PART I

LIVE/ATTENUATED BACTERIA AND VIRUSES AS ANTICANCER AGENTS

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SALMONELLA TYPHIMURIUM MUTANTS SELECTED TO GROW ONLY IN TUMORS TO ERADICATE THEM IN NUDE MOUSE MODELS

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INTRODUCTION

The use of bacteria in cancer therapy dates back to Coley in the 1890s, when he observed that streptococcal (erysipelas) infection was associated with regression of soft tissue sarcomas. Coley then reported on the efficacy of infecting erysipelas in a series of 10 sarcoma patients (1). Coley's pioneering work was the basis for modern studies using bacteria to treat cancer. In the middle part of the last century, Malmgren et al. (2) showed that anaerobic bacteria had the ability to survive and replicate in necrotic tumor tissue with low oxygen content. Several approaches aimed at utilizing bacteria for cancer therapy have subsequently been described (3–15).

Bifidobacterium longum, an obligate anaerobe, has been shown to selectively grow in hypoxic regions of tumors following i.v. administration. This effect was demonstrated in 7,12-dimethylbenzanthracene-induced rat mammary tumors by Yazawa et al. (14, 15) as well as the Lewis lung carcinoma. Vogelstein et al. (16) created a strain of *Clostridium novyi*, also an obligate anaerobe, which was depleted of its lethal toxin. This strain of *C. novyi* was termed *C. novyi* NT. Following i.v. administration, the *C. novyi* NT spores germinated in the

Emerging Cancer Therapy: Microbial Approaches and Biotechnological Tools, Edited by Arsénio M. Fialho and Ananda M. Chakrabarty
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avascular regions of tumors in mice, causing damage to the surrounding viable tumor (16). Combined with conventional chemotherapy or radiotherapy, intravenous *C. novyi* NT spores caused extensive tumor damage within 24 h (16).

Following attenuation by purine and other auxotrophic mutations, the facultative anaerobe *Salmonella typhimurium* was used for cancer therapy (11, 17, 18). These genetically modified bacteria replicated in tumors to levels more than 1000-fold greater than in normal tissue (11). *S. typhimurium* was further modified genetically by disrupting the *msb*B gene to reduce the incidence of septic shock (11). The *msb*B mutant of *S. typhimurium* has been tested in a Phase I clinical trial to determine its efficacy on metastatic melanoma and metastatic renal cell carcinoma (19). To raise the therapeutic index, *S. typhimurium* was further attenuated by deletion of the *pur*I and *msb*B genes (19). The new strain of *S. typhimurium*, termed VNP20009, could then be safely administered to patients (19). More studies are needed to completely characterize the safety and efficacy of the bacteria and to improve its therapeutic index.

Mengesha et al. utilized *S. typhimurium* as a vector for gene delivery by developing a hypoxia-inducible promoter (HIP-1) to limit gene expression to hypoxic tumors. HIP-1 was able to drive gene expression in bacteria residing in human tumor xenografts implanted in mice (20). Genes linked to the HIP-1 promoter showed selective expression in tumors (20). Yu et al. used green fluorescent protein (GFP)-labeled bacteria to visualize tumor targeting abilities of three pathogens: *Vibrio cholerae*, *S. typhimurium*, and *Listeria monocytogenes* (21, 22).

Targeted Therapy with a *S. typhimurium* Leucine–Arginine-Dependent Strain

We initially developed a mutant of *S. typhimurium*, termed A1, which selectively targeted tumors in nude mouse models (23). In contrast, normal tissue was rapidly cleared of infecting bacteria, even in immunodeficient athymic mice. *S. typhimurium* A1 is auxotrophic (leu/arg dependent) but receives sufficient nutritional support from tumor tissue. After inoculation with wild-type *S. typhimurium*, the mice died within 2 days. The longest lived mice were those inoculated with auxotroph A1 which survived as long as control uninfected mice. The bacteria were labeled with GFP so that their tumor-targeting efficacy could be imaged *in vivo*.

To observe the intracellular replication and virulence of *S. typhimurium*-GFP in a human prostate cancer cell line *in vitro*, the PC-3 human prostate cancer cells were labeled with red fluorescent protein (RFP) in the cytoplasm with retroviral RFP and GFP in the nucleus by means of a vector with GFP fused to histone H2B. The dual-color cancer cells and GFP bacteria have enabled visualization of the interaction between bacteria and cancer cells by fluorescence imaging. The quantitative ability of *S. typhimurium* to kill prostate cancer cells was determined with the MTT method and observed to be dose dependent (23).

Efficacy of *S. typhimurium* A1 on Human Prostate Cancer Growing Subcutaneously in Nude Mice

To observe the interaction of prostate cancer cells with bacteria, we used PC-3 human prostate cancer cell line expressing RFP, so their response to the bacteria could be visualized *in vivo*. To evaluate efficacy of *S. typhimurium* A1, 10 NCR nude mice, 6–8 weeks, were implanted subcutaneously (s.c.) on the mid-right side with 2×10^6 RFP-labeled PC-3 human prostate cancer cells. Bacteria were grown and harvested at late log phase and then diluted in PBS and injected directly into the tail vein (5×10^7 cfu/100 μL PBS). Tumor size was determined from fluorescence imaging at each time point after infection. *S. typhimurium* A1 selectively colonized the PC-3 tumor and suppressed its growth (24).

Isolation of a More Tumor-Virulent Strain of *S. typhimurium* A1, Termed A1-R

To enhance tumor virulence, *S. typhimurium* A1 has been passaged by injection in nude mice transplanted with the HT-29 human colon tumor. Bacteria, expressing GFP, isolated from the infected tumor, were then cultured. The reisolated A1 was termed A1-R. The ability of A1-R to adhere to tumor cells was evaluated in comparison with the parental A1 strain *in vitro*. The number of A1-R bacteria attached to HT-29 human colon cancer cells was approximately six times higher than parental A1.

The virulence of GFP-labeled *S. typhimurium* A1 and A1-R in human prostate cancer cells was compared *in vitro* under fluorescence microscopy. Both strains infected dual-color PC-3 cancer cells. Whereas almost all cells were infected and dead after 2 h with A1-R, it took 24 h to get the same result with A1. Thus, the virulence of A1-R was greatly increased (25).

A1-R Has Enhanced Virulence against Prostate Cancer in Nude Mouse Models

GFP-labeled *S. typhimurium* A1 and A1-R (5×10^7 cfu/100 μL) were administered (i.v.) to PC-3-bearing nude mice. The biodistribution of the bacteria in tumor tissue was determined at day 4. A1-R had 100 times greater colony-forming units in PC-3 tumor tissue than A1. This result also suggested that A1-R has greater tumor virulence than A1 (25).

To compare bacterial infection in the tumor with infection in normal tissue, A1-R bacteria (5×10^7 cfu/100 μL) were administered intravenously in PC-3-bearing nude mice. On day 4 after injection, the tumor, liver, and spleen were removed. The tissues were homogenized and plated on LB agar plates. After overnight growth at 37°C, the colony-forming units were counted. The ratio of tumor to normal tissue was approximately 10^6, indicating a very high degree of tumor targeting by A1-R (25).

Efficacy of A1-R on Breast Tumor Growth in an Orthotopic Nude Mouse Model

Treatment with A1-R resulted in significant efficacy in nude mice with s.c. MARY-X human xenografts. Bacteria (5×10^7 cfu/100 µL) were inoculated intravenously in MARY-X-bearing nude mice. Tumor growth was monitored by caliper measurement in two dimensions. The infected tumors regressed by day 5 after infection, and complete regression occurred by day 25. In orthotopic models of MARY-X, A1-R was also efficacious following a single i.v. injection. The destruction of the tumor in treated mice was visualized by whole-body imaging. The difference in tumor volume between the treated group, which showed quantitative regression, and the control was statistically significant ($p < 0.05$) (24).

The survival of the A1-R-treated animals was prolonged with a 50% survival time of 13 weeks compared with 5 weeks of control animals. Forty percent of the mice survived as long as the control non-tumor-bearing mice. Tumors that were eradicated did not regrow. In contrast, the parental *S. typhimurium* A1 was less effective than A1-R. Tumor growth was only slowed after A1 i.v. injection and not eradicated (24).

Treatment of an Orthotopic Human Pancreatic Tumor in Nude Mice with *S. typhimurium* A1-R

A1-R GFP could invade and replicate intracellularly *in vitro* in XPA1 human pancreatic cancer cells expressing GFP in the nucleus and RFP in the cytoplasm. Intracellular bacterial infection led to cell fragmentation and cell death (26).

On day 0, XPA1 was transplanted on the pancreas of nude mice. On day 7, the tumor was exposed and observed by fluorescence imaging. Tumor size was measured by fluorescent area (mm^2). Three mice were treated with a low concentration of A1-R (10^7 cfu/mL); three were treated with a high concentration (10^8 cfu/mL); and three were used as untreated controls. Tumor volume (mm^3) was calculated with the formula $V = ½ \times (\text{length} \times \text{width}^2)$. The bacteria were injected into the tumor. On day 14, the tumor was exposed again, and the size was measured to determine the efficacy of treatment (26).

Before treatment, the average tumor size (fluorescent area) on day 7 was $3.2 \pm 1.9\,mm^2$ in the untreated group, 3.1 ± 1.4 in the high-bacteria-dose group, and 3.5 ± 0.75 in the low-bacteria-dose group. On day 14, after 7 days of treatment, the tumor fluorescence area was $19.9 \pm 4.3\,mm^2$ in the untreated group, 2.2 ± 0.89 in the high-bacteria-concentration treatment group, and 12.7 ± 6.5 in the low-bacteria-concentration treatment group (26).

We have also demonstrated the efficacy of locally as well as systemically administered A1-R on liver metastasis of pancreatic cancer. Mice treated with A1-R, given locally via intrasplenic injection or systemically via tail vein

injection, had a much lower hepatic and splenic tumor burden as compared to control mice (27). Systemic treatment with intravenous A1-R also increased survival time. All results were statistically significant.

Experimental Lymph Node Metastasis Cured by Specific Targeting by *S. typhimurium* A1-R

A new experimental model of lymph node metastasis was developed. To obtain an experimental metastasis in the axillary lymph node, XPA1-RFP human pancreatic cancer cells were injected into the inguinal lymph node in nude mice. Just after injection, cancer cells were imaged trafficking in the efferent lymph duct to the axillary lymph node. Metastasis in the axillary lymph node was subsequently formed. A1-R bacteria were then injected into the inguinal lymph node to target the axillary lymph node metastasis. Just after bacterial injection, a large amount of bacteria were visualized around the axillary lymph node metastasis. By day 7, all lymph node metastases had been eradicated in contrast to growing metastases in the control group. There were very few bacteria in the lymph node by day 7, and no bacteria were detected after day 10. This route of administration was therefore able to deliver sufficient bacteria to eradicate the lymph node metastasis after which the bacteria became undetectable. The average tumor size (fluorescent area) in the axillary lymph nodes on day 0 was $0.4 \pm 0.19\,mm^2$ in the treatment group and 0.46 ± 0.08 in the untreated group, respectively. On day 7, it was $0\,mm^2$ in the treatment group and 0.98 ± 0.17 in the untreated group (28).

We then tested bacterial therapy strategy for spontaneous lymph node metastasis from a fibrosarcoma tumor growing in the footpad. At first, only A1-R bacteria were injected in the footpad in nude mice in order to determine any adverse effects. No infection, skin necrosis, body weight loss, or animal death was observed. Then, HT-1080-GFP–RFP human fibrosarcoma cells were injected into the footpad of additional nude mice. The presence of popliteal lymph node metastasis was determined by weekly imaging. Once the metastasis was detected, A1-R bacteria were injected s.c. in the footpad. Bacteria are small particles, and when injected s.c., the lymph system immediately collects them from the site of injection. The lymph system is well known as a drainage route for bacterial infection. We observed the injected bacteria trafficking in the lymphatic channel. The popliteal region was exposed just after bacteria injection, and a large amount of GFP bacteria targeting the popliteal lymph node metastasis was observed by fluorescence imaging. Dual-color labeling of the cancer cells distinguished them from the GFP bacteria. After treatment, the popliteal lymph node was observed every week by fluorescence imaging. One mouse was used to image the bacteria by exposing the popliteal lymph node on day 7. GFP bacteria invading the lymph node metastasis were observed. All lymph node metastases shrank, and five out of six were eradicated within 7–21 days after treatment in contrast to growing metastases in the control group (28).

S. typhimurium A1-R Therapy for Experimental Lung Metastasis

To obtain experimental lung metastasis, HT-1080 GFP-RFP cells were injected into the tail vein of experimental nude mice (day 0). On day 4 and day 11, A1-R bacteria were injected into the tail vein. On day 16, all animals were sacrificed, and the lungs were imaged to determine the efficacy of bacteria therapy on lung metastasis. To observe the lung metastasis at lower magnification, an RFP filter was used (excitation 545 nm, emission 570–625 nm). In the bacterial treatment group, only a few cancer cells were observed in contrast to multiple metastases in the control (untreated) group. The number of meta stases on the surface of the lung was significantly lower in the treatment group than in the control group ($p < 0.005$). There were no significance differences between the treated and untreated groups in body weight and primary tumor size (28).

Targeting of Primary Bone Tumor and Lung Metastasis of High-Grade Osteosarcoma in Nude Mice with *S. typhimurium* A1-R

Mice were transplanted with 143B-RFP human osteosarcoma cells in the tibia and developed primary bone tumor and lung metastasis. Seven days after tumor injection, RFP tumor was confirmed inside the tibia. After three weekly injections of bacteria, the bone tumor size and lung metastasis were examined on day 28. The bone tumor size (RFP fluorescence area) was $231.7 \pm 70\,mm^2$ in the untreated group and $94.6 \pm 23\,mm^2$ in the treated group ($p < 0.05$). The lung was excised, and the metastases on the surface were counted. The number of metastasis was 52 ± 30 in the untreated group and 2.3 ± 2.1 in the treated group ($p < 0.05$). *S. typhimurium* A1-R therapy was therefore effective for primary and metastatic osteosarcoma (29).

Targeted Therapy of Spinal Cord Glioma with *S. typhimurium* A1-R

Spinal cord tumors are highly malignant and often lead to paralysis and death mainly due to their infiltrative nature, high recurrence rate, and limited treatment options. In this study, we measured the antitumor efficacy of *S. typhimurium* A1-R, administered systemically or intrathecally, to spinal cord cancer in orthotopic nude mouse models. Tumor fragments of human U87-RFP glioma were implanted by surgical orthotopic implantation into the dorsal site of the spinal cord. Five and 10 days after transplantation, eight mice in each group were treated with A1-R (2×10^7 cfu/200 µL i.v. or 2×10^6 cfu/10 µL intrathecal injection). The untreated mice showed progressive paralysis beginning 6 days after tumor transplantation and developed complete paralysis between 18 and 25 days. The mice treated intravenously with A1-R had an onset of paralysis at approximately 11 days and at day 30, five mice developed complete paralysis, while three other mice had partial paralysis. Mice treated via intrathecal injection of A1-R had an onset of paralysis at approximately 18 days, and one mouse was still not paralyzed at day 30. Only one mouse

developed complete paralysis at day 30 in the intrathecal treatment group. The intrathecally treated animals had a significant increase in survival over the i.v.-treated group as well as the control group (30).

Screening for *Salmonella* Promoters Differentially Activated in the PC-3 Prostate Tumor

We have used a high-throughput method to screen for *S. typhimurium* promoters that are selectively activated in tumors in the mouse. A random library of *S. typhimurium* with DNA cloned upstream of a promoterless GFP were injected intravenously in nude mice with s.c. human PC-3 prostate tumors as well as control nude mice. GFP-positive *S. typhimurium* clones from tumor, spleen, and liver, and *in vitro* growth in LB medium, were isolated by fluorescence-activated cell sorting (FACS). Active promoters in all environments were amplified by PCR and identified by DNA microarray hybridization. Among promoters identified as preferentially induced in tumors, and not induced in any of the other environments (spleen, liver, or *in vitro*), were those of at least five genes known to be controlled by the fumerate and nitrate reduction global regulator (FNR). At least five other genes with unknown regulation were also enriched in tumors. The natural tendency of *S. typhimurium* to target tumors preferentially over other tissues, combined with the use of promoters preferentially induced in the tumor environment versus other environments, may allow the exquisitely tumor-specific expression of fusion proteins on the surface or secreted by *S. typhimurium* for highly selective tumor therapy (31,32).

CONCLUSION

Our goal is to develop tumor-targeting *S. typhimurium* strains that can kill primary and metastatic cancer without toxic effects to the host and without the need for combination with toxic chemotherapy. Toward this goal, we have developed a new strain of *S. typhimurium*, A1-R, that has greatly increased antitumor efficacy but maintains its original auxotrophy for leu–arg that prevents it from mounting a continuous infection in normal tissues. A1-R was able to effect cures in monotherapy on mouse models of metastatic human cancer. We have also identified candidate *S. typhimurium* tumor-specific promoters that may enhance the antitumor efficacy of A1-R by driving expression of toxins that could be excreted in the tumors. Future studies will be aimed to bring bacterial treatment of cancer to the clinic.

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