The Therapeutic Effects of R8-Liposome-BCG-CWS on BBN-Induced Rat Urinary Bladder Carcinoma

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Abstract. *Background: The present gold standard for bladder cancer is Mycobacterium bovis bacillus Calmette-Guérin (BCG) immunotherapy, but serious side-effects are common. We previously reported that C3H/HeN mice vaccinated with a mixture of MBT-2 cells and artificial BCG, octaarginine-modified liposomes incorporating the cell wall of BCG (R8-liposome-BCG-CW), significantly inhibited growth of R8-liposome-BCG-CW pretreated MBT-2 cells. Our aim was to determine if a non-live bacterial agent could be as efficacious as live BCG in a model of bladder cancer. We investigated the suppressive effect of liposome-incorporating cell wall skeleton (BCG-CWS) on N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced urinary bladder carcinogenesis in rats. Materials and Methods: F344 rats were fed with BBN and sodium ascorbate for 8 weeks, after which all rats were confirmed to have excreted atypical epithelial cells in the urine. Rats were administered BCG-CW(1.0 mg/rat) or R8-liposome-BCG-CWS (0.1 or 1.0 mg/rat) intravesically once/week for 8 weeks from week 28 to 35 of the experimental protocol. Results: Rats receiving R8-liposome-BCG-CWS intravesically showed significantly inhibited numbers of tumors, especially those of simple hyperplasia, in comparison with the control rats. Conclusion: R8-liposome-BCG-CWS administration had inhibitory effects on rat bladder carcinogenesis. These results may indicate a novel adoptive immunotherapy against bladder cancers.*

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Intravesical BCG therapy is effective against carcinoma *in situ* and as a prophylaxis against the recurrence of bladder cancer (1-5). In addition to its direct antitumor effect, it is widely recognized that intravesical BCG therapy is more potent in preventing tumor recurrence than is intravesical chemotherapy (6). Although immunotherapy using live BCG is effective, it is not free from fatal side-effects, *e.g.*, high fever, granulomatous prostatitis, pneumonitis, hepatitis, and BCG sepsis (7). To avoid such unfavorable events, it is necessary to develop a more active but less toxic immunotherapeutic agent.

Although the cell wall skeleton of BCG (BCG-CWS) has long been investigated for this purpose, its clinical use is very limited because of solubility and stability difficulties. To overcome these unfavorable physicochemical properties of the BCG-CWS preparation, we have applied octaarginine-modified liposomes (R8-liposomes) as a vector to transport BCG-CWS into the cytoplasm effectively. R8-liposomes were initially developed to transfer highly negatively charged DNA molecules into the cytoplasm by macropinocytosis (8-10). R8-liposomes resemble an envelope-type virus and their surface is modified by anchored R8, a characteristic and efficient cell-penetrating peptide (9).

We have previously reported that R8-liposomes-incorporating mycobacterial cell walls (R8-liposome-BCG-CW) successfully attached to the surface of MBT-2 cells and were efficiently internalized into the cytoplasm within an hour of co-incubation (11). Internalized BCG-CW was then distributed to the lysosomes of the MBT-2 cells and finally has been shown to completely inhibit the growth of MBT-2 tumors *in vivo* (11). Since the structure of BCG-CW is not stable and BCG-CWS is easy to formulate, we changed from R8-liposome-BCG-CW to R8-liposome-BCG-CWS. Furthermore, R8-liposome-BCG-CWS rendered cancer cells more susceptible to cytolysis by lymphokine-activated killer cells (12).

However, the antitumor effect in bladder cancer has not been tested. Thus, the primary purpose of the present study was to examine the antitumor effect of R8-liposome-BCG-CWS as a single agent in a rat model carcinogen-induced of bladder carcinogenesis.

Materials and Methods

Preparation of R8-liposome-BCG-CWS. R8-liposome-BCG-CWS was prepared by a method reported previously (13).

Bladder carcinogenesis induced by BBN in rats. *N*-Butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN) bladder carcinogenesis is considered to be a model for superficial bladder tumor. It has been well described that during bladder cancer development, diffuse hyperplasia at 4 weeks after BBN administration is a reversible change toward cancer, whereas nodular hyperplasia at 8 weeks is an irreversible change (14).

The study comprised 20 8-week-old male Fisher-344 rats (Charles River Japan, Yokohama, Japan). All animals used in the study were handled according to the guidelines approved by the University Council for Animal Care. BBN was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The experimental protocol is summarized in Figure 1. Because sodium ascorbate significantly increases papillary or nodular hyperplasia (PN hyperplasia) (15-16), all rats were given diets containing 5% (w/w) sodium ascorbate for 8 weeks. After treatment for 8 weeks with the sodium ascorbate and 0.05% BBN in drinking water, 20 rats were divided into four groups according to the treatment administered at 28 weeks: group 1, control (phosphate-buffered saline [PBS] only); group 2, BCG-CW only (1.0 mg/rat once weekly for 8 weeks); group 3, R8-liposome-BCG-CWS (0.1mg/rat once weekly for 8 weeks), and group 4 R8-liposome-BCG-CWS (1.0 mg/rat once weekly for 8 weeks).

Histological examination. At the end of treatment, the rats were killed under anesthesia. Before removal of the bladder from each rat, the bladder was intraluminally injected with a buffered formaldehyde solution as pre-fixation for histological analyses. A ligature was placed around the bladder neck to maintain proper distention.

For macroscopic quantitative analysis (number and volume of tumors), each bladder pre-fixed in formaldehyde was carefully opened, the lumen was inspected for grossly visible lesions, and the number of tumors per rat and the volume of each tumor were recorded to calculate the incidence of tumors per group and the mean tumor volume per rat. The volume was calculated as V (mm^3)=4×π× 1 mm (major axis) × s^2 mm^2 (minor axis)/3. A tumor was defined as a lesion of >0.5 mm in diameter.

For microscopic qualitative analysis (bladder histology), the bladder was immersion-fixed in 4% buffered formaldehyde and processed for paraffin sectioning. Three slices from each bladder were embedded in paraffin, sliced into 3 μm sections, and stained with hematoxylin and eosin. The lesions observed in the urinary bladder mucosa were classified as simple hyperplasia, PN hyperplasia, papilloma, or carcinoma, as described previously (17).

Blood analysis. Interferon gamma (IFN-γ) in blood was analyzed in weeks 31 and 35 of the experimental protocol. We measured the serum level of IFN-γ immediately before administration of the specific treatment in each rat group and after 8 and 24 hours. Serum levels of IFN-γ were measured with a Rat IFN-γ Colorimetric ELISA Kit (Thermo Fisher Scientific, Yokohama, Japan).

Statistical analysis. The results are presented as the mean (SEM), and groups were compared using non-parametric Dunnett method with significance indicated at a value of -p<0.05.

Results

All rats completed the 36-week protocol, during which the mean intake of food and fluids was not significantly different among the four groups. Macroscopic appearance of bladders in the four groups is shown Figure 2. Bladders from group 1 (control) showed large tumors, but R8-liposome-BCG-CWS caused marked inhibition in bladder tumor growth.

Significant differences were present between groups 1 and 4 in numbers of tumors per rat (–p<0.05) (Figure 3). The mean number of tumors per rat in group 4 (4.4±1.9) was significantly lower than that in group 1 (20.0±7.6). No significant differences were evident in tumor volume among the four groups, but the total tumor volume in group 4 was less than half that in group 1 (Figure 3).

The incidences of urinary bladder tumors are summarized in Figure 4. Histological examination showed that simple and PN hyperplasia were observed in all bladders of the BBN-treated rats. The incidence of tumors in rats treated with PBS was higher than that in groups treated with BCG-CW and R8-liposome-BCG-CWS. The number of simple hyperplasias in group 4 (6.5±1.8) was significantly lower than that of group1 (24.3±6.1). The number of PN hyperplasias in group 3 was higher than that of the other groups, but statistical differences was not found among the groups. In group 1, the number of bladder papillomas and carcinomas were 1.8±2 and 1.1±1.1, respectively. The incidence of urinary bladder carcinoma in group 4 was less than that in group 1, but the difference was not significant.

IFN-γ. IFN-γ has been shown to be important in the function and maturation of multiple immune cells. IFN-γ is essential for T-helper 1 cell (Th1) immune responses and regulates T-cell differentiation, activation, expansion, homeostasis, and survival. At 31 weeks, rats in group 2 had higher levels of IFN-γ than those in other groups, but statistical differences were not found among the groups (Figure 5). At 35 weeks, IFN-γ secretion was more increased in groups 2 and 4 than in groups 1 and 3, but no significant difference was found among all groups. It is suggested that IFN-γ may be high in the groups with a low number of lesions.

Discussion

The antitumor mechanism of BCG and the role of its CWS components have recently been unveiled. Most of the immunostimulatory activities are associated with the CWS

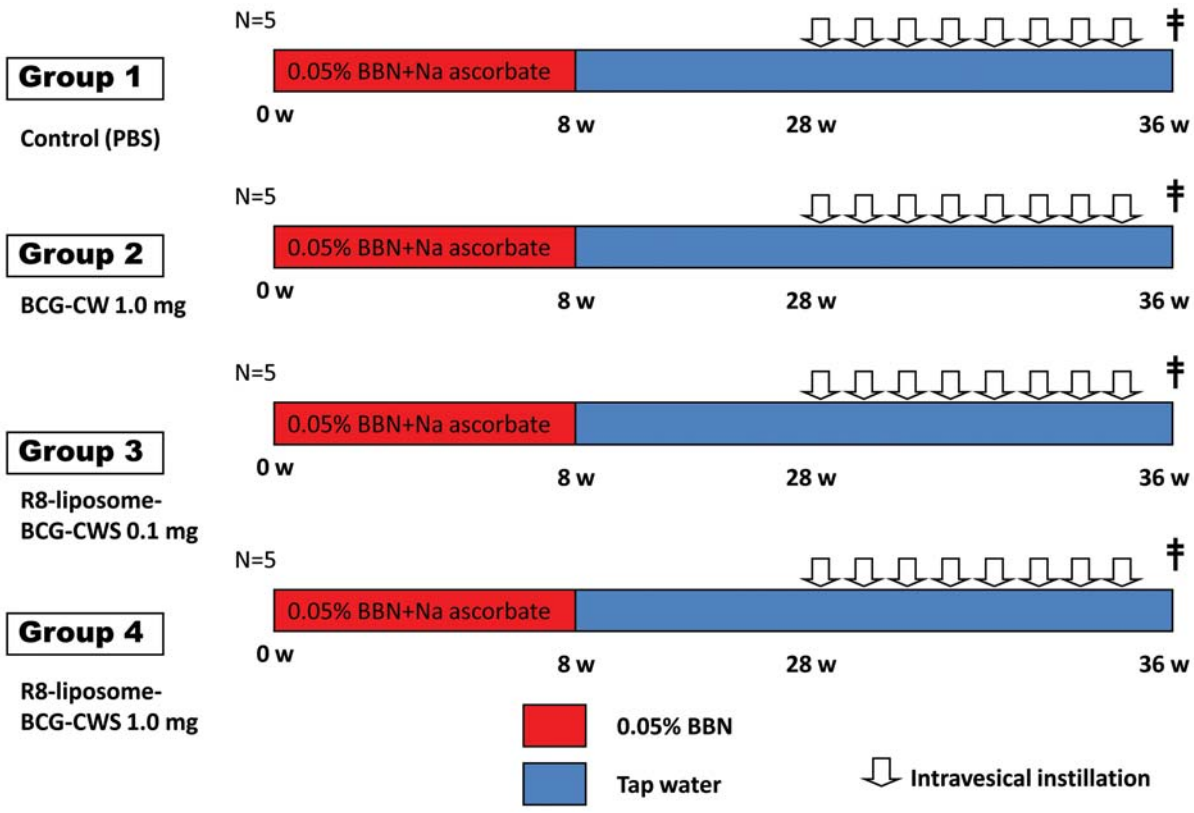

Figure 1. *Treatment protocol: 20 rats were divided into four groups.*

lipids of BCG (18), which involve inflammation and innate and acquired immune responses. However, BCG-CWS itself has difficulty associating with the bladder cancer cells due to its highly hydrophobic properties (19).

We previously reported that R8-liposome-BCG-CW completely inhibited the growth of MBT-2 tumors in C3H/HeN mice, whereas BCG-CW alone did not. In addition, animals vaccinated with a mixture of MBT-2 cells and R8-liposome-BCG-CW showed significant inhibition of the growth of R8-liposome-BCG-CW pretreated MBT-2 cells (11). This suggests that bladder cancer cells, which usually multiply under the condition of immune tolerance from the host, can be recognized through the presence of BCG-related molecules in cancer cells. The aim of the present study was to develop a non-live bacterial agent using a BCG-CWS, which consisted mainly of essential molecules, to induce immune responses (20-22).

The carcinogen BBN was used to induce urinary bladder tumors in the present study (14). Oral administration of BBN dependably induces urinary bladder tumors in a short time. In the rodent model of BBN-induced bladder carcinogenesis, the concentration of the carcinogen and duration of treatment determine the neoplasia in the urinary bladder. Nakanishi *et al*. stated that the lesions observed in the urinary bladder mucosa are classified as simple hyperplasia, PN hyperplasia, or papilloma (17), but cancerous cells were not found in any rat in their experiment (17). Thus, sodium ascorbate was administered to promote urinary bladder carcinogenesis (16). Administration of sodium ascorbate significantly increased the incidence and number of PN hyperplasias. There have been many reports on preneoplastic lesions in rat urinary bladder carcinogenesis (23), and there is strong evidence of correlation between PN hyperplasia and cancer of the urinary bladder. This finding could be related to the induction of cancer in rat urinary bladder by high levels of sodium ascorbate.

The results of the present study showed that both BCG-CW and R8-liposome-BCG-CWS (1.0 mg) inhibited rat bladder carcinogenesis induced by BBN administration. Both drugs, except for low-dose R8-liposome-BCG-CWS (0.1 mg), reduced the incidence of carcinoma and the mean number of tumors to approximately less than half of those of the control group. The formation of PN hyperplasia is thought to be a precursor to transitional cell carcinoma, and both drugs also inhibited PN hyperplasia.

Although the precise mechanism of the chemotherapeutic effect of R8-liposome-BCG-CWS has not been determined,

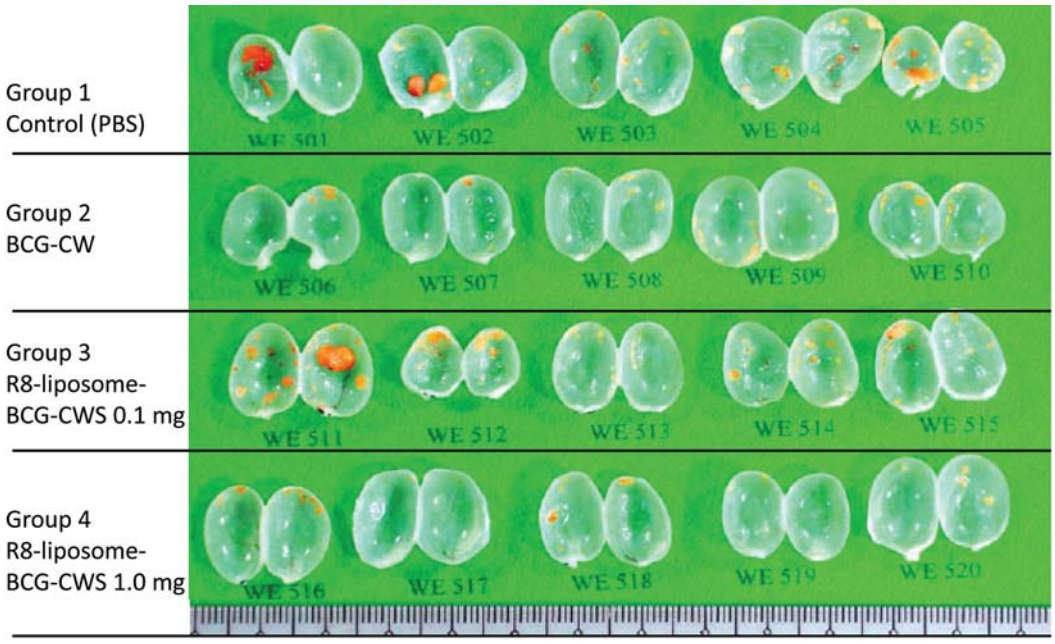

Figure 2. *Macroscopic evaluation at the end of the 36-week protocol. Rats from group 1 had larger bladder tumors, in clear contrast with rats from group 4.*

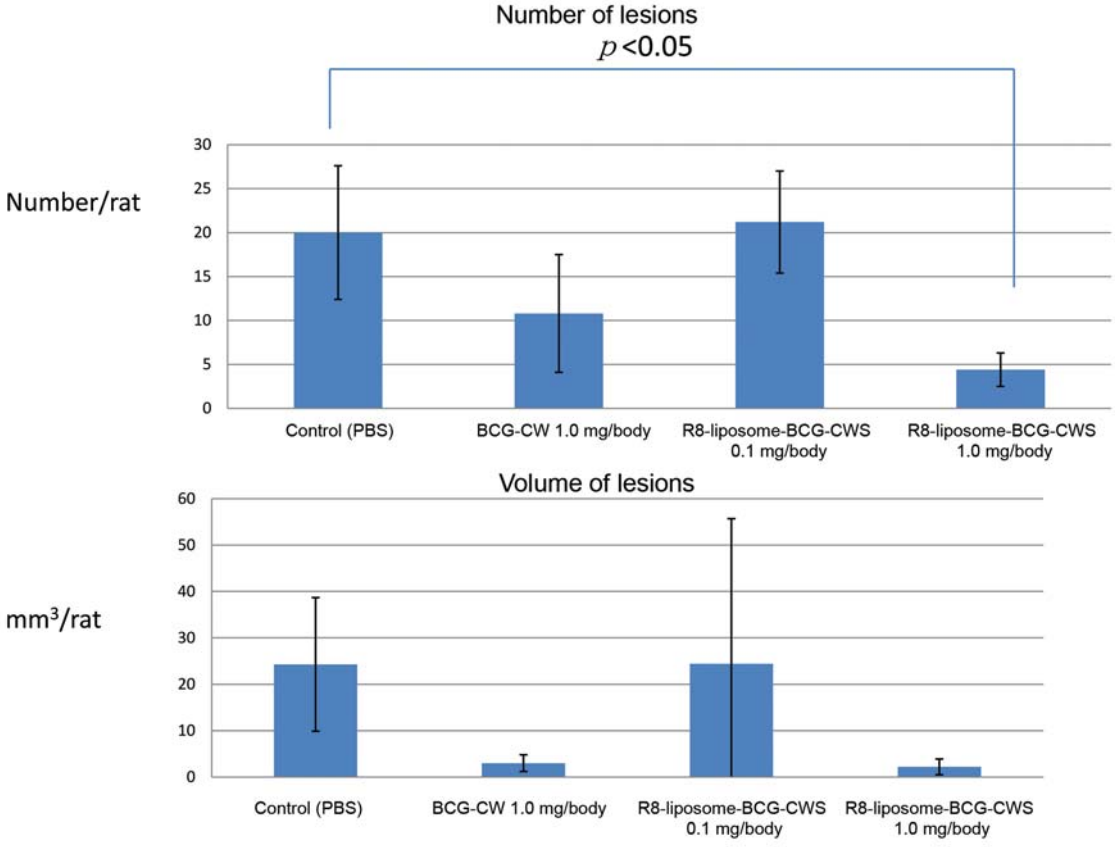

Figure 3. *Mean number of tumors and volume of lesions between the four groups. The number of tumors was significantly lower in the group of R8-liposome-BCG-CWS 1.0 mg/body than in the control group, but the volume of tumors was not significantly different between the four groups.*

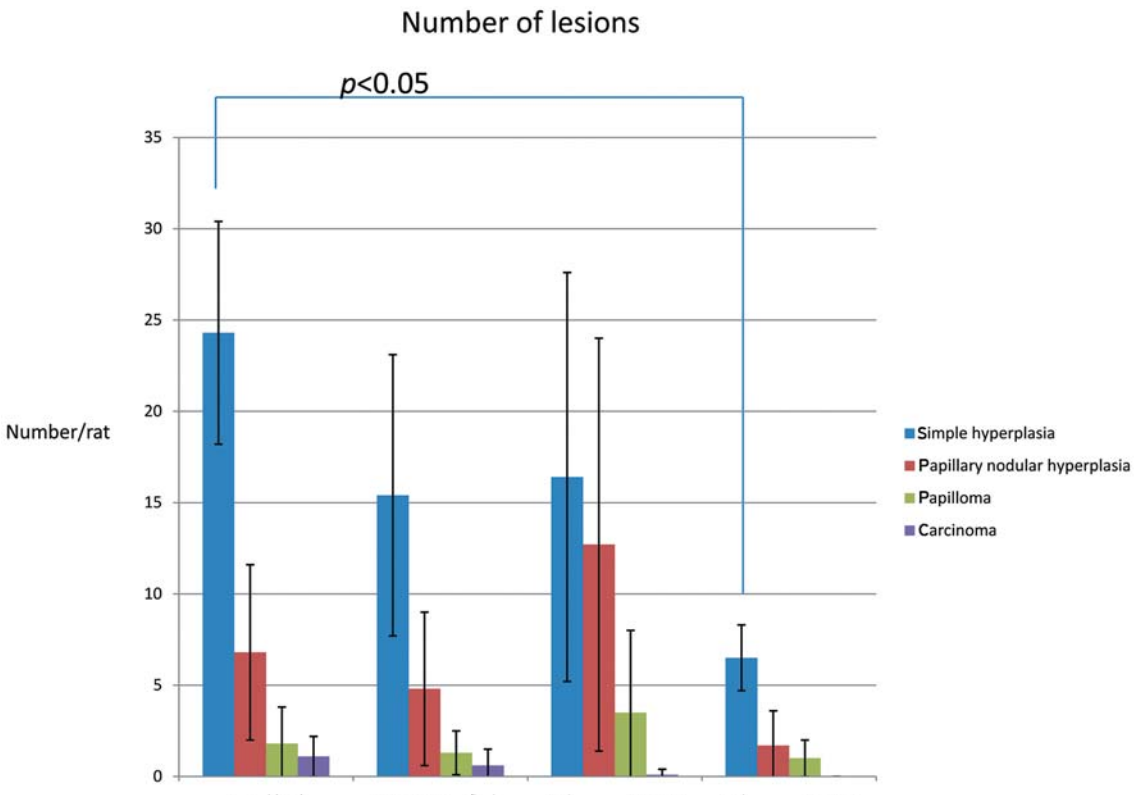

Figure 4. *The histological effect of BCG-CW and R8-liposome-BCG-CWS on rat urinary bladder cancer induced. The mean number of simple hyperplasias was significantly lower in the group of R8-liposome-BCG-CWS 1.0 mg/body than in the control group.*

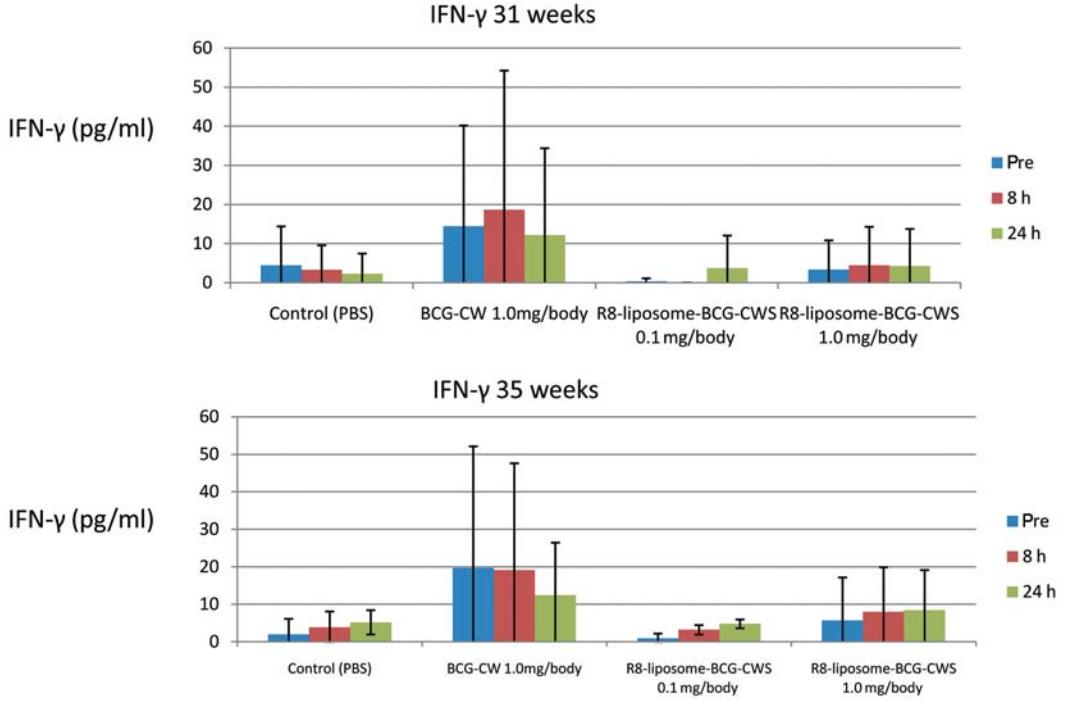

Figure 5. *IFN-γ serum level at 31 weeks and 35 weeks. IFN-γ increased remarkably at 35 weeks in the group receiving BCG-CW and R8-liposome-BCG-CWS 1.0 mg/body intravesically.*

several possible mechanisms were proposed. In our previous study, induction of surface NKG2D (a powerful activating receptor expressed by natural killer cells) ligands by R8-liposome-BCG-CWS rendered cancer cells more susceptible to cytolysis by lymphokine-activated killer cells. T-24 and RT-112 bladder cancer cells, even when cultured singly in the absence of immune cells, can directly respond to R8-liposome-BCG-CWS (12).

IFN-γ has been shown to be important in the function and maturation of multiple immune cells (24). T-Cells, natural killer cells, and natural killer T-cells are the primary producers of IFN-γ which has a myriad of effects in both host defense and immune regulation, including antiviral, antimicrobial, and antitumor activity. IFN-γ also has direct antitumor effects because it is anti-angiogenic, inhibits proliferation, sensitizes tumor cells to apoptosis, up-regulates MHC class I and II expression, and stimulates antitumor immune activity. In a study of recurring superficial transitional bladder carcinoma, it was shown that intravesical instillations of IFN-γ were effective against cancer recurrence (25). Our results are in agreement with the role of INF-γ production in bladder cancer development and the role of IFN-γ activity in cancer prevention.

In conclusion, when given intravesically, R8-liposome-BCG-CWS significantly inhibited rat bladder carcinogenesis. Development of this non-live bacterial agent may contribute to providing a more active and less toxic tool as a future substitute for live BCG in immunotherapy against non-muscle-invasive bladder cancer.

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