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Immunological aspects of intravesical administration of Bacillus Calmette-Guérin (BCG) in the guinea pig

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Summary. The only form of effective immunotherapy for human cancer is the intravesical administration of BCG in the treatment of superficial bladder tumors. In this study BCG was administrered intravesically (once a week for six consecutive weeks) in guinea pigs to investigate the immune response. Both specific immune responses against BCG itself and nonspecific immune responses such as leukocyte subset distribution, mitogenic (ConA, PHA, LPS) lymphocyte stimulation and spontaneous cytotoxic (NK and LAK cell) activity were determined in the regional draining (iliac) lymph node and in the spleen. The PPD skin test became positive after the fourth intravesical instillation of BCG when 1×10^5 culturable particles or higher dosages of BCG were administered. A low dose of 1×10^3 c.p. of BCG induced a positive PPD skin reaction in 2 of 4 animals after six BCG instillations. After six BCG instillation BCG immune lymphocytes were present both in the iliac lymph nodes and in the spleen. No differences in mitogenic responses to ConA, PHA and LPS were observed in iliac lymph nodes or the spleen between placebo or BCG treated animals. In the regional iliac lymph nodes a significantly increased number of leukocytes was present. These lymph node cells showed a significant increase in the expression of MHC class II (Ia) immune response antigen on their surface. In the spleen no differences were observed after BCG administration regarding the number of cells present and in terms of the Ia antigen expression of the leukocytes. The spontaneous cytotoxic (NK cell) activity was not increased after BCG administration. Both in the iliac lymph node cells and in spleen cells lymphokine activated killer (LAK) cell activity could be induced by human recombinant interleukin 2. At day 1 after the sixth intravesical BCG administration, the BCG treated animals showed a significantly higher lymph node induction of LAK cell activity compared to placebo treated animals. Such a difference was not present in the spleen. Our data indicate that, with the exception of the BCG antigen specific reaction, the immunological responses after intravesical BCG administration in the guinea pig are mainly limited to the regional draining (iliac) lymph nodes.

Key words: Intravesical BCG – Guinea pig – Immunostimulation

Introduction

Both the immunostimulatory effect and the antitumor activity of Bacillus Calmette-Guérin (BCG) have been intensively studied (reviewed in [10, 26]). BCG is known to stimulate various parts of the immune system both at the humoral and at the cellular level. Cell mediated immunity seems to be important for the activity of BCG as immunotherapeutic agent against cancer. BCG was reported to induce and/or enhance the activity of cytotoxic T lymphocytes, various activities of macrophages including macrophage cytotoxicity, and the activity of natural killer (NK) cells. The antitumor acitivity of BCG has been clearly demonstrated in laboratory animal tumor systems and in spontaneously occuring tumors in farm animals like bovine ocular squamous cell carcinoma and equine sarcoid tumors of the skin [10, 26]. Although the exact mechanism by which BCG exerts its antitumor activity is not known, it is generaly thought that BCG operates by activation of the immune system. Whether this is merely a kind of adjuvant activity of BCG, or whether intermediate mediators are of importance remains to be established.

Clinically the use of BCG is indicated for superficial bladder cancer. BCG has been used succesfully for prevention of recurrences of superficial bladder tumors after transurethral resection, for residual disease (after incomplete resection) and for carcinoma in situ [4, 13,

15, 16, 23, 28, 39]. BCG is administered by intravesical instillation, once a week for several consecutive weeks or months. It remains in the bladder for 1–2 h, thus providing a close contact between BCG, tumor cells and the epithelial layer of the bladder.

Local administration was found to be of importance for the antitumor activity of BCG in various animal tumor models [3]. In some animal tumor systems local BCG treatment resulted in the induction of tumor immunity [10].

Very little is known about local mechanisms within the bladder after intravesical BCG administration. In man bladder irritability as a result of cystitis was frequently observed [21]. Histologically, mononuclear infiltrates, sometimes accompanied by epithelioid cells (indicating a granulomatous inflammatory reaction) were present [6, 12, 20, 42]. Such infiltration may also reflect an ongoing immune reaction against the BCG, as after repeated intravesical BCG instillation interleukin 2 (IL2) was found in the urine of BCG treated patients [32]. Whether this reflects or is related to the antitumor activity of BCG is unknown.

In order to determine what happens immunologically after intravesical BCG administration, we investigated both the local and systematic immune reactions after repeated instillation of BCG. Both the specific immune reaction to BCG itself and nonspecific immune parameters like mitogenic stimulation and natural killer (NK) cell activity were investigated. As guinea pigs seem to be most comparable to man with regard to their sensitivity for mycobacteria [37], these studies were performed in guinea pigs.

Materials and methods

Animals

Female albino random bred and female inbred Sewall-Wright strain 2 guinea pigs 4- to 6-months of age and weighing about 400 g, were used in the various experiments. The animals were obtained from the laboratory animal facility of the RIVM, Bilthoven, The Netherlands. The animals were fed guinea pig ration and water ad libitum. During the experiments animals were housed individually. Cages were covered with plastic foil to prevent contamination with faeces or urine from neighboring guinea pigs.

Bacillus Calmette-Guérin (BCG)

The BCG preparation was produced by the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands). BCG-RIVM lot 602 containing 6×10^7 culturable particles (c.p.) in 0.54 mg dry weight, was used. The BCG-RIVM was produced in a homogeneously dispersed culture which is harvested at the end of logarithmic growth. The original seedlot for BCG-RIVM was obtained from the Institute Pasteur, Paris, France. BCG-RIVM activated several immune parameters including natural killer (NK) cell activity [9]. The antitumor activity of BCG-RIVM was demonstrated both in laboratory animal tumor systems [9] and in spontaneously occurring bovine ocular squamous cell carcinoma and in equine sarcoid tumors of the skin [17, 18, 25].

Experimental design

BCG was administered intravesically in female guinea pigs at doses as indicated in the tables and figures. After disinfection of the vulva the BCG was instilled into the empty bladder in anaesthesized animals using teflon sheaths (Abbocath-T, 24 gauge, 1202) as a transurethral catheter, and remained in the bladder for 1/2 or 1 hour. The animals were anaesthesized by intramuscular injection of 0.8 ml of a mixture of 20 mg/ml Ketalar®, 2.5 mg/ml Rompun® and 0.05 mg/ml atropine. The BCG was instilled once weekly for six consecutive weeks, a treatment schedule which was used in the first clinical studies reported [22, 27, 39]. Control animals were treated similarly with a placebo preparation.

PPD skin reactivity

In vivo the antigen specific immune response to BCG was determined by injecting 20 µg PPD (purified protein derivate of *Mycobacterium tuberculosis*, produced at the RIVM, Bilthoven, The Netherlands) intradermally in 0.1 ml. At 24 and 48 h after the PPD injection the diameter of the skin reaction, as indicated by redness and swelling, was measured.

Isolation of leukocytes

Leukocytes were obtained generally one week after the last BCG instillation. Animals were killed and the primary draining lymph nodes of the bladder (the iliac lymph node) and the spleen were removed aseptically. Spleen cells were isolated by gently pressing organ fragments through a 60 mesh stainless steel wire screen in Iscove's modified Dulbecco's medium (IMDM, Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% heat inactivated foetal calf serum (FCS, Boehringer FRG), penicillin (100 IU/ml), streptomycin (100 µg/ml) and 5×10^{-5} M 2-mercaptoethanol, further referred to as complete IMDM. After centrifugation erythrocytes were lysed by a buffer containing 8.3 g/l NH_4Cl, 1.0 g/l $KHCO_3$ and 37.2 mg/l EDTA. The spleen cells were washed twice and for in vitro studies resuspended in complete IMDM. Lymph node cells were isolated by gently pressing the lymph nodes between two glass slides. After centrifugation and washing the lymph node cells were resuspended in complete IMDM. Viable nucleated cells were counted by trypan blue (0,5%) exclusion.

In vitro antigen and mitogen stimulation

Leukocytes were cultured (37°C, 5% CO_2) in triplicate in 200 µl complete IMDM in polystyrene 96 well tissue culture clusters with flat bottem wells (Coster, Cambridge, Mass., USA). PPD (5, 10 and 20 µg/ml) and mitogens, ConA (concanavalin A, 1.25, 2.5, 5.0 µg/ml, Miles-Yeda Ltd., Rehovot, Israel), PHA (phytohaemagglutinin, 28, 56 and 112 µg/ml. Wellcome Research Laboratories, Beckenham, UK) and LPS (0.25, 0.5 and 1.0 mg/ml, lipopolysaccharide W of E. coli, 055:B5 Difco Laboratories, Detroit USA) were added. The

highest value obtained with one of the added concentrations, is presented in the tables and figures. After 2- to 4 days the cell cultures were pulse labeled by the addition of 5 μCi methyl-^{3}H-thymidine (specific activity 5 Ci/mmol, Radiochemical Centre, Amersham, Buckinghamshire UK) per ml culture for four hours. Cellular DNA was harvested on glass fiber filters using a multiple cell culture harvester (Titertek). Tritium thymidine incorporation was measured in an ISO cap/300 liquid scintillation counter (Nuclear Inc., Des Plaines, ILL, USA) and expressed as counts per minute (cpm).

In vitro cytotoxicity assay

The spontaneous cytotoxic activity, (natural killer cell activity) and the interleukin 2 induced cytotoxic activity (lymphokine activated killer (LAK) cell activity) from spleen and iliac lymph node leukocytes was determined in a 18 h chromium release assay [2]. In the chromium-release assay K562 a human erythroid leukemia cell line sensitive for both NK and LAK cell activity of guinea pigs was used as target cell. K562 target cells were labeled with ^{51}Cr by incubating 50 μCi per 1×10^6 cells in 0.1 ml for 1 h in a rocking waterbath. After washing (5 times) viable cells were counted by trypan blue exclusion. Target cells (1×10^4 per well) were incubated with effector cells in duplo at E:T cell ratios of 25, 50 and 100. After centrifugation (5 min, 200 g) the microtiter trays (Removawell System, Dynatech Companies, Zug, Switzerland) were incubated for 18 h at 37°C, 5% Co_2 in a humidified atmosphere [11].

After incubation and a second centrifugation step half of the supernatant was collected. The percentage ^{51}Cr-release was determined by measuring the radioactivity in half of the supernatant and half of the supernatant plus sediment. The radioactivity was determined in a gamma counter and expressed as counts per minute (cpm). The percentage release was calculated according to the formula:

$$\% \text{ release} = \frac{2 \times \text{cpm (1/2 supernatant)}}{\text{cpm (sediment + 1/2 supernatant)} + \text{cpm (1/2 supernatant)}}$$

The cytolytic activity was expressed as specific release i.e. percentage experimental release minus percentage spontaneous release. The spontaneous release was determined by incubating ^{51}Cr labeled target cells with medium alone (200 μl) without effector cell addition.

For estimation of lymphokine activated killer cell activity the same assay was used. However, the chromium release assay was performed in the presence of recombinant interleukin 2 (70 U/ml, kindly provided by Biogen, Geneva, Switzerland).

Analysis of lymphoid cell populations

Phenotype characteristics of leukocytes in the draining (iliac) lymph node and spleen were determined by flow cytofluorometric analysis in a Fluorescence Activated Cell Sorter (FACS-Analyser, Becton-Dickinson Immunocytometry Systems, Mountain View, Ca USA). Analysis of the data was performed with Consort 30 software (Becton-Dickinson) on a Hewlett-Packard 9920S computer. For detection of various leukocyte markers the following monoclonal antibodies (MoAb) were used: CT 5 a monoclonal for guinea pig T cells [41], T167 a monoclonal for guinea pig T cells and thymocytes (Burger, personal communication), 31D2 a monoclonal detecting guinea pig B cells reacting with IgM (Burger, personal communication), 305 a monoclonal detecting guinea pig macrophages [24] and 25E3, a monoclonal detecting Ia antigens on guinea pig cells [5]. CT5 was kindly provided by Dr. R.J. Scheper (Free University, Amsterdam The Netherlands), T167, 31D2 and 305 were kindly provided by Dr. R. Burger (Heidelberg, FRG) and 25E3 was kindly provided by Dr. E. Shevach (New York, USA).

Spleen and iliac lymph node cells were obtained as mentioned above, CT5, T167, 31D2 and 305 were diluted 1:200, and 25E3 was diluted 1:500. After washing the antibody coated cells were incubated for 45 min at 4°C with rabbit anti-mouse-FITC diluted 1:40 in PBS, 10 μg/10^6 cells (RAM-FITC, F(ab)$_2$, Cappel, Cooper Biomedical Inc., Malvern, PA, USA).

After washing for removal of surplus RAM-FITC for analysis 750 μl PBS (phosphate buffered saline) was added to the cells. From each sample 10^4 cells were analysed in the FACS. For control on non specific binding a commercially available control ascites was used (Cappel). The data obtained with the FACS measurements were corrected for the non antigen specific binding of the control ascites by subtracting the percentage positive cells binding the control ascites from the percentage cells reacting with the various specific monoclonal antibodies. The non specific binding of the control ascites was in the range 20 to 30% For control on the specific activity of the monoclonal antibodies an Ia positive guinea pig B cell leukemia (EN-L2C), thymocytes and peritoneal exsudate cells (PEC) were used.

Statistical analysis

For differences between BCG and placebo treated animals two sided significances were determined with Student's t-test. In case of insufficient homogeneity of variances, the Welch correction with respect to the degrees of freedom was applied.

Results

Antigen specific reaction to BCG

Initially several dose response studies were performed in which BCG, in doses ranging from 1×10^3 to 5×10^7 c.p. was administered once a week for six consecutive weeks. During these studies PPD skin reactions were monitored. At week 3, after 2 BCG instillations, no positive PPD skin reactions were detected (Table 1). However, with the exception of the doses 1×10^3 c.p., after four BCG instillations all the other doses, 1×10^5 c.p. and higher, resulted in positive PPD skin reactions. Only one animal in the different dosage groups did not show a positive skin reaction (diameter $\geq$ 10 mm) after four BCG instillations. After six intravesical BCG instillations at 7 days after last instillation all dosages induced a positive PPD skin reaction, although in the lower dosages investigated a smaller reaction size was noted together with the presence of non-reacting animals (Table 1). In Fig. 1 results are presented in which the DTH reaction, measured at 24 h after PPD injection, was followed for a period of 12 weeks. All BCG treated (5×10^7 c.p. intravesically) animals became positive at 4 weeks after start of the treatment (after four BCG instillations) and remained positive during

Table 1. Induction of delayed type hypersensitivity (DTH) skin reaction after intravesical administration of BCG[a]

Dosage c.p.	Number of instillations		
	2	4	6
1×10^3	0 ± 0 (0/4)[b]	0 ± 0 (0/4)	7.8 ± 2.9 (2/4)
1×10^5	0 ± 0 (0/4)	13.2 ± 9.5 (3/4)	11.1 ± 3.0 (3/4)
5×10^6	0 ± 0 (0/4)	13.1 ± 2.6 (4/4)	12.5 ± 2.0 (4/4)
1×10^7	1.0 ± 3.8 (1/14)	14.1 ± 5.1 (13/14)	14.6 ± 1.7 (11/11)
5×10^7	0 ± 0 (0/9)	13.4 ± 5.5 (8/9)	15.0 ± 2.2 (9/9)
Placebo	0 ± 0 (0/20)	0.5 ± 2.2 (1/20)	2.9 ± 5.8 (3/17)

[a] BCG was administered once weekly for six consecutive weeks. The DTH reaction was measured at 24 h after intradermal injection of 20 μg PPD, one week after the previous BCG instillation

[b] diameter of redness, mean ± s.d., within parentheses, number of animals with positive reaction (diameter ⩾ 10 mm) versus number of animals tested. Combined results of four experiments are presented

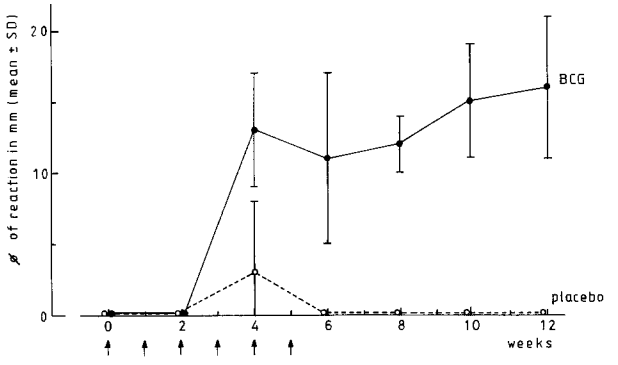

Fig. 1. † = administration of 5×10^7 c.p. BCG c.q. placebo. Delayed type hypersensitivity reaction to PPD in guinea pigs after intravesical administration of BCG

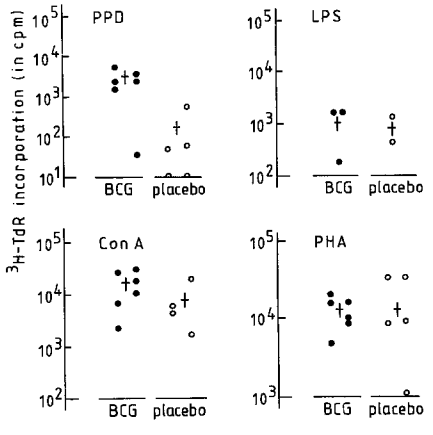

Fig. 2. † = mean; 5×10^7 c.p. BCG, once weekly for six consecutive weeks. In vitro assay at week 7, one week after last BCG instillation. Effect of intravesical BCG administration on in vitro antigen and mitogen response of regional (iliac) lymph node cells

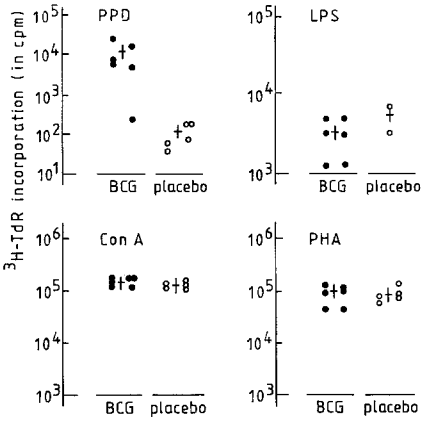

Fig. 3. † = mean; 5×10^7 c.p. BCG, once weekly for six consecutive weeks. In vitro assay at week 7, one week after last BCG instillation. Effect of intravesical BCG administration on in vitro antigen and mitogen response of spleen cells

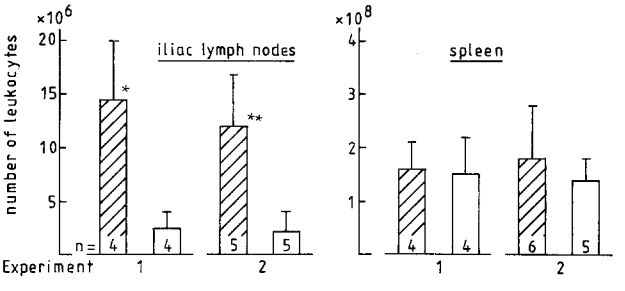

Fig. 4. ▨ = BCG; □ = placebo; * $P<0.05$ ** $P<0.01$ (vs placebo). Effect of intravesical BCG administration on numbers of leukocytes in regional (iliac) lymph nodes and spleen

the observation period of 12 weeks. BCG immune lymphocytes were present both in the regional draining (iliac) lymph node and the spleen at one week after the last BCG instillation (Fig. 2 and 3). The presence of the BCG immune lymphocytes was demonstrated in the in vitro antigen stimulation assay using purified protein derivative (PPD) of *Mycobacterium tuberculosis* as recognition antigen.

Table 2. Phenotype of leukocyte populations in regional (iliac) lymph node and spleen of guinea pigs after intravesical administration (once a week for six consecutive weeks) of BCG

Organ	Treatment	Monoclonal antibodies[a]				
		CT5	T167	31D2	305	25E3
Iliac lymph node	Placebo (2)[b]	34.0 ± 5.7[c]	64.5 ± 6.6	15.1 ± 2.3	2.5 ± 1.3	22.3 ± 1.5
	BCG (3)	52.7 ± 6.7[d]	68.8 ± 7.6	19.4 ± 5.3	0.6 ± 3.7	35.8 ± 2.0[e]
Spleen	Placebo (4)	50.2 ± 5.7	56.1 ± 7.6	19.4 ± 1.3	16.0 ± 12.3	36.3 ± 3.5
	BCG (3)	55.5 ± 5.9	60.0 ± 12.9	16.5 ± 19.5	24.5 ± 30.4	37.8 ± 4.4

[a] Monoclonal antibodies used as markers for guinea pig leukocytes: CT5: T cells, T167: T cells and thymocytes, 31D2: B cells, 305. macrophages, 25E3: Ia antigens
[b] Within parentheses number of samples evaluated; for placebo treated animals lymph nodes of four animals were pooled; for BCG treated animals lymph nodes of individual animals were evaluated
[c] Percent cells positively reacting with monoclonal antibodies as determined with FACS analysis (see Materials and methods)
[d] $P < 0.05$
[e] $P < 0.01$ respectively, Student's *t*-test, two sided

Table 3. Natural killer (NK) cell activity in regional draining (iliac) lymph node and spleen of guinea pigs after intravesical BCG administration[a]

Iliac lymph node	Day 1[b]	Day 4	Day 7
Placebo	3.3 ± 1.5 (7)[c]	2.1 ± 1.9 (7)	2.8 ± 1.6 (6)
BCG[d]	4.1 ± 2.5 (8)	2.6 ± 2.3 (8)	1.7 ± 1.0 (7)
Spleen	Day 1	Day 4	Day 7
Placebo	41.8 ± 12.5 (7)	28.7 ± 14.0 (8)	37.7 ± 10.5 (8)
BCG	45.4 ± 7.4 (8)	28.8 ± 12.1 (8)	47.6 ± 12.1 (8)

[a] NK cell activity was maesured in a 18 h chromium-51 release assay using K562 target cells
[b] Indicated are the days after the last BCG instillation
[c] Percentage specific chromium-51 release, mean ± s.d (number of animals) at effector to target cell ratio 100:1
[d] BCG, 5×10^7 c.p., was administered once weekly for six consecutive weeks

Non antigen specific reactions after BCG administration

The non antigen specific responses of lymphocytes of the iliac lymph node and of the spleen were studied with a B cell mitogen (LPS) and two T cell mitogens (ConA and PHA). One representative from four experiments is presented in Fig. 2 and 3. No differences in responses were observed between placebo and BCG treated animals. However, a significant increase ($P<0.05$ and $P<0.01$, Students-*t*-test, two sided) was noted in the number of leukocytes harvested from the regional lymph node after six weeks treatment with BCG (Fig.4). In contrast, such an increase in cell number was not found in the spleen. Using various monoclonal antibodies (see materials and methods) for the detection of guinea pig T cells, B cells and macrophages, it was found that the subpopulation of the leukocytes in the iliac lymph nodes expressing the CT5 antigen was significantly ($P<0.05$, Student's-*t*-test, two sided) increased (Table 2). In addition, there was a two sided significant ($P<0.01$) increase in the expression of Ia antigens on the leukocytes as detected by the 25E3 monoclonal antibody (Table 2). Only a very low number of macrophages was detected by the 305 monoclonal antibody after the repeated BCG instillations. This is in contrast to our previous histological observations in which epithelioid cells from the monocyte/macrophage series were present in the iliac lymph nodes after six weeks treatment with BCG [42]. In the spleen no differences were found between the leukocyte subpopulations and the expression of Ia antigen on the spleen cells.

The non antigen specific spontaneous cytotoxicity (NK cell activity) was measured in a 18 h ^{51}Cr release

Table 4. Lymphokine activated killer (LAK) cell activity in regional draining (iliac) lymph node and spleen of guinea pigs after intravesical BCG administration[a]

Iliac lymph node	Day 1[b]	Day 4	Day 7
Placebo	16.7 ± 3.9 (7)[c]	13.0 ± 6.0 (8)	14.2 ± 9.8 (8)
BCG[d]	25.2 ± 9.2 (8)[e]	12.3 ± 8.2 (8)	9.2 ± 4.6 (7)
Spleen	**Day 1**	**Day 4**	**Day 7**
Placebo	69.1 ± 7.0 (7)	53.4 ± 11.6 (8)	58.4 ± 6.2 (8)
BCG	68.7 ± 2.8 (8)	56.8 ± 6.4 (8)	62.6 ± 5.4 (8)

[a] LAK cell activity was determined in a 18 h chromium-51 release assay with the addition of recombinant interleukin 2 (70 units/ml) using K562 target cells. The IL2 presence induced for all cell populations at all days investigated a significantly (at least $P<0.02$, Student's t-test, two sided) increased cytotoxic activity (Compare results of Table 4 with Table 3)

[b] Indicated are the days after the last BCG instillation

[c] Percentage specifc chromium-51 release, mean ± s.d. (number of animals) at effector to target cell ratio 100:1

[d] BCG, 5×10^7 c.p., was administered once weekly for six consecutive weeks

[e] At day 1 significantly ($P<0.05$, Student's-t-test, two sided) increased inducation of activity compared to placebo treated animals

assay with K562 target cells. In the iliac lymph nodes, both after placebo and BCG treatment, a low NK cell activity was observed at various times (day 1, 4 and 7) after the sixth BCG instillation (Table 3). In the spleen no differences in NK cell activity were present after intravesical BCG treatment compared to placebo treated animals (Table 3). Both in the iliac lymph node and in the spleen lymphokine activated killer (LAK) cell activity could be induced by adding recombinant interleukin 2 to the NK cell test system. In both organs at all days tested a significantly ($P<0.01$, and in two cases $P<0.02$, Students-t-test, two sided) increased NK cell activity was measured (compare results presented in Tables 3 and 4). At day 1 after the last BCG instillation, the BCG treated animals showed a significantly higher response in lymph node to co-culture with interleukin 2 compared to placebo treated animals ($P<0.05$, Students-t-test two sided).

Discussion

In our studies of the induction of enhanced immune responses after intravesical BCG administration, most non-antigen specific immune reactions, such as lymphocyte motogen responses and natural killer cell activity, were not increased when compared to placebo treated animals. The major difference between BCG and placebo treated animals was the induction of BCG antigen specific immune reactions, exemplified by the positive PPD skin tests, and the lymphocyte response in lymph nodes and in the spleen, with in vitro antigen stimulation assay using purified protein derivative of *Mycobacterium tuberculosis.*

The BCG specific reactivity was obtained after the intravescial administration of BCG in an intact bladder as no intracavitary pretreatment damage was induced. Ratliff et al. [33] demonstrated in a murine model that attachment of BCG to the bladder wall was related to damage of the epithelial layer. When the bladder epithelium was coagulated locally, the attachment of BCG was increased significantly. Histopathologically we were not able to find damage of the epithelial layer covering the bladder wall [42]. Although in these studies bladder damage could not definitively be excluded, it was unlikely that the treatment induced bladder damage. In this respect our data confirm the high sensitivity of the guinea pig for infection with mycobacteria [37]. Even in the lowest doses used (1×10^3 c.p. BCG) a systemic immune reaction to BCG was obtained, as indicated by the positive PPD skin reaction. For the induction of a positive PPD skin reaction, however, repeated BCG instillations were needed. After two BCG instillations almost all animals were negative, whereas after four BCG instillations all, except two in the lowest dose administered (1×10^3 c.p.), animals became positive. Although we did not follow the positive PPD skin reaction for a long time, two months after the six week course of BCG, the animals still had a positive skin reaction to PPD.

The capability of the BCG vaccine, used for intravesical administration, to induce a delayed type hypersensitivity skin reaction seems to be very important. In a murine bladder tumor system it was found that the

antitumor effect of BCG was correlated with the viability and growth rate of BCG. Immunologically, a correlation was observed with positive footpad reactions to PPD and augmentation of NK cell activity; low viability BCG did not induce either reaction [40]. In clinical studies of bladder cancer patients a correlation was found between the PPD skin reaction and therapeutic results [16, 23, 43]. A similar correlation was observed after intralesional BCG immunotherapy in spontaneously occurring bovine ocular squamous cell carcinoma [19]. Considering the relation between therapeutic effect and viability of the BCG both in the murine bladder tumor system [40], and in bladder cancer patients [16], not only the viability of the vaccine but also the total dose of BCG administered might be responsible for the resulting data. In the murine system low and high viability BCG were only investigated at equal doses in micrograms and not in doses of culturable particles administered [40]. Although differences between BCG preparations may be present, the differences observed by Shapiro et al. [40] may also reflect the dependency of the assay system to a dose expressed in culturable particles instead of the weight of BCG. Such a dependecy on dosage in culturable particles was previously demonstrated for the activation of splenic macrophages [38].

Our results predominantly indicated local activation of the draining iliac lymph node. Systemically, as measured by the immunologic activity of spleen cells, a part from the induction of BCG immune lymphocytes no increased activity was observed after the six week course of intravesical BCG administration. Due to an influx or proliferation of leukocytes an increased number of cells was present in the iliac lymph node. On these leukocytes an increased expression of the MHC class II (Ia) immune response antigen was present. Using monoclonal antibodies detecting guinea pig T lymphocytes with T167 a high number of T cells was detected, in contrast to CT5. Also after BCG administation an increase with CT5 was noted but not with T167. Both monoclonal antibodies were reproted to act as pan T markers for guinea pig T cells ([41] Burger, Personal communication). In the spleen cell population our data for CT5 and T167 were more in accordance. We have no explanation for the differences observed. However, it might be that the T167 monoclonal antibody showed some cross reaction with guinea pig B-cell (Burger, personal communication). With MoAb 305, detecting guinea pig macrophages, probably the presence of monocytes/macrophages was underestimated. Histologically the increase in size of the iliac lymph node is partly due to the BCG induced granuloma of monocyte/macrophage origin [30, 42]. MoAb 305 was produced in mice immunized with adherent peritoneal cells, and reacts with mature, elicited, and activated macrophages [24]. Part of the lymph node macrophages in our study were probably mature monocytes/macrophages further differentiated to epithelioid cells forming granulomas [1], which might not be recognised by the 305 MoAb.

In contrast to studies in mice [40] we did not find an increased natural killer (NK) cell activity in the guinea pig after intravesical BCG administration, which might partly be due to the differences in methods used (damaged versus non damaged bladder) for the BCG instillation. Our data for NK cell activity are in the same range as previously reported [2]. At day 1 after the last BCG instillation we found an increased susceptibility of iliac lymph node cells for the induction of enhanced NK cell activity (LAK cells) by interleukin 2. In contrast to data reported by Zwilling et al. [44], our results clearly demonstrate that guinea pig lymph node cells can be significantly activated for cytotoxicity by interleukin 2.

Although initially correlations were found between the antitumor activity in a murine bladder tumor system, and the NK cell activity [40], additional studies indicated that NK cells are not a major contribution to the antitumor activity after intravesical BCG [34]. Of major importance seems to be the thymus dependent immune response to BCG [31]. This thymus dependent immune respose to BCG was also reported to be needed for the antitumor activity of BCG after local BCG administration in murine and guinea pig tumor systems [7, 14, 35]. In the bladder wall repeated antigen contact may lead to a delayed type hypersensitivity reaction [8]. Probably the mononuclear infiltrates, present in the bladder wall after repeated BCG administration [6, 12, 20, 42], might be interpreted as a delayed type hypersensitivity reaction. One of the lymphokines involved in the delayed type hypersensitivity reaction is interleukin 2. In this respect it is noteworthy that in bladder cancer patients during the six week BCG course interleukin 2 could be detected in the urine (unpublished observations, [32]. Besides T cell proliferation interleukin 2 induces lymphokine activated killer (LAK) cells which are able to lyse a broad range of tumor target cells (reviewed by Rosenberg [36]. For the in vivo antitumor activity both NK derived and T cell derived LAK cells were found to be responsible depending on the immunogenicity of the tumor used [29].

For the effect of BCG in the treatment of superficial bladder cancer in different systems various indications were found for the activity of BCG. However, although several suggestions were made, both in experimental animal tumor systems and for clinical studies in man, more knowledge is needed on the mechanism of the antitumor activity of BCG in superficial bladder cancer.

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