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Effects of sequential intravesical administration of mitomycin C and bacillus Calmette-Guérin on the immune response in the guinea pig bladder

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Abstract It has been suggested that intravesical treatment with mitomycin C (MMC) before instillation of bacillus Calmette-Guérin (BCG) improves the antitumor activity of BCG in human bladder cancer. Therefore, we studied the immunological effects of sequential intravesical treatment with MMC and BCG in the guinea pig. Four weekly intravesical instillations with MMC preceded six weekly intravesical BCG instillations. The delayed-type hypersensitivity (DTH) skin reaction evoked by tuberculin purified protein derivative (PPD) in guinea pigs receiving BCG intravesically appeared slightly earlier in animals pretreated intravesically with MMC than in phosphate-buffered saline (PBS)-pretreated animals. However, after completing BCG instillations no differences in DTH reaction were observed between these treatment groups. The extent of the local inflammatory reaction in the bladder wall, as well as the parameters measured in the draining iliacal lymph nodes (i.e., the weight, the number of leukocytes, and the composition of leukocyte subpopulations), did not differ in animals treated with BCG alone or in combination with MMC. A slight increase in the MHC class II expression on the bladder urothelium was shown if MMC and BCG treatment was combined. The adherence of mycobacteria to the bladder wall, measured using ^{3}H-labeled mycobacteria, dit not differ between MMC/BCG- and BCG-treated animals. We conclude that MMC does not enhance the immune response against mycobacteria. Therefore, we hypothesize that a possible increased antitumor activity by the combination of MMC and BCG might be due to separate, rather than synergistic, effects of the drugs, namely a cytostatic effect of MMC on tumor cells and a local immune response in the bladder evoked by BCG.

Key words Mitomycin C · Bacillus Calmette-Guérin · Superficial bladder cancer · Guinea pig

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Intravesical chemotherapy or immunotherapy is used as adjuvant treatment after transurethral resection (TUR) of superficial bladder tumors in man. Intravesical chemotherapy with thiotepa [11, 34] epodyl [18], doxorubicin (DXR) [19], or mitomycin C (MMC) [3, 15, 17, 25] and immunotherapy with bacillus Calmette-Guérin (BCG) [1, 4, 5, 8, 33] or interleukin-2 (IL-2) [14] have been studied. In a number of studies the efficacy of chemotherapy and immunotherapy were compared. BCG was shown to be superior to thiotepa [22] and DXR [20, 22]. In two studies MMC appeared to be equally effective to BCG [7]. However, in these studies BCG was probably given at a suboptimal dose. As MMC and BCG have different effector mechanisms, we expect that sequential administration of MMC and BCG may cause an additive antitumor effect. This hypothesis was based on two arguments. First, Ratliff and coworkers [12, 24] have shown that the binding of mycobacteria to the bladder wall is important in evoking an immune response and antitumor activity in the bladder. One of the factors thought to be essential in the mycobacterial attachment is fibronection. It is present in the bladder after damage by electrocautery during TUR. Chemical cystitis induced by a cytostatic drug such as MMC may also cause expression of fibronectin on the bladder surface. Thus MMC treatment may ficilitate adherence of BCG to the bladder wall, and consequently stimulate the induction of the immune response. Whether

fibronectin is the only factor involved in the adherence of mycobacteria to the bladder wall is not known [29]. The second reason for sequential administration of MMC and BCG is that MMC may cause a decrease in T-suppressor activity and thus increase the immune response against antigens [6].

To study the antitumor activity in humans a phase II study (EORTC GU GROUP protocol no. 30897; Van der Meijden et al.) was started, in which patients whith a marker lesion were treated sequentially with intravesical MMC and intravesical BCG. In this paper we describe a study on the mechanisms of the presumed synergistic activity of both drugs in the guinea pig. In previous studies we showed that in most animals intravesical BCG treatment induced a delayed-type hypersensitivity (DTH) reaction [30, 31] against tuberculin purified protein derivative (PPD). Instillation of BCG induced hyperplasia of regional lymph nodes [30, 31] and an inflammatory reaction in the bladder wall [9, 30]. These parameters were also used to study the effect of sequential administration of MMC and BCG. Additionally, the adherence of mycobacteria to the bladder wall was investigated by intravesical instillation of [^{3}H]uracil-labeled BCG.

Materials and methods

Animals

Female Sewall-Wright inbred strain 2 guinea pigs were obtained from the National Cancer Institute, Frederick Cancer Research Facility, Frederick Md., United States. The animals were caged in groups of six or less and fed a guinea pig ration (Hope Farms, Woerden, The Netherlands) and water ad libitum. The guinea pigs were used at an age of 2–3 months, weighing 400–600 g.

Drugs

Mitomycin C

Reconstitution of mitomycin C (MMC; Christiaens BV; Belgium) with sterile water resulted in a concentration of 1 mg/ml. MMC was administered intravesically once a week for 4 consecutive weeks with the animal under general anaesthesia with Ketalar (20 mg/kg body w., Parke Davis, Madrid, Spain), Rompun (2.5 mg/kg body w., Bayer Leverkusen, Germany), and atropine (0.05 mg/kg body w.) (KRA). Before and after every instillation the bladder was emptied by gently pressing the bladder between the fingertips. Each instillation lasted for 1 h.

Bacillus Calmette-Guérin

Bacillus Calmette-Guérin was produced for cancer immunotherapy of the bladder at the National Institute of Public Health and Environmental Protection (NIPHEP). One milliliter BCG (1×10^7 or 5×10^7 culturable particles, c.p., per milliliter) was administered intravesically 6 times once a week with the animal under general anaesthesia using KRA. Control animals received placebo intravesically, which consisted of polygeline (83.3 mg/ml), glucose (50 mg/ml), and Tween 80 (0.05 mg/ml). BCG instillations started 7 days after the last MMC instillation. One week after the final BCG instillation guinea pigs were put to death and the bladders removed.

Radiolabeling of BCG

The freeze-dried pellet of BCG was reconstituted and cultured for 8 days in Tween-albumin medium at 37°C. Subsequently the BCG suspension was diluted to approximately 3×10^8 c.p./ml in Tween-albumin medium and cultured for an additional 3 days at 37°C in the presence of [^{3}H]uracil (final concentration, 100 μCi/ml). After washing three times with phosphate-buffered saline (PBS) to remove excess [^{3}H]uracil, a suspension containing 3×10^8 c.p. BCG/ml was obtained and used immediately for intravesical instillation.

In the radiolabeling experiments guinea pigs received four weekly instillations of MMC followed by one instillation of ^{3}H-BCG 7 days after the last MMC instillation. Naive animals were given unlabeled BCG. In another group the bladders of animals were damaged by electrocautery before the instillation of ^{3}H-BCG as previously described [29]. Immediately after the 1-h BCG instillation animals were put to death and bladders removed. Bladders were turned inside out and washed carefully 5 times extensively in PBS to remove free BCG. Washed bladders were placed in Soluene (Soluene Tissue Solubilizer, Packard Instruments, Groningen, The Netherlands) for 24 h at 37°C to dissolve the tissue. The amount of ^{3}H-BCG as a measure for the adherence of BCG to the bladder wall was determined in a liquid scintillation counter (Tri-carb 1500, Groningen, The Netherlands).

Delayed-type hypersenitivity response

The in vivo antigen-specific immune response to BCG was determined by intradermal injection of 20 μg tuberculin-PPD (purified protein derivative of *Mycobacterium tuberculosis,* produced at the NIPHEP) in 0.1 ml PBS. The DTH response to tuberculin-PPD was determined after 24 h by measurement of the diameter in millimeters of the erythematous skin reaction.

(Immuno)histochemical analysis of the bladder wall

After removal from the peritoneal cavity, bladders were transurethrally filled with either 10% formalin sublimate or 3% gelatin. Subsequently the bladders were embedded in paraffin (Paraplast, Monoject Scientific, Kildare, Ireland) or frozen in liquid nitrogen respectively. Sections (4 μm for paraffin embedded or 5–10 μm for frozen tissue) were prepared from three different levels of the bladder (cranial, medial, and caudal parts) and routinely stained with hematoxylin and eosin (H & E). On paraffin sections the number of infiltrates in the bladder wall was determined in H & E-stained sections and in sections stained with the monoclonal antibody CT5, reacting with T lymphocytes. (CT5 was kindly provided by Dr. R. Scheper, Pathological Institute, Free University Hospital, Amsterdam, The Netherlands.) Cryostat sections prepared from tissue frozen in liquid nitrogen were used for indirect immunoperioxidase staining [26] with the monoclonal antibody 27E7 specific for (guinea pig) MHC class II antigens (a generous gift from Dr. E. Shevach, National Institute of Allergy and Infections Diseases, Bethesda, Md., USA).

Immunofluorescence staining

The immunofluorescence staining for flow-cytofluorometric analysis was performed as described previously [27, 31] with minor modifications. The following monoclonal antibodies (mAbs) were used: α-Pan T cell (CT5, 1:1000), α-T suppressor/cytotox. (CT6, 1:10000), α-T helper/inducer (CT7, 1:200), α-(IgM)B cell (31D2, 1:500), α-(IgB)B cell (α-IgG 1:50, Nordic, Tilburg, The Netherlands), α-macrophage (305, 1:100), and α-MHC II antigens (27E7, 1:200). (CT6 and CT7 were kindly provided by Dr. R. Scheper and mAbs 31D2 and 305 were a generous gift from Dr. R. Burger, Institute for Immunology, University of Heidelberg, Germany). Leukocytes in spleen and lymph nodes were characterized by flow-cytofluorometric analysis in a fluorescence-activated cell sorter (Becton Dickinson Immunocytometry Systems, Mountain View,

Table 1 Effect of MMC on the DTH reaction to tuberculin-PPD after six weekly intravesical instillations of BCG in guinea pigs

	DTH on day:		
	28	42	56
PBS + placebo[a]	0[d]	0	0
MMC[b] + placebo	0	0	0
PBS + 1×10^7 c.p. BCG[c]	0	1(10.0)[e]	3(16.0 ± 2.0)
MMC + 1×10^7 c.p. BCG	0	2(12.0 ± 2.8)	4(17.3 ± 2.2)
PBS + 5×10^7 c.p. BCG	0	0	5(15.0 ± 1.9)
MMC + 5×10^7 c.p. BCG	0	1(13.0)	4(15.0 ± 0.8)

[a] BCG constituent; polygeline, glucose, and Tween 80
[b] MMC was given intravesically (1 mg/ml) once a week for four consecutive weeks starting on day 0
[c] BCG was given once a week for six consecutive weeks starting 7 days after the final MMC instillation (day 28)
[d] Total number of animals showing a positive DTH reaction. The DTH reaction was considered positive when the diameter of the induration was ≥10 mm. Number of animals per treatment group was five
[e] Mean (± standard deviation) on the diameter of induration in the animals with positive DTH reaction

Table 2 Effect of MMC and BCG on the weight and number of viable leukocytes of the iliac lymph node

	Weight of lymph node (mg)	Number of leukocytes ($\times10^6$)
PBS + placebo[a]	28 ± 10[d]	0.70 ± 0.54
MMC[b] + placebo	17 ± 3	0.15 ± 0.12
PBS + 1×10^7 c.p. BCG[c]	40 ± 16	3.53 ± 2.20
MMC + 1×10^7 c.p. BCG	40 ± 13	3.15 ± 1.81
PBS + 5×10^7 c.p. BCG	40 ± 10	3.96 ± 2.01
MMC + 5×10^7 c.p. BCG	45 ± 7	2.72 ± 1.68

[a] BCG constituent; polygeline, glucose, and Tween 80
[b] Intravesical administration of MMC (1 mg/ml) once a week for 4 consecutive weeks
[c] BCG was given intravesically once a week for 6 weeks, starting 7 days after the last MMC instillation
[d] Weight and number of viable leukocytes were determined 7 days after the final BCG instillation (day 70) in three separate experiments. Data of one representative experiment are depicted. Five animals were used per observation; mean (±SE) weight per lymph node and mean number of leukocytes per lymph node are presented. No significant differences between PBS- and MMC-pretreated animal groups were found when tested with Student's *t*-test

Calif., USA). Analysis of the data was performed with Consort 30 software (Becton Dickinson) on a Hewlett-Packard 9920 S computer. Data were calculated for 10^4 cells after gating the dot plots of the volume-sidescatter diagram.

Statistics

Student's two-tailed *t*-test with the Welch correction in case of insufficient homogeneity of variances was applied to the data.

Results

Effects of MMC on the DTH response to tubercullin PPD

Previous studies in our laboratory showed that intravesical administration of BCG in guinea pigs induced a DTH reaction in the skin against tuberculin-PPD. In the present study we investigated whether MMC can further enhance the immune response elicited by intravesical BCG instillations.

As shown in Table 1, 14 days after the first BCG instillation (on day 42) only a small number of animals developed a positive DTH reaction to tuberculin-PPD in the skin, i.e., the diameter of the induration was 10 mm or more. At this time point the number of animals with a positive response to tuberculin PPD was slightly higher in MMC/BCG-treated animals than in animals treated with BCG alone. Four weeks (day 56) after the first BCG instillation most of the animals exerted a positive skin reaction in all BCG-treated animals. Results of the DTH reaction at week 5 and 6 were not relevant, as control animals (PBS/placebo) also became PPD-positive. This was probably a result of repeated weekly skin injections with tuberculin-PPD. Table 1 showed that MMC pretreatment did not have a significant stimulatory effect on the DTH reaction after BCG administration. We concluded that intravesical application of MMC dit not enhance the systemic immune respones of the host against BCG.

Effects of MMC on the iliac lymph node

The regional lymph nodes of the bladder in the guinea pig are located iliacally. We investigated whether MMC treatment increased the stimulatory effects of BCG in the iliac lymph nodes. Therefore, the weight of the lymph nodes and the number as well as the subtypes of viable leukocytes present were determined. Table 2 shows that treatment with MMC alone did not alter the weight or the number of viable leukocytes of the lymph node significantly when compared with PBS-treated animals. Subsequent intravesical instillations with BCG (either 1×10^7 or 5×10^7 c.p.) resulted in an increase in weight in all animals and in an increased number of leukocytes in PBS-pretreated animals. However, MMC did not cause a further increase.

To investigate the composition of leukocyte subpopulations of the lymph nodes, single cell suspensions were incubated with monoclonal antibodies (mAbs) reacting with different subpopulations of T or B cells, or with macrophages. Table 3 shows the percentages of fluorescence-positive cells as measured in a Facscan. The larger part of the leukocytes populating the lymph nodes were T cells (CT5-positive), predominantly T-helper/inducer (CT7) and approximately 20% T-cytotoxic/suppressor (CT6) cells. B cells detected with α-IgG or 31D2 (IgM), and macrophages (305) were usually present only in minor quantities. When comparing groups receiving PBS/BCG with those receiving MMC/BCG treatment no reproducible differences in the percentages of subpopulations of leukocytes were observed. Increasing the dose of BCG

Table 3 Characterization of subpopulations of lymph node leukocytes[a] obtained from animals treated with MMC and/or BCG

	Percentage of cells reacting with monoclonal antibodies[e]					
	Pan-T	T-c/s	T-h/i	IgG	IgM	MΦ
PBS + placebo[b]	66.4	16.6	47.8	1.0	4.6	0.4
MMC[c] + placebo	76.3	19.0	60.1	2.6	10.1	0
PBS + 1×10^7 c.p. BCG[d]	77.3	22.5	48.3	0	5.1	0
MMC + 1×10^7 c.p. BCG	76.0	36.6	57.0	0	7.3	0
PBS + 5×10^7 c.p. BCG	77.2	20.2	56.6	2.8	11.0	2.1
MMC + 5×10^7 c.p. BCG	80.9	17.7	49.1	0	4.2	0.8

[a] Lymph node leukocytes obtained from five animals per treatment group were pooled and tested for antigen expression using the indicated antibodies

[b] BCG constituent; polygeline, glucose, and Tween 80

[c] Intravesical administration of MMC (1 mg/ml) once a week for 4 consecutive weeks

[d] BCG was given intravesically once a week for 6 weeks, starting 7 days after the last MMC instillation

[e] Monoclonal antibodies reacting with Pan-T cells (CT5), T-cytotoxic/suppressor cells (T-c/s; CT6), T-helper/inducer cells (T-h/i; CT7), IgG (α-IgG), and IgM (31D2) B cells and macrophages (MΦ; 305). Positive reaction was measured as the percentage of fluorescence-positive cells (FITC label conjugated to RαM antibody) in a Facsan

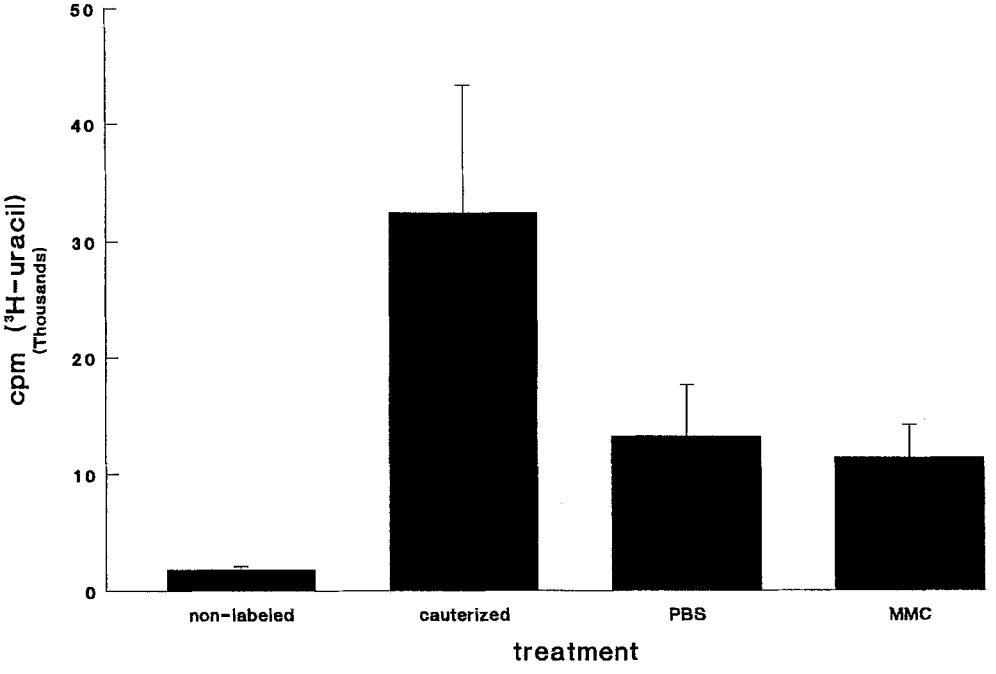

Fig. 1 Adherence of ^{3}H-BCG to the guinea pig bladder. BCG was instilled for 1 h in guinea pig bladders and radioactive counts were taken as a standard for BCG attachment to the bladder wall. Two groups of animals were pretreated with either PBS or MMC and subsequently received ^{3}H-BCG. Bladders of another group of animals were damaged by electrocautery before intravesical instillation with ^{3}H-BCG. Animals receiving unlabeled BCG served as negative controls. *Bars* standard errors. Number of animals per treatment group was five. No significant differences between treatment groups were found with Student's *t*-test

from 1×10^7 c.p. to 5×10^7 c.p. did not lead to a change in the subpopulations.

Attachment of BCG to the bladder wall

In order to invetigate whether BCG adheres to the bladder mucosa and whether this adherence can be enhanced by MMC pretreatment, we radiolabeled BCG with [^{3}H]uracil. First, we determined the optimal concentration of [^{3}H]uracil required to obtain detectable levels of [^{3}H]uracil. Adding 120 μCi [^{3}H]uracil/ml BCG (3×10^8 c.p.) resulted in the highest cpm (data not shown) and this protocol was used for radiolabeling of BCG.

Subsequently radiolabeled ^{3}H-BCG was used for intravesical instillations in PBS or MMC-pretreated guinea pigs. Two control groups were used: the first consisted of guinea pigs recieving unlabeled BCG, while in the second group animals received ^{3}H-BCG after electrocautery. The amount of detected [^{3}H]uracil (expressed in cpm) was used as a measure for BCG attachment to the bladder wall. The ^{3}H content of individual bladders and the mean of the detected cpm per treatment group is depicted in Fig. 1. Adherence of ^{3}H-BCG to the undamaged bladder wall was demonstrated in animals pretreated intravesically with PBS. No significant differences in the amount of [^{3}H]uracil detected in bladders pretreated with PBS or with MMC could be demonstrated. A second experiment confirmed these results (data not shown). However, after electrocautery, which causes severe damage of the urothelium, ^{3}H-BCG adherence to the bladder wall was increased compared with PBS-pretreated bladders.

Table 4 Number of lymphocytic infiltrates in the bladder wall after MMC and/or BCG intravesical instillations

	Pan-T cells		H & E	
	Slides[d]	Infiltrates[e]	Slides[d]	Infiltrates[e]
PBS + placebo[a]	12	0	12	0
MMC[b] + placebo	15	0	15	0
PBS + 1×10^7 c.p. BCG[c]	15	5.4 ± 5.2	15	5.3 ± 6.5
MMC + 1×10^7 c.p. BCG	12	2.4 ± 3.0	12	2.3 ± 4.0
PBS + 5×10^7 c.p. BCG	15	7.8 ± 5.7	15	7.2 ± 6.1
MMC + 5×10^7 c.p. BCG	15	7.5 ± 6.1	15	7.1 ± 6.3

[a] BCG constituent; polygeline, glucose, and Tween 80
[b] Intravesical administration of MMC (1 mg/ml) once a week for 4 consecutive weeks
[c] BCG was given intravesically once a week for 6 weeks, starting 7 days after the last MMC instillation
[d] Number of slides read. For each bladder ($n = 5$) slides were made from three levels (cranial, middle, and caudal part of the bladder)
[e] Number of infiltrates, (i.e., a cluster of lymphocytes) were counted in paraffin-embedded sections after routinely staining with H-& E or staining with mAb CT5 (staining all T cells). Bladders were isolated 7 days after the final BCG instillation (day 70). Data from one representative experiment are presented. Mean number of infiltrates per treatment group ± standard error is shown. In each treatment group five animals were used. Data were tested with Student's *t*-test, but no significant differences in the number of infiltrates were demonstrated between PBS- and MMC-pretreated animals

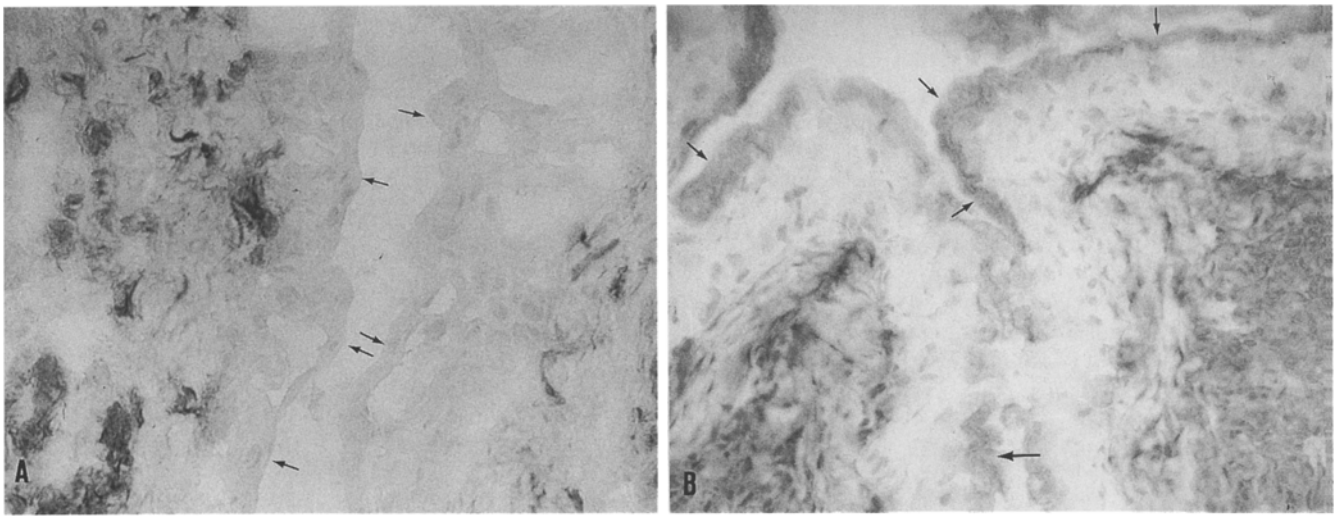

Fig. 2A, B MHC class II expression on bladder urothelium, ×225. **A** No expression of MHC class II molecules in bladder urothelium (→) of PBS/placebo-treated guinea pigs. **B** Expression of MHC class II molecules (→) on the bladder urothelium of MMC/BCG-treated animals. mAb 27E7 was used for the detection of MHC class II molecules

(Immuno)histochemical analysis of the bladder urothelium

The amount of BCG-induced lymphocytic infiltrates (i.e., clusters of predominantly T lymphocytes) in the bladder wall was studied after PBS or MMC (pre)treatment. The number of infiltrates was considered to be a measure of the intensity of the immune reaction evoked in the bladder wall by intravesical treatment of BCG.

Bladder tissue embedded in paraffin was histologically examined after incubation with an α-Pan T cell mAb (CT5) or routine H & E staining. The intravesical administration of BCG induced a moderate inflammatory reaction in the bladder wall. The inflammatory reaction generally consisted of mononuclear cell infiltrates, varying in size from an accumulation of several cells (with a minimum of five cells) to large cell clusters. Small and large infiltrates were observed equally in all sections. The number of infiltrates varied considerably between animals in the same group (Table 4). In bladders of animals treated intravesically with PBS/placebo no infiltrates were observed. Bladders of animals treated with BCG (1×10^7 or 5×10^7 c.p.) generally showed scattered focal infiltrates. However, intravesical pretreatment with MMC did not increase the number or size of infiltrates significantly.

Immunohistological analysis (data not shown) of MHC class II antigens in bladder wall sections showed an induction of MHC class II antigens by intravesical BCG instillations. Pretreatment with MMC slightly increased the percentage of MHC class II positive urothelial cells. Furthermore, the urothelium directly overlying large infiltrates expressed in most cases MHC class II anitgens (Fig. 2), probably indicating a local induction of MHC class II antigens, e.g., by lymphokines.

Discussion

In this study the DTH reaction against tuberculin-PPD (Table 1) was not significantly enhanced after sequential intravesical administration of MMC and BCG. BCG is known to induce an increase in the number of infiltrates in the bladder wall [9]. The sequential application of MMC and BCG, however, did not result in a further increase in the number of infiltrates (Table 4). In the iliac lymph node we found no significant changes after intravesical combination of MMC and BCG (Tables 2, 3). Thus, BCG seems to have a local effect on the bladder wall, while MMC does not have an additional effect on the overall immune response against BCG.

Significant changes in the bladder wall were observed after intravesical of MMC [10]. In patients with superficial bladder cancer the previous tumor or biospsy sites frequently appeared as ragged necrotic areas [25], which implied a direct (cytostatic) effect on the damaged urothelium. Wientjes et al. [32] showed that MMC did not penetrate into the deeper layers of the bladder wall. Erosion of the bladder wall and damage of the protective mucin layer by MMC could lead to enhanced bacterial adherence [2, 16, 24]. Enhanced attachment of BCG to the bladder urothelium is thought to be fibronectin(FN)-mediated [13]. FN is a component of the fibrin clot in the process of wound healing and is also distributed in the bladder wall. In normal undamaged bladders FN is present in the basal layer and in the submucosa and is exposed only after disruption of the urothelium, e.g., after TUR in patients with bladder cancer. Kavoussi et al. [16] showed that chemical substances, such as Adriamycin, are capable of mediating FN-mediated BCG attachment. BCG adherence to FN on the luminal surface of the bladder appeared to be a requisite first step for the induction of an immune response and, possibly, the expression of antitumor activity. Thus, an increase of the adherence of mycobacteria to urothelium damaged by MMC may facilitate the immunological response to BCG, which might result in an improved antitumor activity. In this study the adherence of BCG to the bladder wall was investigated with radiolabeled BCG. No significant differences in BCG adherence between PBS/BCG- and MMC/BCG-treated guinea pig bladders (Fig. 1) were observed. We hypothesize that MMC apparently did not damage the mucin layer to such an extent that FN was exposed.

There are several lines of evidence that local administration of low doses of selective cytostatic drugs at the site of antigenic stimulation may potentiate the generation of T-effector cells, as detected by the DTH measurements [21, 28]. This immunopotentiations is probably due to selective elemination of suppressor cell function and the augmentation of dendritic cells. In our experiments we found only a tendency (not significant) towards an earlier detection of the DTH response to tuberculin-PPD. If MMC does not pentrate the bladder wall no systemic elimination of T-suppressor cells can be expected. In all parameters measured no increased response to BCG was observed, indicating that MMC did not enhance the responce to BCG. After BCG therapy in bladder cancer patients a cellular infiltrate of predominantly T cells and macrophages was found in the bladder wall. Intravesical therapy with MMC, however, did not result in an cellular infiltrate in the urothelium [23]. In the same study MHC class II expression was observed after intravesical BCG; but not MMC, instilations. In our study also MHC class II expression was demonstrated after BCG administration, while MMC showed a slight additional effect.

In summary, the immune reaction evoked in the bladder wall and measured by the above parameters is not significantly enhanced after sequential administration of MMC and BCG compared with BCG treatment alone.

There is a lack of data for bladder cancer patients treated with chemotherapeutic drugs followed by immunotherapy using BCG. A phase II trial to test this protocol is presently underway. If such a combination is found to lead to a prolongation of the disease-free period compared with monotherapy, then our data suggest that this process may not depend upon an increased mycobacterial adherence to the bladder wall. Also no enhancement of the immune response is observed after local application of MMC. We hypothesize that the expected superiority of MMC/BCG combination therapy over monotherapy with BCG in cancer patients might rather be the result of the additive effects of two individual mechanisms of action, namely the cytostatic effects of MMC directly on the tumor cells and a local immune response against tumor cells in the bladder mediated by BCB.

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References

1. Badalament RA, Herr HW, Wong GY, Genecco C, Pinsky CM, Whitmore WF, Fair WR, Oetggen HF (1987) A prospective randomized trial of maintenance versus non-maintenance intravesical Bacillus Calmette-Guérin therapy of superficial bladder cancer. J Clin Oncol 5:441
2. Badalament RA, Franklin GL, Page CM, Dasani BM, Wientjes MG, Drago JR (1992) Enhancement of bacillus Calmette-Guérin attachment to the urothelium by removal of the rabbit bladder mucin layer. J Urol 147:482
3. Bracken B, Johnson D, Von Eschenbach A, Swanson D, De Furia D, Crook S (1980) Role of intravesical mitomycin in management of superficial bladder tumors. Urology 16:11
4. Brosman SA, Lamm DL, Van Der Meijden APM, Debruyne FMJ (1989) A practical guide to the use of intravesical BCG for the management of stage Ta, T1, CIS, transitional cell cancer. In: Debruyne FMJ, Denis L, Van der Meijden APM (eds) EORTC Genito-Urinary Monograph 6: BCG in superficial bladder cancer. Alan R. Liss, New York, p 311
5. Catalona WJ, Hudson MA, Gillen DP, Andriole GL, Ratliff TL (1987) Risk and benefits of repeated courses of intravesical Bacillus Calmette-Guérin therapy for superficial bladder cancer. J Urol 137:220

6. Claessen AME, Bloemena E, Bril H, Meijer CJ, Scheper RJ (1992) Locoregional administration of etoposide, but not of interleukin-2, facilitates active specific immunization in guinea pigs with advanced carcinoma. Cancer Res 52:2440
7. Debruyne FMJ, Van der Meijden APM, Geboers ADH, Franssen MPH, Van Leeuwen MJW, Steerenberg PA, De Jong WH, Ruitenberg EJ (1988) BCG (RIVM) versus mitomycin intravesical therapy in superficial bladder cancer. First results of randomized prospective trial. Suppl Urology 31:20
8. De Jager R, Guinan P, Lamm D, Khanna O, Brosman S, De Kernion J, Williams R, Richardson BA, Muenz L, Reitsma D, Hanna MG (1991) Long-term complete remission in bladder carcinoma in situ with intravesical Tice Bacillus Calmette-Guérin. Overview analysis of six phase II clinical trials. Urology 38:507
9. De Jong WH, De Boer EC, Van der Meijden APM, Steerenberg PA, Debruyne FMJ (1991) Intravesical BCG administration in the guinea pig. II Immunohistochemical characterization of cellular infiltrate after one or two cycles of intravesical BCG. Virchows Arch [Cell Pathol] 61:159
10. Friedman D, Mooppan UMM, Rosen Y, Kim H (1991) The effect of intravesical instillations of thiotepa, mitomycin C, and adriamycin on normal bladder urothelium: an experimental study in rats. J Urol 145:1060
11. Heney NM, Koontz WW, Barton B, Soloway M, Trump DL, Hazra T, Weinstein RS (1988) Intravesical thiotepa versus mitomycin C in patients with Ta, T1 and Tis transitional cell carcinoma of the bladder. A phase III prospective randomized study. J Urol 140:1390
12. Hudson MA, Ritchey JK, Catalona WJ, Brown EJ, Ratliff TL (1990) Comparison of the fibronectin-binding ability and antitumor efficacy of various Mycobacteria. Cancer Res 50:3843
13. Hudson MA, Yuan JJ, Catalona WJ, Ratliff TL (1990) Adverse impact of fibrin clot inhibitors on intravesical bacillus Calmette-Guérin therapy for superficial bladder cancer. J Urol 144:1362
14. Huland E, Huland H (1989) Local continuous high dose Interleukin 2: a new therapeutic model for the treatment of advanced bladder carcinoma. Cancer Res 49:5469
15. Huland H, Otto U, Droese M, Kloeppel G (1984) Longterm mitomycin C instillation after resection of superficial ladder carcinoma: influence on recurrence, progression, and survival. J Urol 132:27
16. Kavoussi LR, Brown EJ, Ritchey JK, Ratliff TL (1990) Fibronectin-mediated Calmette-Guérin bacillus attachment to murine bladder mucosa. Requirement for the expression of an antitumor response. J Clin Invest 85:62
17. Koontz WW, Heney NM, Soloway MS, Kluskens L, Hazra TA, Trump DL, Barton B (1985) Mitomycin C for patients who have failed on thiotepa. Urology 26:30
18. Kurth KH, Maksimovic PA, Hop WCJ, Schröder FH, Bakker NJ (1983) Single-dose intravesical epodyl after TUR of Ta transitional cell bladder carcinoma. World J Urol 1:89
19. Kurth KH, Schroeder FH, Tunn U, Ay R, Pavone-Macaluso M, Debruyne F, De Pauw M, Dalesio O, Ten Kate F (1984) Adjuvant chemotherapy of superficial transitional cell bladder carcinoma: preliminary results of an European Organization for research on treatment of cancer. Randomized trial comparing doxorubicin hydrochloride, ethoglucid and transurethral resection alone. J Urol 132:258
20. Lamm DL, Blumenstein BA, Crawford ED, Montie JE, Scardino P, Grossman HB, Stanisic TH, Smith JA, Sullivan J, Sarosdy MF, Crissman JA, Coltman CA (1991) A randomized trial of intravesical doxorubicin and immunotherapy with bacillus Calmette-Guérin for transitional-cell carcinoma of the bladder. N Engl J Med 325:1205
21. Limpens J, Garssen J, Germeraad WThV, Scheper RJ (1990) Enhancing effects of locally administered cytostatic drugs on T effector cell functions in mice. Int J Immunopharmacol 12:77
22. Martinez-Pineiro JA, Jimenez León J, Martinez-Pineiro Jr, Fiter L, Mosteiro JA, Navarro J, García Matres MJ, Cárcamo P (1989) Intravesical therapy comparing BCG, Adriamycin and Thiotepa in 200 patients with superficial bladder cancer: a randomized prospective study. In: Debruyne FMJ, Denis L, Van Der Meijden APM (eds) EORTC Genito-Urinary Group Monograph 6: BCG in superficial bladder cancer. Liss, New York, p 237
23. Prescott S, James K, Busuttil A, Hargreave TB, Chisholm GD, Smyth JF (1989) HLA-DR expression by high grade superficial bladder cancer treated with BCG. Br J Urol 63:264
24. Ratliff TL, Palmer JO, McGarr JA, Brown EJ (1987) Intravesical Bacillus Calmette-Guérin therapy for murine bladder tumors: initiation of the response by fibronectin-mediated attachment of Bacillus Calmette-Guérin. Cancer Res 47:1762
25. Soloway MS, Ford KS (1983) Subsequent tumor analysis of 36 patients who have received intravesical mitomycin-C for superficial bladder cancer. J Urol 130:74
26. Steerenberg PA, De Jong WH, Elgersma A, Burger R; Poels LG, Claessen AME, Den Otter W, Ruitenberg EJ (1990) Tumor infiltrating leukocytes (tils) during progressive tumor growth and BCG-mediated tumor regression. Virchows Arch [Cell Pathol] 59:185
27. Steerenberg PA, De Jong WH, Geerse E, De Graaf A, Scheper RJ, Den Otter W, Ruitenberg EJ (1991) MHC class II antigen expression on leukocyte subpopulations in the draining lymph node and tumor in the early phase of BCG induced regression. Cancer Immunol Immunother 33:189
28. Tan BTG; Limpens J, Koken M, Valster H, Scheper RJ (1986) Local administration of various cytostatic drugs after subcutaneous immunization enhanced type hypersensitivity reaction to sheep red blood cells in mice. Scan J Immunol 23:605
29. Teppema JS, De Boer EC, Steerenberg PA, Van Der Meijden APM (1992) Morphological aspects of the interaction of Bacillus Calmette-Guérin with urothelial bladder cells in vivo and in vitro: relevance for antitumor activity? Urol Res 20:219
30. Van Der Meijden APM, De Jong WH, Steerenberg PA, Walvoort HC, De Boer EC, Debruyne FMJ, Ruitenberg E (1988) Intravesical BCG administration in the guinea pig. A histomorphological study. Virchows Arch [Cell Pathol] 55:207
31. Van Der Meijden APM, De Jong WH, De Boer EC, Steerenberg PA, Debruyne FMJ, Ruitenberg EJ (1989) Immunological aspects of intravesical administration of bacillus Calmette-Guérin (BCG) in the guinea pig. Urol Res 17:47
32. Wientjes MG, Dalton JT, Badalament RA, Drago JR, Au JL (1991) Baldder wall penetration of intravesical mitomycin C in dogs. Cancer Res 51:4347
33. Witjes JA, Van Der Meijden APM, Debruyne FMJ (1990) Use of intravesical Bacillus Calmette-Guérin in the treatment of superficial transitional cell carcinoma of the bladder: an overview. Urol Int 45:129
34. Zincke H, Utz DL, Taylor WF, Myers RP, Leary FJ (1983) Influence of thiotepa and doxorubicin at time of transurethral surgical treatment of bladder cancer in tumor recurrence: a prospective, randomized, controlled trial. J Urol 129:505