Primer Dosing of *S. typhimurium* A1-R Potentiates Tumor-Targeting and Efficacy in Immunocompetent Mice

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Abstract. *We developed the tumor-targeting strain Salmonella typhimuium A1-R (A1-R) and have shown it to be active against a number of tumor types in nude mice. However, in immunocompetent mice, dosing of A1-R has to be adjusted to avoid toxicity. In the present study, we developed a strategy to maximize efficacy and minimize toxicity for A1-R tumor-targeting in immunocompetent mice implanted with the Lewis lung carcinoma. A small primer dose of A1-R was first administered (1×10^6 colony forming unit [cfu] i.v.) followed by a high dose (1×10^7 cfu i.v.) four hours later. The primer-dose strategy resulted in smaller tumors and no observable side-effects compared to treatment with high-dose-alone. The serum level of tumor necrosis factor (TNF-α) was elevated in the mice treated with primer dose compared to mice only given the high dose. Tumor vessel destruction was enhanced by primer dosing of A1-R in immuno-competent transgenic mice expressing the nestin-driven green fluorescent protein, which is selectively expressed in nascent blood vessels. The primer-dose may activate TNF-α and other cytokines in the mouse, necessary for invasion of the tumor by the bacteria, as well as enhance tumor vessel destruction, thereby allowing a subsequent therapeutic dose to be effective and safe. The results of the present study suggest effective future clinical strategies of bacterial treatment of cancer.*

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We previously developed a strain of *Salmonella typhimurium*, termed A1, which selectively targets tumors, inhibited their growth, and, in some cases, eradicated them, in mouse models of human cancer, without overt toxicity. *S. typhimurium* A1 is auxotrophic (leu/arg-dependent), but receives sufficient nutritional support from tumor tissue. These bacteria effected PC-3 human prostate cancer growth inhibition and regression of subcutaneous xenografts in nude mice (1). To increase the tumor-targeting capability of *S. typhimurium* A1, the strain was re-isolated after infection of a human colon tumor growing in nude mice and termed *S. typhimurium* A1-R (A1-R). The selected strain A1-R had enhanced efficacy in the treatment of orthotopic mouse models of human breast cancer (2, 3), prostate cancer (4), osteosarcoma (5), pancreatic cancer (6-8), fibrosarcoma (6), lung cancer (9) and brain cancer (10), as well as spinal cord glioma (11).

A1-R destroys tumor blood vessels and this is enhanced in tumors with high vascularity (12). Leschner *et al.* (13) observed a rapid increase of tumor necrosis factor (TNF-α) in blood, in addition to other pro-inflammatory cytokines, after *S. typhimurium* treatment of tumors. This treatment induced a great influx of blood into the tumors by vascular disruption, after which bacteria were flushed into the tumor along with the blood (13).

The studies described above used nude-mouse models which are T-cell deficient. It is, however, very important to determine the anticancer efficacy of A1-R in an immunocompetent host as a bridge to application in the clinic. Bolus treatment was toxic to the immunocompetent host, in contrast to nude mice. However, lower weekly doses and metronomic doses were well-tolerated by the immunocompetent host. Weekly intravenous injection with 2×10^7 bacteria or twice a week injection with 1×10^7 bacteria significantly inhibited metastasis formation while bolus injection was toxic (9).

In the present study, a new treatment strategy was developed for bacterial tumor targeting in immunecompetent mice, whereby a small primer dose of A1-R was first administered, followed by a high-dose four hours later. The primer-dose strategy resulted in greater antitumor efficacy with no observable side-effects and thus has potential for future clinical applications.

Materials and Methods

Establishment of red fluorescent protein (RFP)-expressing Lewis lung carcinoma (LLC) cell line. For RFP gene transduction of cancer cells, 70% confluent LLC cells were used. In brief, cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67-RFP cells and RPMI-1640 medium (Irvine Scientific, Santa Ana, CA, USA) containing 10% fetal bovine serum (FBS) (Omega Scientific, San Diego, CA, USA) for 72 h. Cells were harvested with trypsin/EDTA 72 h post-transduction and subcultured at a ratio of 1:15.

Cell culture. LLC-RFP cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone Laboratories, Logan, UT, USA), supplemented with 8% FBS, 1% penicillin/streptomycin, and 250 μg/ml G418 (Invitrogen, Carlsbad, CA).

Growth of S. typhimurium A1-R for treatment. *S. typhimurium* A1-R was grown in Luria Bertani (LB) medium (Fisher Sci., Hanover Park, IL, USA) and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with phosphate buffered saline (PBS) (Omega Sci., San Diego, CA, USA) and then diluted in PBS. Bacteria were then ready for administration to mice (1, 2, 4).

Mice. Female C57/BLG mice (AntiCancer Inc., San Diego, CA, USA), age 6 weeks, were used for *S. typhimurium* A1-R efficacy studies. Nestin-driven-green fluorescent protein (ND-GFP) transgenic mice (AntiCancer Inc.) were used for tumor vessel destruction determination. Mice were fed with an autoclaved laboratory rodent diet. All animal studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance of number A3873-1.

Subcutaneous inoculation of LLC-RFP. Mice were anesthetized with a ketamine mixture (10 μl ketamine HCl, 7.6 μl xylazine, 2.4 μl acepromazine maleate, and 10 μl H_2O) *via s.c.* injection. After anesthesia, 50 μl of a suspension containing 5×10^5 LLC-RFP cells were injected subcutaneously into the left flank of C57 mice for efficacy studies or ND-GFP mice for tumor-vessel destruction studies.

Primer-dose bacterial therapy. Two weeks after inoculation, mice (n=5) bearing subcutaneous tumors, were treated with *S. typhimurium* A1-R (1×10^6 colony forming units [cfu]/200 μl PBS) *i.v. via* the tail vein as a primer dose. PBS (*i.v.*) was used as a control. Four hours after the primer dose, both control and primer dose-treated mice were treated with a high dose of *S. typhimurium* A1-R (1×10^7 cfu/200 μl PBS). Primer-dose, or PBS-only, followed by a high-dose was administered once a week for four weeks. The treatment scheme is shown in Figure 1. After four weeks administration of bacterial therapy, mice were sacrificed. Tumors were removed and their size was measured.

TNF-α. Tumor-bearing C57 mice were used for TNF-α determination. Blood samples were obtained at various time points after the *S. typhimurium* A1-R docing. TNF-α was measured with a mouse TNF-α enzyme-linked immunosobent assay (ELISA) kit (Invitrogen, Carlsbad, CA, USA).

Histology. Twenty-four hours after the high dose, mice were sacrificed. Tumors were removed and embedded in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. Frozen sections were made at 7 μm and observed by fluorescence microscopy.

Fluorescence imaging. An Olympus OV100 Small Animal Imaging System (Olympus, Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus), was used for imaging (14).

Results

Dose-limiting toxicity of S. typhimurium A1-R. Immunocompetent mice without tumors were tested for *S. typhimurium* A1-R dose-limiting toxicity after *i.v.* administration (Figure 2). In the group treated with 5×10^7 cfu/200 μl, 80% of the mice were dead by seven days after injection. In the group treated with 2×10^7 cfu/200 μl, 20% of the mice were dead by two days after injection. In the group treated with 1×10^7 cfu/200 μl, none of the mice died during the observation period. The main treatment dose of *S. typhimurium* was therefore chosen as 1×10^7 cfu/200 μl.

Efficacy of primer dosing of S. typhimurium A1-R. After four weeks' bacterial administration, tumor volumes in each group were measured. The tumor volume in the primer-dose group was significantly smaller than in the high-dose-only control group (the average tumor sizes, for n=5 mice for each group, were 2555 mm^3 for control *vs*. 685 mm^3 for the primer-dose group, $p<0.01$) (Figure 3).

Primer-dose elevated uptake of bacteria in tumors. Mice treated with the primer dose had greater uptake of bacteria in tumors than did the control group (Figure 4A). However, the uptake of bacteria in the spleen was similar in both groups (Figure 4A).

Primer-dose elevated TNF-α in serum. The serum concentration of TNF-α in the primer dose group was significantly increased 24 h after the primer-dose treatment compared to after four hours ($p<0.05$) (Figure 4B). However, there was no difference in serum TNF-α in the high-dose-only control group between four and 24 h (Figure 4B).

Administration of a primer-dose promoted destruction of vessels in tumors. In the high-dose-only control group, intact ND-GFP vessels were observed in the tumor. However, in the primer dose group, some of the ND-GFP-positive vessels were destroyed (Figure 4C).

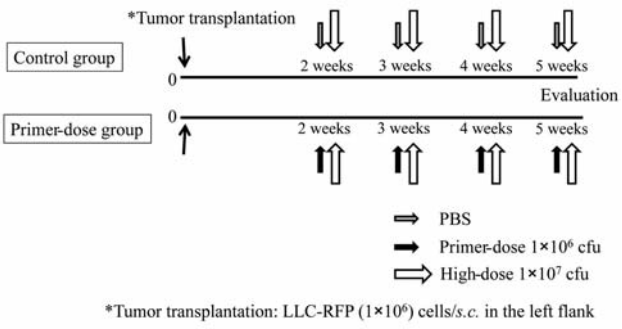

Figure 1. *Schematic representation of primer schedule of bacterial therapy. Two weeks after tumor transplantation, mice bearing tumors were distributed into two groups and received a primer-dose (1×10^6 cfu) followed by a high-dose (1×10^7 cfu), or control followed by high-dose.*

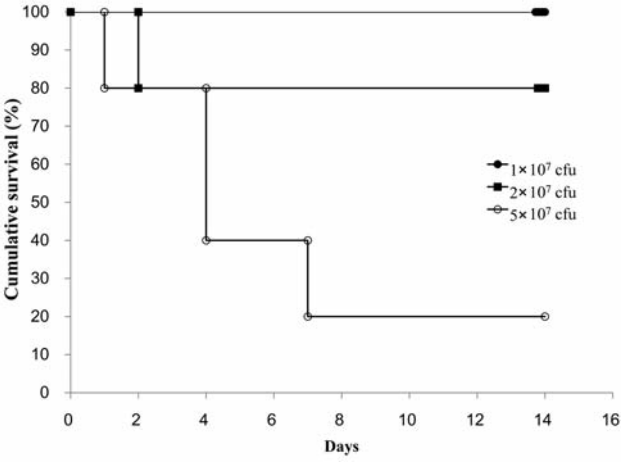

Figure 2. *Dose-limiting toxicity determination of S. typhimurium A1-R in immunocompetent. C57/BL6 mice without tumors were tested. The mice were injected intravenously with increasing doses of bacteria (1×10^7 cfu/200 μl; 2×10^7 cfu/200 μl; and 5×10^7 cfu/200 μl). The cumulative survival rates were compared within the three different groups to determine the optimal dosage of the bacteria. The treatment dose of S. typhimurium A1-R was chosen as 1×10^7 cfu/200 μl, due to lack of toxicity.*

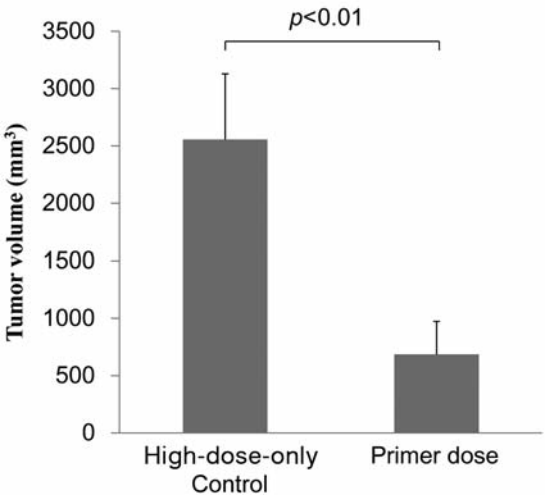

Figure 3. *Administration of a primer-dose of S. typhimurium A1-R increases its efficacy on Lewis lung carcinoma expressing red fluorescent protein (LLC-RFP) in immunocompetent mice. The tumor volumes in each group were compared. The tumor volume in the primer-dose group was significantly less than in the high-dose-only control group ($p<0.01$). Statistical analysis was performed using the Student's t-test.*

These results suggest that the primer dose can boost the efficacy of *S. typhimurium* A1-R on LLC-RFP in immunocompetent mice, by elevating tumor targeting of the bacteria, raising the serum level of TNF-α, and increasing destruction of tumor blood vessels.

Discussion

There have been reports since the early 19th century that patients with cancer who had bacterial infections sometimes had spontaneous regression of their tumors. By the end of the 19th century, Coley treated patients with cancer with bacteria and later used bacterial extracts (Coley's toxins) to treat such cancer patients. After Coley's death in 1936, bacterial treatment fell out of favor and was not allowed by the Food and Drug Administration (FDA) (3, 15).

Anaerobic bacteria can selectively grow in hypoxic and necrotic areas of tumors in animal models (16-31). Yazawa *et al*. (29, 30) showed that the anaerobic bacterium *Bifidobacterim longum* was able to selectively grow in the hypoxic regions of solid tumors. Vogelstein and co-workers (32) created a strain of *Clostridium novyi,* depleted of its lethal toxin, which grew in necrotic regions of tumors in mice and destroyed the surrounding viable tumor cells, but which needed combined chemotherapy for better efficacy (32).

S. typhimurium, a facultative anaerobe that can grow in necrotic as well as viable regions of tumors (3, 33), was previously multiply attenuated by purine and other auxotrophic mutations and was used for cancer therapy (27, 34, 35). These bacteria replicated in tumors by more than 1,000-fold compared with normal tissues (27). *Salmonella* lipid A was also genetically modified by disrupting the *msbB* gene to reduce septic shock (27). The *msbB* mutant of *S. typhimurium*, termed VNP20009, which also has additional multiple auxotrophic mutations, has been tested in a Phase 1 clinical trial to determine its safety and efficacy on metastatic melanoma (36). However, VNP20009 may have limited efficacy due to over attenuation. *S. typhimurium* A1-R has only auxotrophic mutations for leu and arg, and is thus not

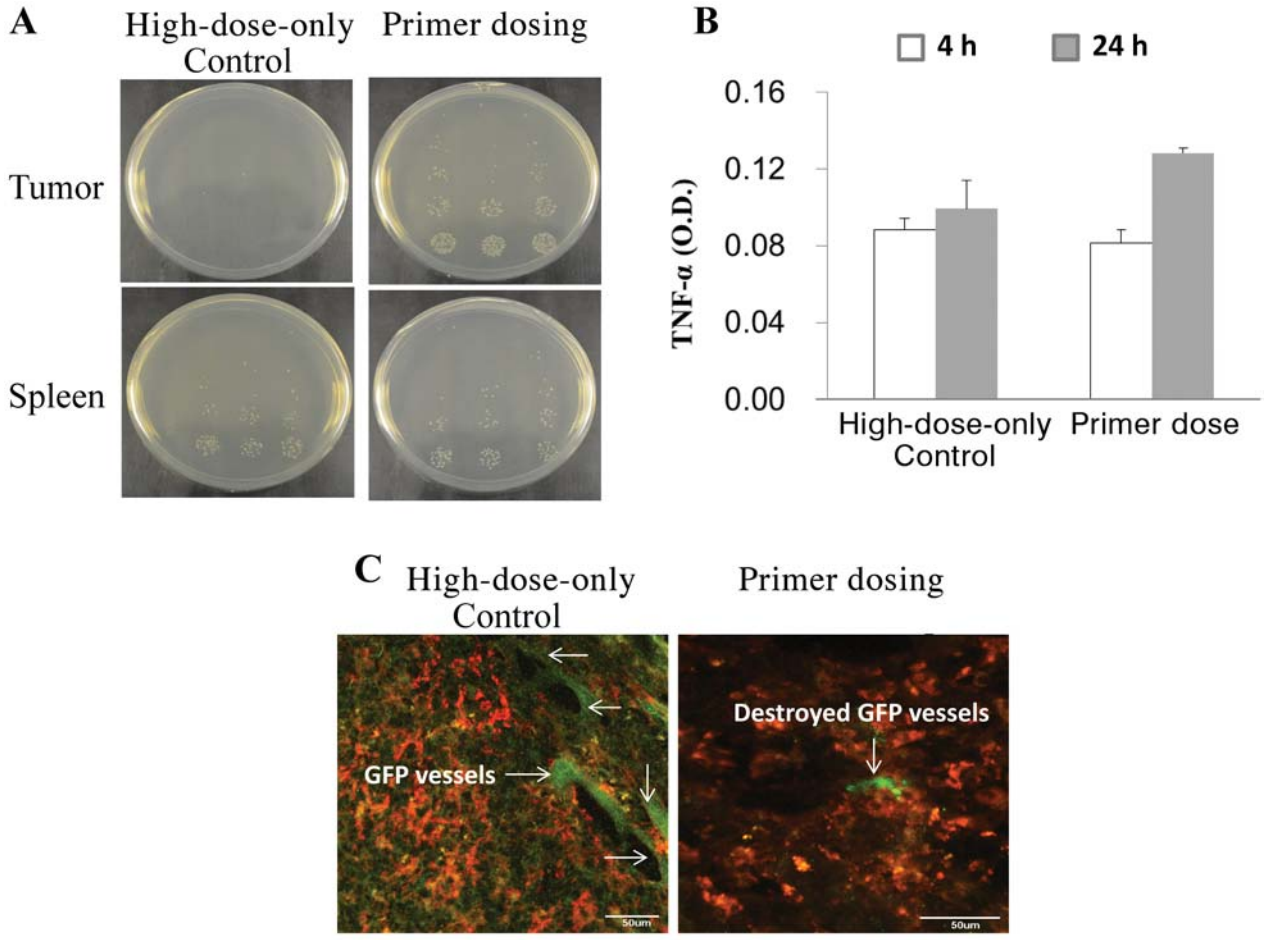

Figure 4. *Administration of a primer dose elevated uptake of bacteria in the tumor and the level of TNF-α in the serum and promoted destruction of tumor blood vessels. A: Representative images of colony assay of bacteria isolated from tumor and spleen. Mice treated with the primer dose had greater uptake of bacteria in the tumor than did those of the high-dose-only control group. B: The serum level of TNF-α was significantly increased 24 h after the primer dose compared to that after 4 h ($p<0.05$). In contrast, there was no difference in TNF-α in the high-dose-only control group between 4 and 24 h. Statistical analysis was performed using the Student's t-test. C: In the high-dose-only control group, intact blood vessels (ND-GFP) were observed in the tumor. However, in the primer-dose group, many ND-GFP-positive tumor blood vessels were destroyed. Bars=50 μm.*

over-attenuated, but does not mount a continuous infection in normal tissues. In addition, A1-R was selected for increased virulency by *in vivo* tumor passage (2).

In the present study, we developed a strategy to maximize efficacy and minimize toxicity for A1-R tumor-targeting in immunocompetent mice implanted with LLC using a small primer dose followed by a high dose. The primer-dose strategy resulted in smaller tumors and no observable side-effects compared to high-dose-alone treatment. The serum level of TNF-α was elevated in the mice treated with primer-dose compared to mice given the high-dose alone. Tumor vessel destruction was also enhanced by primer dosing. The results of the present study suggest future effective clinical strategies of bacterial treatment of cancer.

Conflicts of Interest

None of the authors have any conflicts of interest in regard to this study.

Acknowledgements

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