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Monotherapy with a tumor-targeting mutant of *Salmonella typhimurium* cures orthotopic metastatic mouse models of human prostate cancer

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Bacterial infection occasionally has a marked therapeutic effect on malignancies, as noted as early as the 19th century. Recently, there have been attempts to develop cancer treatment by using tumor-targeting bacteria. These treatments were developed to deliver therapeutic molecules specifically to tumors. Researchers used anaerobic microorganisms that preferentially grew in necrotic tumor areas. However, the resulting tumor killing was, at best, limited. We have developed a far more effective bacterial cancer therapy by targeting viable tumor tissue by using *Salmonella typhimurium* leu-arg auxotrophs. Although these bacteria grow in viable as well as necrotic areas of tumors, the nutritional auxotrophy severely restricts growth in normal tissue. In the current study, we measured the antitumor efficacy of the *S. typhimurium* A1-R mutant, which is auxotrophic for leu-arg and has increased antitumor virulence selected by tumor passage. A1-R was used to treat metastatic PC-3 human prostate tumors that had been orthotopically implanted in nude mice. GFP was used to image tumor and metastatic growth. Of the 10 mice with the PC-3 tumors that were injected weekly with *S. typhimurium* A1-R, 7 were alive and well at the time the last untreated mouse died. Four A1-R-treated mice remain alive and well 6 months after implantation. Ten additional nontumor-bearing mice were injected weekly to determine the toxicity of *S. typhimurium* A1-R. No toxic effects were observed. The approach described here, where bacterial monotherapy effectively treats metastatic prostate tumors, is a significant improvement over previous bacterial tumor-therapy strategies that require combination with toxic chemotherapy.

In the 1890s, Coley observed that certain cancer patients with postoperative bacterial infection were cured of their tumors (1). It has been known since the 1940s that anaerobic bacteria can selectively grow in hypoxic and necrotic areas of tumors (2–17). Recently, a number of novel approaches to developing tumor-therapeutic bacteria have been described.

Yazawa *et al.* (18) demonstrated that the anaerobic bacterium *Bifidobacterium longum* could selectively germinate and grow in the hypoxic regions of solid tumors after i.v. injection. *B. longum* is a nonpathogenic Gram-positive bacterium found in the lower small intestine and large intestine of humans and other mammals. Yazawa *et al.* showed that *B. longum* selectively grew in 7,12-dimethylbenzanthracene-induced rat mammary tumors after i.v. administration.

Vogelstein *et al.* (19) compared *B. longum* and *Clostridium novyi*, an obligate anaerobic, for their capacity to grow within transplanted tumors. Vogelstein *et al.* created a strain of *C. novyi* depleted of its lethal toxin termed *C. novyi* NT. They showed that i.v.-administered *C. novyi* NT spores germinated within the avascular regions of tumors in mice and destroyed surrounding viable tumor cells (19). *C. novyi* NT grew to a greater extent in the B16 melanoma than *B. longum*. When *C. novyi* NT spores were administered together with conventional chemotherapeutic drugs or radioactivity, extensive hemorrhagic necrosis of tumors often developed within 24 h, resulting in significant and prolonged antitumor effects (19, 20). Vogelstein *et al.* (1, 20) subsequently observed that *C. novyi* NT treatment of tumors elicits an immune response that can, in combination with chemotherapy, result in tumor cure by using syngeneic models with immunocompetent mice.

In another approach, attenuated *Salmonella typhimurium* strains, which are facultative anaerobes, have been described as anticancer agents (21). *Salmonella* strains have been shown to preferentially amplify within tumors and express prodrug-converting enzymes such as the herpes simplex thymidine kinase (21). Initially, *S. typhimurium* was attenuated by auxotrophic mutations (22, 23). The internal tumor environment apparently meets the auxotrophic requirements of the mutated *Salmonella*, where they replicated 1,000 times the concentration found in normal tissues (21). Within the tumor, there may be areas of hypoxia that favor the growth of facultative anaerobes. In addition, the presence of tumor necrosis provides additional nutrients, such as purines, required by the organism. Genetic modification of the *Salmonella* lipid A was done to reduce septic shock (21). Disruption of the *Salmonella* msbB gene reduced TNFα induction and increased the LD₅₀ of *Salmonella* by 10,000-fold (21). The *msbB* mutant prevents the addition of a terminal myristyl group to the lipid A domain (21). Melanomas in mice treated with this *Salmonella* were 6% the size of tumors in untreated controls (23). Attenuated strains of *Salmonella* have also been modified to secrete immunostimulatory cytokines that may have the potential to stimulate host antitumor responses (24).

Recently, the lipid A-attenuated *S. typhimurium* has been evaluated in a phase I clinical trial (25). Twenty-four patients with metastatic melanoma and one patient with metastatic renal cell carcinoma received i.v. bolus infusions containing 10⁶ to 10⁹ cfu/m² of the lipid A-mutant *Salmonella* (VNP20009). To increase safety and reduce toxicity, *S. typhimurium* was attenuated by deletion of both the *purF* and *msbB* genes. The *purF* mutant requires an external source of adenine (25). The resulting VNP20009 strain of *S. typhimurium* could be safely administered to patients. At the highest tolerated dose, tumor colonization was observed. Additional studies are required to reduce dose-related toxicity and improve tumor localization (25) to obtain efficacy.

To increase the potential of attenuated *Salmonella* as a gene-delivery vector for cancer treatment, Mengesha *et al.* (26) developed a hypoxia-inducible promoter (HIP-1) to limit gene expression specifically to the tumor. Mengesha *et al.* demonstrated that HIP-1 can drive hypoxia-mediated gene expression...
We initially developed an S. typhimurium strain, A1, which grows in tumor xenografts. In sharp contrast, normal tissue rapidly clears these bacteria even in immunodeficient athymic mice. S. typhimurium A1 is auxotrophic (leu/arg-dependent), but apparently receives sufficient support from the necrotic tissue to grow locally. In vivo, the bacteria caused PC-3 tumor inhibition and regression of s.c. xenografts (31) visualized by whole-body imaging. By day 15, S. typhimurium A1 was undetectable in the liver, lung, spleen, and kidney, but it continued to proliferate in the PC-3 tumor, which stopped growing. The S. typhimurium A1 strain grew throughout the tumor, including viable malignant tissue, which may account, in part, for the strain’s unique antitumor efficacy (31). This result is in marked contrast to results with anaerobic bacteria that grew only in tumor-necrotic areas.

To increase the tumor-targeting capability of S. typhimurium A1, the strain was reisolated after infection of a human colon tumor growing in nude mice. The tumor-isolated strain, A1-R, had increased targeting for tumor cells in vivo as well as in vitro compared to A1. Treatment with A1-R resulted in highly effective tumor targeting, including viable tumor tissue and significant tumor shrinkage in mice with s.c. or orthotopic human breast cancer xerographs. Survival of the treated animals was significantly prolonged (32).

In the present study, we report that S. typhimurium A1-R, administered systemically, can cure metastatic human prostate cancer in orthotopic mouse models, suggesting the clinical potential of this cancer-therapeutic approach.

Results

Anti-Tumor Virulence of S. typhimurium A1-R in Vitro. Dual-color PC-3 tumor cells, growing on plates, were labeled with GFP in the nucleus and RFP in the cytoplasm. Fluorescence microscopy showed their interaction with the GFP-expressing S. typhimurium A1-R. The bacteria attached to the cells within 30 min. The infected cells initially rounded up. At 60 min after infection, cell membranes appeared broken, and the cytoplasm had begun to fragment. The nucleus also fragmented, and by 200 min the red fluorescent cytoplasm completely dispersed (Fig. 1).

Primary Tumor Growth Suppression in Vivo. PC-3 tumor cells were transplanted orthotopically into nude mice. In the first experiment, the animals were injected with A1-R at day 16 after tumor...
transplantation. The second injection of A1-R was given 2 weeks later. Tumor growth in the treated animals slowed compared to the untreated group (Fig. 2). However, after these two doses of A1-R, the tumors resumed growth and eventually killed the animals.

In a second experiment, tumor-bearing animals were inoculated with bacteria weekly, and efficacy was enhanced dramatically (Fig. 3). At day 30 after tumor transplantation, the tumor size in the treated group was $\sim$75% smaller than the untreated group ($n = 10; P = 0.04$). (B) Imaging of tumor growth in eight mice of the untreated group at day 30. (C) Imaging of tumor growth of 10 mice in the A1-R-treated group at day 30.

**Metastasis Suppression.** The PC-3 tumor orthotopic model is highly metastatic. Metastasis occurred by day 21 after tumor transplantation in the untreated animals. By day 30, metastases spread to the peritoneal cavity and other organs, and the mice were very sick. In contrast, tumors in the treated group grew slowly or disappeared (Fig. 3 B and C). Most untreated animals died of metastatic disease at 4 to 5 weeks after tumor transplantation. However, in the weekly treated group, metastasis was suppressed (Fig. 4). Treating animals only twice initially inhibited tumor growth, but tumor growth later resumed, and metastasis eventually resulted in the animals’ death. These data show the importance of weekly treatment.

**Survival Efficacy.** The PC-3 tumor orthotopic model results in rapid death of untreated animals. All 10 untreated mice with orthotopic PC-3 tumors died within 6 weeks of tumor implantation (Fig. 5A). However, weekly bacteria treatment greatly prolonged survival of the tumor-bearing mice. Of the 10 mice with the orthotopic PC-3 tumors treated with A1-R, seven were alive at the time the last untreated mouse died, and four eventually appeared cured.

**Cures.** Forty percent of the tumor-bearing mice apparently cured of detectable disease by weekly bacteria treatment were examined further (Fig. 5A). Tumors and metastases were imaged in real time to determine cures. It took 10 to 12 weekly injections of bacteria to completely cure the mice. After treatment stopped, tumors did not recur. In contrast, after two injections, only 1 of 10 mice was cured (Fig. 5B). Further study will examine the effects of different protocols.

**Safety of Bacterial Treatment.** Nontumor-bearing mice were treated with weekly injections of bacteria to establish the toxicity of bacteria treatment. Body weight and survival time were monitored with no deaths observed. The weekly bacteria treatment had no effect on body weight and no apparent toxicity (Fig. 6). These results indicated that the animals well tolerated the weekly infection with *S. typhimurium* A1-R.
Discussion

From the results described above, the following conclusions can be reached:

1. PC-3 human prostate tumor growth and metastasis in nude mice were targeted by i.v.-administered *S. typhimurium* A1-R, a leu-arg auxotroph.
2. A1-R treatment of PC-3 tumors resulted in a doubling of the 50% survival time of the treated mice.
3. Four of the 10 A1-R-treated mice with PC-3 orthotopic tumors were cured.
5. A1-R treatment resulted in cures of metastatic cancer without combination with toxic chemotherapy, which distinguishes our approach to bacterial therapy of tumors by using a facultative anaerobic from those using obligate anaerobes such as Clostridia.

Future experiments will involve designing bacteria focusing on tumor-environment-specific gene expression of the infecting bacteria. The particular auxotroph, A1-R, although effective, may not be optimum for all tumor types, and we will examine further mutations. We will eventually consider whether therapy can be individualized by tailoring bacteria to target individual patient tumors.

Materials and Methods

**GFP Gene Transfection of S. typhimurium** (31). *S. typhimurium* (ATCC 14028) was grown at 37°C to midlogarithmic phase in liquid LB and harvested at 4°C. Bacteria (2.0 x 10^9) in 40 µl of 10% glycerol were mixed with 2 µl of the pGFP (Clontech, Mountain View, CA) vector containing the hr-GFP gene (Stratagene, La Jolla, CA) and placed on ice for 5 min before electroporation with a Gene Pulser apparatus (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Electroporation was done at 1.8 kV with the pulse controller at 1.000-Ω parallel resistance.

**Induction of Bacterial Mutations with Nitrosoguanidine (NTG) and Selection for Auxotrophs** (31). Freshly prepared NTG (1 mg/ml in sterile water) was added to the washed culture to a final concentration of 100 µg/ml in Tris-maleic acid buffer (pH 6.0). The bacteria were incubated with NTG for 30 min. The NTG-treated cells were grown in nutrient broth to express any mutations that were induced. Bacterial colonies were replica-plated in supplemented minimal agar plates containing specific amino acids to identify the requirements of the auxotrophs. Auxotroph A1, which required leu and arg, was identified.

**Reisolation of S. typhimurium A1** (32). S. *typhimurium* A1 auxotrophs expressing GFP were reisolated as follows: The A1 bacteria were injected into the tail vein of an HT-29 human colon tumor-bearing nude mouse. Three days after infection, the tumor tissue was removed from the infected mouse. The tumor tissue was then homogenized and diluted with PBS. The resulting supernatant of the tumor tissue was cultured in LB agar plates at 37°C overnight. The bacteria colony with the brightest green fluorescence was picked up and cultured in 5 ml of LB medium. This strain was termed “A1-R.”

**Preparation of Bacteria** (32). The A1-R bacteria were grown overnight on LB medium and then diluted 1:10 in LB medium. Bacteria were harvested at late-log phase, washed with PBS, and then diluted in PBS. Bacteria were then injected into the tail vein of nude mice (5 x 10^7 cfu per 100 µl PBS).

**RFP or GFP Gene Transduction of the PC-3 Human Prostate Cancer Cell Line** (31). For RFP or GFP gene transduction, ~60% confluent PC-3 human prostate cancer cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 packaging cells and F12K medium containing 7% FBS (Gemini, Calabasas, CA) for 72 h. The packaging cells produced the pLNCX2 vector (Clontech) containing the DsRed2-expressing RFP or the pLEIN vector containing GFP and the neomycin resistance gene. GFP- or GFP-expressing PC-3 cancer cells were selected in medium containing 200 to 1,000 µg/ml neomycin.

**Fig. 5.** Survival efficacy of *S. typhimurium* A1-R. (A) Survival efficacy of *S. typhimurium* A1-R with weekly treatment (n = 10). (B) Survival efficacy of *S. typhimurium* A1-R with twice-only treatment (n = 10).

**Fig. 6.** Toxicity of *S. typhimurium* A1-R. Nontumor-bearing mice were tested for the possible toxicity of *S. typhimurium* A1-R. The mice were injected i.v. weekly with the bacteria (5 x 10^7 cfu). The survival time and body weight were compared with the untreated mice. A1-R-infected animals treated with repeated weekly injections of *S. typhimurium* A1-R survived as long as untreated mice with no significant body weight change.
increased in a stepwise manner. Clones expressing RFP or GFP were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ) by trypsin/EDTA and were amplified and transferred by conventional culture methods in the absence of selective agent.

Establishment of Dual-Color Cells Expressing GFP and RFP. For establishing dual-color cells, PC-3-RFP cells were then incubated with a 1:1 precipitated mixture of retroviral supernatants of PT67 packaging cells expressing GFP linked to histone H2B (33) and culture medium. The histone H2B-GFP fusion gene was inserted into the pLHCX vector (Clontech) that also contains the hygromycin resistance gene. To select the double transfectants, cells were incubated with hygromycin 2 h after transfection. The level of hygromycin was increased stepwise up to 400 μg/mL. These sublines stably expressed GFP in the nucleus and RFP in cytoplasm.

Surgical Orthotopic Implantation of Prostate Tumors. Tumor fragments (1 mm³) were prepared from a PC-3-GFP tumor growing s.c. in nude mice. Two tumor fragments were implanted by surgical orthotopic implantation in the lateral lobe of the prostate, which was exposed after a lower midline abdominal incision. After proper exposure of the bladder and prostate, the capsule of the prostate was opened and the two tumor fragments (1 mm³) were inserted into the capsule. The capsule was then closed with an 8–0 surgical suture in one layer. The animals were kept under isoflurane anesthesia during surgery. The wall was closed with a 6–0 surgical suture in one layer. The incision in the abdominal wall was closed with an 8–0 surgical suture. The incision in the abdominal cavity was measured.

Bacterial Infection. When the implanted tumors were externally imaginable, 10 mice were administered S. typhimurium A1-R (5 × 10⁷ i.v. S. typhimurium A1-R i.v. weekly, and survival time was compared above. Ten of the 20 mice served as controls and were followed until death without treatment. Ten mice were administered S. typhimurium A1-R i.v. weekly, and survival time was compared to the untreated mice. Progressive tumor enlargement and metastasis were externally imaged by their GFP fluorescence.

Host Safety. Ten nontumor-bearing mice served as controls for the possible toxicity of S. typhimurium A1-R. The mice were weekly injected i.v. with the bacteria (5 × 10⁷ cfu). Survival time after bacteria infection was monitored and body weight was measured.

Imaging in Live Mice. The Olympus OV100 Small Animal Imaging System (Olympus) containing an MT-20 light source (Olympus) and DF70 CCD camera (Olympus) was used for imaging in live mice. The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging as well as microimaging with high light-gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Five individually optimized objective lenses, parcentered and parfocal, provide a 10⁴-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has the lenses mounted on an automated turret with a high magnification range of ×1.6 to ×16 and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images were captured directly on a PC (Fujitsu Siemens, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 and Cell (Olympus) (33).

Analysis of Antitumor Efficacy. Twenty mice were implanted orthotopically with PC-3 tumors expressing GFP as described above. Ten of the 20 mice served as controls and were followed until death without treatment. Ten mice were administered S. typhimurium A1-R i.v. weekly, and survival time was compared to the untreated mice. Progressive tumor enlargement and metastasis were externally imaged by their GFP fluorescence.