Interactions of BCG with urothelial tumor cells in immunotherapy for superficial bladder cancer

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BCG immunotherapy for superficial bladder cancer. A review

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Introduction

Bladder cancer is detected in 75\% of patients at an early, superficial stage. The majority of the superficial urothelial cell carcinomas (TCC) consists of papillary bladder carcinomas (Fig. IA), while the remaining (10 \%) are high grade mucosal lesions called carcinoma-in-situ (CIS), characterized by their diffuse surface-spreading behavior [1]. The standard treatment of papillary TCC is trans urethral resection (TUR) followed by intravesical instillation with adjuvant, chemo- or immunotherapeutic drugs [2,3]. For treatment of the surgically non-accessible CIS these latter modalities are the only bladder sparing options available. Although the therapeutic efficacy is well recognized, a high recurrence rate of 60-70 \% and a progression rate of 15 \%
Th1 response
IL-2, IL-12, IFN-γ, TNF-β

Antigen presentation
Cytokine production
Th1 type response
Cell-mediated tumor cell eradication
Cell-mediated immune response or cell-mediated immunity
Adherence & phagocytosis
Processing antigen
Cytokine production
BCG + Bladder

Side effects
Immune response
Cell-mediated immune response
Inflammation
Natural Killer cell
Bladder cancer cell
Dendritic cell
CD4+ T-cell
APC properties
G1, G2, G3
Grade
TCC
Antigen presenting cell
CD4+ T-cell
dictate the need for lifelong follow-up and treatment [4].

Application of adjuvant agents after surgery was introduced to reduce the risk for recurrence and progression of TCC. After many years of experience with chemotherapeutic agents, more recent, comparative trials indicate that immunotherapy with Bacillus Calmette-Guérin (BCG) is superior to chemotherapy in patients with intermediate and high risk for recurrence. Nowadays it is generally accepted that prophylactic BCG treatment is the preferred form of treatment for patients with more aggressive high-grade disease. In these patients with a high risk for progression or recurrence the benefits out weight the burden of side effects [2,5,6]. Moreover, BCG seems to exert a better anti-tumor effect in CIS and high-grade cancer [2,7]. The recent introduction of an extended BCG treatment protocol, known as maintenance immunotherapy, seems to lead to a further increase of efficacy [5,6].

BCG, an attenuated strain of *Mycobacterium bovis* (Fig. IB), was developed over the period 1908 - 1921 by Calmette and Guérin with the intention to generate a vaccine against tuberculosis. Nowadays, several, commercially available strains of BCG, such as Connaught, Evans, Glaxo, Pasteur, RIVM, Tice, and Tokyo 172, have been extensively studied for (adjuvant) treatment of TCC. Although these strains are genetically slightly different, the clinical efficacy appears to be comparable [8-10]. Unfortunately, treatment with BCG, installed as a viable, living organism, has some severe disadvantages, since it can cause infections, ranging from bothersome cystitis in the majority of patients, to sepsis eventually leading to death in rare cases [11].

Ever since 1976, when Morales and colleagues first described the use of intravesical BCG for superficial bladder cancer [12], investigators try to understand the working mechanism of BCG, as an anti-tumor modality. Both BCG treatment regimen and dose are historically determined. Arbitrary, BCG therapy consists of a single course of six weekly intravesical
instillations with commercially available preparations. Despite its success, 30-50% of patients either fail to respond or relapse within the first 5 years of treatment, while toxicity associated with BCG remains a serious point of concern.

This review intends to present an overview of the current state of "BCG research". Special attention will be paid to the interaction between BCG and the bladder wall, including a possible role of the urothelial cells in the cascade of events leading to BCG-associated tumor cell clearance (and side-effects). As an underdeveloped field of research, the significance of an interaction between potentially hostile bacteria and epithelial cells is a relatively unknown aspect of the host immune-defense system. Better knowledge about this interaction might open new avenues for improvement of the BCG immunotherapy and, in addition, might generate important clinically applicable information for other fields of medicine.

For an actual overview on the practical, clinical aspects of the immunotherapy for TCC with BCG (and other biological response modifiers), the reader is referred to various, recently published papers [2,3,13].

Details of the mode of action of BCG

Nowadays, it is generally assumed that the BCG-induced anti-tumor activity is critically dominated by the local nonspecific immunological reaction reflecting the activity of immunocompetent cells [14,15]. It should be realized however that preceding the activation of the local immune system, some possibly rate-limiting events needed to be executed. The sequential order of these initial events has been addressed only to a limited extent.

Interaction of BCG with the bladder wall

Interaction of BCG with the luminal surface of the bladder is the first step for BCG to accomplish. Accumulation of BCG near the bladder wall as well as its adherence may be limiting processes for an adequate, clinical response. Suboptimal binding of BCG to the bladder has been raised to explain the absence of clinical response in a subgroup of patients [16-18]. In the guinea pig the actual number of BCG organisms found in the bladder wall after BCG instillations represents only a fraction of the number of instilled bacilli [17]. Furthermore, experimental modulation of intraluminal BCG attachment has been connected to considerable effects on the anti-tumor efficacy of
BCG or BCG-induced immune response in animal models [19-21]. Systematic analysis of the interaction of BCG with the bladder wall has not been accomplished, probably due to the poor recognition of various involved processes, biological as well as physicochemical. The process of interaction should be divided in non-specific, physicochemical and specific, receptor-ligand mediated events.

**Physicochemical interaction**

The bladder surface on the luminal side is covered with a layer of hydrophilic, highly sulphated glycosaminoglycans (GAGs), the so-called GAG-layer that protects the bladder from toxic compounds and microorganisms [22,23]. Both this GAG-layer and the BCG cell wall are highly negatively charged (the zeta potentials) [18,24]. As outlined by Schamhart et al. these conditions prescribe that BCG bacteria accumulate or dock, *without adherence*, at a close (70 - 100 Å) distance, the so-called docking distance, from the bladder wall (Fig. 1C) [18,25]. In addition to this non-specific, *reversible* adsorption, physicochemical considerations predict a low probability of *irreversible* adherence of BCG to the bladder wall, due to the high electrostatic, repellant force between the respective surfaces. The observed, in animals, low abundance of BCG adherence to the uninjured bladder wall and the dependency of BCG adherence to diluent properties (pH, salt concentration) seem to be in accord with these theoretical considerations [16,17,26,27]. Damage of the GAG-layer and urothelium may lower the negative charge of the bladder wall, leading to an increased BCG docking and adherence, as observed in a murine BCG model after electrocautery damage of the bladder [16]. Interestingly, the presented physicochemical analysis provides a solid base for the observation that the polyvalent polysaccharide, pentosanpolysulphate (PPS), enhances the binding of BCG and BCG-induced immune response in the guinea pig bladder (Fig. 2) [20,21].

**Specific, receptor-ligand mediated events**

In addition to non-specific interaction, more specific mechanisms seem to be involved in BCG adherence. A crucial binding of BCG to fibronectin (FN) in the bladder mucosa has been postulated [17,28]. FN is part of the extracellular matrix, is equally distributed on normal and malignant urothelium and a soluble form can be found in urine [29]. Binding of BCG to the murine bladder was impaired with anti-FN antibodies or addition of soluble FN [28]. Furthermore, BCG bacteria possess a receptor with high affinity for the collagen domain of FN, the fibronectin attachment protein (FAP). Intravesical addition of FAP fragments abrogates BCG attachment.
Figure 2. The influence of intravesical pentosan polysulphate (PPS) pretreatment on BCG-induced effects in guinea pigs. PPD skin reaction, number of infiltrates in the bladder wall and number of cells per 2 iliac lymph nodes were determined after intravesical placebo (PBS) and BCG treatment (BCG), and pretreatment with PPS followed by BCG treatment (PPS + BCG) [20].

and abolishes the BCG anti-tumor effect in an orthotopic, murine bladder cancer model [30]. These data and the observation that the clinical effect of BCG therapy is related to the degree of FN expression on normal mucosa suggest a specific FN-mediated adherence of BCG to the bladder wall [31]. Following TUR, in 70% of patients the level of urinary FN is high during 2 weeks, while BCG instillation induces an additional, strong rise in urinary FN levels within hours. It has been suggested that the high soluble fibronectin level in the urine of patients after TUR and during BCG treatment is a defense mechanism of the body in order to prevent bacteria from invading the tissue. The originally bound fibronectin is shed as a result of elastase released by polymorphonuclear leucocytes. Inefficacy of BCG instillations when starting too soon after TUR may result [32,33]. Several investigators pointed out, however, that the experimental, animal data were largely obtained with pre-injured bladders, contradictory to the clinical
situations bladders, and suggested a fortuitous relationship between the efficacy of BCG-therapy and fibronectin [17,32].

**Internalization of BCG and phenotypical alterations of urothelial cells**

**Internalization of BCG in urothelial cells**

There are now several reports showing that urothelial cells are capable of internalization of BCG [34-39]. BCG internalization in vitro is time and dose dependent and can already be shown to occur after a 15-min incubation period. In a spheroid, 3-D model, BCG was found to penetrate and internalized in cells, 4 cell-layers deep, whereas normal urothelial cells in this system did not internalize BCG [39]. This latter observation is in accord with studies in guinea pigs showing no BCG in or adherence to normal urothelial cells [27]. In malignant cells, BCG internalization appeared to be cell differentiation-dependent. Contrary to well-differentiated (G1) bladder tumor cell lines, poorly differentiated (G3) cell lines exhibit significant internalization of BCG (Fig. 3) [36].

**Figure 3.** Internalization of BCG and BCG-induced IL-6 production in a series of well and poorly differentiated human bladder cancer cell lines: SBC-2 and SBC-7 (grade 1), T24, TCC-SUP and J82 (grade 3) [37].
Clinically, these data may be related to observations that show a better response to BCG treatment of high grade compared to low-grade tumors (Table 1) [7].

Table 1. Recurrence rate /100 patients-months in patients with superficial bladder cancer, following TUR with and without adjuvant intravesical BCG instillations.

<table>
<thead>
<tr>
<th></th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUR</td>
<td>1.5</td>
<td>3.67</td>
<td>20.83</td>
</tr>
<tr>
<td></td>
<td>ns</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.002</td>
</tr>
<tr>
<td>TUR + BCG</td>
<td>1.18</td>
<td>1.02</td>
<td>1.26</td>
</tr>
</tbody>
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Data from Melekos et al. [7]; ns = not significant.

Knowledge about the mechanism of BCG internalization is scarce. As for the interaction of BCG with the extracellular matrix, an intermediate role of FN has been put forward. Urothelial cells express an integrin (α5β1 receptor) with affinity for FN [30,40]. Pretreatment of T24 cells, a human TCC cell line, with anti-FN or anti-α5β1-receptor antibodies resulted in an impaired BCG attachment to and internalization in these cells. It has been suggested that FN acts as a bridging molecule by binding to this urothelial cell and mycobacterial receptors. Interestingly, interleukin (IL)-6, a cytokine generally found after BCG instillation, upregulates the α5β1 receptor, and thereby may influence BCG binding [41]. However, other investigators were not able to inhibit BCG internalization with anti-FN antibodies [36,42]. Although these conflicting results may be due to different experimental conditions or applied techniques, the current available data do not suggest a mandatory role of FN in BCG internalization. It seems that BCG internalization is mediated by additional molecule(s), possibly co-expressed with FN, such as heparan sulphate-containing proteoglycans that interact with the mycobacterial heparin-binding hemagglutinin adhesin (HBHA) [35,36,42,43]. Regardless the specific, receptor-mediated mechanism(s) of internalization, intracellular BCG is transported within phagosomes to become fused with lysosomes to form phagolysosomes, although recent evidence shows that intracellular, live, BCG can interfere with the fusion of phagosomes with lysosomes [44]. However, the majority of BCG in commercially available BCG preparations consists of dead BCG and
fragments. Most BCG particles will thus be degraded and mycobacterial glycoproteins and lipoproteins are transported to the cell surface [45].

Patient studies, analyzing bladder washings after BCG instillations, revealed vigorous phagocytosis of BCG by leukocytes. With respect to urinary urothelial cells, Becich et al. did, whereas Teppema et al. did not find internalized BCG in urothelial cells [27,34]. Accepting the inability of normal urothelial cells to internalize BCG, these conflicting observations may be explained by the presence or absence of residual urinary tumor cells in the limited number of patients included in both studies. Some of the BCG particles probably escape intracellular degradation. At 6 days after instillation intact BCG organisms were still observed electron microscopically [27,34]. Using the polymerase chain reaction methodology for mycobacterial 16S ribosomal DNA, BCG was detected in 32% of bladder biopsies 1 week after BCG instillation. After one year BCG was still detectable in 4% of the patients [46].

In summary, BCG particles can be internalized and processed by residual, especially high-grade tumor cells. As a consequence, the possibility that BCG introduces phenotypical alterations of TCC cells that affect tumoricidal effector mechanisms has been subject of study. Nowadays a multitude of effects ranging from a direct anti-proliferative/cytotoxic effect of BCG, to a role in the initiation and/or modulation of the host immune response, and to an increase of susceptibility of tumor cells, have been proposed.

**Cytotoxic effects of BCG on BCG-internalizing urothelial cells**

*In vitro* studies with a series of human TCC cell lines show that BCG exhibits direct, dose-dependent cytolytic, anti-proliferative and anti-motility effects [47-52]. In general, the inhibitory effects on cell proliferation were most pronounced in highly dedifferentiated (grade 3) cell lines. Today, the causal mechanism by which BCG interferes with these phenomena is not known. In bladder tumor cell lines internalized BCG causes an increase of intracellular, cytotoxic nitric oxide (NO), while patients treated with BCG showed a high production of NO in the bladder and an upregulation of urothelial-associated nitric oxide synthase (NOS) [38,53]. BCG-stimulated inducible NOS and the concomitant NO production, at high concentration, may cause DNA damage, and cytostatic and cytotoxic effects [54]. Possible accumulation of DNA damage may be related to the observation that, contrary to non-treated patients, urothelial cells of BCG-treated patients express regulatory genes related to DNA repair, knowingly wild type p53 and P21waf1/cip1 [55]. Whether these observations represent an indirect or
direct cytotoxic effect of BCG in situ remains unclear, since IFN-γ, a well-known modulator of NOS and NO production, is produced in high quantities during BCG therapy.

**BCG-internalizing urothelial cells and the initiation / modulation of the immune response**

Activation of the host immune system has been considered an exclusive characteristic of professional antigen-presenting cells (APC), like dendritic cells and macrophages. New insights emphasize a possible, additional but causal role of the interaction between epithelial cells and bacteria in the (early) initiation of the immunological cascade [14,56-58]. An encounter between airway epithelial cells and microorganisms, like *E. coli* or *Citrobacter*, is accompanied by the expression of a broad range of immunomodulating molecules [59-61]. These findings challenge the traditional view of the epithelium as solely a passive physical barrier to the external environment. Regarding the interaction between BCG and urothelial cells, similar observations have been made. Urothelial tumor cells are now considered as active participants in the cytokine-mediated initiation and/or regulation of the early immune response during BCG therapy [14]. Interaction of BCG with urothelial cells can be a fruitful model to study this new concept of the bacterial-associated immune response of the host.

**The initial immune response to BCG.**

The local immune response to bacteria, including live BCG, is complex, but the presentation of bacterial antigens, peptide fragments, by APC to T-helper cells is the pivotal interaction [14]. Derived from an extensive series of papers, it appears that, after internalization and processing by APC, processed BCG antigen(s) become associated with MHC class II molecules. The antigen-MHC class II complex is expressed at the cell surface to be recognized by CD4+ T-helper lymphocytes via the T cell receptor (TCR) molecule. Along with this binding, the interaction between the APC and CD4+ T-cell is only fully accomplished by an additional series of co-stimulatory, but essential, interacting molecules. Among others, binding of CD4 to MHC class II, lymphocyte function associated antigen-3 (LFA-3) to intercellular adhesion molecule-1 (ICAM-1) and CD28 (CD4+ cell) to B7 enhance the conjugation of the cells and promote T-cell activation signals. It is of importance to appreciate that generation and “fine-tuning” of both APC and T-cells need additional signals, provided by soluble cytokines, such as IL-2, IL-6 and IFN-γ.
Depending on many nonspecific factors, including antigen dose, type of APC and the expression of the mentioned membrane-bound co-stimulatory signals of the T-helper cells, a so-called Th1-type and, to some degree, a Th2-type response develops during BCG treatment [15]. The Th1 or cell-mediated immune response, and the Th2 or humoral immune response, are characterized by the patterns of cytokines, secreted by the CD4+ T-helper cells following antigen-specific stimulation. As fully established in mouse models, the Th1 or Th2 responses are characterized by the accumulation of primarily IL-2, IL-12, IFN-γ and TNF-β or IL-4, IL-5, IL-6 and IL-10, respectively. The recognition that this description of the two types of responses reflects the human immune response and that they are regulated in a reciprocal fashion, critically regulated by the cytokines, represent a major advance in the field of anti-tumor cytotoxicity mechanisms [62].

The outlined accumulated information about the APC–T-helper cell interaction and development of the cell-mediated immune (Th1 response) and humoral immune (Th2 response) has been used to study the urothelium before and after BCG therapy and human TCC cell lines to provide insights of a possible role of TCC cells in antigen presentation and initiation of the immune response.

**Uroepithelial tumor cells and antigen-presenting properties.**

Immunocompetence of the host is essential for effective BCG therapy [63]. APC, such as dendritic cells and macrophages, and CD4+ T-lymphocytes play a crucial role in the anti-tumor effect [64-66]. In addition, evidence has emerged indicating an important role of epithelial, TCC cancer cells. Lattime and co-workers were the first to provide evidence that (mouse) bladder tumor cells, MB49, were capable of BCG antigen presentation to CD4+ T-cells, via MHC class II molecules [67]. These initial observations were extended for another murine cell line, MBT-2, and a panel of human cell lines. Beside MHC class II, constitutive and BCG-induced expression of the major co-stimulatory molecules ICAM-1 and B7-1 were observed in these cell lines. Furthermore, it has been observed that these antigen-presentation factors are enhanced in high grade, but not in low grade, TCC cell lines (Fig. 1D) [68-70]. (Quantitative) immunohistochemistry has confirmed the in vitro findings. Serial bladder biopsies and urinary cytopsins, taken before and after BCG therapy, revealed an upregulation of MHC class II and ICAM-1 expression of urothelial tumor cells [71-73]. Compared with normal urothelium, untreated patients with bladder cancer had significantly less macrophages, and less MHC class II expression, fitting in the theory of host “immune escape” [74]. BCG seems to restore the local immune response. In addition to this classical presentation of antigens, recently non-MHC-encoded, CD1 restricted presentation of antigens of lipid or glycolipid nature.
has been recognized [44]. Although for BCG no data are available, Sieling et al. observed the presence of CD4⁺ CD1-restricted T-cells in patients suffering from *M. leprae* [75]. These cells produce IFN-γ, but not IL-4. BCG-therapy related research has revealed an increased CD1 expression of TCC cell lines in the presence of live BCG [70] and a virtual absence of urinary IL-4 during BCG treatment [76]. From these findings it is tempting to hypothesize that CD1-facilitated BCG antigen presentation contributes to the development of BCG immunity.

The outlined results indicate that epithelial TCC cells gain the phenotypical characteristics and functioning of antigen-presenting cells in the presence of BCG. These functions strongly suggest that the BCG-high grade tumor cell interaction act in cohort with the BCG-professional APC interaction (Fig. 1E). Clinical observations seem to confirm this conclusion, since BCG therapy seems to exert a better anti-tumor effect in high-grade bladder cancer (Table 1).

**Secretion of cytokines by urothelial tumor cells**

Following intravesical BCG instillations there is an increase of the urinary level of more than a dozen cytokines, such as IL-1, IL-2, IL-6, IL-8, IL-12, IL-18, TNF-α and IFN-γ [77-84]. Undoubtedly, the major cell sources are the immunocompetent cells, but urothelial cells seem to contribute to a significant degree. *In vitro* studies with human TCC cells revealed a BCG-induced upregulation of the cytokine IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and IFN-α, but not IFN-γ, IL-2, IL-4 and IL-12 (Fig. 1F, G) [37,85-88]. Of all cytokines produced by TCC cell lines, the cytokines IL-6 and IL-8 are highly prominent. As a consequence, their role in the induced immune response will be discussed here in some detail.

*Interleukin-6.*

Within the challenged immune system, several cell types including APC produce IL-6. It is a multifunctional cytokine critically involved in the acute phase response. T-cell proliferation B-cell maturation, macrophage maturation, and cytotoxic T-cell differentiation. In combination with IL-1, IL-2 and IFN-γ, IL-6 induces expression of the IL-2 receptor (IL-2R), showing its role in the activation of resting T-cells [89]. Furthermore, IL-6 seems to contribute to MHC-nonrestricted cytotoxic activity by inducing NK cell proliferation. Nowadays, NK cells are considered as essential, cytotoxic effector cells during BCG therapy (Fig. 1H) [90].
Although the high constitutive and BCG-induced production of IL-6 by high grade TCC cell lines is well recognized, the possible contribution of IL-6 produced by bladder cancer cells to the BCG-induced immune response remains to be established. In vitro studies revealed that IL-6 mRNA upregulation and IL-6 production depend on the dose, the incubation period, the internalization of BCG and the TCC cell grade (Fig. 3) [37,85,87,91]. Enhancement of the IL-6 synthesis of TCC cells is not absolutely specific for BCG and has been found for Escherichia coli and Citrobacter freundii too [61]. However, the relative strong capability of BCG to induce IL-6 in TCC cell lines was indicated after comparison with other bacteria or bacterial cell extracts, like Listeria, E. coli, S. faecalis, and cell (wall) extracts of Nocardia rubra or M. phlei [85,92]. IL-6 production requires a minimal BCG exposure time of 0.5-1 h, while maximum production is reached after an incubation period of 2-3 h. These data seem to be in accordance with clinical practice.

IL-6 is considered a major source of early Th1/Th2 control during CD4+ T cell activation as it attributes to the promotion of the Th2 response and simultaneously inhibition of Th1 polarization (Fig. 1G). As recently shown by Diehl et al. IL-6 activates the production of IL-4 by CD4+ T-cells and their differentiation into Th2 effector cells. Moreover, it inhibits Th1 differentiation by interference with IFN-γ signaling and the development of Th1 cells [93]. The abundant IL-6 response during BCG therapy and virtually absence of urinary IL-4 seem to be however in conflict with the recognition that the presence of BCG primarily induces a Th1 response. The absence of IL-4 and a high production of IFN-γ may prevent or contradict the Th2 promoting effect of IL-6 [94,95]. The absence of IL-4 production during therapy with live BCG suggests an important role of a CD1 restricted presentation of antigens of lipid or glycolipid nature, since CD4+ CD1-restricted T-cells produce IFN-γ, but not IL-4 [70-75].

Interleukin-8.

IL-8. IL-8 is a proinflammatory cytokine with strong chemotactic properties, attracting T lymphocytes and neutrophils. De Boer and associates observed that, compared to urinary IL-2 and IL-6, IL-8 is rapidly, already after the first instillation, induced during BCG therapy [81]. Dendritic cells, macrophages and a number of other cells, including TCC cell lines [87], produce IL-8. The early urinary IL-8 production in vivo may indicate the significance of an interaction of BCG bacilli with (residual) bladder cancer cells in the initiation of the host immune response.
**Cell-mediated anti-tumor effects: the effector cells**

The final step in the eradication of tumor cells consists of mobilization, activation and an interaction of cytotoxic effector cells with the tumor cells (Fig. 1H) [63-65,96]. *In vitro* studies have provided some evidence that nonspecific cytolytic cells (natural killer cells, BCG-activated killer cells, lymphokine-activated killer cells) are involved. Brandau *et al.* pointed to a key role for natural killer (NK) cells [90]. NK cells are a special population of mononuclear cells. They recognize 'self'-peptides presented by MHC class I molecules on the surface of cells. A cell not displaying these peptides in a correct way is attacked and killed by NK cells [97]. In untreated bladder cancer patients a loss or alteration of MHC class I expression is seen in tumor cells [74]. BCG infected cells present BCG glycoprotein and lipoprotein antigens on their MHC class I molecule. [45] This may be a trigger for NK cells to attack BCG infected urothelial tumor cells. BCG therapy in the murine model, using NK cell deficient mice, is reported to be ineffective [90]. However, in studies regarding the presence of NK cells after BCG therapy, relatively few NK cells were seen 3 weeks after the last instillation of a six-week course [72,74]. It would be interesting to know if NK cells are more abundant earlier, during the BCG course. Apart from these cells, *in vitro* studies have indicated other cell types that may play a role in the final step of eradication, such as BCG activated killer cells (BAK), lymphokine activated killer cells (LAK), macrophage activated killer cells (MAK) and cytotoxic T-lymphocytes [63,98-106]. However, in humans strong direct evidence about the actual effector cell(s) is lacking. The recent acknowledgement of effector cells that recognize mycobacterial (glyco)lipid antigens through non-polymorphic MHC molecules, such as CD1, may provide new insights in the true nature of the cytolytic effector cells involved in tumor cell eradication during BCG treatment [44,98].

**Future directions for clinical improvement**

This section considers the currently available knowledge, collected from clinical as well as experimental studies, about the supposed optimal conditions necessary for BCG therapy to act against bladder cancer cells. Actual clinical experience, gathered over a period of many years, is considered as a valuable source for clinically orientated fundamental research. Mutual interaction is an absolute prerequisite to define the directions of research at which potentially fruitful improvements, aiming at efficacy with minimal side effects, can be established.
**BCG preparation.**
Clinical as well as experimental observations have been interpreted as evidence for an absolute necessity to treat with live organisms in order to provoke a local immune and clinical response [98,107,108]. Recent, experimental information challenges however the absolute necessity for viable bacteria during each of the 6 weekly instillations. Compared to the standard regimen, initial instillations (3 times) with viable BCG followed by 3 instillations with killed BCG resulted in a highly comparable cytokine response in a mouse model [109]. Provided that the “degree” of BCG-induced immune activity is related to clinical efficacy, such an approach may reduce toxicity while maintaining anti-tumor activity.

**Mode of administration.**
In the past various modes of administration have been proposed, but contact between BCG and the target organ to be treated was shown to be crucial for anti-tumor effect. Consequently, intravesical administration of BCG is now considered as the optimal route [3,63]. This condition is convincingly underscored with upper urinary tract carcinoma that requires, for effective treatment, the introduction of BCG through a nephrostomy catheter or ureter catheter [110,111]. These observations illustrate that the mode of action should be considered first of all as a local immune activation, requiring intense contact. Research efforts have been directed to improvement of adherence of BCG to the bladder wall [19,20,25]. Preliminary results suggest that an improvement can be accomplished by altering the physical-chemical conditions of the diluent or addition of polyvalent polysaccharides.

**Dose of BCG.**
In an attempt to reduce toxicity BCG dose reduction have been evaluated in several clinical trials, but no definite conclusions about efficacy as well as toxicity can be made yet. Several randomized, multicenter, prospective trials indicate a decreased incidence of side effects without significant overall differences in recurrence or progression rate after BCG administration at a reduced dose [112-115]. Accepting viability of BCG organism as crucial for clinical efficacy [107], it is important to realize that the lack of well-defined BCG preparations, in terms of viable BCG content, makes comparisons between different studies virtually impossible. The content of viable BCG, expressed as colony-forming units (cfu), may differ from $6 \times 10^6$ to $1 \times 10^{12}$ per BCG ampoule, depending on the strain, lot and period of storage. The frequently used characterization by dry weight, largely determined by the extremely high content of subcellular debris and dead bacilli [116,117], does not represent the content of viable bacilli.
Treatment schedule.

Patients may benefit, with a reduction of recurrence and progression, from some form of maintenance therapy, consisting of repeated courses of BCG [5,6,118-120]. Although no definite proof has been presented and the optimal schedule of these intensive treatment protocols is still debated, maintenance schedules are currently used in many centers. Surprisingly and in contrast to the current tendency to increase the number of instillations, recent, preliminary experimental evidence questioned the requirement for a total of 6 instillations during the BCG induction course. In a mouse model, a highly comparable degree of immunological activity, reflected by the Th1 response, was observed after a total of two intravesical BCG instillations, at week 1 and week 6. Accepting a causal relation between the BCG-induced Th1 immune response and anti-tumor activity, these results suggest that, during the induction course, the intermediate (weeks 2, 3, 4 and 5) BCG instillations may be omitted without loss of therapeutic efficacy [121]. Based on the lymphoproliferative response of PBMNC by BCG-derived antigens, Zlotta et al. suggested that the number of BCG instillations may be reduced in those patients that are reactive to BCG antigens prior to therapy [122]. To what extent intermediate instillations can be omitted during maintenance therapy, notable for its side effects, remains to be established.

Tuberculostatic agent

In order to diminish side effects, specific treatment by isoniazid (INH), a tuberculostatic drug, preceding BCG instillation, has been considered and studied in a limited number of clinical trials [123,124]. INH interferes with the production of Mycobacterial cell wall-associated saturated fatty acids, leading to cell lysis if the bacterium is dividing [125]. Application of INH assumes however that INH does not affect, at any level, the complex BCG-induced host response. Detailed data on potentially interfering effects of INH on these successive steps are scarce or absent. Bevers et al. [126], accepting a critical role of tumor cells in the BCG-induced cascade of immunological events, studied the affects of INH on various characteristics of human bladder cancer cells, grown in vitro. No effects of INH were found on BCG-induced upregulation of various cytokines in these cells. The results of animal studies were, however, conflicting, revealing either no effects of INH or an impairment of BCG-induced reactions [127-129]. In a study with a small number of patients lacking however satisfactory statistical power, no impairment by INH of the local immune stimulation after BCG instillation was found [130]. Clinical studies indicated no interference with efficacy in patients with intermediate and high-risk bladder cancer [124,131]. However, no reduction of the side effects of BCG with INH was found, while in contrast INH-associated liver function disturbance was encountered.
Consequently, the prophylactic use of INH was considered as impracticable [123,124]. Durek and associates determined the effects of modern antibacterial chemotherapeutics on BCG viability, but the potential effects of these drugs on the BCG-induced host response are only scarcely addressed [132,133].

**Subcellular fractions of BCG, recombinant BCG and other mycobacteria**

The need of viable BCG for a highly effective initiation of a local immune response and anti-tumor activity has been generally accepted [63,134]. However, the potential use of non-viable, killed BCG, subcellular BCG fractions, modified BCG and non-BCG approaches have retained interest over the years to reduce side effects.

**Non-viable Mycobacteria.** Recent studies explored the cytotoxic effect of more or less defined components of BCG and other mycobacterial species. Investigations with human mononuclear cells *in vitro* showed that several subcellular fractions of BCG are capable to induce the production of cytokines, like IL-12 and IFN-γ and enhance non-MHC-restricted cytotoxicity against T24 bladder tumor cells. [135,136]. The therapeutic efficacy of these BCG-derived fractions has not been addressed yet. Interestingly, a cell wall-DNA complex (MCC) of another *Mycobacterium, M. phlei*, appears to induce apoptosis in human TCC cell lines, while intravesically administrated MCC provoked an inflammatory response and anti-tumor action in the orthotopic MBT-2 mouse model, although it was less than that of BCG [92,137]. In patients with carcinoma in situ, a 60 % success rate at 12 weeks was reported after instillations with these cell wall-DNA complexes, suspended in mineral oil droplets [138].

**Modification of BCG.** An interesting new approach comprehends the introduction of genetically engineered BCG, capable of secreting relevant cytokines, such as human interferon-alpha 2B, IL-2 or IL-18 [15,139-141]. Provided the establishment of an improved contribution to immunotherapeutic protocols, dose reduction of recombinant BCG may be considered to diminish side effects.

**Non-BCG approaches.** Research is ongoing for new immunomodulators with anti-tumor activity for superficial bladder cancer [2,142]. A whole spectrum of immunomodulating agents has been proposed. These so-called biological response modifiers range from the noted MCC to *Nocardia rubra* cell-wall skeletons, *Allium sativum* (garlic), *Lactobacillus* species, Keyhole Limpet Hemocyanin and recombinant cytokines [48,143-151]. The promising results of some of these agents justify randomized trials, which should compare clinical efficacy and toxicity of these substances with BCG. Among others and despite the existence of contrary data, some studies suggest the clinical feasibility (without attending major side effects) of
simultaneous intravesical instillation of IFN-α and low dose BCG, or recombinant human IL-2 alone [148,149]. To date, however, the clinical feasibility, efficacy and toxicity, as either a single or additional treatment, remains a challenge.

**Prognostic markers.**

Non-invasive, urinary markers that predict immediate and long-term response to BCG would provide, among others, an efficient tool to monitor attempts to optimize BCG treatment. Several independent research groups found an association between BCG-induced urinary cytokine production, such as IL-2, IL-8 and IL-18, and the clinical response in patients and evidence has been presented that urinary cytokine production during BCG therapy can predict the clinical response [82,83,151]. On the other hand, Jackson et al. were neither able to identify a prognostic value of any cytokine [94]. As noted by these latter authors, these conflicting results may be due to differences in methodology, indicating the urgent necessity of a “consensus method” with regard to sampling (time and period), pre-analytical work-up, assay method and inter-laboratory standards [82].

**Summary and conclusions**

Illustrated in Fig. 1, the current insight of the mode of action of BCG, ranging from its introduction into the bladder towards killing of residual tumor cells, has revealed a complex sequence of processes. BCG accumulates near the bladder wall, followed by adherence and passage through the protecting outer GAG layer. Subsequently BCG is internalized and processed by professional antigen-presenting cells (APC) and tumor cells. These processes alter the gene expression of these cells, accumulating in the presentation of BCG antigens, and secretion of particular cytokines (also termed interleukins). BCG antigens are presented via MHC class II molecules to CD4+ T-cells and via MHC class I molecules to CD8+ T-cells. Lipid and glycolipid BCG antigens can be presented to CD4+ and CD8+ T-cells in a non MHC-restricted, CD1 restricted fashion. Production of chemokines, such as IL-8, secreted partly by BCG-internalized tumor cells, contributes to the local activation of the immune system and invasion of activated leukocytes and mononuclear cells into the bladder wall. These developments provide the condition for a Th1 cell-mediated immune response, associated with a series of particular cytokines, such as interferon [IFN]-γ, IL-2, IL-12 and tumor necrosis factor [TNF]-β. This specific cytokine profile promotes delayed-type-hypersensitivity (DTH) response,
cytotoxic cell response, and macrophage activation or cellular immune inflammatory reaction. Since the ultimate effects of bacteria are not always predictable and depend on both constitutive and induced bacterial and host components, an upregulation of the humoral immune response or Th2 response, noted by the cytokines IL-6, and IL-10, may occur to some degree and adversely affects the functioning of the cell-mediated immune response. The Th1 cytokine profile enables recruitment and maturation of cytotoxic effector cells. No definite statements can be made yet about the actual effector cell(s), but a crucial, cytotoxic role of NK cells has been proposed. In addition, some of the cytokines, and BCG itself may exhibit a direct cytotoxic effect on tumor cells.

In 27 years of major research efforts, understanding of the mode of action underlying BCG therapy for bladder carcinoma is obviously much improved. Yet the jigsaw is not complete and many details wait unraveling [14,15,96,142]. For example, to what extent, if any, acute inflammation resulting in cystitis and a humoral response contribute to the anti-tumor effect of BCG has not been fully established, illustrating the lack of complete understanding. However, if successful, the reward might be a better, evidence-based BCG immunotherapy with optimal clinical efficacy and minimal occurrence of side effects in the form of an optimal BCG dose and treatment schedule, genetically engineered BCG, or particular antigenic molecule(s) that trigger immunological anti-tumor activity in a well-controlled manner.
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