Scand. J. Lab. Anim. Sci. No. 1. 2002. Vol. 29

A rat model for the immune response to the intrauterine administration of BCG

by *M. Kanter*[1], *M. Yoruk*[1]*, *A. Koc*[1], *I. Meral*[2] & *H.H. Timurkan*[3]
[1]Department of Histology and Embryology, [2]Department of Physiology, [3]Department of Reproduction, Faculty of Veterinary Medicine, Yuzuncu Yil University, 65080, Kampus, Van, Turkey. e-mail: myoruk@hotmail.com

Summary
This study was designed to investigate the changes in the numbers of lymphocytes, macrophages and plasma cells in the uterus and ileocecal lymph nodes of rats exposed to the intrauterine administration of Bacillus-Calmette Guérin (BCG). Thirty female Wistar Albino rats, age 6 months and weighing between 200-250 g, were assigned to the two experimental groups BCG treated and controls (n=15). The intrauterine BCG injections were made using laparatomy in the diestrous cycle under Rompun and Ketalar anesthesia. 0.1 ml BCG were injected for each into cornu uteri while the control group received 0.1 ml sterile saline in the same place. Two weeks later, the rats in both groups were anesthetized with ether and decapitated. Uterus and ileocecal lymph nodes were processed to determine α naphthyl acid esterase (ANAE) - positive T lymphocytes and macrophages. The plasma cells were stained with the methyl green-pyronin method. It was found that the numbers of lymphocytes, macrophages and plasma cells on the uterus increased ($P<0.01$) in BCG treated rats. In addition, the number of these cells also increased in the ileocecal lymph nodes indicating the presence of an immune response to the intrauterine BCG administration. It is concluded that although the rat was chosen as a model and BCG was given by the process of laparatomy in this study, intracervical administration of BCG in the uterus should be studied clinically in cases of immune deficiency disorders related to the uterus, such as endometritis, myometritis, pyometra, endometriosis, infertility and implantation problems of domestic animals, to see if there is an increase in the immune response.

Introduction
Bacillus-Calmette Guérin (BCG) has been accepted as the most effective immunostimulating agent used against superficial bladder cancer in immunotherapy. However, its mechanism of action remains incompletely understood (*Morales et al., 1976*; *Lamm et al., 1980*; *Brosmon et al., 1982*; *Van Der Meijden et al, 1988; O'Donnell et al., 1999*). The effectiveness of local administration of BCG has been suggested to be important for the induction of the inflammatory and immune responses (*Teppema et al., 1992*).
The effects of BCG on the bladder have been studied by many workers. Guinan et al. (1986) have demonstrated that the numbers of T cells increased in the bladder wall after repeated BCG instillations. By using immunohistochemical staining, De Jong et al. (1991) have showed that the numbers of T cells increased in the bladder wall after intra vesical BCG administration in guinea pigs. Widely scattered B cells and macrophages were also present, but were much fewer in number than T cells.
BCG is also a potent and persistent inducer of lymphocyte trapping, particularly in regional lymph nodes, where it also markedly enhances the trapping response to a subsequent antigenic challenge (*Zatz, 1976*). It has been suggested that BCG activates both macrophages and T cells in vitro *(Mokyr et al., 1975)*, and that its effects are mediated in vivo by T cell systems *(Ariyan and Gershon, 1973; Mackaness et al., 1973; Lagrange and Mackaness, 1975)*. It has been also reported that B lymphocytes increase in lymphoid organs following the intraperitonal administration of BCG *(Meyer et al., 1979)*.
Although the effects of BCG on the bladder,

which has lumen and lymphoid organs have been well-documented. there is not much information about the effect of BCG on the uterus. which also has lumen. Thus. this study was designed to investigate the quantitative changes in the numbers of lymphocytes, macrophages and plasma cells in the uterus of rats exposed to the intrauterine administration of BCG. In addition, to be able to investigate the immune response to the BCG administration, the numbers of these cells in the ileocecal lymph nodes were also examined.

Materials and Methods

Thirty female Wistar Albino rats, age 6 months and weighing between 200-250 g, were used. The rats were assigned to the two experimental groups, BCG treated and controls (n=15), and fed with standard feed and water ad libitum. In order to maintain their biological rhythms stable, 12 h artificial and 12 h dark was applied. All rats received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

The intrauterine BCG injections were made with the process of laparatomy in the diestrous cycle. The diestrous cycle was preferred for BCG injection because the numbers of T lymphocytes, macrophages and plasma cells are fewer in this cycle than in other cycles of estrous *(Yalçın and Kanter, 2000)*. The Vaginal smear method was used to determine the diestrous cycle (*Mallenby et al., 1993; Kanter et al., 1996*). The rats were premedicated with 11.66 mg/kg Rompun intramuscularly (i.m.) (Xylazin hydrochloride 23.32 mg/ml. Bayer). and 5 minute later Ketalar (1.5 ml/kg) was applied i.m. (Ketamin hydrochloride 50 mg/ml. Eczacıbası) for general anesthesia. The abdomen was entered through a midline caudal abdominal laparotomy. 0.1 ml BCG were injected for each into cornu uteri and control group received 0.1 ml sterile saline in the same place.

Two weeks after injections, rats in both groups were anesthetized with a high dose of ether and decapitated. Uterus and ileocecal lymph nodes were processed to determine α naphthyl acid esterase (ANAE) - positive T lymphocytes and macrophages. Briefly, the uterus and ileocecal lymph nodes were removed and fixed in previously cooled formalin-sucrose solution at + 4 ^{0}C for 22 h and then kept at + 4 ^{0}C in Holt solution for 22 h *(Mueller et al., 1975)*. Ten µm thin slides obtained using a cryostat (Microm, Germany) were transferred into the formalin-gelatin covered glass. The materials were left to dry at room temperature in order to determine ANAE positive lymphocytes *(Mueller et al., 1975)* and macrophages *(Zicca et al., 1981)*. The slides were stained in an incubation solution described by Mueller et al (1975) at pH 7.2 for 5 min. After ANAE staining, the slides were washed and processed for nucleus staining for five minutes with 1 % methyl-green dissolved in acetate buffer (pH 4.2). Water was removed by standard processing techniques. The tissue samples were fixed in formalin-alcohol fixation solution for 24 h. The routine histological tissue processing protocol was applied to the tissue samples. Methyl-green pyronine staining was applied to 6 µm-thick slides *(Bancroft and Cook, 1984)* obtained using rotary microtome (Leica 2135, Germany). Samples were examined through a research microscope (Nikon Optiphot 2, Japan).

In order to determine the numerical distribution of cells in the tissue samples, 20 areas in the endometrium and myometrium of the uterus and the medulla of the lymph nodes were chosen randomly and the cells were counted in each area. The cell (100 square mm) densities in each area were calculated and recorded as cell number/mm^2. The data were expressed as means with standard deviations (SD). Student's *t*-test was used to compare BCG treated vs. control rats.

Results

T lymphocytes in the endometrium were identified by ANAE enzyme staining with the numerous specific brown granules around large nuclei. Although the numbers of T lymphocytes of both groups were almost the same in the endometrium close to the epithelium, they were higher ($P<0.01$) in BCG treated rats than in controls in the endometrium close to the myometrium (Table 1 and Fig. 1). In addition, the T lymphocyte number

in the myometrium was also increased ($P<0.01$) by BCG treatment (Table 1 and Fig. 1).

Macrophages could not be seen in the endometrium. These cells, however, were easily identified by their diffuse browner coloration in the myometrium. The macrophage number in the myometrium was increased ($P<0.01$) by BCG treatment (Table 1 and Fig. 2).

Similarly the number of plasma cells stained with the methyl green-pyronin in the endometrium was higher ($P<0.01$) in BCG treated rats than controls (Table 1 and Fig. 3). In the myometrium, their numbers were few and not different between experimental groups (Table 1).

T lymphocytes n lymph nodes were recognized with one or two specific brown granules by ANAE enzyme staining. Macrophages, on the other hand, were easily identifiable by their darker coloration. The lymph nodes of rats treated with BCG had a much higher ($P<0.01$) number of T lymphocyte, macrophage and plasma cells than those of controls (Table 2).

Table 1. The numbers (cell number/mm^2) of T cells, macrophages and plasma cells in the endometrium and myometrium of control and BCG treated rats. The data is expressed as mean ± standard deviation (n=15).

Cells	Control		BCG treated	
	Endometrium	Myometrium	Endometrium	Myometrium
T cells	16.26 ± 2.01	3.54 ±0.78	62.13 ± 2.23*	15.87 ± 2.63*
Macrophages	-	32.53 ± 1.59	-	87.06 ± 2.12*
Plasma cells	11.73 ± 1.79	1.34 ± 0.27	43.06 ± 1.75*	1.42 ± 0.31

*: $P<0.01$ compared to control

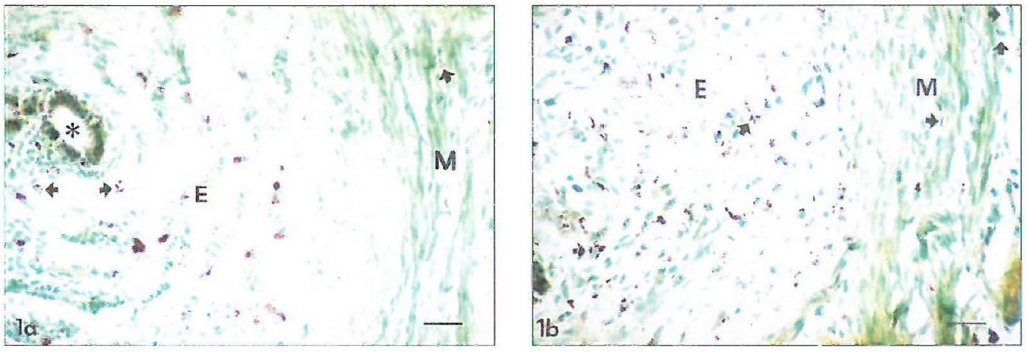

Fig. 1. The distribution of T cells in the endometrium and myometrium of the control (a) and the BCG treated (b) rats. E: Endometrium, M: Myometrium. *: Uterine gland, →: T cells. ANAE, Bar = 28 µm.

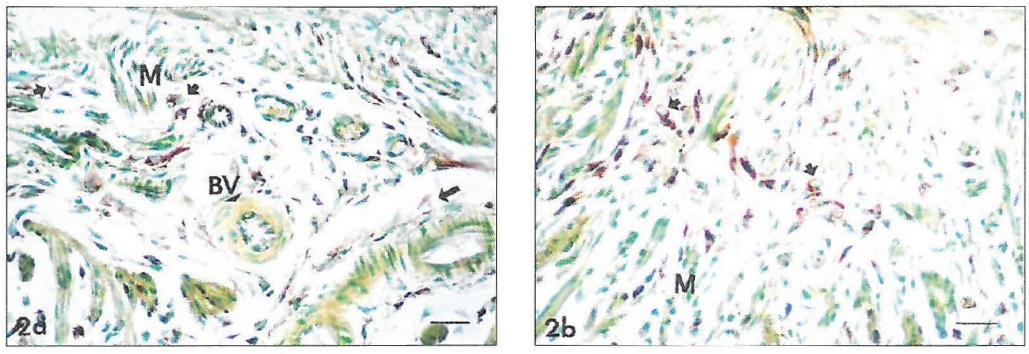

Fig. 2. The distribution of macrophages in the myometrium of the control (a) and the BCG treated (b) rats. M: Myometrium, BV: Blood vessels, →: macrophages. ANAE, Bar = 28 μm.

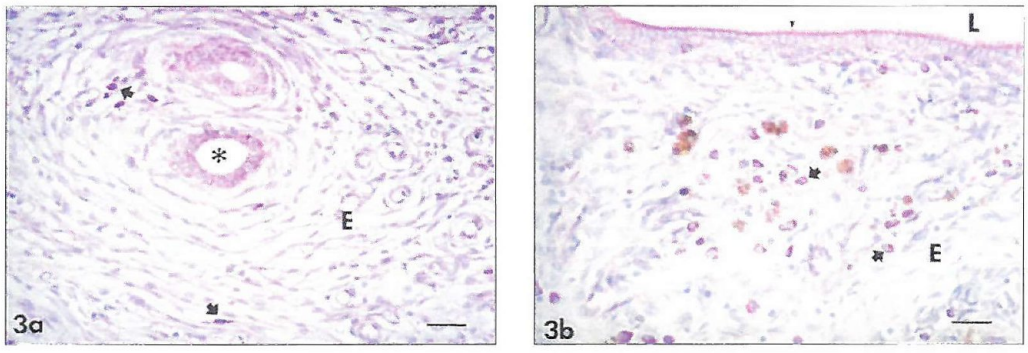

Fig. 3. The distribution of plasma cells in the endometrium of the control (a) and the BCG treated (b) rats. E: Endometrium, L: Lumen, *: Uterine gland, →: plasma cells, ▶: uterine epithelium. Methyl green-pyronin, Bar = 28 μm

Table 2. The numbers (cell number/mm^2) of T cells, macrophages and plasma cells in the medulla of lymph nodes of control and BCG treated rats. The data is expressed as mean ± standard deviation (n=15).

Cells	Control	BCG treated
T cells	59.11 ± 5.34	180.24 ± 21.33*
Macrophages	26.16 ± 1.54	69.44 ± 5.62*
Plasma cells	48.35 ± 5.73	534.09 ± 49.80*

*: P<0.01 compared to control

Discussion

It is generally assumed that BCG operates by activating the immune system *(Leong et al., 1990)*. The humoral and cellular immunity of BCG on the bladder has been demonstrated by many workers. Immunohistochemical and flow-cytofluorometric studies have shown that T lymphocytes, monocytes/macrophages and polymorphonuclear leukocyte increased in the bladder wall in response to intravesical BCG treatment *(De Boer et al., 1991)*. It has been also demonstrated that after intravesical BCG treatment, the bladder mucosa was prone to inflammatory changes showing granulomatous lesions with lymphocyte, macrophage, and plasma cell infiltrations together with urothelial dysplasia *(Morales et al., 1976; Morales, 1980)*.

Yakhnitsa et al. (1988) have studied the reaction of lymphocytes in the endometrium to the intrauterine administration of antigens, and showed an increase in immune activity in the endometrium of rats. However, the present study is the first one that demonstrated the quantitative changes in the numbers of lymphocytes, macrophages and plasma cells on the endometrium and also myometrium of BCG treated rats. In addition, increased numbers of lymphocytes, macrophages and plasma cells in the ileocecal lymph nodes indicated the presence of an immune response to the BCG administration. Our result is consistent with the results of Meyer (1979) and Mokyr et al., (1975) who showed an increase in the numbers of B lymphocytes, macrophages and T cells in lymphoid organs following BCG administration.

In the present study, the rat has been chosen as a model, and it has been demonstrated that BCG increased the humoral and cellular immunity in the uterus. Although BCG was given by the process of laparatomy in this study, intracervical administration of BCG in the uterus should now be studied clinically in cases of immune deficiency disorders related to the uterus, such as endometritis, myometritis, pyometra, endometriosis, infertility and implantation problems of domestic animals, to see whether it increases the immune response.

References

Ariyan S & KR Gershon: Augmentation of the adoptive transfer of specific tumor immunity by non-specifically immunized macrophages. J Natl Cancer Inst 1973, *51*, 1145-1148.

Bancroft JD & HC Cook : Manual of histological techniques. Churchill Livingstone, New York, 1984.

Brosman SA: Experience with Bacillus Calmette-Guérin in patients with superficial bladder carcinoma. J Urol 1982, 27-30.

Davies M: Bacillus Calmette-Guérin as an antitumor agent. The interaction with cells of the mammalian immune system. Biochim Biophys Acta 1982, *651*,143-47.

De Boer EC, WH De Jong, APM Van Der Meijen, PA Streenberg, F Witjes, PDJ Vegt, FMJ Debruyne & EJ Ruitenberg : Leukocytes in the urine after intravesical BCG treatment for superficial bladder cancer. A flow citofluorometric analysis. Urol Res 1991, *19*, 45-50.

Guinan P, M Shaw & V Ray: Histopathology of BCG and Thiotepa treated bladders. Urol Res 1986, *14*, 211-215.

Kanter M, E Öztaş & C Dalçık: Sıçan, fare ve kobaylarda gebeliğin ilk gününü tayin etmede vaginal smear yönteminin kullanılması. Van

Tıp Derg. 1996, *3*, 112-116.

Kelly MT: Activation of guinea pig macrophages by cell walls of Mycobacterium bovis, strain BCG. Cell Immunol 1976, *26*, 254-63.

Lagrange PH & GB Mackaness: A stable form of delayed type hypersensitivity. J Exp Med 1975, *141*, 82-96.

Lamm DL, DE Thor, SC Harris, JA Reyna, VD Stoydill, & HM Radwim: Baciilus Calmette-Guérin immunotherapy of superficial bladder cancer. J Urol 1980, *74*, 38-42.

Leong AS-Y, P Wannakrairot, J Jose & J Milios : Bacillus Calmette Guérin-treated superficial bladder cancer: correlation of morphology with immunophenotyping. J Pathol (Lond) 1990, *162*, 35-41.

Mackaness GB, DJ Auclair & PH Lagrange: Immunopotentiation with BCG. I. Immune response to different strains and preparations. J Natl Cancer Inst. 1973, *51*,1655-67.

Mallenby J, J Dwyer, C Hawkins, C Hitchen: Effects of experimental limbic epilepsy on the estrus cycle. Epilepsia 1993, *34*, 220-227.

Meyer EM, K Wilmsmann, W Schalke & E Grundmann : Quantification of BCG-induced reactions of T and B areas in peripheral lymphoid organs of young adult BALB/c mice. A histometrical and autoradiographical study. Path Res Pract 1979, *164*,127-140.

Mokyr MB & MS Mitchell: Activation of lymphoid cells by BCG in vitro. Cell Immunol 1975, *15*, 264.

Morales A, D Eindinger & AW Bruce: Intracavitary Bacillus Calmette Guérin in the treatment of superficial bladder tumors. J Urol 1976, *116*, 180-183.

Morales A: Treatment of carcinoma in situ of the bladder with BCG. Cancer Immunol Immunother 1980, *9*, 69-72.

Mueller J, GB Del Re, H Buerki, HU Keller, MW Hess & H Cottier: Nonspecific acid esterase activity: A criterion for differentiation of the T and B lymphocytes in mouse lymph nodes. Eur J Immun 1975, *5*, 270-74.

O'Donnell MA, Y Luo, X Chen, A Szilvasi, SE Hunter & SK Clinton: Role of IL-12 in the induction and potentiation of IFN-γ in response to Bacillus Calmette Guérin. J Immunol 1999, *163*, 4246-4252.

Ten Dam HG, K Toman, KL Hitze & J Guld: Present knowledge of immunization against tuberculosis. Bull Wld Hlth Org 1976, *54*, 255-69.

Teppema JS, EC De Boer, PA Streenberg & APM Van Der Meijden : Morphological aspects of the interaction of Bacillus Calmette-Guérin with urothelial bladder cells in vivo and in vitro: relevance for antitumor activitiy? Urol Res 1992, *20*, 219-228.

Van Der Meijden APM, WII De Jong, PA Steerenberg, HC Walvoort, EC De Boer, FMJ Debruijne & EJ Ruitenberg: Intravesical BCG administration in the guinea pig. A histomorphological study. Virchows Arch [B] 1988, *55*, 207-215.

Yakhnista AG, SB Stefanov & EV Artyukh: Reaction of lymphocytes in endometrium to the intrauterine administration of antigens. Arch Anat Hist Embr 1988, *64*, 74-77.

Yalçın A & M Kanter: Sıçanlarda östrus siklusunda endometriyum dokusunun hücresel ve sıvısal savunma sistemi hücreleri üzerinde histokimyasal ve histometrik araştırmalar. YYU Sağ. Bil. Derg. 2000, *6*, 77-83.

Zatz MM: Effects of BCG on lymphocyte trapping. J Immunol 1976; *116*, 1587-1691.

Zicca A, A Leprini, A Cadoni, AT Franzi, M Ferrarini & CE Grossi: Ultra - structural localization of alpha naphtyl acid esterase in human T lymphocytes. Am J Pathol 1981, *105*, 40-46.