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Anti-tumor effect of intravesical instillation of OK432 against rat bladder tumors induced by N-butyl-N-(4-hydroxybutyl) nitrosamine

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Summary. The anti-tumor effect of OK432 instilled into the bladder was evaluated in rat bladder tumors induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN). In experiment I, the rate of the natural killer (NK) activity was determined with cells from spleen and mesenteric lymph nodes. Intravesical OK432 instillation enhanced NK activity; however, this activity was not dose-dependent and was not augmented by OK432 inoculation into the foot pad. In experiment 2, the therapeutic effect of intravesical OK432 instillation was examined in rat bladder tumors induced by BBN. OK432 was instilled weekly for six weeks. Rats given BBN for 10 weeks were divided into six groups: 1) control; 2) saline; 3) OK432 0.05 KE/ml; 4) OK432 0.05 KE/ml bladder instillation with 0.01 KE/ml foot pad inoculation; 5) OK432 0.05 KE/ml, every other week; and 6) OK432 0.5 KE/ml. Weekly OK432 instillation significantly reduced tumor weight and the incidence of tumor development; however, this inhibition was not dose-dependent and was not enhanced by OK432 inoculation into the foot pad. In rats given OK432 weekly, the augmentation of NK activity and increase in tissue infiltrating lymphocytes were significant. These results suggest that intravesical OK432 instillation is effective in the management of superficial bladder tumors. The study further emphasizes that the dose and method of administration are critical variables in determining the efficacy of immunotherapy.

Key words: Bladder cancer-OK432-bladder – Instillation-NK activity

Superficial bladder tumors, which represent 70 to 75 percent of all bladder malignancies, have a variable potential for recurrence, progression to muscle invasion and subsequent metastasis [30]. At least 50% of patients will have recurrence or develope a new tumor, with a variable propensity for invasive disease despite complete resection of the initial tumor [19].

Recent evidence suggestsd that intravesical instillation of bacille Calmette-Guerin (BCG) may be an effective treatment for superficial bladder tumors [7, 12, 17]. The mechanism by which BCG mediates antitumor activity is not yet clear, but it may be predominantly a host-mediated immunity [15, 25]. In spite of its success in bladder tumor models, BCG does have drawbacks such as severe cystitis and the potential hazard of systemic toxicity because of the live vaccine [13, 21]. These drawbacksd of BCG prompted us to evaluate other biological response modifiers (BRM) with possibly less severe side effects and greater tumoricidal activiery [6, 14]. OK432, derived from a virulent strain of *Streptococcus pyogenes*, is used widely as an immunomodulator in Japan [37]. OK432 is not a viable organism and its mechanism of action is mainly through host-mediated immunity [36]. Specifically, OK432 has been reported to promote the induction of cytotoxic T lymphocytes against syngeneic tumor cells [5], and to active cytotoxic macrophages, natural killer (NK) cells [27], natural killer (NK) cells [22], and lymphokine-activated killer (LAK) cells [28]. We have already demonstrated the effect of OK432 as a BRM against bladder tumors [32, 33]. In this study, we examined the anti-tumor effect of intravesicular OK432 instillation against BBN-induced rat bladder tumors.

Materials and methods

Chemicals

BBN was purchased from Izumi Co., Yokohama, Japan. OK432 as an immunomodulator was kindly supplied by Chugai Pharmaceutical Co., Tokyo, Japan.

Animals

A total of eighty-two six-week-old female Wistar rats (CLEA Japan, Inc., Japan) were used in this study. The animals were housed four rats per cage at a room temperature of 23 ± 2°C, with a 12 h light-dark cycle.

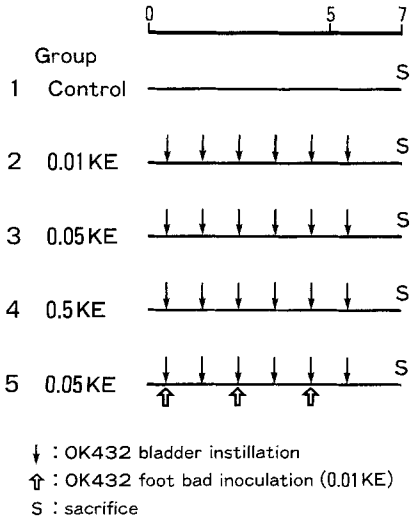


Fig. 1. Protocol of experiment 1

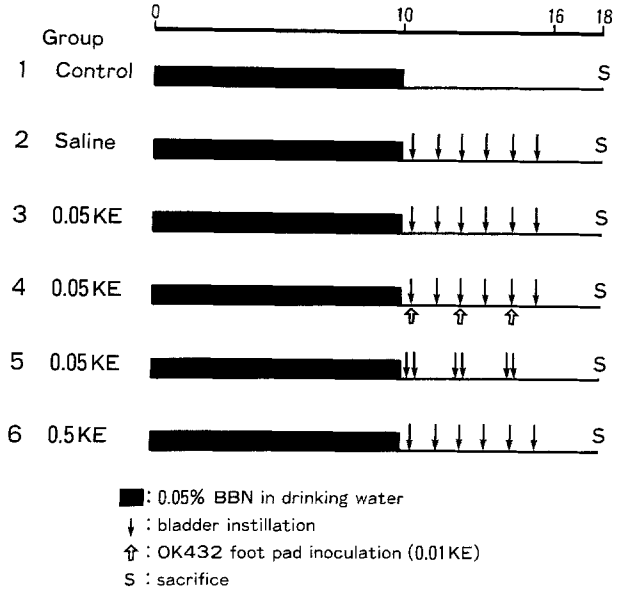


Fig. 2. Protocol of experiment 2

Experiment 1

Rats were given drinking water without BBN and divided into the following five groups (Fig. 1): group 1, no treatment; group 2, 0.01 KE/ml OK432 by bladder instillation; group 3, 0.05 KE/ml OK432 by bladder instillation; group 4, 0.5 KE/ml OK432 by bladder instillation; group 5, 0.05 KE/ml OK432 by bladder instillation plus 0.01 KE/ml OK432 by foot pad inoculation. One milliliter of OK432 solution was instilled through a catheter into the bladder of rats for 6 weeks under nembutal anesthesia.

Before and 1 h after the instillation, lthe bladder contents were emptied by light abdominal massage so that the instilled compounds were retained for 1 h. The volume used for foot pad inoculation was 0.3 ml. The rats were killed 7 weeks after the beginning of the experiment.

Experiment 2

Rats were given drinking water containing 0.05% BBN for 10 weeks. After BBN treatment, they were divided into six groups (Fig. 2): group 1, no treatment; group 2, physiological saline by bladder instillation; group 3, 0.05 KE/ml OK432 by bladder instilation; group 4, 0.05 KE/ml OK432 by bladder instillation plus 0.01 KE/ml OK432 by foot pad inoculation; group 5, 0.05 KE/ml OK432 by bladder instillation, every other week; group 6, 0.5 KE/ml OK432 by bladder instillation. The methods for injecting drugs into the bladder (6 times in total) and inoculating the foot pad were the same as those in experiment 1. The rats were killed 18 weeks after the beginning of the experiment.

Histological observations

After weighting, the bladders were fixed with a 10% solution of phosphate-buffered formalin (pH 7.4) and stained with hematoxylin and eosin (H&E) for histological examination.

Infiltration of tissue infiltrating lymphocytes (TIL)

The degree of TIL in sections stained with H&E was graded as slight, moderte or severe, as previously reported [33, 34].

Preparation of effector cells

Effector cells were taken from spleens and mesenteric lymph nodes, which were removed, minced with scissors and teased through 200-mesh stainless steel. The cells were then suspended in 0.84% ammonium chloride at 37°C for 5 min to lyse red blood cells, then washed twice in RPMI 1640 (Flow Laboratories, USA).

Cytotoxicity assay

NK activity was determined via a 4 h ^{51}Cr release assay as previously reported [32]. Briefly, target cells, YAC-1 cells [26] in RPMI 1640 containing 10% fetal calf serum (FCS; Flow Laboratories) were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, England) ate 37° C for 1 h. To 96-well microplates (Corning, Inc., USA) were added 100 μl of ^{51}Cr-labeled cells and 100 μl of effector cells. The effector: target cell (E/T) ratio was 50:1. After incubation for 4 h, the plate was centrifuged for 4 min. The supernatant was collected by the Titertek collection system (Flow Laboratories), and radioactivity was determined by gamma counter (cpm exp.). Spontaneous release was determined by intubation of target cells without the presence of effectors (cpm spont. release), and maximum release was determined by the addition of 5% Triton X-100 (cpm max. release). Specific release was calculated by the following formula:

$$\% \text{ Specific release} = \frac{\text{cpm exp.} - \text{cpm spont. release}}{\text{cpm max. release} - \text{cpm spont. release}} \times 100$$

Statistical methods

Student's t-test or the Chi-square test was performed to analyze differences. The level of statistical significance was set at 5% unless otherwise indicated.

Table 1. Effect of treatment on NK activity (experiment 1)

Exp. 1 Group	Treatment	Effective no. of rats	NK activity (%)	
			Spleen	MLN
1	Control	4	9.1 ± 1.5	2.5 ± 1.3
2	0.01 KE	4	14.4 ± 1.3*	
3	0.05 KE	4	18.0 ± 0.5*	12.8 ± 0.9*
4	0.5 KE	4	12.8 ± 1.0**	
5	0.05 KE foot pad inoculation	4	13.6 ± 0.9*	

mean ± S.D.
MLN: mesenteric lymph node
* $P < 0.01$; ** $P < 0.05$; the differences were compared to control

Table 2. Body weight

Exp. 2 Group	Treatment	Effective no. of rats	Body weight (gm.)
1	Control	9	198.9 ± 13.6[a]
2	Saline	5	194.0 ± 8.9
3	0.05 KE	10	197.0 ± 14.2
4	0.05 KE foot pad inoculation	10	201.0 ± 8.8
5	0.05 KE every other week	8	203.8 ± 9.2
6	0.05 KE	8	201.3 ± 3.5

[a] mean ± S.D.
all cases: not significant

Table 3. Effect of treatment on tumor size (bladder weight)

Exp. 2 Group	Treatment	Effective no. of rats	Bladder weight (gm.)
1	Control	9	0.167 ± 0.012[a]
2	Saline	5	0.171 ± 0.015
3	0.05 KE	10	0.150 ± 0.010*
4	0.05 KE foot pad inoculation	10	0.160 ± 0.013
5	0.05 KE every other week	8	0.163 ± 0.010
6	0.05 KE	8	0.157 ± 0.014

[a] mean ± S.D.
* $P < 0.01$; control vs. group 3

Results

Experiment 1

Only 5 rats died during experiments 1 and 2. The anesthesia given for intravesical instillation caused the deaths. Inexperiment 1, the effect of the bladder instillation of OK432 on NK activity in the normal rat was studied (Table 1). When spleen cells were used as effector cells, NK activity was significantly increased in groups 2, 3, 4 and 5. The level was highest in group 3 (0.05 KE, once a week); thus, foot pad inoculation did not enhance NK activity. In addition, the rate of augmention of NK activity was not dose-dependent. When mesenteric lymph nodes were used for effector cells, the NK activity in group 3 was significantly greater than that in the control group.

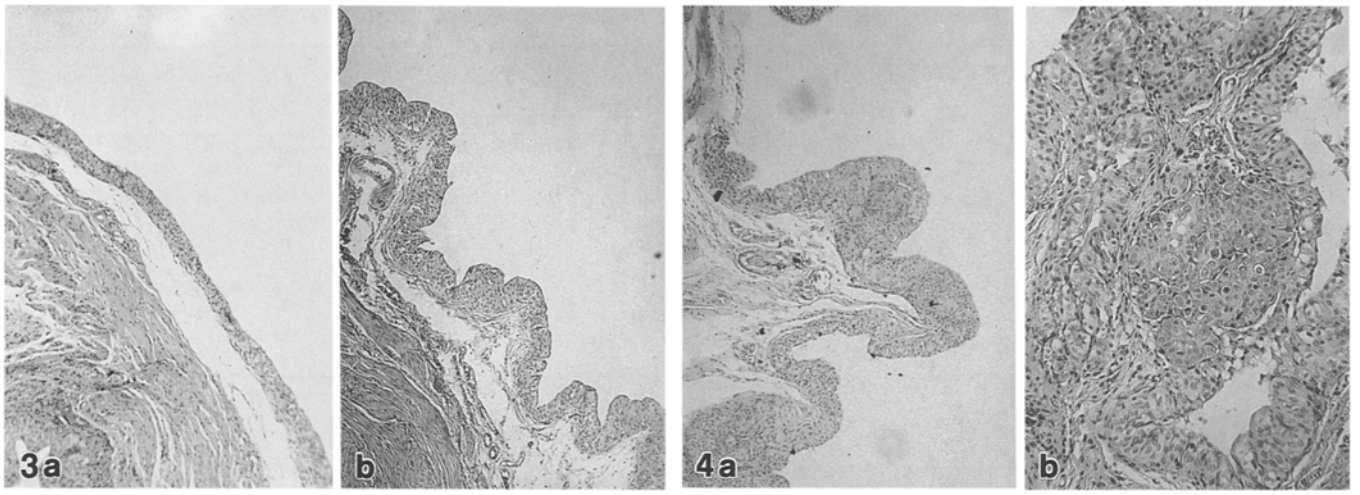
Experiment 2

The anti-tumor effect of OK432 instilled into the bladder against BBN-induced rat bladder tumors was examined. With reference to the results of experiment 1, the intravesical instillation of OK432 was performed according to the schedule shown in Fig. 2. There was no intergroup difference in the mean body weights of rats at the end of the experiment (Table 2). At sacrifice (at 18 weeks) the bladder weight (tumor weight) was determined in each group (Table 3). The weight of the bladder was low with intravesical OK432 instillation, but a significant difference was observed only in group 3 (0.05 KE, once a week). Interestingly, the weight of the bladder was the greatest in group 2 (saline administration group).

Table 4. Histological findings in rat urinary bladder at 18 weeks

Exp. 2 Group	Treatment	Effective no. of rats rats	Histological findings (%)			
			Simple hyperplasia	PN hyperplasia	Papilloma	Cancer
1	Control	9			3(33.3)	6(66.7)
2	Saline	5			2(40.0)	3(60.0)
3	0.05 KE	10	1(10.0)	5(50.0)	3(30.0)	1(10.0)*
4	0.05 KE food pad inoculation	10		5(50.0)	4(40.0)	1(10.0)*
5	0.05 KE every other week	8		1(12.5)	4(50.0)	3(37.5)
6	0.5 KE	8			5(62.5)	3(37.5)

* $P < 0.05$; controls vs. groups 3, 4



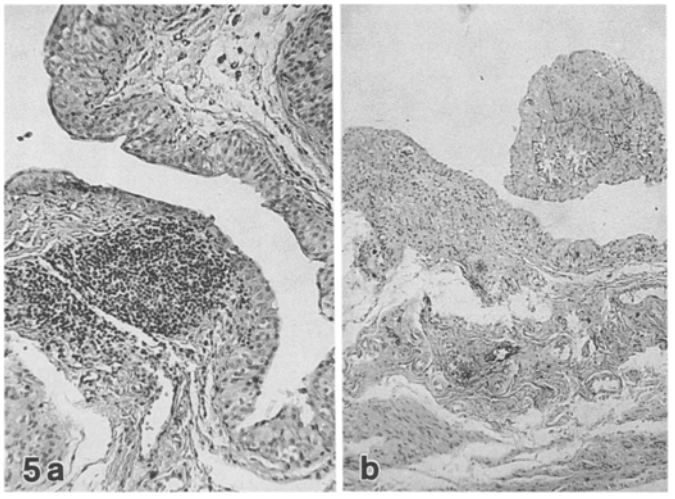


Fig. 3. **a** Simple hyperplasia (H&E ×100) and **b** papillary or nodular (PN) hyperplasia (H&E ×100)

Fig. 4. **a** Papilloma (H&E ×100) and **b** cancer (H&E ×200)

Fig. 5. **a** Increased tissue lymphocytes (H&E ×200) and **b** exfoliation of cancer cells (H&E ×100) were found in OK432 treated rats

Table 5. Infiltrated rates of TIL (tissue infiltrating lymphocytes)

Infiltrated rates of TIL	Treatment (Exp. 2)	
	Control	OK432 (Group 3)
slight	6	
moderate	3	2
severe		8*

* = $P < 0.01$; control vs. OK432

Table 6. Effect of treatment on NK activity (experiment 2)

Exp. 2 Group	Treatment	Effective no. of rats	NK activity (%)
			Spleen
1	Control	4	12.7 ± 0.6[a]
3	0.05 KE	4	26.8 + 1.7*

[a] mean ± S.D.
* = $P < 0.01$; control vs. group 3

At the end of the 10th week, immediately prior to the instillation of drugs into the bladder, 7 rats which had received BBN were examined for lesion development. Papilloma was detected in 5, while cancer and papillary or nodular hyperplasia (PN hyperplasia) were detected in 1 rat each. Histological changes in the urinary bladder epithelium (Figs. 3 and 4) consisted of simple hyperplasia, PN hyperplasia (Fig. 3) and papilloma, and cancer (Fig. 4). PN hyperplasia has been considered to be a reversible or irreversible epithelium to normal mucosa. Table 4 summarizes the bladder lesions found at week 18. Among the 9 rats in group 1 (control), papilloma was detected in 3 (3.3%) and cancer in 6 (66.7%).

Approximately the same results as those in group 1 were obtained in group 2 (saline administration group). In contrast, the incidence of cancer in groups 3 and 4 was only 10%, and the differences compared with group 1 were significant ($P < 0.05$). No inhibition of cancer from additional OK432 foot pad inoculation was found in group 4. The incidence of cancer in groups 5 (administration every other week) and 6 (administration of high dose of OK432) was higher than that in group 3 and 4. In all

groups, the infiltration of cancer was confined to the mucosal layers and was not found in the muscular layers.

In the group given OK432 by bladder instillation, stromal edema, vascular dilation and exfoliation of cancer cells (Fig. 5) were found. Concomitantly, the infiltration of tissue lymphocytes was marked (Fig. 5). TIL infiltration in groups 1 and 3 is presented in Table 5. TIL infiltration in group 3 (OK432) was significantly greater than in group 1 (control). The NK activity of spleen cells in groups 1 and 3 was also investigated (Table 6). The NK activity in group 3 was increased 26.8%, significantly different from group 1 (12.7%, $P < 0.01$).

Discussion

The recurrence of bladder tumor after transurethral resection is well known, and 15 to 20 percent of those with superficial bladder tumors eventually suffer an invasive carcinoma [31]. Recent evidence suggests that intravesical instillation with bacille Calmette-Guerin (BCG) may be an effective treatment for superficial bladder tumors. [7, 12, 17]. The mechanism by which BCG mediates antitumor activity is still not clear, but it may be predominantly through host-mediated immunity [15, 25]. Severe side effects and complications, including pulmonary infection and granulomatous hepatitis have, however, been reported [13, 21]. OK432 is a bacterial product, not a viabled organism [37]. Its mechanism of action is mainly through host-mediated immunity [36]. OK432 has been reported to promote the induction of autologous killing activity [2] and to active NK cells both in humans [36] and animals [22]. We have already demonstrated the effect of OK432 as a biological response modifiers (BRM) against bladder tumors [32, 33, 35]. In fact, Fujita [3] reported the therapeutic effect of intravesial OK432 instillation against superficial bladder tumors. In the present study, we examined the antitumor effect of intravesical OK432 instillation against rat bladder tumors induced by BBN. We adopted the BBN-induced rat bladder tumor as an animal model, since this model seemed to be an appropriate model for studying superficial bladder tumors [8, 20].

In the saline instillation model, the tumor weight increased compared with the control, although not significantly. Koseki et al. [11] reported that transurethral catheterization induced neoplasms in bladder epithelium. Regardless of catheterization, however, inhibited tumor weight and appearance were significant in group 3 (0.05 KE, once a week). The incidence of cancer in group 6 (high dose of OK432) was somewhat higher than that in group 3. These findings suggest that, unike anticancer drugs, special attention should be paid to the optimal dose and method of BRM administration in immunotherapy [14, 35]. Furthermore, the additional inhibition of cancer induced by OK432 inoculation into the foot pad was not seen in this model. Herr et al. [4] reported that, with intravesical BCG instillation, there was no difference in therapeutic effect with and without intradermal BCG inoculation. We also experienced in human bladder tumors that OK432 injection into the bladder, without intradermal inoculation, had the capacity to produce systemic sensitization [32, 33].

Recently, considerable attention had been focused on NK cells because of their tumoricidal capacity [16, 18], but the origin and nature of these cells remain controversial. NK cells are believed to be most important during early tumor development [18, 32]. In the present study, augmented NK activity was found in the spleen of OK432-treated rats (experiments 1 and 2). These results suggest that NK activity may be associated with the therapeutic results obtained with OK432 immunotherapy. Some studies on BCG immunotherapy have found that the augmentation of NK cells was responsible for the inhibition of bladder tumors [10, 15, 23, 29]. The mechanism by which OK432 enhances NK cell activity is unknown. However, it seems that OK432-activated macrophages produce interferon, which subsequently activates NK cells [5, 22, 27, 36]. In experiment 1, the rate of augmentation of NK activity was not dose-dependent, and no additional effect was induced by foot pad inoculation. Yagita et al. [38] reported that lymphokine-activated killer (LAK) generation is also inhibited by high doses of OK432. Intravesical OK432 instillation, without foot pad inoculation, induced systemic sensitization of NK activity. Simultaneously, NK activity of the mesenteric lymph nodes was also augmented. Ratliff et al. [24], using the mouse model, reported that BCG given by bladder instillation induced the augmentation of NK activity in inguinal lymph node cells. There are few NK cells in regional lymph nodes of patients with bladder tumors, but the NK activity of these lymphocytes can be augmented by in vitro OK432 cultivation [35].

On the other hand, TIL was remarkably incrased in rats given OK432 by bladder instillation. We reported that OK432 given by bladder injection induced the infiltration of tissue lymphocytes (T, NK cells) in human bladder tumor and augmented the anti-tumor activity yielded by these lymphocytes [32, 33]. Kelly et al. [9] also demonstraterd that the local inflammatory response to BCG in bladder biopsy specimens was significantly correlated with the antitumor response to BCG therapy. In the bladder instillation of BRM, we believe local immunocompetence mediated by TIL and lymph nodes plays a major role in the host-tumor interaction [1, 9, 32–35].

In conclusion, our results show that intravesical OK432 instillation significantly inhibits rat bladder tumors induced by BBN. At present, we are carrying out a prospective randomized study of OK432 instillation for human bladder tumors.

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