



Bacillus Calmette-Guérin immunotherapy—increasing dose as a means of improving therapy?

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Abstract: Bladder cancer is not life threatening but it is characterized by frequent recurrences which may progress to muscle invasive disease. The standard therapy for intermediate and high grade non muscle invasive bladder cancer is tumor removal followed by *Mycobacterium bovis*, *Bacillus Calmette-Guérin* (BCG) immunotherapy. This consists of weekly intravesical instillations of BCG that are divided into induction and maintenance phases. BCG immunotherapy stimulates the immune system and this leads to tumor removal. While BCG immunotherapy is regarded as the most successful immunotherapy, it is associated with side-effects that can in some cases be so severe that patients cannot complete this therapy. Those who fail to complete therapy are more likely to have a recurrence. Some 30–50% of patients will have a recurrence despite therapy. Thus most clinical and laboratory analyses are aimed at improving the response to BCG immunotherapy and trying to identify those will respond to therapy from non-responders. These are not trivial problems as clinical studies on BCG immunotherapy are not all similar. These studies are conducted in different countries and use different BCG strains, doses and schedules of therapy such as frequency of induction and maintenance instillations. Patient genetic polymorphisms and tumor characteristics are also known to impact response to therapy. These differences do make improving BCG therapy challenging. A recent study has proposed a novel strategy of dose increase to improve BCG induced cytotoxicity. The study is discussed in the context of our current knowledge of the response to BCG immunotherapy.

Keywords: Bladder; cancer; *Bacillus Calmette-Guérin* (BCG); immunotherapy

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Bladder cancer

According to Globocan 2012, bladder cancer is the eleventh most common cancer worldwide. It occurs more commonly in Europe, North America, North Africa and Western Asia (1) with a greater occurrence in males rather than females and in older people. The majority (75–85%) of bladder cancers are confined to the mucosa (Ta or carcinoma *in situ*) or submucosa (T1). Though the incidence rate of bladder cancer is decreasing worldwide (1), it still poses a significant problem as patients who have bladder cancer are prone to frequent recurrences. These

may eventually progress to muscle invasive disease. The cause of recurrence has been attributed to either remnant tumor cells missed at surgery or precancerous lesions that later develop into cancer. Without therapy the majority (88%) of patients with bladder cancer are likely to have a recurrence (2).

The gold standard treatment for intermediate and high grade non-muscle invasive bladder cancer is surgical removal of the tumor followed by *Mycobacterium bovis*, *Bacillus Calmette-Guérin* (BCG) immunotherapy. It is the most successful immunotherapy in clinical practice and reduces the incidence of recurrence (2,3).

BCG immunotherapy

The first use of BCG immunotherapy for the therapy of bladder cancer was by Morales in 1976 (4). Morales developed this trial based on the studies of several researchers who showed in animals that BCG instilled in the bladder could induce immune activation (5,6). Since then many clinical trials have been performed. The therapy consists of weekly intravesical instillations of BCG. It is believed that the instilled BCG induces a non-specific immune response in the bladder (7) in an attempt to remove the bacteria which inadvertently removes the remnant tumor cells and/or precancerous cells that give rise to recurrent tumors. Almost every type of immune cell has been shown to play a role in the immune response to BCG therapy and this is well presented in the review by Redelman-Sidi *et al.* (8). However there are still a significant number of patients (30–50%) who do not respond to therapy and why this is so is not known. The dose of BCG, frequency of BCG instillations, BCG strain and patient genetics are all important variables that impact patient response to therapy.

Frequency of BCG instillations

The South West Oncology Group (SWOG) recommends six weekly instillations of BCG (induction phase) followed by three instillations BCG (maintenance therapy) every 3 months for 3 years (9). The latest meta analyses show that better recurrence and progression free survival are associated with maintenance therapy (10). But the long therapeutic schedule can be burdensome for patients.

There is evidence that prior BCG vaccination and the presence of pre-existing immunity results in improved recurrence free survival (11). Zlotta *et al.* assayed lympho-proliferation against mycobacterial antigens in patients receiving BCG immunotherapy pre therapy and weekly post BCG therapy (12). They found that subjects with reactivity to mycobacterial antigens pre BCG immunotherapy had maximal lympho-proliferation after the 3rd or 4th week of BCG instillations. For those without any reactivity pre-BCG therapy maximal proliferation was observed at 6 weeks. This was confirmed in mice where prior vaccination with BCG and confirmation of the generation of BCG T cells correlated with improved response to BCG immunotherapy (11).

The impact of the frequency of BCG dosing was evaluated by de Boer *et al.* who showed in mice that

reducing the number of BCG instillations from 6 to 2 (13) did not reduce efficacy in terms of cytokine gene expression. Normal mice instilled with BCG at the 1st and 6th week resulted in similar cytokine gene expression as mice given a weekly dose of BCG for 6 weeks. However, they did not evaluate this therapeutic schedule in tumor bearing mice.

BCG strains

The original *Mycobacterium bovis* vaccine strain was generated by Calmette and Guérin, in the early 20th century, by maintaining the bacteria in continuous culture (230 passages) over a period of 13 years from 1908–1921. This resulted in attenuation of the strain. By 1924 BCG was distributed to several countries. But as the technical ability to lyophilize BCG and/or store samples at –80 °C were not available until the 1960s, BCG was maintained in continuous culture leading to further attenuation and differences between the strains in terms of antigenic potential (14) and loss of T cell epitopes (15). These are probably a cause of the variability of the efficacy of the vaccine strains. For bladder cancer therapy several different BCG strains have been utilized but very few comparative studies have been performed on their efficacy.

In a randomized Phase III trial conducted in Switzerland the efficacy of BCG Connaught and TICE was evaluated on 142 patients. There was a median follow-up of 25 months and the 5 years recurrence free survival was 74% for Connaught and 48% for TICE (P=0.0108) (16). The poorer performance of TICE may be attributed to its lower survival in cells (17). Another trial compared BCG Tokyo (BCG Japan) and Connaught in patients with carcinoma-in-situ (CIS), but due to the lack of the Connaught strain this trial did not achieve significance (18). The 2-year recurrence-free survival was 73.2% and 68.8%, for Tokyo and Connaught, respectively. BCG Japan has better survival in macrophages being a strain that produces methoxymycolate (14). BCG Japan, is the most attenuated BCG strain and is associated with the least complications; strongest tuberculin reactivity and the best viability after lyophilisation (11,16). Trials with other BCG strains are small and underpowered to determine efficacy differences. BCG sub-strains used in clinical therapy include BCG Pasteur, Glaxo, Moscow, Moreau, A Frappier, S African, Copenhagen, Romanian, RIVM/1 (19). Good outcomes have been obtained with BCG Moreau (19) and BCG Pasteur Danish strain 1331 for patients with TIS (19). BCG RIVM was as effective as mitomycin C for patients with pTa, pT1 and CIS (20).

BCG interaction with cancer cell lines

A comparison of eight BCG sub-strains (Japan, Moreau, Russia, Connaught, Danish, Glaxo, Phipps and Tice) for their ability to kill bladder cancer cells and induce cytokine production revealed that Russia (an early strain) and Connaught (a late strain) were the most effective at killing tumor cells and inducing cytokine production (17). A comparison of Moreau, Tice and RIVM for direct anti-proliferative effects on T24 cells and indirect effects via activation of dendritic cells (DC) and peripheral blood mononuclear cell (PBMCs) showed that these three strains had similar indirect effects on the bladder cancer cells but little direct effects (21).

It is known that BCG interacts with the fibronectin receptor ($\alpha 5\beta 1$ integrins) (22). Binding to $\alpha 5\beta 1$ integrins triggers p21 dependant cell arrest and reduces apoptosis (23). BCG increased HMGB1 secretion (24) in bladder cancer cell lines and BCG internalization modulated cellular redox levels (25). The viability of BCG modulates the amount of reactive oxygen species generated (26,27) and this is related to BCG induced cell death.

The relevance of BCG effects on human bladder cancer cell lines is questionable because BCG therapy commences after removal of the tumor mass. Thus there should be only a few cancer cells present except in the case of TIS.

Immune cells modulate response to BCG

Analysis of patient tissue has shown the importance of immune cells in the bladder environment. Increased CD4⁺ and GATA3⁺ T-cells in the tumor environment was associated with increased recurrence free survival post-BCG immunotherapy (28) while Tissue associated macrophages (TAMs), T regulatory cells (Tregs) and T-bet⁺ T-cells are associated with poor outcomes (28,29). The importance of the Tregs was confirmed in a clinical study using anti-CTLA4 blockade which resulted in increased CD4⁺ICOS^{hi} IFN γ expressing T cells over Treg cells in the bladder and peripheral blood (30). But after CTLA4 blockade more of the CD4⁺ICOS^{hi} T cells could recognize tumor antigens (31).

Dose of BCG

The normal dose of BCG used, termed standard dose contains between 1×10^9 – 1×10^{12} colony forming units (CFU) of lyophilized BCG depending on the strain used. But the

viability of BCG in lyophilized preparations for different strains also varies. Some patients are unable to tolerate standard dose BCG and thus 1/3 (32) and 1/6 (33) doses of BCG have been evaluated for immunotherapy as well as 1/3 dose BCG and IFN α (34). The lowering of BCG dose reduces side-effects and adverse events. In clinical trials, standard dose and 1/3 dose BCG seem to have similar outcomes (35).

Shah *et al.*'s study takes a different view of the dosage of BCG rather than decreasing the dose of BCG, they considered the effect of increasing the dose of BCG (36). They report that increasing the ratio of BCG to bladder cancer cells resulted in increased adherence; NF κ B function and cytokine production with a plateau around 200:1. When they examined cell death by necrosis the amount of necrosis started to decrease at higher doses. But a higher ratio of BCG to cells resulted in better outcomes in mice with bladder tumors. They evaluated three instillations of BCG given at a 3–4 days intervals. The short duration of the animal studies performed by Shah *et al.* means it is difficult to determine the efficacy of this strategy. The authors do acknowledge that the use of a higher dose of BCG has to be considered in the context of likelihood of adverse events. In a Phase III trial evaluating TICE some 20% of patients had to stop therapy due to side-effects (37) during the maintenance phase. In this trial patients received 2×10^8 – 5×10^8 CFU of TICE. Increasing the amount of BCG used in intravesical therapy will increase side-effects.

This work also disregards the impact of immune cells which are known to be major players in the modulation of the response to BCG. The dose of bacteria and timing of the exposure are known to differentially modulate immune cells. For Lactobacillus species exposure of DC to a higher dose of Lactobacilli results in tolerance rather than immune activation (38).

Genetic control of BCG survival in macrophages and patient response to therapy

A further complication is that genetic differences could influence response to BCG as well. Skamene *et al.* were the first to show that the ability of host macrophages to respond to BCG was controlled by the BCG gene later identified as the natural resistance associated macrophage protein 1 (Nramp1) gene (39,40). Kadhim *et al.* demonstrated that the Nramp1 gene controlled response to BCG immunotherapy in a murine orthotopic model of bladder cancer (41) using BCG sensitive and resistant mice strains. The BCG sensitive

mice respond to BCG immunotherapy. BCG survival in macrophages from BCG sensitive and BCG resistant mice is related to the production of nitric oxide (NO) (38). The BCG resistant cells produce more NO. How this is linked to the NRAMP gene is not known?

As a consequence of these results, single nucleotide polymorphisms (SNPs) in the NRAMP1 gene have been evaluated in patients with respect to the response to BCG therapy. The NRAMP1, SNP analyses have produced contradictory results (42-44) in bladder cancer patients. This is likely due to differences in genotype expression at polymorphic sites in different human populations. Polymorphisms in a variety of cytokine genes (45,46); DNA repair enzymes (47), FASL (48) oxidative stress response genes (49,50) have been evaluated as well.

A recent study has shown that besides germline mutations somatic cell differences such as E2F4 expression in tumor versus normal tissue was predictive of the response to BCG (51). Predictive signatures have been found in T1 tumors that correlated with response to BCG therapy (52).

Conclusions

Shah *et al.* have introduced an interesting concept that an increased BCG dose would improve response to BCG immunotherapy. However more work needs to be performed taking into account the importance of immune cells, BCG strain differences and patient genetic factors before it can be determined if this would be of clinical benefit. If a higher dose of BCG is to be evaluated it may be a better strategy to reduce the dosing schedule as suggested by de Boer *et al.* (13). Forty years since the introduction of BCG immunotherapy we are still trying to understand how it works. Because without understanding, it is not possible to improve or optimise this therapy. The quest is on-going to determine the optimal dose; dosing schedule; BCG strain and adjuvant therapy.

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