) which included DNaseI digestion. One gram of total RNA preparation was reversely transcribed with cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) using oligo (dT)23.

Quantitative RT-PCR: cDNA was diluted 1:4 and 2.5 μL were used as template in 25 μL TaqMan-PCR-mix according to the manufacturer's protocol (Eurogentec, Seraing, Belgium). MgCl₂ was added to reach a final concentration of 5 mM and cycler conditions were 95°C/10 min, 40 cycles of 95°C/15 and 60°C/1min. Primers, probes and annealing temperatures are listed in Table III; primers and probes were purchased at MWG Biotech. Fluorogenic probes (FAM) were obtained from Eurogentec, Belgium. Specificity of RT-PCR was controlled by no template and no RT controls. PCR-efficiencies for all reactions were similar (~0.95-1.0) and threshold values were normalized to β-actin. Quantitative PCR results are expressed as relative induction towards the housekeeping gene β-actin.

Evaluation of direct pro-apoptotic effect of CpG-ODN.

FACS-Analysis and fluorescence microscopy after stimulation of tumor cells by CpG-ODN. MB-49 cells (10⁶) were pre-incubated for 24 h in 6-well cell culture plates in 2 mL of culture medium. Cells were then stimulated for 4 h by either PTO-CpG 1668, PTO-CpG 1668 or PO-CpG 1668 at a concentration of 3 μM per well. In a control well, an equivalent volume of phosphate buffered saline (PBS) was added for 4 h. The cells were washed and centrifuged and stained by annexin-V-fluorescein with propidium iodide nuclear counterstaining (Annexin V Fluos Staining Kit; Roche, Germany). FACS Analysis (PAS II, Partec, Münster, Germany) and fluorescence microscopy were then performed at an excitation wavelength of 488 nm (emission wavelengths of 518 and 617 nm). Single (green) surface staining for annexin V indicates apoptosis, whereas double (green and red) staining indicates leaking necrotic cells.

Cytokine expression in orthotopic bladder cancer after CpG-ODN treatment: After sacrificing the mice on the last day of the instillation schedule (Table II), bladders were immediately removed and stored in liquid nitrogen. RNA-extraction and cDNA-synthesis were performed as described above.

Quantitative RT-PCR for IL-10, IL-12, IFNγ and TGF-β was performed as described above, the primers and fluorogenic probes were purchased from MWG BioTech (Table III). For better quantification of cytokine expression in the different groups, mRNA copy numbers for each sample were calculated after correction for β-actin expression.

Results

CH3/He-N mouse model – CpG-ODN effects in healthy urinary bladder. Catheterization and instillation was feasible in all animals. No animal showed acute or long-term toxicity after instillation of the different agents. No weight loss occurred and no hematuria was detectable.
**Histology.** After a single installation of an immunostimulative CpG-ODN an inflammatory response of the submucosal layer was detectable, including lymphocyte infiltration and congestion of the submucosal tissue (Figure 1C). Triple instillation resulted in a marked increase of the described inflammatory signs with development of multiple, follicle-like aggregations of mononuclear cells, beginning infiltration of the mucosa by lymphocytes and the appearance of apoptotic cells within the mucosal layer (Figure 1D).

Animals in group 3 (GpC-sequence) and PBS controls showed no differences as compared with completely untreated animals (Figure 1A and B). Thus, the observed effects seem to be highly specific for CpG-ODN.

**Fluorescence microscopy.** After 1 hour of FITC-CpG-ODN installation, the mucosal surface was intensively stained by fluorescent material (Figure 2B and D). No reproducible FITC-staining was detectable in the deeper layers of the bladder wall. Untreated control animals showed no significant endogenous fluorescent activity, implying the FITC-labelled ODN as being responsible for mucosal fluorescence.

**Immunohistochemistry.** After 2 hours of contact time of biotin-labelled ODN and bladder mucosa, the bladder wall stained highly positively for biotin. Biotin-positive material was present almost exclusively in the submucosa (Figure 2C) and was continuously distributed. Control animals were completely biotin negative (Figure 2A).

**TLR-expression on MB 49 marine bladder cancer cells.** Figure 3 shows the amplification plots of TLR-2, -4, and especially -9. It is clearly shown that there was minimal, but at least some expression of TLR-2 and TLR-4, but no TLR-9 expression at all in MB-49 cells. Thus, a direct effect of CpG-ODN on the tumor cells in the in vivo model can be excluded due to the lack of adequate CpG-ODN receptor.

**Direct pro-apoptotic effect on MB-49 cells in culture.** Figure 4A shows the percentage of necrotic and apoptotic cells after stimulation by various agents. Both phosphothioate and phosphodiester (PO) CpG-ODN failed to induce apoptosis as compared to PBS or non-stimulative GpC-ODN. Looking at both apoptosis and necrosis, CpG-ODN appears to have the least pronounced effect on cell survival. This observation is confirmed by the fluorescence microscopy findings, showing no isolated annexin-V staining indicative of apoptosis. Red and green double staining of the visible cells indicate necrosis.

**Discussion**

TCC is a tumor entity that is amenable to an immuno-therapeutic approach and there is probably a close connection between the anti-neoplastic and preventive effect of BCG and its content of bacterial DNA. BCG has been investigated for cancer therapy since the 1960s. Several groups showed antitumor activity and modulation of tumor resistance in animal and cell culture models and clinical research (30-32). The search for the active component of BCG led to a DNA-rich fraction, called MY-1, which activated NK-cells and induced IFNγ production by murine spleen cells and peripheral blood lymphocytes (33). DNase-digest of MY-1 abrogated these effects while RNAse digest did not affect the induced immunological responses. Toxic or anaphylactic effects of MY-1 were not relevant. Extensive research on DNA sequences in MY-1 found 15- to 30-mer ODNs with a central CG-motif and palindromic 5’ and 3’
Figure 1. All sections: HE ×40; A (Group 4)/B (Group 3): No structural or architectural changes as compared to untreated, native bladder wall. C (Group 1): nested, multiple round cell infiltrations of the submucosa with concomitant edema. D (Group 2): follicular infiltrates of lymphocytes and other mononuclear cells, predominantly located in the submucosa, lymphocytes infiltrating the urothelium, disseminated apoptosis of urothelial cells.

Figure 2. A: Avidin-biotin complex staining in untreated murine bladder; propidium iodide nuclear counterstaining ×40; no relevant endogenous biotin-staining. C: Avidin-biotin complex staining after 1 hour of biotynilated CpG-ODN instillation ×20: almost continuous biotin-positive staining of the submucosa. B (×40) and D (×10), fluorescence microscopy at 518 nm: murine bladder wall after 1 hour of FITC-labelled CpG-ODN: FITC-positive material adherent to urothelium and apparently reaching the deeper mucosal layers.
Figure 3. Expressions of TLR-2, -4 and -9 towards β-actin; red and green lines displaying no-RT controls.

Figure 4. Results of FACS analysis and fluorescence microscopy after stimulation of MB-49 cells by different stimulatory and non-stimulatory ODNs: A: Diagram shows the percentage of necrotic and apoptotic cells after 4 h of stimulation. Values are medians of 5 consecutive experiments. B and C: MB-49 cells without stimulation under white light and fluorescence microscopy. D: Typical double staining after stimulation by all tested ODNs.
flanking regions to be responsible for the immunological potency of the substance (33). Moreover, short, synthetic CG-motifs were able to imitate the immunostimulatory effects of BCG-DNA. Interestingly, unmethylated CG-motifs or “islands” are abundantly prevalent in prokaryotic DNA but comparably rare in eukaryotic and especially vertebrate DNA.

The purpose of the present study was to investigate the local effects of immunostimulatory DNA after single and repeated instillation into the native and tumor-bearing murine bladder. Furthermore, the aim was also to investigate if there is relevant uptake of CpG-ODN into the bladder wall. The intention was to evaluate a possible direct effect of CpG-ODN on tumor cells and to examine the effects of CpG-ODN on the cytokine milieu in an orthotopic murine bladder cancer model.

It was possible to demonstrate that CpG-ODN adheres to the intact urothelium and penetrates the mucosal surface of the urinary bladder within ≤2 h. A possible explanation for the phenomenon that biotin-labelled CpG-ODN passes the urothelial layer quickly and FITC-labelled ODN does not might be the larger molecular size of the latter. The biotin-labelled substance seems to be deposited within the submucosa. To the Authors’ knowledge this is the first report on intravesical administration and the local kinetics of CpG-ODN. It was also possible to demonstrate that the agent comes into contact with deeper layers of the urothelium and of the submucosa, i.e. interaction with cells of the immune system or tumor cells is possible. Previous damage or alteration of the mucosal surface does not seem to be necessary.

Histological examination of the bladders showed that immunostimulative DNA exerts an inflammatory response within the bladder wall already after a single 2 h instillation. This response is more pronounced after triple instillation with lymphocytic infiltrations, apoptoses of urothelial cells and lymphocytic infiltration of the mucosa itself. GpC-ODN and PBS-treated controls show no inflammatory response at all, i.e. neither anesthesia, manipulation nor the phosphorothioate backbone modification seem to be responsible for the observed phenomena. The site (submucosa) and the histomorphological pattern (follicular aggregations of predominantly mononuclear cells) of the induced changes appear to be similar to the BCG-induced changes described in the literature (30, 32).

Recent studies have shown immunostimulatory DNA to be an effective immunomodulator with the ability to bias an immunological response towards a Th-1 direction (34). CpG-ODN is able to activate antigen-presenting cells (APC) including differentiation and maturation of dendritic cells. There is an up-regulation of IL-12, IFNγ and TNF-α on the mRNA and protein level and of MHC-class II protein. B-cell proliferation and IL-6 secretion is induced and B-cells are protected from apoptotic cell death (34, 36). Interestingly, T-cells are not directly stimulated by CpG-ODN, but after

Figure 5. Quantitative real time RT-PCR for IL-10, IL-12, IFNγ and TGF-β; Group designation: Table II. The results represent the mean of 5 animals in each group.
crosslinking of T-cells via the T-cell receptor, stimulating ODN can act as a co-stimulant to induce IL-2 secretion and differentiation into cytolytic effector cells (4, 5). Animal studies have shown that CpG-ODN have the potential to initiate an anti-neoplastic immune response (8-11, 36).

In the orthotopic bladder cancer model, a pronounced up-regulation of IL-12 and IFNγ was demonstrated; these cytokines are involved in a Th-1-type immune response, the type of response that is assumed to be essential in BCG action. However, an up-regulation of IL-10 and TGF-β was also observed, cytokines that are essential for regulation or even down-regulation of acute inflammation. The control groups 1 (tumor bearing bladder, untreated) and 4 (native bladder) showed, that overexpression of all cytokines are probably treatment effects. The high expression of IL-10 and TGF-β might be interpreted as reactive. This appears to be plausible as immunocompetent animals were sacrificed after quite a long period of repeated CpG-ODN challenges, giving the immune system enough time to mount a counter-regulatory response towards the artificially induced Th-1 reaction.

Recently, TLR-9 was detected in human benign urothelial cells (37) and in a high percentage of lung cancers (38). To date, the relevance of a direct, non-immunologically mediated effect of CpG-ODN is unclear. To rule out any direct effect of CpG-ODN on bladder cancer in the orthotopic model, TLR-9 expression on the MB-49 cell line and a pro-apoptotic effect of CpG-ODN on these cells in vitro were excluded. Immune cells or even a complete competent immune system appear to be necessary to induce any CpG-mediated anti-neoplastic effect. Recently, there is accumulating data on the expression pattern of toll-like receptors. It becomes more and more evident that TLRs are expressed not only in immune cells. Other tissues and even tumor cells do express toll-like receptors and TLR-9, however the impact of this phenomenon on the therapeutic potential of TLR-agonists is unclear today (39, 40).

Intravesical therapy of non-muscle invasive transitional cell carcinoma of the bladder is the only indication for BCG that is approved by the FDA and European authorities. As early as 1976, Morales et al. had proven the high efficacy of BCG in treating and preventing recurrent superficial bladder cancer with limited and tolerable local, and only rare cases of systemic, toxicity (41). Today, intravesical BCG instillation is the therapy of choice in the prevention of superficial bladder cancer with high risk for recurrence or progression (17). Large scale randomized studies and meta-analyses are available proving the superiority of BCG to other substances in terms of recurrence and progression (18). New instillation schedules with maintenance of instillation prophylaxis for up to 3 years seem to improve results despite the higher toxicity (42).

The mode of action of BCG has not yet been elucidated, but the research of the last 20 years has revealed immunological properties and reproducible effects of BCG that seem to be essential for its anti-neoplastic and preventive effects. BCG effects in the bladder wall appear to be T-cell-mediated, because athymic nude mice are not responsive to a BCG challenge but they regain their ability to react adequately to BCG instillation after transfer of homologous T-cells (23). The urinary bladder is able to mount a delayed-type hypersensitivity reaction in response to BCG and CD4+ cells have shown to be essential for this reaction. During BCG therapy, the CD4+CD8+ ratio changes from 1:2 before therapy to 2:1 after (22, 24).

The mononuclear and polymorphonuclear infiltrations after BCG instillations are found predominantly in the submucosa forming a pattern of so-called BCG-induced granulomas. Infiltration of mucosa and muscle is less pronounced. This parallels the histological and immunohistochemical findings. CpG-ODNs seem to be located in the submucosa after intravesical instillation and the immunological response caused by these CpG-ODN affects the submucosal layer as well.

Examination of local cytokine expression and of urinary cytokine excretion revealed high levels of IL-1, IL-2, TNF-α and IFNγ as early as 2 hours after the first installation of BCG. IL-1 did not discriminate between healthy subjects with uncomplicated cystitis and BCG-induced cystitis, whereas IL-2, TNF-α and IFNγ seemed to be specific for a BCG response (20, 25, 26).

In conclusion, it was possible to show that immunostimulative DNA, a substance class with remarkable local and systemic immunological effects, is able to exert an inflammatory response in the native murine urinary bladder after intravesical instillation. In an orthotopic tumor model, up-regulation of Th-1 and Th-2 cytokines parallels the histological and clinical effects recently described (8, 9). Investigations into human bladder cancer will have to determine the therapeutic potential of CpG-ODN in this disease.

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