Analysis of the Immunologic Mechanism of Intravesical Bacillus Calmette-Guerin Therapy for Superficial Bladder Tumors: Distribution and Function of Immune Cells

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Intravesical bacillus Calmette-Guerin (BCG) administration has been used as an adjuvant therapy after transurethral resection for superficial bladder cancer, but the exact mechanisms of its antitumor activity are not yet known. The aim of this study was to characterize the immunologic aspects of antiumor activity of BCG using an animal model. C3H/He inbred mice and murine bladder tumor cell line, MBT-2 were used. The changes in immune cells such as helper T cells, suppressor T cells, macrophages and natural killer cells in the bladder and spleen were analysed by immunohistochemical method in intravesical BCG instilled in normal bladder, MBT-2 implanted after electrocauterization of the bladder mucosa and MBT-2 implanted and intravesical BCG treated group. The changes in natural killer cell activity of the splenocytes and peritoneal lymphocytes were evaluated using ^{51}chromium release assay at regular time intervals following intraperitoneal BCG instillation. The prophylactic anticancer effect was evaluated by observing the tumor growth in the intravesically BCG treated group after intravesical MBT-2 implantation. In immunohistochemical examination, a remarkable infiltration of macrophage and helper T cell was observed in the lamina propria of the bladder, and the helper and suppressor T cells ratio (Th/Ts ratio) was increased after intravesical BCG therapy. In ^{51}chromium release assay, enhanced natural killer cell activity of the splenocytes and peritoneal lymphocytes was observed after intraperitoneal BCG inoculation. The growth of implanted tumor was suppressed following intravesical instillation of BCG. These results suggest that the antitumor activity of BCG is not related to the simple inflammatory reaction but to the local and systemic immune response in which helper T lymphocytes and mononuclear cells play an important role.

Key Words: MBT-2, Bacillus Clamette-Guerin, Anticancer mechanism

INTRODUCTION

Transitional cell carcinoma of the bladder is the most common urologic malignant tumor in Korea and superficial bladder tumors comprise over 70% (Ahn and Lee, 1986). Although most superficial bladder tumors are managed effectively by transurethral resection, the high recurrence rate of 50-70% and progression to invasive tumor in 5-15% are troublesome problems (Cutler et al., 1982: Heney et al., 1983: Lee et al., 1991). Prevention of recurrences and progression of the superficial bladder tumor is the main problem to be solved by the urologists. Intravesical instillation of vari-

ous anticancer chemotherapeutics and immune modulating agents have been used to solve such problems of the superficial bladder tumor. Thio TEPA, bleomycin, adriamycin, mitomycin C, epodyl, bacillus Calmette-Guerin (BCG) were used and BCG has been reported to be the most effective agent (Lee et al., 1991: Brosman, 1982, 1985: Morales et al., 1976: Lamm et al., 1980.)

Since its first successful clinical trial by Morales et al. in 1976, BCG has been used widely. But the exact mechanism of the prophylactic and/or therapeutic anticancer effect of BCG is not well known. Whether the antitumor activity is mediated by the systemic immune mechanism or by local inflammatory reaction is not clearly defined.

The objective of this study is to elucidate the mechanism of the anticancer effect of BCG on bladder tumors. For this purpose, we used MBT-2 cell line (Soloway, 1977), originally derived from chemically induced murine bladder tumor. From the experimental data, the exact mechanism of the anticancer effect of BCG could be determined and a proper method of BCG treatment could be suggested.

MATERIALS AND METHODS

Materials

Animals. Six to eight week old female C3H/He mice were used (Seoul National University Laboratory Animal Center) throughout the study. They were housed 10 mice per cage and fed with laboratory chow (Samyang Co.) and water ad libitum.

BCG. Lyophilized Pasteur strain BCG (Korean National Tuberculosis Association) was used. BCG contained 4×10^6 colony forming units (CFU) of live tuberculosis bacteria per mg.

Tumor. Mouse bladder tumor, MBT-2 was kindly provided by Prof. Akaza, Department Of Urology, Tsukuba University, Japan, who was provided with the MBT-2 by Prof. Soloway, University of Tennesse, Memphis, TN. USA.

Tissue culture media. RPMI-1640 (Gibco, Grand Island, NY) containing 10mM HEPES buffer, 300 μg/ml glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY) was used in all experiments.

Methods

Effect of BCG on immune cell distribution in the bladder wall and spleen

Experimental design. The mice were randomly divided into 2 groups. Control group (Group I) con-

sisted of 30 mice receiving 0.1ml of normal saline intravesically every week for 4 consecutive weeks. The intravesical BCG treated group (Group II) consisted of 30 mice which received 10^7 CFU of BCG intravesically every week for 4 consecutive weeks. In both groups, 5 mice were sacrificed at 1, 3, 7, 14, 21 days after treatment.

Immunohistochemical staining. Changes in distribution of T cell subsets, natural killer cells and macrophages were examined by immunohistochemical staining. Immediately after sacrifice by cervical dislocation, 0.1ml of 15% sucrose-phosphate buffered saline was instilled into the bladder. The bladder and spleen of the animal were removed aseptically. Removed tissues were soaked in 4°C 15, 20, 25% sucrose-phosphate buffered solution (0.05M, pH 7.2) serially for 16 to 20 hours. Thereafter the tissues were imbeded into OCT compound (Lab-Tek Products, Division Miles Lab. Inc.) and frozen sections were made at -20°C with 5μm thickness. Tissue sections were mounted on slide glass and dried sufficiently for 1-2 hours at room temperature, they were fixed with cold acetone (-10°C) for 10 minutes. As a primary antibody, rat anti-L3T4 monoclonal antibody (IgG2a, Becton-Dickinson Immunocytometry Systems) for helper T cells, rat anti-Lyt-2 monoclonal antibody (IgG2a, Becton-Dickinson Immunocytometry Systems) for suppressor T cells, rat anti-CD11b monoclonal antibody (IgG1, Behringer Manheim) for macrophages, and rabbit anti-asialo GMI polyclonal antibody (Waco Pure Chemical Industries Ltd.) for natural killer cells were used. These primary antibodies were diluted 1:100 with phosphate buffered saline (PBS) and reacted for 30 minutes at room temperature. As a secondary antibody, biotinylated goat anti-Rat-IgG (Sigma Chemical Co.) was used after 1:200 dilution with PBS and reacted for 30 minutes at room temperature. Then avidin biotin peroxidase complex (ABC, Vector Lab.) was reacted for 30 minutes at room temperature. Color reaction was made by 0.0025% 3.3'-diaminobenzidine (DAB) and 0.04% nickel chloride. Wet mounting was done with glycerogel (DAKO Corp.) and dried for 1 day after color reaction. Slides were sealed with permount to prevent discolorization.

Light microscopic examination. The number and distribution of helper and suppressor T cells, macrophages and natural killer cells were examined under light microscope. Antibody positive cells were counted using 1cm² lattice under the microscope with a magnification of X400 in 3 to 5 fields.

Effect of BCG on natural killer cell activity

Experimental design. The intraperitoneal BCG treat-

ed group (Group III) consisted of 20 mice which received 10^8 CFU of BCG intraperitoneally. Among them five mice were sacrificed at 5, 10, 15, 20 days respectively after BCG therapy. The control group (5 mice) were also sacrificed.

Preparation of effector cells. Effector cells were collected from the spleen and peritoneal exudates. Spleens were removed aseptically and teased gently in PBS containing 5 units/ml heparin. The suspension was filtered through 100 micron stainless steel mesh to remove cell debris. The cells were washed and suspended in 0.84% ammonium chloride solution at 37°C for 5 minutes to lyse red blood cells (RBC) and washed twice with RPMI-1640 medium. To obtain peritoneal lymphocytes, 5ml of PBS containing 5 units/ml heparin was injected into the peritoneal cavity. After gentle agitation, the fluid was aspirated. The process was repeated 3 to 5 times and the exudates were pooled. The peritoneal exudate was filtered and RBCs were lysed and resuspended in media. Both the spleen and peritoneal exudate cells were suspended in culture flasks and kept horizontally for 1 hour at 37°C in a humidified atmosphere containing 5% carbon dioxide. The nonadherent cells were collected, pelleted and resuspended in RPMI-1640 medium. The cells were then counted by trypan blue dye exclusion and kept on ice until use.

In vitro cytotoxicity assay. Four hour ^{51}Cr-release assay was used. YAC-1 cells were used as target cells to assess natural killer cell activity. Target cells were adjusted to 10^7/ml in RPMI-1640 containing 10% FBS and 10^6 cells were incubated with 100μCi of Na_2 $^{51}CrO_4$ (1mCi/ml, NEZ 030S, NEN, USA) at 37°C for 1 hour. The cells were then washed three times, counted and resuspended in RPMI-1640 containing 10% FBS. 100μl of effector cells and 100μl of ^{51}Cr-labelled target cells were added to the wells of a U bottom microtiter plate. After 4 hours incubation, the plate was centrifuged for 15 minutes and 100μl supernatant aliquots were removed with a micropipette and radioactivity was measured in a gamma counter (Packard, USA). The effector and target ratios in splenocytes were 100:1, 50:1, 25:1, 12:1 and 50:1, 25:1, 12:1, 6:1 in peritoneal lymphocytes. The spontaneous release was obtained when the labelled target cells were incubated in medium alone, and maximum release was obtained by incubation of the labelled cells with 5% triton X-100 (Sigma, USA). The results were calculated with the formula:

per cent cytotoxicity =

$$\frac{\text{test release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

Antitumor effect of BCG on intravesically implanted bladder tumor

Experimental design. The intravesical MBT-2 implanted group (Group IV) consisted of 50 mice. 10^6 viable cells in 0.1ml suspension were implanted into the bladder following electrocauterization of bladder mucosa (Soloway, 1977). In the 50 mice of the BCG treated group following intravesical MBT-2 implantation (Group V), 10^6 CFU of BCG were instilled intravesicaly 1 day and 8 days after intravesical tumor implantation.

Observation. Growth suppression effect of BCG was evaluated by weekly sacrifice of 5 mice for 4 consecutive weeks after intravesical MBT-2 implantation, and changes of immune cells in bladder and spleen were observed by immunohistochemical staining.

Statistical analysis

T-test, Fisher's exact test, Mann-Whitney test, chi-square test, Wilcoxon rank sum test and Kruskal-Wallis one way anova test were used using SPSS/PC+ program in a personal computer.

RESULTS

Effect of BCG on immune cell distribution in the bladder wall and spleen

Changes in the bladder. In the control group (Group I), helper T cells, suppressor T cells, natural killer cells and macrophages were distributed sporadically in mucosa, especially in lamina propria (Fig. 1). No significant changes were found with time after normal saline instillation. In the intravesical BCG treated group (Group II), helper T cells, natural killer cells and macrophages except suppressor T cells increased in general. Helper T cells and macrophages increased markedly 14 days and 7 days after BCG therapy, respectively (Fig. 2). The helper T and suppressor T cells ratio (Th/Ts ratio) increased with time. It was 2.9 at 7 days and 6.5 at 21 days after BCG therapy compared to 1.2 in the control group (Fig. 3). Most of the helper T cells were found in lamina propria. Macrophages, compared to helper T cells or suppressor T cells, were found in mucosal layer as well as in lamina propria. Natural killer cells showed a similar pattern in the BCG treated group compared to controls, but increased after 14 days following BCG therapy.

Changes in the spleen. In the control group, most of the helper T cells were found at the inner periarterial lymphatic sheath (PALS), and suppressor T cells were confined to a limited area near the central arterioles. Most of the natural killer cells and macro-
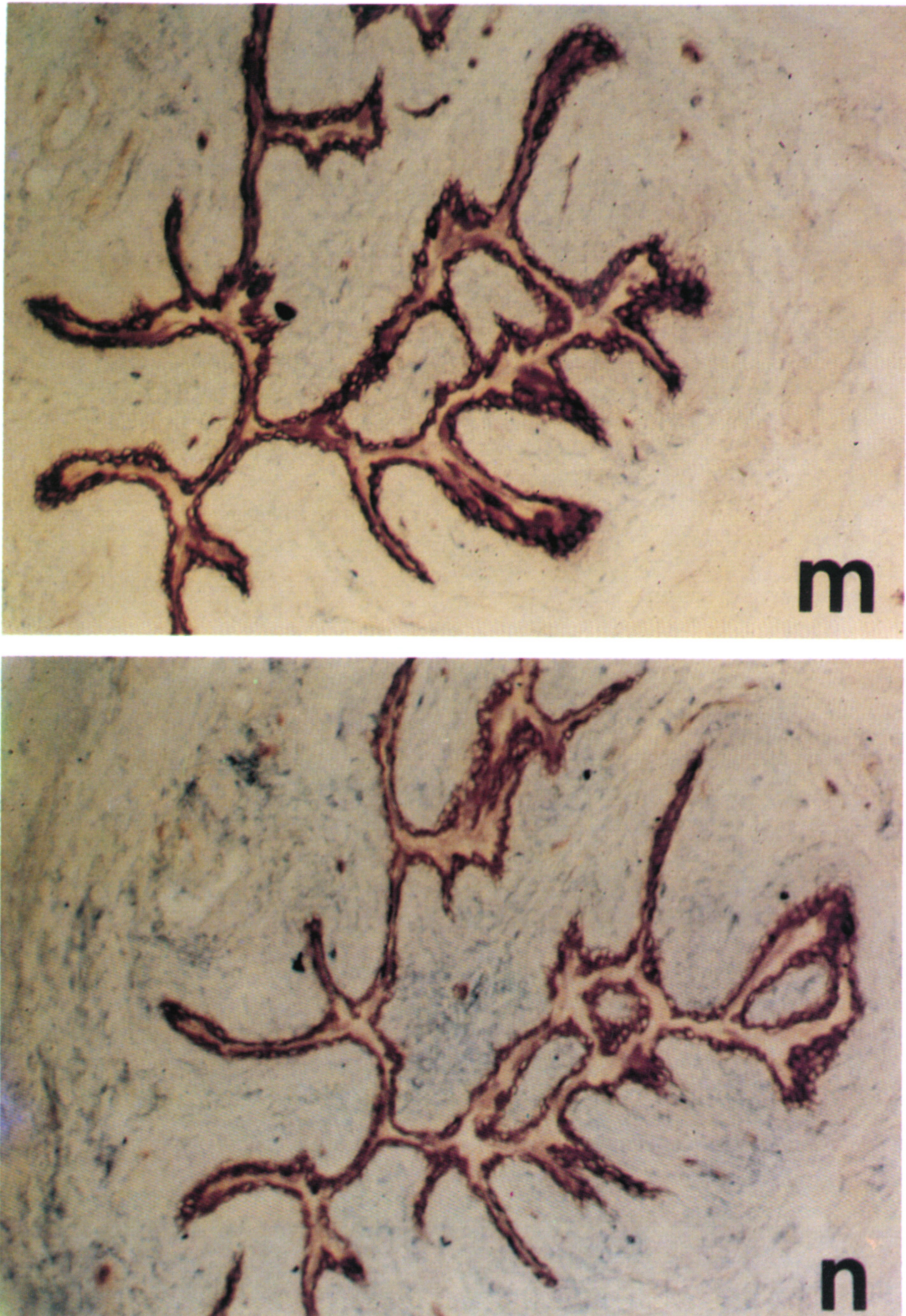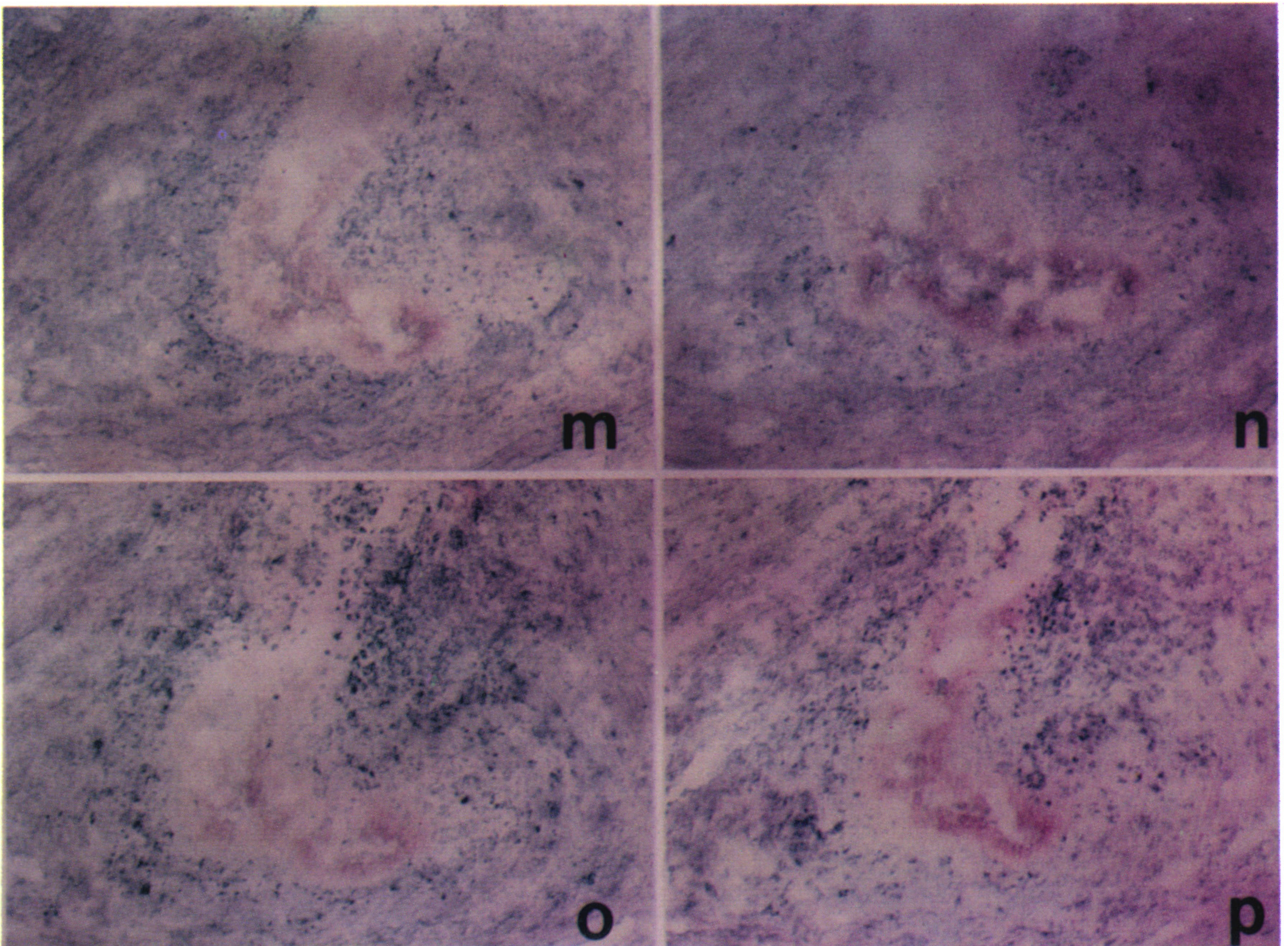
Fig. 1. Immunohistochemical staining pattern for the several immune cells in the adjacent bladder sections of normal control group.

m: L3T4 positive cells (x40)

A few positive cells (black) are scattered mainly in the lamina propria. Mucosal epithelial cells are stained in dark red.

n: Lyt-2 positive cells (x40)

Few positive cells (black) are observed in the lamina propria.

Fig. 2. Immunohistochemical staining pattern for the several immune cells in the adjacent bladder sections of intravesical BCG instilled group.

m: L3T4 positive cells (x100)

Relatively many positive cells (black) are observed in the lamina propria just below the mucosal epithelial layer (faintly red).

n: Lyt-2 positive cells (x100)

A few positive cells (black) are scattered in the lamina propria.

o: Anti-Macrophage positive cells (x100)

Quite many positive cells (black) are present mainly over the lamina propria. Some positive cells, however, invaded into the mucosal epithelial layer.

p: Anti-NK positive cells (x100)

Several positive cells (black) are scattered mainly over the lamina propria. A few positive cells are observed in the mucosal epithelial layer (faintly red).

phages were distributed in the red pulp, with few in the white pulp. After BCG therapy, suppressor T cells showed little difference, but helper T cells increased at inner PALS and extended to outer PALS. These changes were accentuated with time after BCG therapy. Natural killer cells and macrophages were distributed in the red pulp, as in the control group. The number of natural killer cells changed little, but the number of macrophages increased moderately at 21 days after BCG therapy.

Effect of BCG on natural killer cell activity

NK activity of the splenocytes increased and reached a peak at 10 days after intraperitoneal BCG therapy (Table 1). NK activity of the peritoneal lymphocytes also increased and reached a plateau at 10 days after BCG therapy. Enhanced NK activity induced by intraperitoneal BCG was more prominent in peritoneal lymphocytes than splenocytes.
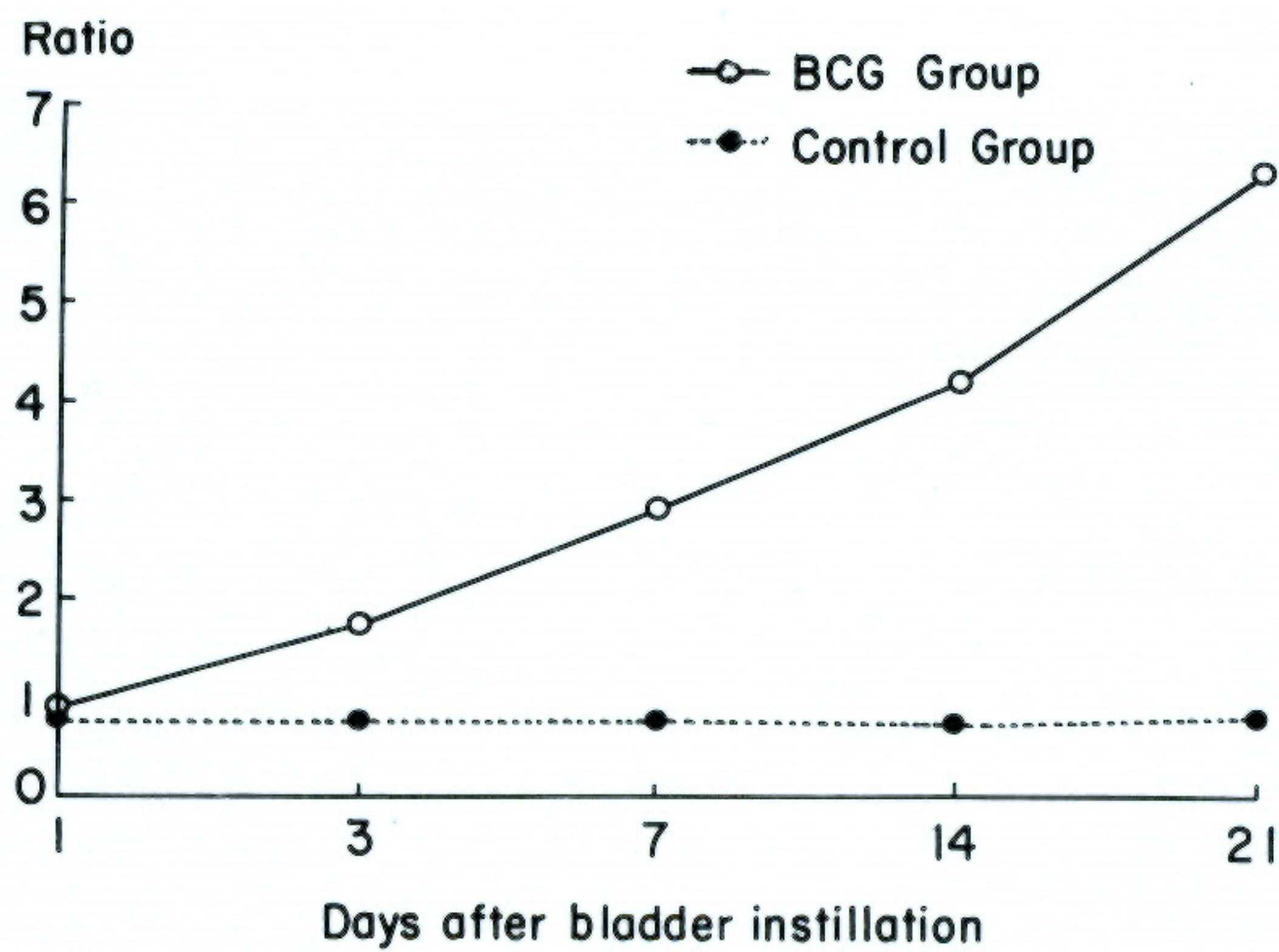
Fig. 3. Change of Th/Ts cell ratio in control and BCG group.

Antitumor effect of BCG on intravesically implanted bladder tumor

Antitumor effect. Antitumor effect of BCG in intravesical tumor implantation model is shown on table 2. The size of the implanted tumor was markedly decreased in the BCG treated group after intravesical MBT-2 implantation (Group V) compared to the MBT-2 implanted only group (Group IV).

Immunologic study. In the intravesical MBT-2 implanted group (Group IV), immunohistochemical staining of the bladder showed that helper, suppressor T

cells, macrophages and natural killer cells increased in the bladder wall around the tumor. Markedly increased T cells and natural killer cells were found especially adjacent to the tumor. In the intravesical BCG treated group after MBT-2 implantation (Group V), helper T cells, macrophages, natural killer cells increased in general compared to the intravesical MBT-2 implanted group (Fig. 4). Macrophages increased markedly at 2 weeks after tumor implantation and declined thereafter, and natural killer cells increased steadily at 3, 4 weeks. There was no difference in supressor T cells between the BCG treated group after intravesical MBT-2 implantation (Group V) and the MBT-2 implanted only group (Group IV).

In the spleen, distribution of supressor T cells in the intravesical MBT-2 implanted group was similar to the normal control group (Fig. 5), and helper T cells were depleted markedly in almost all of the spleen tissues after 2 weeks following tumor implantation. There was no difference in natural killer cells and macrophages between the intravesical MBT-2 group and controls. In the BCG treated group after intravesical MBT-2 implantation, helper T cells increased markedly in the white pulp after 2 weeks compared with the tumor implanted only group. But there was no difference in suppressor T cells (Fig. 6) as well as in macrophages and natural killer cells between two groups.

**Table 1.** NK activity after intraperitoneal BCG inoculation

Days after BCG	0	5	10	15	20
Splenocyte					
1:100	4.31 ± 0.96	6.70 ± 0.87*	8.54 ± 1.82*	5.00 ± 1.33	8.29 ± 2.04*
1:50	1.60 ± 0.45	4.06 ± 0.87	5.12 ± 1.34	4.66 ± 1.35	6.12 ± 1.54
1:25	0.39 ± 0.26	1.15 ± 0.57	2.71 ± 0.62	4.27 ± 1.21	6.52 ± 1.62
1:12	0.09 ± 0.62	2.11 ± 0.63	1.85 ± 0.38	4.48 ± 1.02	6.41 ± 1.82
Peritoneal exudate cell					
1:50	4.68 ± 0.75	5.28 ± 0.98	12.07 ± 1.61*	10.84 ± 1.24*	12.31 ± 1.90*
1:25	2.98 ± 0.56	4.18 ± 1.06	7.11 ± 2.05	6.12 ± 1.59	7.28 ± 2.61
1:12	2.52 ± 0.39	1.19 ± 0.62	5.09 ± 1.20	7.62 ± 1.92	6.47 ± 1.38
1:6	0.81 ± 0.32	1.32 ± 0.52	4.72 ± 1.00	5.92 ± 1.24	6.03 ± 1.53

*$p < 0.05$ by Mann-Whitney test

Table 2. Reduction of tumor growth by intravesical BCG instillation

Group	Average tumor diameter (mm) $\pm$ S.D.		
	2 weeks	3 weeks	4 weeks
Tumor implanted	3.5 ± 1.3	11.3 ± 4.3	14.1 ± 4.7
BCG treated after tumor implantation	1.2 ± 0.7 *	5.5 ± 2.3 *	9.6 ± 3.8 *

*$p < 0.05$ by T-test
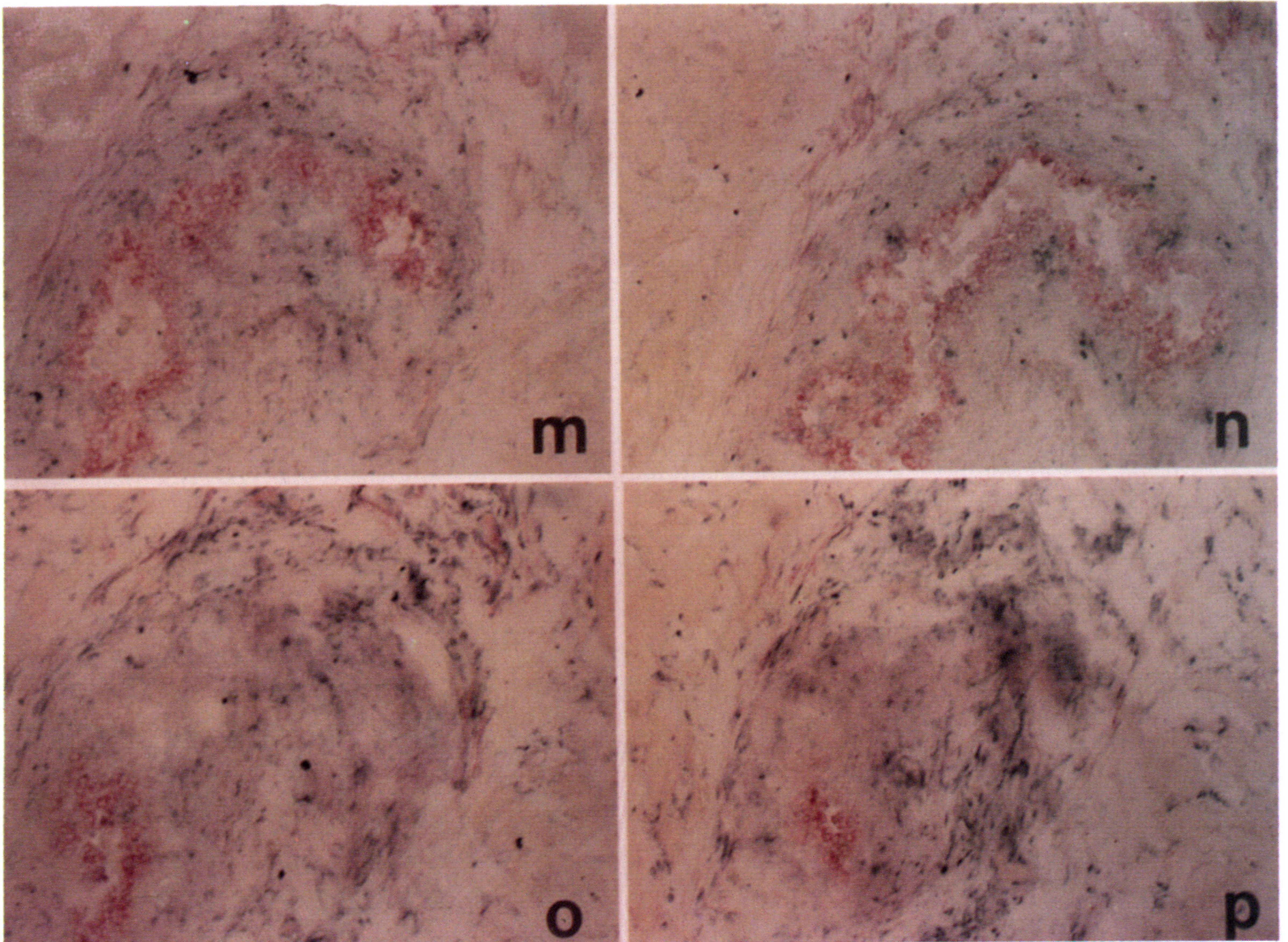
Fig. 4. Immunohistochemical staining pattern for the several immune cells in the adjacent bladder sections of BCG treated group affer intravesical MBT-2 implantation.

m: L3T4 positive cells (x100)

Relatively many positive cells (black) are scattered over the lamina propria

n: Lyt-2 positive cells (x100)

A few positive cells (black) are scattered in the lamina propria

o: Anti-Macrophage positive cells (x100)

Positive cells (black) are observed in the lamina propria near the muscle layer

p: Anti-NK positive cells (x100)

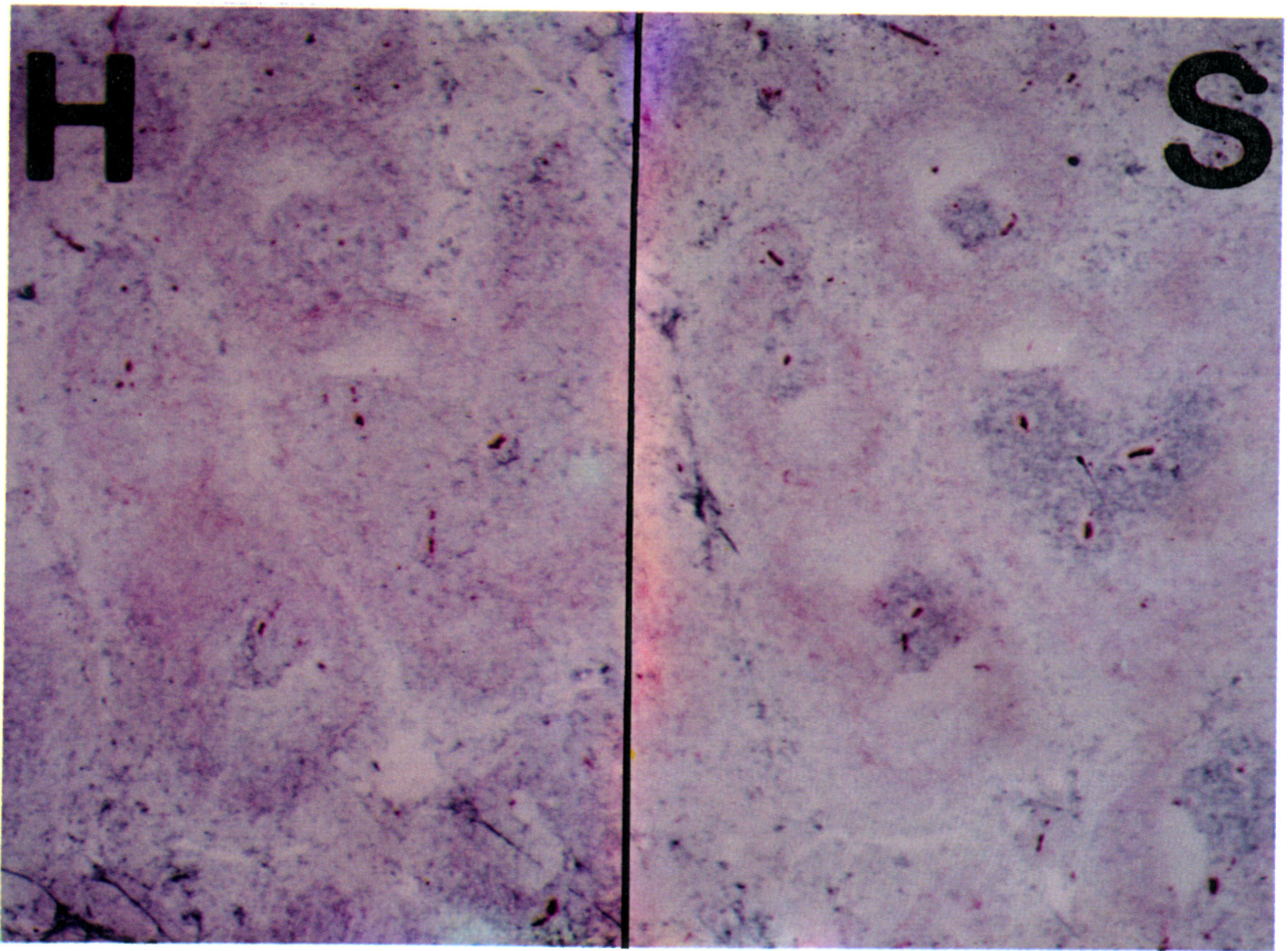Many positive cells (black) are observed in the lamina propria.

Some positive cells are observed among the muscle fiber.

DISCUSSION

The MBT-2 model for experimental study on bladder tumors was established by Soloway (1977). MBT-2 is a mouse bladder tumor induced by oral administration of chemical carcinogen FANFT to inbred C3H/He Mouse for 8 to 10 months. MBT-2 is similar to human bladder tumor in many aspects: it has a close relation with chemical carcinogens, histological characteristics are similar, it has weak immunogenecity, and shows the same results to chemotherapy or BCG therapy. Therefore many studies have been undertaken to evaluate the antitumor effects of intravesical instillation of various chemotherapeutic agents and BCG using C3H/He mouse and its syngeneic bladder tumor, MBT-2 (Lee et al., 1987: Morales and Pang, 1986: Pang and Morales, 1982).

As superficial bladder tumor arises in the bladder,

Fig. 5. Immunohistochemical staining for T cell subsets in the mouse spleen of intravesical MBT-2 implanted group.

    h: L3T4 positive cells (x40)

        Few positive cells (black) are scattered in the inner PALS, suggesting that helper T cells are markedly depleted in the T cell zone.
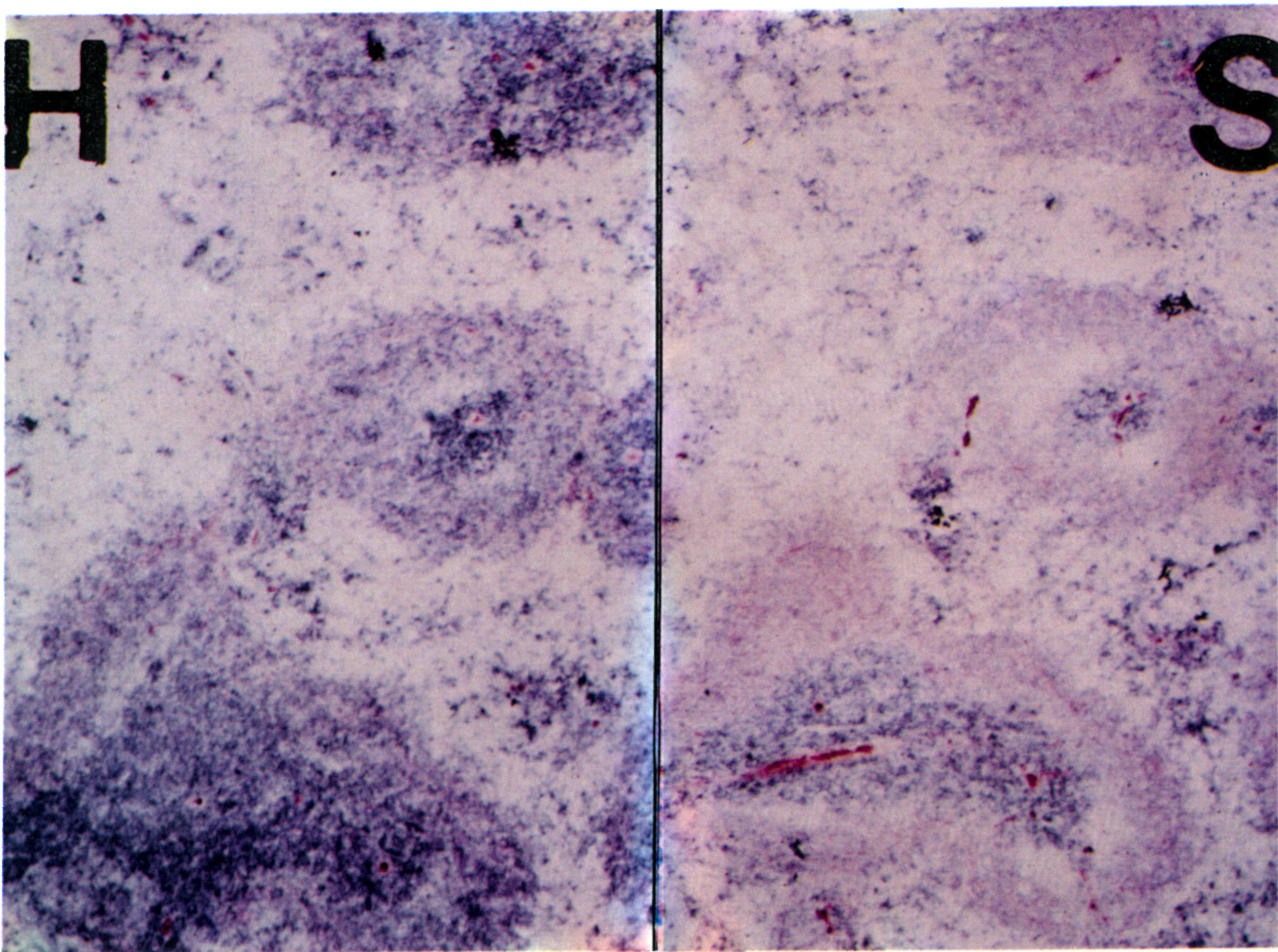
    s: Lyt-2 positive cells (x40)

        Few positive cells are well localized in the inner PALS.

it is usual to perform animal experiments after intravesical implantation of MBT-2. Various methods of implanting tumor cells in the bladder were introduced. We injured the mucosa by electrocautery prior to implantation so that the tumors could be implanted focally in the bladder mucosa in this study. And we did not use chemicals to avoid any possible immunologic reactions. Although the electrocautery method has the advantages such as focal implantation of tumors and saving time because tumors grow in 2 to 4 weeks after implantation, it also has some disadvantages. The technique is not easy to reproduce and the tumor implantation rate is not even. In some cases tumors may be implanted extravesically and intraperitoneal seedings occur not infrequently. Actually in this study the intravesical tumor implantation rate was 55% and suc-

cessful tumor implantation could be identified by laparotomy at 2 weeks after MBT-2 inoculation. We excluded mice without successful implantation to prevent any possible distortion of the results.

The exact mechanism of the antitumor effect of BCG is not clarified yet, but much evidence has been accumulated to support the view that immunologic mechanisms participate. In 1974, Zbar suggested that the antitumor activity of BCG is the immune reaction. He reported that intimate contact of BCG with tumor cells is necessay for the antitumor effect of BCG, the host should develop delayed hypersensitivity reaction to bacillus antigen, tumor burden should be small, and an appropriate number of live bacilli is necessary. Haaff et al (1986) reported increased interleukin-2 in the urine of patients who received intravesical BCG

Fig. 6. Immunohistochemical staining for T cell subsets in the mouse spleen of BCG treated group after intravesical MBT-2 implantation.

    h: L3T4 positive cells (x40)

        Many positive cells (black) are congregated over the inner PALS and some positive cells are scattered in the red pulp.

    s: Lyt-2 positive cells (x40)

        The positive cells are clearly localized in the inner PALS.

therapy and suggested systemic immune response following BCG instillation. Guinan et al (1986) noticed infiltration of mononuclear cells, especially T lymphocytes in the bladder mucosa after intravesical BCG inoculation in animal experiments and suggested that the mechanism of the antitumor effect of BCG would not be a simple local inflammatory reaction but had some relation to T cell mediated immune reaction. Ratliff et al (1987) observed that intravesical BCG therapy in athymic nude mice had no antitumor effect and antitumor effect was restored after transplantation of T lymphocytes from normal mice. They strongly suggested that the antitumor effect of BCG depends on T lymphocytes.

In order to clarify a part of its mechanisms in this

study, we observed the changes in the immune system not only in the bladder wall but also in the spleen at regular time interval after intravesical BCG therapy using C3H/He mice. And we also observed changes after BCG therapy following intravesical MBT-2 implantation to simulate clinical situations. Increment of helper T cells was prominent in bladder mucosa, and the helper T cells and suppressor T cells ratio increased markedly after BCG therapy. Helper T cells, macrophages and natural killer cells increased in general after BCG therapy in bladder tumor implanted mice compared to tumor implanted mice without BCG therapy. We assumed from these observations that the antitumor mechanism of BCG is partly mediated by T lymphocytes, as other authors have sug-

gested.

There are many reports which support the view that not only a local immune reaction but also systemic immune response participate in the immunologic mechanisms of the antitumor effect of BCG. Antibody against BCG was detected in the serum after intravesical BCG instillation (Winter and Lamm, 1981), and antibody titer and duration of antibody positivity had a close relation to the treatment results. Pang and Morales (1982) reported the antitumor effect of intraperitoneally administered BCG on subcutaneously implanted bladder tumors, and Morales and Pang (1986) also reported a markedly decreased tumor implantation rate and growth by intraperitoneal administration of BCG before tumor implantation. Shapiro et al (1983) reported that the antitumor effect of BCG had a close relation with natural killer cell activities and PPD skin test, and natural killer cell activity increased as the concentration of BCG increased. In our study, helper T cells increased in the spleen after intravesical BCG therapy with or without bladder tumor implantation into the bladder. Also, natural killer cell activity of the splenocytes as well as the peritoneal lymphocytes increased following intraperitoneal treatment of BCG. These observations suggest that systemic immune responses as well as local immune reactions play an important role in the mechanism of the antitumor effect of BCG.

Although the mechanisms of the antitumor effect of BCG were not completely clarified from this study, at least part of it was uncovered. Further studies of this kind will be necessary for a precise understanding of the mechanisms of BCG therapy.

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