

RECENT KNOWLEDGE ON BCG'S MECHANISM OF ACTION IN THE TREATMENT OF SUPERFICIAL BLADDER CANCER

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ABSTRACT

Intravesical Bacillus Calmette-Guérin (BCG) against superficial bladder carcinoma recurrences is regarded as the most successful immunotherapy to date. However, the mode of action has not been fully elucidated yet. The aim of this review is to provide a conclusive overview on this complex field and to give detailed information on several aspects of relevance for the understanding of the involved immune mechanisms.

The BCG-induced inflammation after intravesical immunotherapy in patients obviously differs from non-specific inflammation by its quality and its subclinical duration. The pronounced infiltration of the bladder wall by immunocompetent cells together with the secretion of cytokines into the urine point toward the intense local immune activation after BCG. In-vitro models of BCG-induced cytotoxicity have shown powerful and selective effector mechanisms. Orthotopic animal models gave valuable information, confirming and extending ex-vivo and in-vitro data. These approaches led to a hypothesis of BCG-induced tumor control, which will be the platform for further improvements of this highly effective anticancer immunotherapy.

Key words: BCG, bladder neoplasms, immunotherapy, mycobacterium bovis, mechanism
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INTRODUCTION

In 1976, Morales, Eidinger & Bruce were the first to report on successful treatment of superficial bladder cancer (SBC) with BCG (1). Since then, BCG became the treatment of choice for high-risk superficial bladder cancer in most countries of the world and is given at an annual rate of approximately 1 million. Intravesical BCG therapy is regarded as the most successful immunotherapy to date (2,3) and not only is superior to intravesical chemotherapy with regard to the recurrence rate of SBC (4-6) but also beneficially acts on the progression rate of this tumor (7-9) and, obviously, also positively influences survival in high-risk patients (8,9).

Ever since the immuno-activating properties of BCG were discovered, investigations have been carried out to ascertain the functional mechanism. All investigations to date have shown that not one single functional mechanism, but a whole series of immu-

nological phenomena are involved. For this reason, the following contribution is only able to provide an overview of the most important findings from this interesting area of research.

GENETIC BACKGROUND OF BCG

What is BCG? The life attenuated Bacillus Calmette-Guérin (BCG) vaccine for the prevention of disease associated with mycobacterium tuberculosis was derived from the closely related virulent tubercle bacillus mycobacterium bovis (10). Although the BCG vaccine has been one of the most widely used vaccines in the world for over 40 years, the genetic basis of BCG attenuation had never been elucidated. The current vaccine was originally developed by Calmette and Guérin who passaged a strain of M. bovis 230 times in vitro between 1908 and 1921. The resulting vaccine was thought to have struck a balance between reduced virulence and preserved im-

munogenicity. However, because of the inability to preserve viable bacteria (such as by freezing), this live vaccine required continued passage, eventually resulting in a profusion of phenotypically different daughter strains that are collectively known as BCG. By the time lyophilized seed lots of BCG vaccines were created in the 1960s, these vaccines had been separately propagated through about 1,000 additional passages (depending on the daughter strain), usually under the very conditions that effected the original attenuation. To better understand the differences between *M. tuberculosis*, *M. bovis* and the various BCG daughter strains their genomic compositions were

Table 1 – Genetic information about distribution of deletions (RD) in virulent *M. bovis* and BCG strains. Parentheses after *M. bovis* indicate how many strains of virulent *M. bovis* are missing the genetic element (modified after reference 11).

Deletion Region	Strains where Missing
RD1	All BCG strains
RD2	BCG-strains: -Danish, -Prague, -Glaxo, -Frappier, -Connaught, -Phipps, -Tice, -Pasteur
RD3	<i>M. bovis</i> (3/8)
RD4	<i>M. bovis</i> (8/8)
RD5	<i>M. bovis</i> (8/8)
RD6	<i>M. bovis</i> (8/8)
RD7	<i>M. bovis</i> (8/8)
RD8	BCG-Frappier, BCG-Connaught
RD9	<i>M. bovis</i> (8/8)
RD10	<i>M. bovis</i> (8/8)
RD11	<i>M. bovis</i> (8/8)
RD12	<i>M. bovis</i> (8/8)
RD13	<i>M. bovis</i> (4/8)
RD14	BCG Pasteur
RD15	<i>M. bovis</i> (8/8)
RD16	BCG-Moreau

studied recently by performing comparative hybridization experiments on a DNA microarray (11). By this method 11 regions of a virulent *M. tuberculosis* strain were found that were absent from one or more virulent strains of *M. bovis*. Five additional regions representing 38 open reading frames were present in *M. bovis*, but absent from some or all BCG strains. This was seen as evidence for the ongoing evolution of BCG strains since their original derivation. Furthermore, contemporary BCG vaccines were compared to their progenitor strain. Because this strain was lost during World War I, the origin of current BCG vaccines could only be inferred through an evolutionary approach. Behr et al. (11) curated a collection of BCG daughter strains representing this global dissemination for the purpose of performing genomic comparisons. Altogether 16 regions were found as deleted in BCG strains as compared to virulent TBC. Of the 16 deletion regions, nine are missing from BCG and all virulent *M. bovis* strains tested, two are missing from BCG and some of the strains of *M. bovis*, one is missing from all BCG strains, and four are missing only from certain BCG strains (Table-1).

To further address the differences specific to BCG the authors assumed that regions of a virulent TBC present in *M. bovis* strains and absent only from BCG were deleted during the derivation and maintenance of BCG vaccines in various vaccine facilities around the world. If this was true, then regions of *M. bovis* missing from BCG strains would indicate unidirectional genetic events from which the phylogeny of BCG strains can be inferred. Because some BCG daughter strains were obtained directly from the Institute Pasteur while other strains were derived from another vaccine facility, it was possible to reconstruct the genealogy of BCG strains and determine when and where BCG specific deletions occurred (Figure-1). In comparison with *M. bovis*, all BCG vaccines lack one region (RD1) that presumably was lost during the 1908-1921 attenuation (10). Another deletion (RD2) occurred at the Institute Pasteur between 1927 and 1931. A further deletion (RD14) specific to BCG-Pasteur indicates an event in 1938, and before the lyophilization of BCG Pasteur 1173 in 1961. The losses of RD8 in Montreal (between

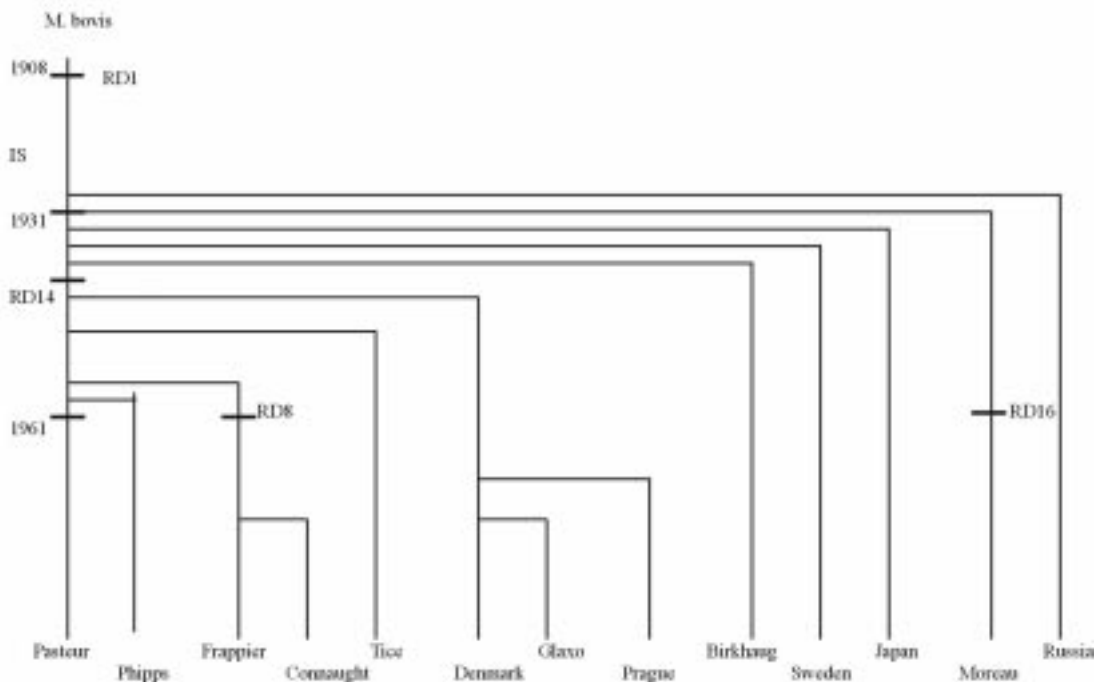


Figure 1 – Genetic genealogy of BCG (modified after reference 11). RD: region of genetic deletion; IS: insertion element.

1937 and 1948) and RD16 in Uruguay or Brazil (after 1925) indicate that ongoing evolution of BCG strains was not confined to Institute Pasteur. A historical review of the BCG literature revealed reports of decreasing virulence in the Institute Pasteur at various times, consistent with the documented ongoing evolution (11).

Thus, BCG significantly differs from *M. bovis*, from which it was originally derived. Attenuation led to the elimination of most virulence genes, which made BCG a famous vaccine against tuberculosis and a well-known immunostimulant with a very advantageous risk-to-benefit ratio.

ANIMAL EXPERIMENTS: BACKGROUND AND DEVELOPMENT

Old et al. were able to observe the inhibition of growth of implanted sarcomas, carcinomas and ascites tumors in mice following pre-treatment with BCG (12). The systematic work of Zbar et al. at the National Cancer Institute, USA, was of vital importance to the further development of BCG applications in oncology (13-17). Using the model of a guinea pig

hepatocarcinoma, Zbar et al. were able to demonstrate that administration of tumor cells with BCG leads to a significant inhibition of tumor growth. Intralesional injections of BCG led to involution of tumors already formed, and also of the lymph node metastases. Furthermore, animals that had become tumor-free after treatment with BCG had developed immunity to the hepatocarcinoma, as re-administration of tumor cells led to no further tumor growth.

From their investigations, Zbar et al. have devised basic rules for the optimum immunotherapy of tumors with BCG (14-17): 1)- localized tumors respond to BCG immunotherapy better than generalized tumors; 2)- the tumor mass must be as small as possible before commencement of immunotherapy; 3)- a direct contact of long duration between the tumor cells and the BCG should be ensured; 4)- in the animal experimental models tested, the optimum effective dose of BCG for local or intratumoral application was 10^6 - 10^8 colony forming units (CFU).

Similar favorable results were reported by Baldwin & Pimm on rat sarcomas (18) and by Bartlett et al. (19,20) on spontaneously originating

or carcinogenically induced murine tumors. The growth-inhibiting effect of the BCG treatment of mice, rat and guinea pig tumors has been observed in numerous other investigations. In many cases, systemic pre-treatment (prophylaxis) was already found to lead to a significant inhibition of tumor growth. Sparks et al. were able to demonstrate that an intravesical BCG injection before surgical removal of the spontaneously originating metastasizing mammary adenocarcinoma in rats not only significantly extended the median survival time, but that a significant number of animals remained tumor-free over the long term (21). Therefore, after primary therapy of the tumor had been carried out, BCG therapy was able to eliminate the occult minimal residual tumor ("minimal residual disease").

From these investigations, it was concluded that high doses (10^6 - 10^8 CFU) of living BCG microbes were necessary to produce an anti-tumor effect. Differences between the various BCG strains were found in some studies, but it was not possible to determine conclusively whether the different therapy results in animal experiments were attributable to this (22,23).

INTRAVESICAL IMMUNOTHERAPY IN THE RODENT BLADDER MODEL

Using the orthotopic bladder carcinoma model in mice, Ratliff et al. could significantly contribute to the explanation of the mode of action of intravesical immunotherapy. By means of implantation of syngeneic bladder carcinoma cells into the bladders of normal, immunocompetent mice, these authors examined an animal model which was very close to reality and which was highly relevant for intravesical therapy. These authors were able to establish the significance of fibronectin for the adhesion of BCG to the bladder wall (24-28) Ratliff et al. initially conducted investigations into the binding of BCG to various surfaces. They were able to show that BCG adheres *in vitro* almost selectively to fibronectin-coated wells. This binding could be inhibited using fibronectin antibodies, and also by soluble fibronectin (26,27). No differences were seen when comparing various commercially available BCG preparations (26). Furthermore, the authors were able

to establish in animal experiments (24) that BCG is retained in the mouse bladder after instillation only if the bladder wall had been damaged by either physical or chemical means before instillation. This binding could be inhibited by soluble fibronectin or anti-fibronectin antibodies. Moreover, it was evident that after inhibition of BCG binding, the typical delayed-type hypersensitivity (DTH) reaction did not occur and that no anti-tumoral activity was generated. From these investigations, it was possible to conclude that, at least in animal experiments, fibronectin is essential for the binding of BCG to the bladder wall.

Guinan et al. established a quantitative difference in the cellular infiltrate of the urinary bladder wall after intravesical instillation of thiotepa and BCG (29). They postulated a T-cell dependent functional mechanism, which Ratliff et al. were able to prove. Athymic nude mice who did not form T-cells were not able to generate a BCG-induced tumor defense, which then became possible after the transfer of homologous T-cells (30).

These results were further confirmed in an animal model by Ratliff et al. who showed, using immunocompetent mice, that the depletion of both $CD4^+$ (T-helper) cells and $CD8^+$ (T-suppressor) cells completely inhibited the BCG-induced anti-tumoral effect on implanted bladder tumor cells (31). In this system, $CD4^+$ and $CD8^+$ cells appeared to be necessary for producing a DTH immune response.

INVESTIGATIONS IN HUMANS: EX-VIVO

While it was initially suspected that a non-specific effect within the context of an eroding cystitis provided a therapeutic momentum for the effect of BCG (32) most authors nowadays agree to an involvement of the immune system. In our systematic investigations on patients treated with BCG we were able to demonstrate the local immune response in the bladder by immunohistological analysis of the infiltrating cellular subpopulations (33) and of local cytokines determined directly in the bladder wall (34), as well as by examining the patients' urine with regard to cytokine secretion (35). In this way, we characterized both the acute and also the long-term

persisting local immune response of patients to the intravesical immunotherapy.

Immunohistology

Biopsies from the bladder wall before and after BCG were analyzed by means of immunohistology by several groups (29,36-47).

To give an insight into the development of the local inflammatory response against BCG, data from our analyses are given here. During routine follow-up, "cold-cup" biopsies were obtained before instillation and immediately after the sixth instillation, and then at three-monthly intervals. Before therapy, such biopsies showed relatively few mononuclear cells in the bladder. The local ratio of T-helper/T-suppressor cells in the bladder wall before the start of therapy was about 1:2. After the sixth instillation of BCG, a massive inflammatory reaction of the entire bladder wall ensued. In all biopsies, aggregates of immunocompetent cells which had developed under the treatment could be detected, corresponding with the so-called BCG-induced granulomas (41,48) (Figure-2)

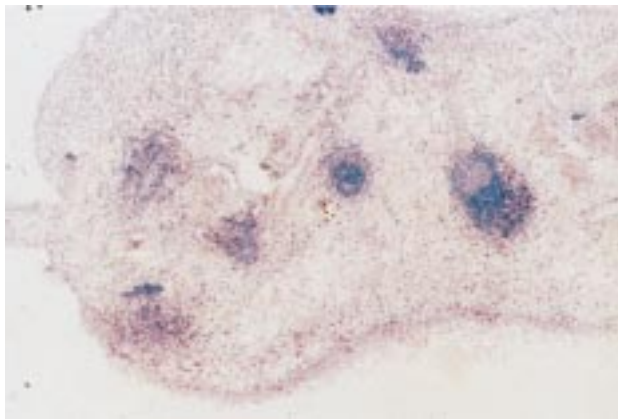


Figure 2 – Biopsy of a urinary bladder 3 months after intravesical BCG therapy. Typical picture with several suburothelial BCG-induced granulomas visible.

These follicle-like structures were found almost exclusively in the highly vascularized submucosa. The infiltrate of mononuclear cells in the bladder wall consisted mainly of T-cells after completion of the intravesical BCG therapy, with a distinct predominance of CD4⁺ (so-called T-helper/inducer) cells compared with CD8⁺ (so-called T-suppressor/

cytotoxicity) cells in the granuloma (Figure-3). The ratio of CD4⁺/CD8⁺ T-cells detectable in the submucosa was 2:1, which represents a reversal of the original ratio found in the normal bladder. The immunohistological characteristics remained unchanged through later examinations at 6, 9 and 12 months after start of therapy.

These investigations point to a stimulation of the local immune system in the bladder which differs significantly from non-specific cystitis in terms of its duration and also in qualitative terms: while in the case of non-specific cystitis, or even of cystitis induced by cytostatic drugs, a mainly granulocytic infiltration is found, an almost exclusive increase in mononuclear, immunocompetent cells (lymphocytes and macrophages) was found after BCG treatment. Increased number of immunocompetent mononuclear cells could be detected to express so-called activation markers (IL-2-R and HLA-DR). This infiltrate of activated cells persists for at least 12 months, mainly in suburothelial granulomas.

The numbers of infiltrating mononuclear cells into the bladder wall was also measured by Honda et al. (49), who found lymphocytes and γ/δ cells significantly increased after treatment compared with numbers before treatment (although the correlation with regard to γ/δ cells was not statistically significant). The localization of pro-inflammatory cytokines was measured by immunohistology (50). Among the few patients studied no single cytokine or cytokine profile was associated with clinical response to BCG therapy. Bladder-wash derived lymphocytes were studied by Bruno et al. (51). The addition of BCG on bladder-wash derived lymphocytes expanded in-vitro enhanced their proliferation suggesting that this population was sensitized against BCG. This hypothesis was confirmed by analysis of T cell receptor restriction patterns showing that bladder lymphocytes from patients under BCG were oligoclonal. Bladder-wash derived lymphocytes were also analyzed by the group from Esuvaranathan (52). They found an increasing trend in the percentage of CD 3⁺ T cells with each weekly intravesical instillation and the proportion of CD 3⁺ T cells expressing the γ/δ T cell receptor was significantly higher in patients receiving standard dose BCG than those receiving low dose BCG.

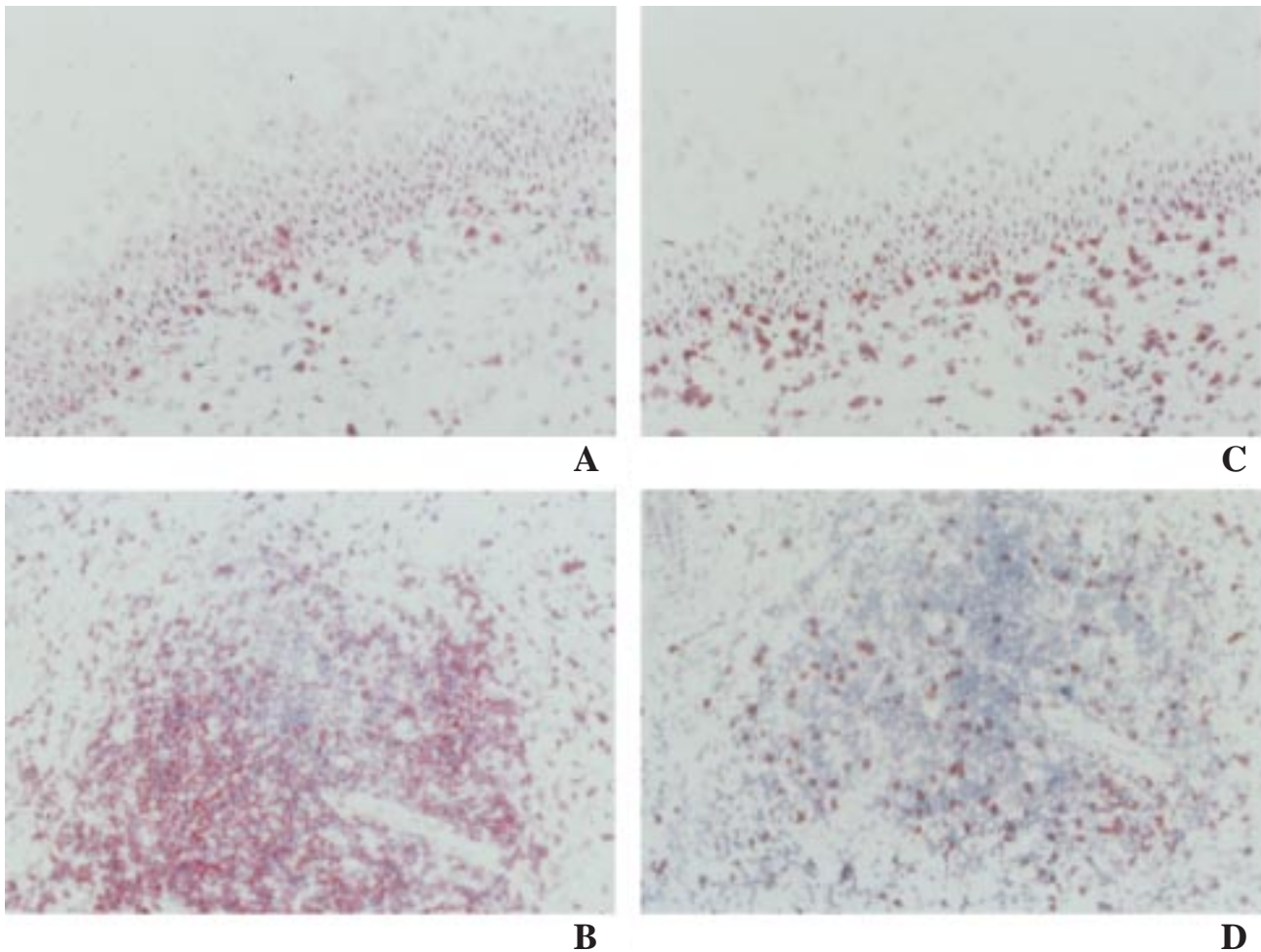


Figure 3 – Dynamics of the immune reaction after BCG. Biopsies of a normal urinary bladder (upper row) compared to biopsies from BCG-treated bladders (lower row). A and B, staining against CD4⁺ T-helper cells; C and D, staining against CD8⁺ T-suppressor/cytotoxic cells. Before therapy, only relatively few immunocompetent cells (A,C) are visible. After BCG, abundant mononuclear cells are visible within granulomas (B,D), with a clear predominance of T-helper cells (B) as compared to T-suppressor/cytotoxic cells (D).

Cytokine Secretion into the Urine

Further investigations to determine the secretion of cytokines into the urine were performed from others and from our group (35,53-57). Within 24 hours after BCG instillation, considerable quantities of inflammatory cytokines such as interleukin (IL-1), interleukin-2 (IL-2) and tumor necrosis factor (TNF) were found in the urine(35). The cytokine titers in the urine differed considerably from one patient to another after BCG instillation - both with regard to the temporal maximum and the total level of 24-hour secretions. Significantly elevated values were detected only 2 hours after the instillation of BCG, with a maximum

after 2-8 hours. The elevated titers returned to normal within 24-hours. In the control group, consisting of patients with non-specific cystitis, only minute amounts of cytokines could be detected in the urine of individual patients. Comparison of the 2 groups showed a highly significant difference between the BCG group and the control group. These investigations confirmed the results of others with regard to IL-2 secretion (58) and furthermore described for the first time the secretion of IL-1- β and TNF into the urine of patients after BCG treatment. The presence of IL-1 in the urine of healthy and febrile subjects has already been demonstrated (35,59). However, the secretion of IL-2 and TNF fol-

lowing BCG treatment represents a qualitatively and quantitatively very different reaction compared with non-specific cystitis. Neither IL-2 (58,60) nor TNF have been found in the urine of healthy individuals and IL-2 has been detected in the urine of patients with non-specific cystitis in minute amounts only. IL-2 is mainly produced by activated T-helper cells. It acts specifically in the proliferation and differentiation of T-lymphocytes. Its effect on tumor cells is mainly based on the generation of lymphokine-activating killer cells (LAK cells). TNF- α is mainly formed by activated macrophages (61,62) and, alongside other effects, possesses both a direct cytotoxic effect on tumor cells as well as an inhibitory effect on the vascularization of tumors, imposing macroscopically as a hemorrhagic necrosis (12).

A special attention was paid by some groups to interleukin-8 (IL-8) due to its rapid onset in the immune response to intravesical BCG (57,63). Due to its appearance already after the first instillation and its stability, IL-8 seemed an attractive candidate for investigation of its prognostic value for clinical response to BCG. However, in analyzing the predictive potency of IL-8 Rabinowitz et al. (64) found no association between the direction of change in interleukin-8/creatinine ratio and response to intravesical BCG making this cytokine probably not an useful marker for predicting the response to BCG.

Analysis of Peripheral Blood Parameters

Few groups have looked at the antibody response against BCG or against defined mycobacterial antigens after BCG intravesical therapy with regard to systemic immune activation and the possible inherent specificity of this immune response (65-69). In an early study, van der Sloot et al. have characterized the antibody response in the urine and peripheral blood after BCG (66,70).

Zlotta et al. looked into the proliferative T cell response to mycobacterial antigens of bladder carcinoma patients before any treatment to detect possible cross-reactivity between mycobacterial antigens and tumors (71). They showed that patients with superficial bladder carcinoma demonstrated an increased lymphoproliferation against mycobacterial antigens before intravesical BCG as compared to control subjects, suggesting indeed a possible existence

of bladder carcinoma antigens cross-reactive with mycobacterial antigens (67-69). Furthermore, the serum antibody response against several heat shock proteins was analyzed in patients receiving BCG (68) showing indeed an antibody response against various heat shock proteins.

A very interesting way of predicting the response to BCG was found by Kempfer et al. from Israel (72). These authors analyzed the induction of interleukin-2 and interferon- γ mRNA in peripheral blood mononuclear cells during BCG treatment. They could show that independent of tumor type, induction of interleukin-2 mRNA was observed for patients who responded with remission, but not for those who relapsed ($p = 0,0001$). Multivariate logistic analysis showed that inducibility of IL-2 mRNA was the discriminating parameter, which yielded a predictive value of 97 % for remission.

The relevance of all these phenomena with regard to the anti-tumoral efficacy of BCG was not clarified until further, *in vitro* investigations had been carried out, which gave a complex picture on the mode of action of BCG.

IN VITRO INVESTIGATIONS

The *ex-vivo* investigations described above have resulted in a substantial qualitative and quantitative characterization of the local immune response to intravesical BCG therapy in humans. This reaction represents a complex immune response of the body to the local BCG instillation. Further analysis of these phenomena as possible effector mechanisms against bladder tumors required dissection of the immune response into several individual events, and it was necessary to investigate these events *in vitro* independently of one another (73).

The Direct Cytotoxic Activity of BCG

In order to rule out the possibility that BCG, as in the case of cytostatic drugs, acts purely as a cytotoxic agent, the direct cytotoxic activity of BCG towards bladder tumor cells was investigated (73,74): co-incubation of increasing concentrations of BCG with several target cell lines showed no significant cytotoxicity. Thus, under the known clini-

cal conditions, a direct cytotoxic effect of BCG on bladder tumor cells can be ruled out as a major functional principle.

Humoral Mechanisms

A further possible mechanism was addressed by Popas et al. (75), who suggested that BCG might activate anti-angiogenic pathways. The group collected urine samples and determined the urinary output of anti-angiogenic IP10 and interferon-gamma during 12 post-treatment hours. In all cases, significant titers of these chemo- and monokines were detected. The in-vitro response to stimulation of human transitional and endothelial cells with BCG or interferon confirmed these data. The results suggested that anti-angiogenesis might indeed be a further factor of BCG's mode of action. The next step downwards was analyzed by a Swedish group (76). As cytokines may induce nitric oxide (NO) by the enzyme nitric oxide synthase (NOS) and as NO exerts cytotoxic effects in tumor cells, this mechanism was further analyzed with regard to BCG. Induction of NOS activity in the human urinary bladder after BCG treatment was determined and the presence of NOS was localized in urothelial cells by immunohistochemistry. NO was shown to exert cytotoxic effects on bladder cancer cells. Therefore, NO might be involved in cytotoxicity induced by BCG.

Cellular Mechanisms Involved in BCG Immunotherapy

Unstimulated mononuclear cells. Immunocompetent cells can be found in the bladder wall even before BCG instillation - albeit in considerably smaller numbers. In order to clarify the extent to which these non-stimulated immune cells (whose cytotoxicity resides mainly in the natural killer (NK) cell fraction) are able to kill urothelial carcinoma cells, the cytotoxicity of these cells was investigated (77,78): all five urothelial carcinoma cell lines tested were virtually resistant. These findings also underline the value of results obtained from cell lines for the in vivo situation in humans resistant to unstimulated NK cells. The cell line used as a control, the erythroleukaemia cell line K562, known to

be NK-sensitive, showed a distinct sensitivity in the same experiment. Not only those bladder tumor cell lines quoted, but also short-term cultures of newly resected papillary bladder tumors showed the same resistance to non-activated immune cells (79), which is an indication of the inability of the immune system without adequate stimulation to control a tumor once it has become established.

Lymphokine-Activated Killer Cells (LAK Cells)

Through co-incubation with IL-2, immune cells can be stimulated to produce so-called lymphokine-activated killer (LAK) cell cytotoxicity. As IL-2 could be detected in the urine as well as in the bladder wall after BCG treatment, it appeared possible that these killer cells were generated. For this reason, the cytotoxicity of LAK cells towards bladder tumor cells was tested. The results of these investigations showed that a marked cytotoxicity towards bladder tumor cells could be induced by IL-2 and IFN- γ (77,80). This mechanism can therefore be considered as a possible functional principle for intravesical immunotherapy using BCG.

BCG-Activated Killer Cells (BAK Cells)

In order to demonstrate further BCG-induced effects on immunocompetent cells, activation by BCG itself was tested (74,81): after pre-incubation of immune cells with BCG over several days, it was possible to induce a considerable degree of cytotoxicity to all bladder tumor cell lines tested so far (82). We named this apparently autonomous BCG-activated killer cell phenomenon "BAK" cytotoxicity. Further experiments showed that only living BCG bacteria were able to induce BAK cells, whereas dead bacteria or cell-free fragments were ineffective - corresponding to the situation in vivo. Also, a dose and time optimum was determined for BCG efficacy in vitro.

Characterization of BAK Cells

Further characterization of the effector cells was the next step taken in our investigations (82,83). The cytotoxicity of LAK cells shows a well-known maximum after 2-3 days of stimulation with IL-2

(84), while BAK-cell cytotoxicity continued to increase to day seven. In contrast to LAK cells, BAK cells could not be generated from CD4⁺- and CD8⁺-depleted cell populations. Because of these and other differences, it was concluded that BAK cells represent a different cell population and are activated via a different route than LAK cells. Experiments followed in which this unique cytotoxic BAK effector cell was further characterized: we were able to demonstrate that neither macrophages nor CD4⁺ T-cells come into question as effector cells, but that cells expressing CD8⁺ and CD56⁺ antigens on their surface are responsible for this BCG-induced cytotoxicity. The induction of BAK cytotoxicity, on the other hand, is apparently very complex, as all detectable mononuclear subpopulations, also those in the bladder wall, play a part: both the depletion of CD4⁺ and CD8⁺ cells and the depletion of macrophages prevents, concentration dependent, the generation of BAK cells (85).

To understand BCG-induced activation of effector lymphocytes more precisely we investigated the lytic pathways of human BAK cells and compared BAK cell cytotoxicity with LAK cell cytotoxicity (86). Perforin and Fas Ligand (FasL) are the major cytolytic molecules of cytotoxic lymphocytes. Our results demonstrate that BAK- and LAK cells showed an increased expression of perforin and FasL as compared to unstimulated controls. Killing of T-24 bladder tumor cells by BAK and LAK cells was predominantly mediated via perforin as demonstrated by a drastically reduced lysis in the presence of specific inhibitors. In contrast, lysis and membrane disintegration of target cells by BAK and LAK cells could not be blocked with an inhibitory anti-FasL antibody. We concluded that cellular mediators of BCG effector mechanisms, such as BAK and LAK cells, kill their targets via perforin and independent of the FasL-pathway. This has potential clinical relevance as BCG-therapy would not be impaired by FasL-resistance of target cells, which recently has been described for some tumors. As perforin is the cytotoxic effector molecule typically used by Natural Killer (NK) cells, these data suggested involvement of these effector cells in the mode of action of BCG. We further elucidated the

role of NK cells in BCG-induced cellular cytotoxicity (87): Magnetic depletion experiments and fluorescence-activated cell sorting revealed that NK cells were the major effector cell population in vitro, indeed. To confirm the role of NK cells in vivo we studied a syngeneic orthotopic murine bladder cancer model and compared BCG-immunotherapy in C57BL6 wild-type mice, NK-deficient beige mice and mice treated with anti-NK1.1 monoclonal antibody targeting NK cells. Four weekly instillations of viable, commercially available BCG significantly prolonged survival in wild-type mice with bladder cancer as compared to control mice treated with solvent alone. In contrast, BCG-therapy was completely ineffective in NK-deficient beige mice, and mice treated with anti-NK1.1 monoclonal antibody. Altogether, these findings suggested a key role for NK cells during BCG-immunotherapy.

CONCLUSION

A complex local immune response involving humoral and cellular immune mechanisms is induced by BCG in the human bladder (Figure-4). Long-term follow-up examinations showed a predominance of the T-helper/inducer cell population and the persistence of inflammatory (Th1-type) cytokines within

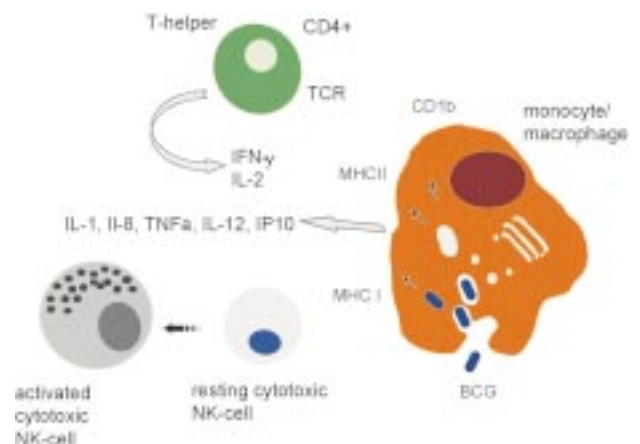


Figure 4 – Hypothesis of the mode of action of BCG against bladder carcinoma: Supported by CD4⁺ T-helper cells, BCG mycobacteria induce a Th1-polarized cytokine secretion in macrophages/monocytes. These cytokines activate resting Natural Killer (NK) cells, which kill their malignant target via perforin.

the bladder wall. These sub-clinical local inflammatory signs persist for a long time within the so-called BCG-induced granulomas, which might have an important role in the recurrence-free status of the patient. The function of this prolonged inflammation seems to provide immature effector cells with a continuous level of activating cytokines (such as IL-2, Ifn- γ , and Il-12). In vitro, at least two cellular cytotoxic effector mechanisms had been determined. Next to the well-known LAK-cell cytotoxicity, a further cytotoxic phenomenon could be characterized, which was termed "the BCG-activated killer (BAK) cell phenomenon". Recent investigations convincingly proved that the effector cells involved are activated NK cells, which are known to selectively kill malignant targets. Thus, the search for effector mechanisms involved in BCG immunotherapy has opened a new and exiting field.

An important clinical aspect of these investigations is to decipher which part of the complex immune response is involved in the killing of the tumor, and which parts contribute to the side-effects involved with BCG immunotherapy. Enhancing the further and suppressing the latter reaction would lead to an even more effective therapy.

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