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Antitumor Effect of VNP20009, an Attenuated *Salmonella*, in Murine Tumor Models

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VNP20009, a genetically modified strain of *Salmonella typhimurium* with deletions in the *msbB* and *purI* loci, exhibited antitumor activities when given systemically to tumor-bearing mice. VNP20009 inhibited the growth of subcutaneously implanted B16F10 murine melanoma, and the human tumor xenografts Lox, DLD-1, A549, WiDr, HTB177, and MDA-MB-231. A single intravenous injection of VNP20009, at doses ranging from 1×10^4 to 3×10^6 cfu/mouse, produced tumor growth inhibitions of 57–95%. Tumor volume doubling time, another indicator for tumor growth inhibition, also significantly increased in mice treated with VNP20009. Using mice with immune system deficiencies, we also demonstrated that the antitumor effects of VNP20009 did not depend on the presence of T and B cells. In addition, VNP20009, given intravenously, inhibited the growth of lung metastases in mice. Only live bacteria showed the antitumor effect.

Key words: VNP20009; *Salmonella typhimurium*; Murine tumor model; Antitumor effect

It has long been known that tumor regression occasionally occurs in patients with bacterial infections (1). An infection arising from a tumor may be the first clinical manifestation of neoplastic disease (2). Numerous clinical reports describe patients with tumors infected by bacteria, frequently *Salmonella* (3–6). For example, Giel (4) described an abscess in a pheochromocytoma containing 90 cc of thick, yellow pus encapsulated by a thin sphere consisting of a few layers of tumor cells. The bacteria were later identified as *S. typhimurium.*

The use of bacterial products for cancer treatment dates back to the early 1900s. William B. Coley, who was then a surgeon at Memorial Hospital, now Memorial Sloan-Kettering Cancer Center, observed that patients with sarcoma responded better after surgery if they developed severe postoperative infections. Coley later developed a regimen containing bacterial cell wall components for the treatment of cancer (7). The grain-positive bacterium *Clostridium* was evaluated as an anticancer agent in clinical trials in the 1970s. Although in many cases *Clostridium* was recovered from tumors and oncolysis was observed, these clinical trials were subsequently discontinued because they failed to produce clinical benefits to patients (8). Bacille Calmette-Guerin (BCG[2]), another viable bacterium, is being used for the treatment of superficial bladder carcinoma in humans. BCG, an attenuated, avirulent strain of *Mycobacterium bovis*, is administered by urethral catheterization at periodic intervals for up to 24 months. In patients with bladder cancer, BCG treatment achieves complete responses in greater than 60% of the patients (9).

These reports suggest that bacteria could serve as anticancer agents if their virulence were controlled. *S. typhimurium*, if attenuated, can be safely administered to animals to retard tumor growth in murine tumor models. Avirulent strains of *Salmonella* have been developed as vaccines for the prevention of bacterial infections (10,11). A viable *S. typhi* vaccine, Ty21a, has been approved for the prevention of typhoid fever in humans (12). In addition to serving as vaccines against Salmonellosis, attenuated strains of *Salmonella* have been used for expressing and delivering heterologous proteins to the immune system. This approach may eventually be developed for combating infections and cancer (13,14). Using an *aro4*-mutant SL3225, Eisenstein et al. (14) reported tumor inhibition of a plasmacytoma by either intraperitoneal or intralesional injection of the attenuated bacteria. Similar results have been obtained by Pawelek et al. (15) using other auxotrophic mutants of *S. typhimurium.* To overcome the propensity of grain-negative bacteria to induce septic shock in animals, Low et al. (16) constructed an attenuated *S. typhimurium* by deleting the *msbB* gene, which encodes the enzyme involved in the terminal myristoylation of lipid A. The mutated bacteria lost the capability to induce tumor necrosis factor-α (TNF-α), both in vitro and in vivo, and their pathogenicity was greatly reduced. These bacteria also exhibited the characteristics of preferential accumulation in tumors and inhibition of tumor growth.

We report here that VNP20009, an attenuated strain of *S. typhimurium* with deletions in the *msbB* and *purI* loci, suppresses the growth of subcutaneously implanted tumors and lung metastases. We also show that the antitumor effects of VNP20009 do not depend on the pres-

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[2]Abbreviations used: BCG, Bacille Calmette-Guerin; TNF-α, tumor necrosis factor α; cfu, colony forming unit.

ence of T and B vells, and only live bacteria exhibit the antitumor effect.

MATERIALS AND METHODS

Cell Culture Conditions

All cell lines were maintained at 37 ± 2°C in a humidified atmosphere containing 5% CO_2. B16F10 murine melanoma, DLD-1 human colon carcinoma, A549 human lung carcinoma cells, HTB177 human lung carcinoma cells, Lox human melanoma, and MDA-MB-231 human breast carcinoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. WiDr human colon carcinoma cells were maintained in Eagle's minimum essential medium, containing Earle's salts, and supplemented with both nonessential amino acids and 10% fetal bovine serum.

Tumor Cell Implantation

Solid tumor models were obtained by SC injection of tumor cells in the right hind flank of C57BL/6, athymic nude, or SCID mice. For tumor implantation, cells were detached from the flask by trypsinization, washed, and suspended in PBS to a cell density of 5×10^6 cells/ml (B16F10 cells), 7×10^7 cells/ml (DLD-1 cells), 5×10^7 cells/ml (WiDr, A549, HTB177 cells), and 4.5×10^7 cells/ml (MDA-MB-231 cells). A 0.1 ml bolus of cell suspension, giving a total of 5×10^5 cells (B16F10), 7×10^6 cells (DLD-1), 5×10^6 cells (WiDr, A549, HTB177), or a 0.2 ml bolus, giving a total of 9×10^6 cells (MDA-MB-231), was injected SC into the right flank. Animals were immediately randomized and arranged into groups of 5–10 animals per group. Experimental lung metastases were produced by IV injection of 1.5×10^5 B16F10 murine melanoma cells through the lateral tail vein into C57BL/6 and athymic nude mice. Animals were identified by ear tags.

Treatment of Mice With VNP20009

VNP20009 was grown as a liquid culture to an OD_{600} = 0.8 by suspending a single colony into 25 ml of Luria-Bertani (LB) broth. The suspension was diluted in PBS before use, based on 1 OD = 1×10^9 colony forming unit (cfu)/ml. Solid tumors were staged for 6–24 days prior to bacterial inoculation. A 0.2-ml bolus of the PBS-diluted bacterial suspension was administered IV via the lateral tail vein. Each mouse received approximately 1×10^4 to 3×10^6 cfu of VNP20009. Mice bearing lung metastases were treated with a 0.2-ml bolus of live VNP20009 (containing 2×10^6 cfu) or dead VNP20009 (containing 2×10^8 cfu) 5 days after tumor inoculation. Actual doses were determined by plating the dosing solution on LB agar plates, and colonies were enumerated after overnight incubation at 37°C. Cyclophosphamide (Sigma, Milwaukee, WI), used as a positive control, was given IP at a dosing schedule of 200 mg/kg, once weekly for 3 weeks. In some experiments, SCID mice receiving VNP20009 also received oral ciprofloxacin, 100 mg/kg, twice daily for 5 days. In comparison studies, killed VNP20009 was prepared by autoclaving the bacteria for 20 min, and lack of viability was confirmed by plating.

Tumor Measurements

Tumor size was determined from measurements obtained with electronic calipers along three axes: length (L), width (W), and height (H). The volume of the tumor was calculated using the following formula: tumor volume = (L × W × H)/2. The mean tumor volume and standard deviation of all animals comprising each group were determined. Tumor volume of individual animals from control and treated groups at the end of experiment was also analyzed with the Student's *t*-test. Lungs with metastases taken from mice were weighed and photographed.

Histopathological Studies

To analyze mechanisms involved in the observed antitumor effects, B16F10 tumors that were treated with PBS or VNP20009 were removed from mice 3–11 days after treatment and fixed with 4% buffered formalin. The specimens were embedded in paraffin, and 6-μm-thick sections were stained sequentially with H&E for microscopic examination.

Animal Care

All animals were treated, fed, housed, and handled according to guidelines established by the National Research Council. The animals used in this study were obtained from Charles River Laboratories (Wilmington, MA). Animals selected in this study were as uniform in age and weight as possible. They were approximately 8–10 weeks of age, and their body weights ranged from 18 to 22 g. Animals were housed in plastic cages with stainless steel covers and were identified by an ear tag. Five mice were housed in each cage. Mouse Chow food was available ad libitum via food hoppers. Tap water was provided in glass bottles ad libitum. All animals were kept in a well-ventilated room where a 12-h light/12-h dark photoperiod was maintained. Room temperature was maintained at 72 ± 2°F. At the end of the study, animals were euthanized in a dry ice-containing chamber.

RESULTS

The ability of VNP20009 to inhibit tumor growth was examined over a dose range of 1×10^4 to 1×10^6 cfu/mouse in the SC B16F10 melanoma model. Mice were administered VNP20009 IV on day 7, and tumor volumes were measured on days 10, 13, 17, and 20 (Fig. 1). The differences observed between individual groups were deemed significant when analyzed by the Student's two-tailed *t*-test. All doses were found to give significant antitumor activity ($P < 0.01$). A single dose of VNP20009 at 1×10^5 cfu/mouse produced responses equivalent to, or better than, the antitumor drug cyclophosphamide, which was given three times during the course of treatment.

Figure 2 demonstrates that VNP20009 inhibited the growth of B16F10 melanoma implanted into immunocompetent C57BL/6 (Fig. 2A), athymic nude (Fig. 2B), or SCID mice (Fig. 2C). A single injection of VNP20009 produced a tumor growth inhibition of 85% or more re-

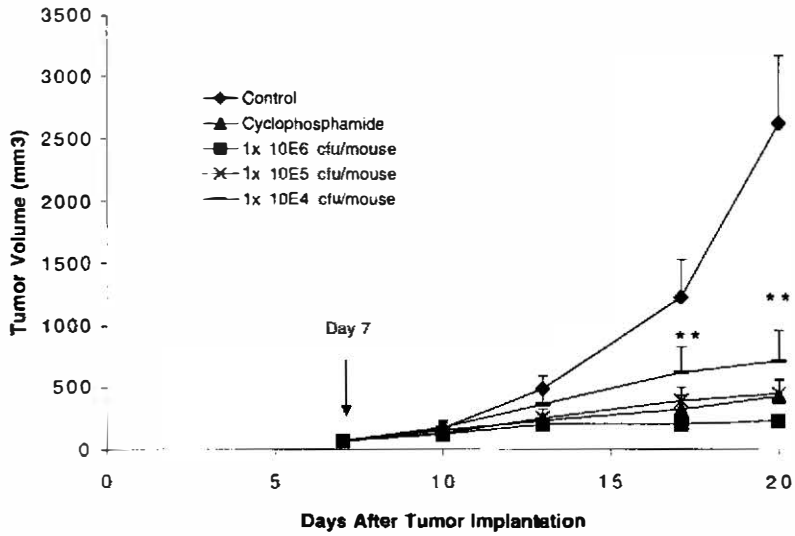

Figure 1. Tumor growth inhibition of B16F10 melanoma by VNP20009. A single dose of VNP20009 (from 1×10^4 to 1×10^6 cfu/mouse) was injected IV into mice 7 days after tumor implantation. Cyclophosphamide was given IP at 200 mpk on days 7, 14, and 21. $^{**}P < 0.01$).

gardless of strain of mice used. SCID mice were sensitive to VNP20009 treatment, and started dying 7 days after bacterial injection when no antibiotics were given. No mortality or weight loss was noted in C57B/6 or athymic nude mice treated with VNP20009. Significant antitumor activities ($P < 0.01$, t-test) were recorded in all the treated groups.

Athymic nude mice bearing A549, WiDr, or DLD-1 tumors were treated with VNP20009 at a dose of 2×10^6 cfu/mouse on day 6 (A549), day 8 (WiDr), or day 13 (DLD-1). A single injection of VNP20009 caused significant and sustained tumor growth inhibitions of 54%, 65%, and 74% for A549, WiDr, and DLD-1, respectively (Fig. 3). MDA-MB-231 tumors grew in nude mice and were staged for 24 days before dosing. Similar results were obtained, with a single dose of VNP20009 inhibiting the growth of MDA-MB-231 tumors more than 70%. HTB177 lung carcinoma staged to 11 days after implantation into SCID mice was treated with a dose of 1×10^6 cfu/mouse. This dose of VNP20009 produced a 94% tumor growth inhibition at the 10-day interval (Fig. 3E). The experiment was terminated on day 20 because of the death of animals caused by bacterial infection. VNP20009 inhibited tumor growth regardless of size of tumors at the time of treatment (0.05–0.3 g). It was equally efficacious in inhibiting fast (B16F10, Lox) or slow growing (MDA-MB-231, DLD-1) tumors.

In another experiment using SCID mice bearing Lox human melanoma, the antibiotic ciprofloxacin was given to mice receiving VNP20009. All the SCID mice treated with VNP20009 alone died before day 15 (i.e., 10 days after VNP20009 dosing). In contrast, all the mice survived that received ciprofloxacin 4 days after VNP20009 treatment. In addition, a very significant delay of tumor growth was observed in animals receiving VNP20009 alone or VNP20009 plus ciprofloxacin (Fig. 3F). Tumor volume doubling time in treated animals was significantly increased compared with vehicle-treated controls.

VNP20009, at a dose of 2×10^6 cfu/mouse injected IV, inhibited the growth of lung metastases compared with untreated controls (Fig. 4). Similar experiments carried out in athymic nude, SCID, and beige mice produced comparable results. In addition, only viable bacteria inhibited the growth of lung metastases. When VNP20009 was heat inactivated, even if given at a higher amount, no antitumnor effects were observed (Fig. 5).

Tumor sections were prepared from untreated mice and mice treated with VNP20009 for various days. At least three tumors for each time point and 20 slides from each tumor were examined for the presence or absence of necrosis and infiltrating cells. Sections taken from tumors treated with VNP20009 revealed a massive infiltration of immune cells accompanied with an extensive necrosis in the central part of the tumors. Significant tumor necrosis (over 70% surface of tumor tissue) was found in mice treated with VNP20009 for 7 days, compared with PBS-treated control mice (Fig. 6). The dead and dying tumor cells were stained with bright red color, characterized with an increase in cell size and the disintegration of the nuclei. Infiltrating cells formed a zone surrounding the necrotic center of the tumor (Fig. 6A). Most of infiltrating cells, at higher magnification, were shown to be neutrophils with horseshoe-shaped nuclei (Fig. 6B). In contrast, tumors obtained from mice treated with PBS (Fig. 6C) revealed a relatively low level of necrosis at the center (less than 5% surface of tumor section) and with only a few infiltrating immune cells (Fig. 6D).

DISCUSSION

In previous studies, we have demonstrated that a single dose of YS1629, a *msbB* knockout mutant, is highly effective in inhibiting the growth of B16 murine mela-

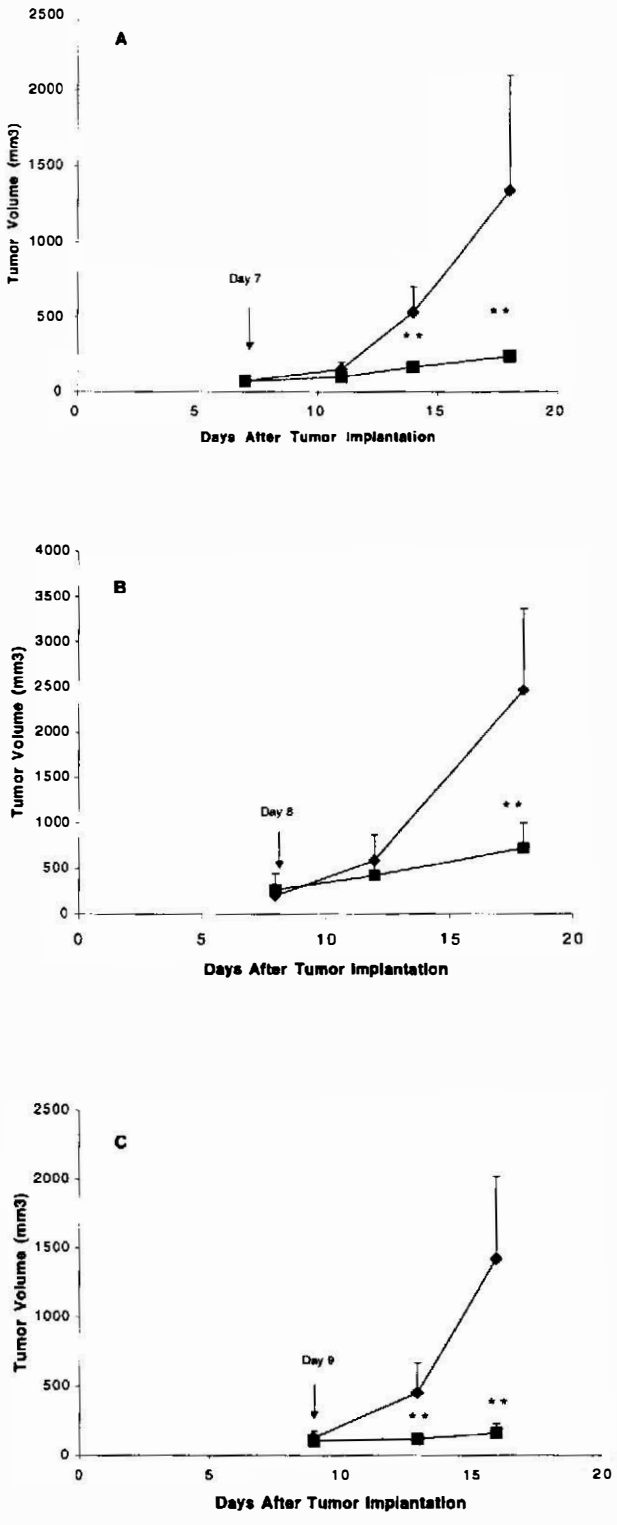

Figure 2. Antitumor effect of VNP20009 on B16F10 melanoma implanted in immunocompetent C57BL/6 (A), athymic nude (B), and SCID mice (C). VNP20009, at a dose of 2×10^6 cfu/mouse was injected IV into tumor-bearing mice 7–9 days after tumor implantation. $^{**}P < 0.01$.

noma (15). In this report, we further demonstrate that a single dose of VNP20009, a tetracycline-sensitive, *msbB* and *purI* knockout mutant, is also capable of suppressing tumor growth in human tumor xenograft models representing melanoma and lung, breast, and colon cancer. Similar to previous observations with B16F10 melanoma, a single dose of VNP20009 inhibited tumor growth, consistent with an ability of VNP20009 to persist within tumor xenografts (17). Only a slight dose–response correlation was observed between VNP20009 levels and tumor inhibition in B16F10 melanoma, because VNP20009 replicates within the tumor, producing a greater than expected potency at lower doses (18). The antitumor activity of VNP20009, at the dose of 1×10^5 cfu/mouse, is superior to that induced by the antitumor drug cyclophosphamide, which has been given at the optimal dosing schedule for this tumor.

Toxicology study of the VNP20009 revealed that SCID mice were approximately 5- to 50-fold less tolerant compared with immune-competent mice (data not shown), which was consistent with the enhanced sensitivity of SCID mice to *Salmonella* infection previously reported with other attenuated *Salmonella* strains (19). Athymic CD-1 nude mice and immune-competent CD-1 mice had similar LD_{50} values for VNP20009 (data not shown). These data suggest that B cells may be involved in the clearance of VNP20009. Without receiving treatment of ciprofloxacin, SCID mice succumbed to bacterial infections and began to die 10 days after VNP20009 dosing. Nevertheless, VNP20009-mediated mortality in SCID mice could be completely prevented by antibiotic administration, without affecting the antitumor efficacy. Number of bacteria in both liver and tumor decreased by 90% to 99% with the current dosing schedule during antibiotic treatment but returned to normal levels a few days after treatment ended (data not shown). Page-Clisson et al. (20) reported similar results that ciprofloxacin could not completely eradicate the persistence of *S. typhimurium* in liver and spleen. It should also be noted that VNP20009 inhibited tumor growth in immunocompromised mice with T-cell and B-cell deficiencies. Using an experimental metastasis model, we demonstrated that the antitumor activity of VNP20009 may not require T cells, B cells, and natural killer cells (unpublished data). Schafer et al. (21) reported that live, attenuated *Salmonella* was capable of inducing tumoricidal macrophages in C3H/HeJ mice, whereas BCG was not effective in that strain of mice. Our finding that only viable VNP20009 shows antitumor effects suggests that the antitumor mechanism of VNP20009 differs from that of BCG.

The mechanisms involved in tumor growth inhibition by VNP20009 are not completely understood. It is possible that some cell wall components of live *S. typhimurium* are cytotoxic to mammalian cells and induce apoptosis in macrophages and granulocytes (22). Galan and his coworkers suggested that a type III secretion system is responsible for the *Salmonella*-induced apoptosis in cultured mammalian cells (23). VNP20009 accumulated at high levels inside the tumor mass, which could induce low levels of cytokines, such as TNF-α in situ that elic-

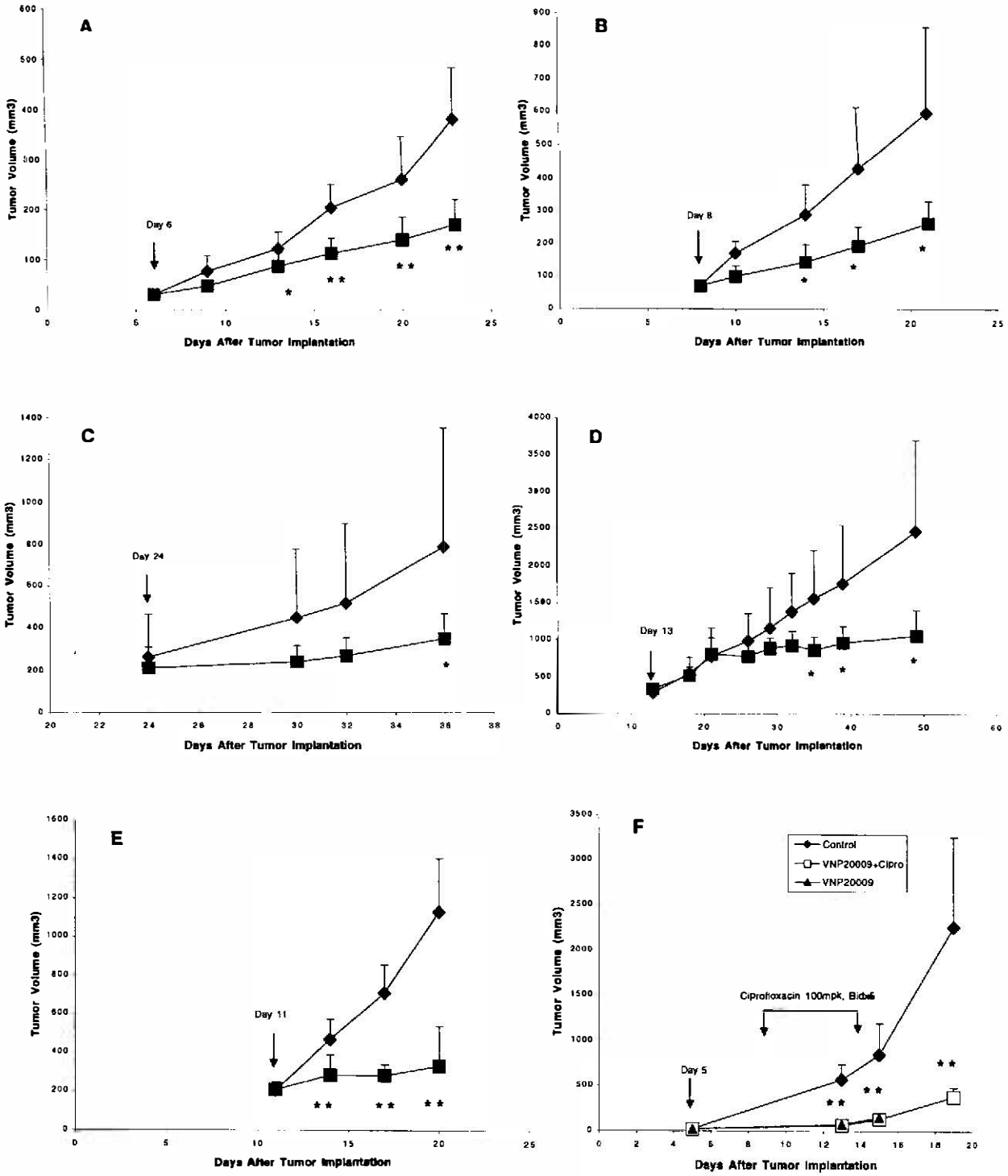

Figure 3. Antitumor effect of VNP20009 on human tumor xenografts. (A) A549, (B) WiDr, (C) MDA-MD-231, (D) DLD-1, (E) HTB177, and (F) Lox cells implanted into either athymic or SCID mice. VNP20009 was given IV at doses ranging from 1×10^6 to 3×10^6 cfu/mouse 6 to 24 days after tumor implantation. $^*P < 0.05$; $^{**}P < 0.01$.

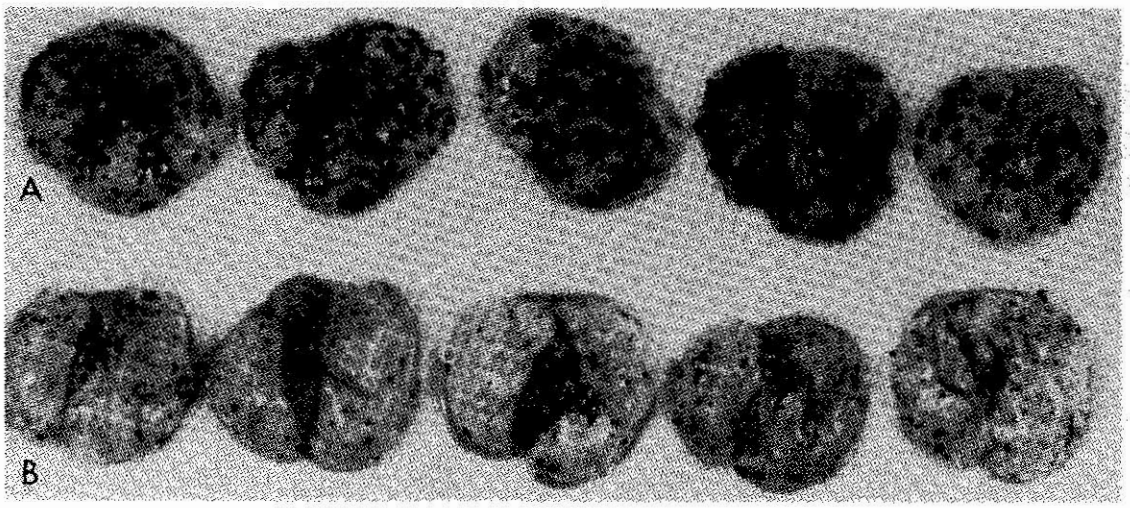

Figure 4. Growth inhibition of lung metastases by VNP20009. A single IV injection of VNP20009 at 2×10^6 cfu/mouse was given to C57BL/6 mice 5 days after inoculation of B16F10 cells. (A) PBS control, (B) VNP20009 treatment.

its an antitumor activity. Other possible mechanisms may include competition between bacteria and tumor cells for nutrients and oxygen. Histolgic evaluation revealed the presence of VNP20009 in both the necrotic center and peripheral portion of the tumor (17). We have further demonstrated in this study that significant tumor necrosis occurred in treated but not untreated mice. In the treated mice, tumor necrosis was also accompanied with a massive infiltration of immune cells including neutrophils. The contribution of these factors to the antitumor activity of VNP20009 is unknown and remains to be elucidated.

Unlike Clostridium, which is an obligate anaerobe and colonized only in hypoxic and necrotic areas of tumors, *S. typhimurium* is a facultative anaerobe and is distributed homogeneously throughout the entire tumor and is accumulated in tumors as small as 0.1 g (24). *Clostridium* accumulated and exerted its oncolytic effects only in large tumors (8,25,26); tumor size ranging from approximately 0.7 g (26) to 1.4 g (25) was needed to allow germination of the bacteria and the subsequent lysis of tumors. Using Sarcoma 180 SC implanted in Taconic Farms Swiss female mice, Thiele et al. (25) reported that tumors of 0.6 g and below were less efficacious in accumulating *Clostridium*. In contrast, we demonstrate here that VNP20009 has antitumor activity against small tumors, with tumor size ranging from 0.05 to 0.3 g at the time of treatment in various murine tumor models. VNP20009 accumulated efficiently in SC implanted B16F10 melanomas with size 1 g or above but antitumor activity has not been evaluated under these conditions. VNP20009 has been attenuated at least 10,000-fold compared with the activity of the parental wild-type *Salmonella* strain in immunocompetent C57BL/6 mice (27). Our studies suggest that T-cell-deficient nude mice are not overly sensitive to VNP20009, in contrast to studies using mice with both T-cell and B-cell deficiency. These results are consistent with a previous report demonstrating that moderate immunodeficiency, resulting from sublethal irradiation of BALB/c mice, does not cause an increase in susceptibility to an attenuated *aro4*-strain of *Salmonella* (28). VNP20009 is well

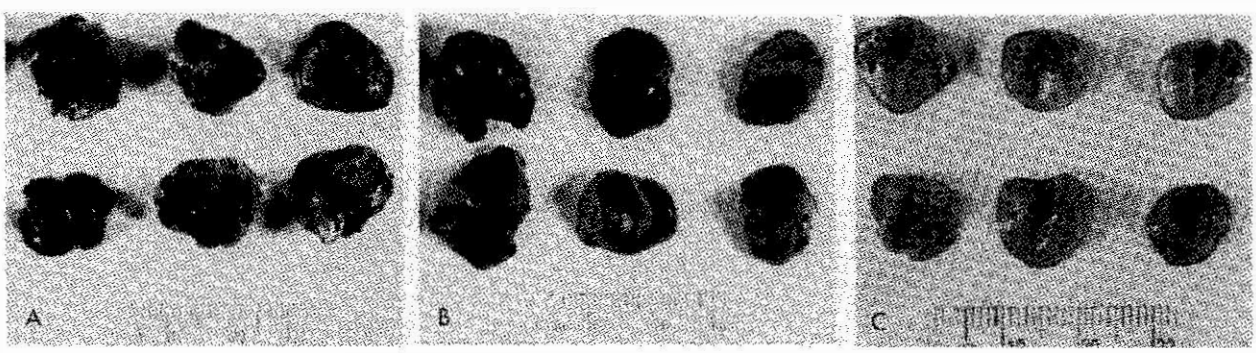

Figure 5. Tumor growth inhibition of lung metastases by live or dead VNP20009. A single IV injection of live VNP20009 at 2×10^6 cfu/mouse or dead VNP20009 at 2×10^8 was given to athymic nude mice 5 days after inoculation of B16F10 cells. (A) PBS control, (B) dead VNP20009 treatment, and (C) live VNP20009 treatment.

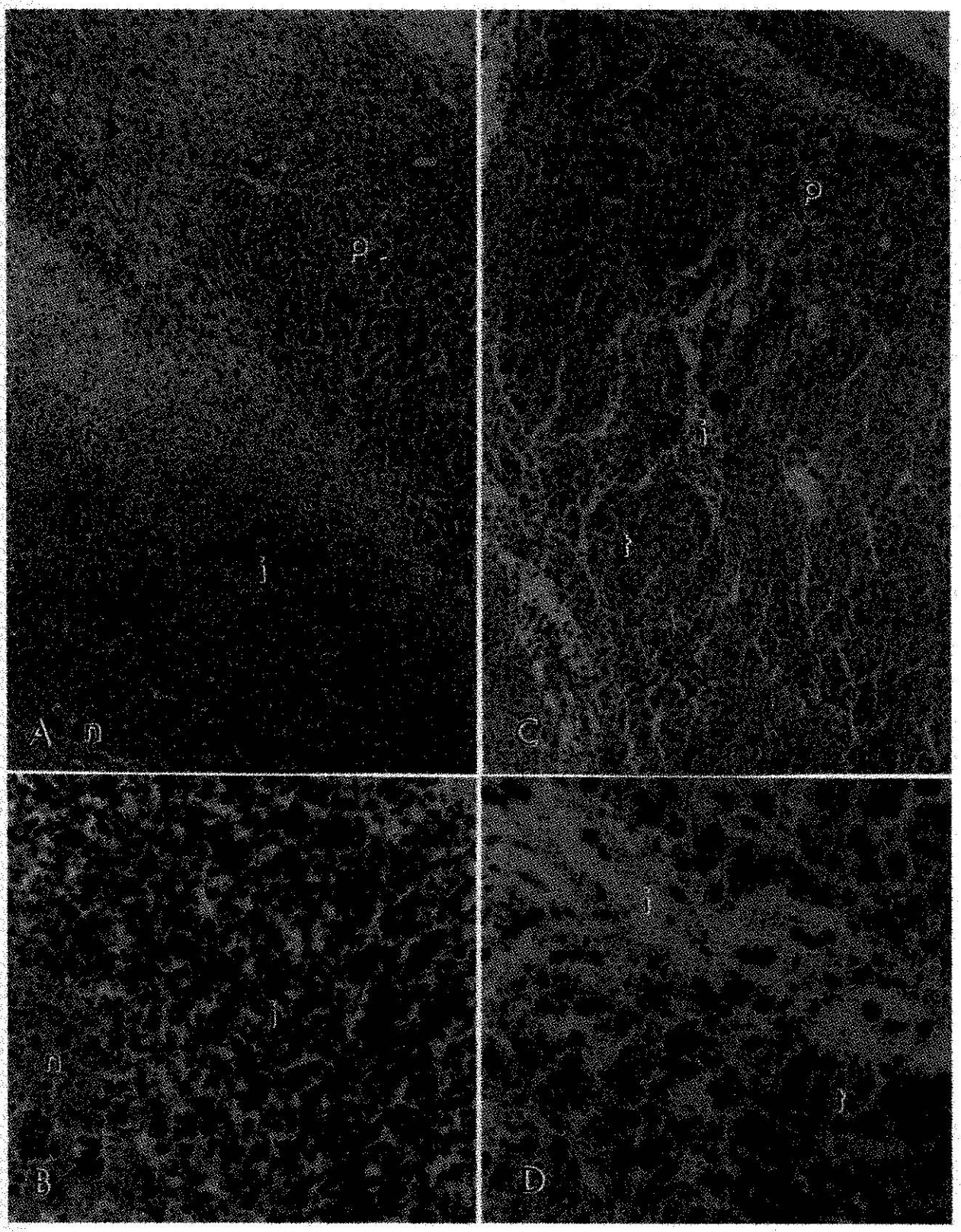

Figure 6. Histologic evaluation of VNP20009 in murine B16F10 melanoma. At low magnification (×100) of VNP20009-treated group (A), tumor was taken from mice treated with VNP20009 at 18 days after tumor implantation and 7 days after bacterial treatment. The periphery of the tumor (p) consisted with tumor cells, host fibroblasts, and infiltrating cells. A layer of infiltrating cells (i) surrounded the dead and dying tumor cells (red color), which represents the necrotic center of the tumor (n). At higher magnification (×400) of VNP20009-treated group (B), most of infiltrating cells were shown to possess horseshoe-shaped nuclei and were identified as neutrophils. In PBS-treated control mice (C), very few necrotic cells (×100) and infiltrating cells (D) were found in the tumor tissue (i) (×400).

tolerated in nonhuman primates; the highest nontoxic dose via IV injection is 2.5×10^8 cfu/kg (18,27). The probability of VNP20009 reverting to wild-type bacteria is unlikely, because VNP20009 was attenuated by deleting both the *msbB* and *purI* genes from the bacterial genome. Because of its potent inhibitory effect in a wide spectrum of human tumor xenografts and its relatively nontoxic characteristics in nonhuman primates, VNP20009 is currently being evaluated as a potential antitumor agent in humans. Moreover, VNP20009 preferentially accumulated in tumor tissues, reaching levels as high as 1×10^9 cfu/g tumor, a concentration 1000 times higher than that found in liver and other normal tissues (17). The use of VNP20009 as a tumor-selective vector to deliver cytosine deaminase to tumors is currently being evaluated in human clinical trials (29).

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