Systemic Administration of an Attenuated, Tumor-Targeting Salmonella typhimurium to Dogs with Spontaneous Neoplasia: Phase I Evaluation

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Abstract

Purpose: Genetically modified bacteria are a potentially powerful anticancer therapy due to their tumor targeting capacity, inherent antitumor activity, and ability to serve as efficient vectors for gene delivery. This study sought to characterize the acute and short-term toxicities and tumor colonization rates of a genetically modified Salmonella typhimurium (VNP20009) in dogs with spontaneous tumors, in the context of a phase I dose escalation trial.

Experimental Design: Forty-one pet dogs with a variety of malignant tumors received weekly or biweekly i.v. infusions of VNP20009, at doses ranging from $1.5 \times 10^5$ to $1 \times 10^8$ cfu/kg. Vital signs and clinicopathologic variables were monitored regularly. Incisional biopsies were obtained before and 1 week following the first infusion for histopathology and bacterial culture.

Results: The nominal maximum tolerated dose was $3 \times 10^7$ cfu/kg, with refractory fever and vomiting being the dose-limiting toxicities. One treatment-related acute death occurred. Bacteria were cultured from tumor tissue in 42% of cases. Thirty-five patients were evaluable for antitumor response. Major antitumor responses were seen in 15% (4 complete response and 2 partial response), and disease stabilization for at least 6 weeks in 10%.

Conclusions: Administration of VNP20009 at doses with acceptable toxicity results in detectable bacterial colonization of tumor tissue and significant antitumor activity in tumor-bearing dogs.

The purposeful infection of tumors with live bacteria or fractions thereof, reported in the 1900s by William Coley, was one of the first nonsurgical cancer therapies. Although significant toxicity and lack of tumor specificity were serious problems, dramatic antitumor responses were reported in some patients (1–3). There has been a resurgence of interest in the use of bacteria as anticancer agents. It has been shown that certain obligate and facultative anaerobic organisms have the capacity to preferentially colonize and replicate within the tumor microenvironment (4, 5).

Anaerobic bacteria have great potential as cancer therapeutics. They may exert antitumor effects via several mechanisms, including direct toxicity to tumor cells via type III secretion, in which cytotoxic peptides are injected directly into the target cell's cytoplasm (6, 7); the facilitation of a potent nonspecific immune reaction; the depletion of essential nutrients; and alteration of the tumor microenvironment as a result of bacterial colonization. Additionally, immunomodulatory effects such as the stimulation of dendritic cell function or alteration of T helper cell polarization could play a role (8, 9). Anaerobic bacteria’s tropism for hypoxic tumor tissue allows them to exert an antitumor effect against cells that are often resistant to other forms of therapy such as chemotherapy and radiation, and their motility allows effective migration and dispersion through tumor tissue and potentially into distant sites. Additionally, bacteria have the potential to serve as excellent vectors for gene delivery due to their large genome size and ease of genetic manipulation (10); transgenes coding for cytokines, antiangiogenic factors, enzymes, and immunogens can all be expressed following bacterial infection (10–15). Finally, their sensitivity to antibiotics provides a ready mechanism for control should adverse effects occur.

Salmonella have been shown to infect and preferentially accumulate within murine tumors, achieving tumor/normal...
tissue ratios of \( \sim 1,000:1 \) (5, 16). Wild-type Salmonella, and in particular Salmonella typhimurium, produces self-limiting enteritis in most healthy adults, infects many mammalian species, and can easily be manipulated to carry therapeutic transgenes. S. typhimurium exists as a facultative anaerobe, allowing it to survive in both oxygenated and hypoxic conditions; thus, it may colonize both small metastatic lesions and larger tumors. S. typhimurium can also be genetically modified to reduce or eliminate pathogenicity in animals and humans.

A S. typhimurium strain has been attenuated by partial deletion of the mmb gene, which is responsible for addition of a terminal myristoyl group to lipid A (17). Lipopolysaccharide (LPS) derived from these lipid A mutants has markedly diminished ability to induce tumor necrosis factor-\( \alpha \) in isolated human monocytes (18, 19), and administration of the intact organism to mice or pigs results in 14% to 33% of the tumor necrosis factor-\( \alpha \) induction seen with wild-type S. typhimurium (18). The bacteria were further attenuated by partial deletion of the purl gene, creating a growth requirement for external sources of purines (17), which may be present in higher concentrations in the interstitial fluids in the tumor microenvironment. The modifications of this attenuated form of S. typhimurium (VNP20009) were accomplished by deletions of large portions of the genes, making reversion to wild-type highly unlikely. These modifications allow for significant dose escalation in mice when compared with the native organism and do not affect its tumor specificity (17).

The VNP20009 mutant Salmonella has shown antitumor activity in a variety of murine syngeneic and xenogeneic models after intraperitoneal or systemic administration (16, 17, 20, 21). It has supra-additive antitumor effects when combined with external beam radiation (22) and tumor-directed transgene expression has been shown after administration of VNP20009 carrying the genes for green fluorescent protein or Escherichia coli cytosine deaminase (10, 23). These findings led to a human pilot trial of administration of genetically modified Salmonella carrying the E. coli cytosine deaminase gene, in which enhanced conversion of 5-fluorocytosine to 5-fluorouracil was detected within the tumor microenvironment in two of three patients treated (11).

A phase I study of VNP20009 in humans with melanoma was recently reported. The maximum tolerated dose was found to be \( 3 \times 10^5 \) cfu/m\(^2\), with thrombocytopenia, anemia, persistent bacteremia, hyperbilirubinemia, diarrhea, vomiting, nausea, elevated alkaline phosphatase, and hypophosphatemia as the dose-limiting toxicities. No antitumor responses were observed, and bacteria were cultured from tumor tissue in 12.5% of patients (24). Altering the kinetics of infusion failed to improve response or colonization in a small number of subsequently treated patients (25).

The dog is an excellent translational model for the investigation of novel antineoplastic therapies. Unlike murine models, dogs are relatively outbred, immunocompetent animals with spontaneously occurring tumors, representing a spectrum of tumor histotypes that have biology similar to that found in humans. The relatively large size of canine tumors, when compared with murine tumors, may more closely approximate human solid tumors with respect to important biological factors such as hypoxia and clonal variation and allows for multiple samplings of tumor tissue over time. Finally, the relatively rapid time course of disease progression, when compared with human cancer, allows for more rapid assessment of therapeutic end points than is possible in many human clinical trials (26, 27).

This study sought to characterize the acute and short-term toxicities and assess tumor colonization following the systemic administration of VNP20009 to dogs with a variety of spontaneous malignant neoplasms, in the context of a phase I dose escalation trial. Preliminary data regarding antitumor efficacy were also generated.

### Materials and Methods

**Patient population.** Client-owned (pet) dogs from the patient population presenting to the Veterinary Medical Teaching Hospital at the University of Wisconsin-Madison were studied. Study participation was offered in cases where standard therapy had failed or had been declined, or in cases of advanced disease where no meaningful standard therapy exists. Dogs were treated as patients of the Veterinary Medical Teaching Hospital and in accordance with the “NIH Guidelines for Care and Use of Laboratory Animals.” Protocol approval was obtained from the Institutional Animal Care and Use Committee and the Campus Biosafety Committee. Signed informed consent and consent to necropsy were obtained from owners before study entry.

Histologic confirmation of diagnosis was obtained in all patients. Staging methods employed varied depending on the histologic type and anatomic site of the tumor and the clinical status of the dog. These included but were not limited to physical examination, complete blood count, serum biochemistry profile, urinalysis, and thoracic radiographs. Dogs were eligible for the study provided they had adequate performance status and hematochemical and serum biochemical variables to undergo therapy, were not receiving immunosuppressive medication or antibiotics, and were free of serious concurrent disease. Dogs in the study had not received chemotherapy or radiotherapy within 3 weeks before study entry nor was concurrent antineoplastic therapy of any form used. All dogs had measurable disease at the time of entry. Tumors were measured by physical assessment (i.e., caliper measurements), or by the serial examination of radiographs or ultrasound. Tumor volume was calculated using the formula \( V = (\pi / 6)lw^2h \), where \( l, w, \) and \( h \) represented tumor diameters in three mutually orthogonal planes. For tumor measurements where only two dimensions were available, the formula \( V = (\pi / 6)lw^2 \) was used, where \( l \) represented the maximal diameter and \( w \) a perpendicular diameter.

**Administration of agent.** The VNP20009 was prepared and packaged in individual vials under GMP conditions and stored at \(-80^\circ\)C until use. Immediately before use, VNP20009 was thawed at room temperature and diluted in sterile 0.9% sodium chloride for injection. The final number of organisms was suspended in 100 mL of 0.9% NaCl and given within 4 hours of preparation.

Patients received VNP20009 as a weekly or biweekly 30-minute or 4-hour i.v. infusion at doses ranging from \( 1.5 \times 10^8 \) to \( 1 \times 10^9 \) cfu/kg. The objective was to enroll cohorts of three dogs per dose level and escalate the dose in half-log increments until more than one third of patients experienced a dose-limiting toxicity. At doses exceeding \( 1.5 \times 10^8 \) cfu/kg, some patients were treated with dexamethasone (0.5 mg/kg s.c.) before VNP20009 infusion, and at 4 and 8 hours following infusion, and/or acetaminophen (10 mg/kg p.o.) before infusion and 4 hours following infusion. An expanded cohort of patients was treated at the highest dose level so that alternate administration and premedication schemes could be evaluated in an attempt to mitigate adverse effects.

**Patient monitoring.** Patients were hospitalized for 24 hours following the first treatment, and vital signs measured at the beginning and end of infusion, then at 0.5, 1, 2, 4, and 24 hours. Complete blood counts and serum biochemistry profiles and incisional tumor biopsies were obtained when possible at 1 and 8 weeks following the first treatment. In the first 13 dogs, blood cultures were obtained from two separate venipuncture sites 7 days following the first treatment.
Patients were rechecked weekly or biweekly. Owners were asked to report adverse events on a weekly basis. When applicable, adverse events were quantified retrospectively according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events v1.0 (28).

Tumor volume was documented at each visit, and tumors were assessed after each treatment for response to therapy, according to standard oncologic criteria. Briefly, complete response (CR) was defined as complete disappearance of all measurable tumor, partial response (PR) as \( \geq 50\% \) reduction in the volume of the tumor, stable disease (SD) as \( <50\% \) increase or \( <50\% \) decrease in volume, and progressive disease (PD) as \( >50\% \) increase in volume. Responses or SD had to persist for a minimum of 6 weeks to be considered clinically meaningful. Incisional biopsies were done on patients experiencing PD to rule out significant inflammation as the cause for tumor enlargement. Dogs experiencing PD without significant evidence of inflammation were considered to have failed and were then eligible for other therapy as deemed appropriate by the attending oncologist. Median progression-free survival (PFS) and median overall survival (OS) were calculated using the Kaplan-Meier product limit method. Differences in PFS and OS between groups were compared using the log-rank test.

**Processing of tumor tissue: Histopathology.** Seven-micrometer sections of pretreatment and posttreatment tumor biopsies were stained with H&E. A single pathologist (R.R.D.), blinded to timing of sample collection or clinical response, evaluated all samples.

**Culture.** Incisional biopsies were shipped overnight on ice from the University of Wisconsin to Vion Pharmaceuticals, Inc. Tumor samples were weighed, transferred into 2 mL ice-cold autoclaved Luria-Bertani (LB) medium, and homogenized. The homogenate was plated onto nine LB plates (200 μL per plate) and incubated at 37°C overnight. One additional aliquot of homogenate (200 μL) was cultured in 5-mL LB medium at 37°C overnight. Colonies were counted after overnight incubation, and the bacterial content expressed as cfu per gram of tumor tissue. Any colony or positive broth culture was verified to be VNP20009 by PCR.

**PCR.** Each colony was suspended in 100 μL Luria-Bertani medium. One microliter of this cell suspension was used in a 25-μL PCR reaction using primers for the msbB locus (forward, 5’-GTGACTCGAGGCTTCGAG-3’ and reverse, 5’-CTGACCGGGCTCTATCCGGC-3’). Controls were present in the form of VNP20009 and wild-type *Salmonella typhimurium* 14028. Cycling conditions were 95°C for 15 minutes (one cycle), 95°C for 1 minute, 55°C for 1 minute, 72°C 2 minutes (35 cycles), and 72°C for 10 minutes (one cycle). PCR products were analyzed by agarose gel electrophoresis, and the products visualized with ethidium bromide. The VNP20009 resulted in a PCR product of 900 bp, whereas wild-type *S. typhimurium* 14028 resulted in a PCR product of 900 bp.

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**Results**

**Patient demographics.** Patient information is presented in Table 1. Seven tumor types were treated. Among soft tissue sarcomas (STS), there were eight anaplastic/undifferentiated sarcomas; five fibrosarcomas; and one each of rhabdomyosarcoma, hemangioendothelioma, and myxosarcoma. Among carcinomas, there were one each of thyroid carcinoma, ceruminous gland carcinoma of the ear canal, tonsillar adenocarcinoma, apocrine gland carcinoma of the anal sac, and undifferentiated carcinoma. The median number of prior nonsurgical treatments was 0, with a range of 0 to 3. Twenty-four dogs had prior surgery, nine had prior radiotherapy, eight had prior chemotherapy, and six had prior investigational immunotherapy or gene therapy. Dose escalations occurred in eight dogs treated at the lowest three doses, and no dose reductions were implemented. A total of 180 treatments were given at doses ranging from \( 1.5 \times 10^5 \) to \( 1 \times 10^8 \) cfu/kg. The median number of treatments per patient was 3, with a range of 1 to 19.

**Adverse effects.** Fever, diarrhea, and vomiting were the most commonly encountered adverse effects. Although generally mild and self-limiting at less than the maximum tolerated dose, these increased in severity with increasing VNP20009 dose, and fever became more profound, prolonged, and unresponsive to antipyretics or alterations of infusion regimen at a dose of \( 1 \times 10^8 \) cfu/kg (Table 2). Temperature typically peaked at 0.5 to 2 hours after infusion. Gastrointestinal signs (fever, diarrhea, and inappetence) also were more frequent with increasing dose but did not exceed grade 2 in any patient.

When compared with pretreatment values, six (15%) dogs developed elevations in hepatic transaminases. Grade 3 elevation occurred in one dog (20%) treated at \( 5 \times 10^7 \) cfu/kg, and grade 3 and 4 elevations occurred in one dog each (8.3%) treated at \( 1 \times 10^8 \) cfu/kg. Fourteen (34%) dogs developed leukocytosis (median WBC count, 25,400/μL; range, 14,500-49,770), and band cells appeared in the peripheral blood or increased in number in 24 (59%) dogs (median, 290 bands/μL; range, 70-6,970). Incidence or magnitude of leukocytosis could not be related to dose. No laboratory abnormalities could any be related to clinical signs of toxicity.

Additional adverse effects occurred in individual dogs. Individual dogs developed glomerulonephritis, clinical hepatotoxicity, pancreatitis, epistaxis, and pyoderma. The dog experiencing glomerulonephritis was diagnosed following 10 VNP20009 infusions at \( 1.5 \times 10^8 \) to \( 5 \times 10^6 \) cfu/kg. The dog experiencing clinical hepatotoxicity developed significant elevations of transaminases and alkaline phosphatase 10 days following the first treatment. This dog was treated concurrently with the nonsteroidal anti-inflammatory drug carprofen, and clinical and laboratory abnormalities resolved with i.v. fluids and carprofen discontinuation. Subsequent VNP20009 treatments at the same dose in this patient were not associated with adverse events.

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**Table 1. Patient characteristics**

| Body weight (kg), median (range) | 30.2 (7-56) |
| Age (y), median (range) | 10 (2-16) |
| Sex | |
| Male | 25 |
| Female | 16 |
| Breed | |
| Mixed breed dog | 9 |
| Golden Retriever | 7 |
| Labrador Retriever | 6 |
| Rottweiler | 3 |
| Beagle | 2 |
| German Shepherd | 2 |
| Other (one each) | 12 |
| Tumor type | |
| STS | 16 |
| Melanoma | 11 |
| Carcinoma | 5 |
| Osteosarcoma | 4 |
| Hemangiosarcoma | 2 |
| Lymphoma | 2 |
| Mast cell tumor | 1 |
any clinical or laboratory signs of hepatotoxicity. Carprofen is known to cause idiosyncratic hepatotoxicity in some dogs (29). The dog that developed pancreatitis presented 4 days following its second treatment at 5 × 10^7 cfu/kg for disseminated mast cell tumor with fever and epistaxis. Profound thrombocytopenia and hyperglycemia were detected, and severe pancreatic edema was documented ultrasonographically. The dog was euthanized 3 days later due to failure to improve with supportive care, and severe pancreatitis was confirmed at necropsy.

One treatment-related acute death occurred at a dose of 1 × 10^8 cfu/kg. The patient was a 10-year-old male Corgi with an anaplastic sarcoma metastatic to lymph node and lung. The dog developed a high fever unresponsive to antipyretics within 3 hours and experienced severe respiratory distress followed by cardiopulmonary arrest 7 hours after the infusion. Thoracic radiographs revealed severe, diffuse pulmonary edema and pleural effusion. Clinical, radiographic, and postmortem findings were consistent with systemic inflammatory response syndrome, and VNP20009 bacteria were cultured from lymph node and normal lung in this patient. A second dog receiving 1 × 10^8 cfu/kg was euthanized at the owner’s request due to persistent abscessation of a site of metastatic melanoma in the ventral cervical area, which led to dyspnea and exposure of the trachea. A third dog treated at 1 × 10^8 cfu/kg developed vegetative endocarditis leading to euthanasia 14 days following treatment. A pure culture of Proteus spp. was cultured from this dog’s urine.

Seven dogs developed tumor abscission or hemorrhage after treatment. This occurred in two dogs with melanoma, two dogs with STS, and three dogs with probable hemangiosarcoma (angiosarcoma). These reactions occurred at a variety of doses. In the dogs with melanoma and STS, tumor abscission occurred within 1 week after the first treatment. VNP20009 organisms were cultured from the abscess in three of these four cases. In each case, treatment was discontinued. In one dog with cutaneous hemangiosarcoma, tumor rupture and hemorrhage occurred within 1 week of the first treatment. In two other dogs with presumed hemangiosarcoma, VNP20009 treatment was initiated for other neoplasms (tonsillar carcinoma and STS), and the patients presented with hemobdemon and ultrasonographic evidence of splenic lesions consistent with hemangiosarcoma.

Although rare in humans, hemangiosarcoma is quite common in dogs and the spleen is the most common site of presentation of this disease in dogs (30).

**Postmortem findings.** Two patients were alive at the time of last evaluation. Among the 39 patients that died, postmortem evaluations were done in 26. Hepatic changes were observed in 10 (38%), consisting of vacuolar hepatopathy in four, fibrotic change in three, passive congestion in two, and lipidosis in one. Renal changes were observed in five (19%), consisting of glomerulonephropathy in three, interstitial fibrosis in one, and basement membrane and tubular mineralization in one. The hepatic and renal changes ranged from mild to severe, and there was no obvious relationship between VNP20009 dose or number of treatments given. There were no repeatable findings in any other organs.

**Histopathology and culture.** Biopsy samples obtained 1 week following the first treatment were evaluable in 16 patients. There were no repeatable changes in tumor cellularity, necrosis, inflammation, or vascularity observed 1 week following treatment. In the first 13 patients, blood cultures were obtained 1 week following the first treatment; VNP20009 was not detected in any samples evaluated. Tumor tissue was evaluated for VNP20009 colonization 1 week following the first treatment in 24 patients. The organism was isolated from tissue homogenates in five (21%), ranging from 1.01 × 10^4 to 4.2 × 10^5 cfu/g (mean, 3.6 × 10^4; median, 9.9 × 10^3) and from bulk broth cultures in 10 (42%). All positive cultures were confirmed by PCR to be VNP20009, based on presence of msbB gene truncation. There was no correlation between VNP20009 dose given and culture results.

**Antitumor response.** Thirty-five of 41 patients were evaluable for response to therapy. All 41 patients were included in intent-to-treat analysis of response rate, PFS, and OS. There were four CRs (10%), two PRs (5%), and four patients (10%) experienced clinically relevant disease stabilization (SD). This yielded an objective response rate of 15% and overall clinical antitumor activity seen in 25%. Responses or disease stabilization occurred at doses of 1.5 × 10^5 cfu/kg (three dogs), 5 × 10^6 cfu/kg (four dogs), and 1 × 10^8 cfu/kg (three dogs). Three additional dogs experienced minor responses (<50% regression) or transient PR. Interestingly, six dogs had evidence of regional disease regression (five of which met the criteria for PR based on duration) in the face of no response or progression of metastatic disease (Fig. 1). One of these dogs had complete regression of a primary oral melanoma in the face of lymph node and pulmonary progression; no evidence of primary melanoma was found grossly or histologically on postmortem evaluations. VNP20009 was cultured from tumor tissue in 50% of responders and 35% of nonresponders (P = 0.64, Fisher’s exact test).

The four dogs experiencing CR included two with metastatic melanoma, one with STS, and one with cutaneous epithelioid lymphoma. One dog with digital melanoma metastatic

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**Table 2.** Incidence and grade of fever in dogs receiving VNP20009

<table>
<thead>
<tr>
<th>Dose (cfu/kg)</th>
<th>No. enrolled</th>
<th>No. evaluable</th>
<th>Grade 1 (%)</th>
<th>Grade 2 (%)</th>
<th>Grade 3 (%)</th>
<th>Grade 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 × 10^6</td>
<td>8</td>
<td>8</td>
<td>3 (37.5)</td>
<td>0 (0)</td>
<td>1 (12.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>5 × 10^6</td>
<td>3</td>
<td>2</td>
<td>1 (50)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1.5 × 10^6</td>
<td>2</td>
<td>1</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>5 × 10^7</td>
<td>9</td>
<td>8</td>
<td>2 (25)</td>
<td>2 (25)</td>
<td>2 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1.5 × 10^7</td>
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<td>1 (50)</td>
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<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>3-5 × 10^7</td>
<td>5</td>
<td>4</td>
<td>1 (25)</td>
<td>1 (25)</td>
<td>1 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1 × 10^8</td>
<td>12</td>
<td>10</td>
<td>0 (0)</td>
<td>1 (10)</td>
<td>4 (40)</td>
<td>1 (25)</td>
</tr>
</tbody>
</table>
to lymph node had complete disease regression following one treatment at $1.5 \times 10^5$ cfu/kg, which persisted for 4.5 months, at which time, brain metastasis was documented by magnetic resonance imaging. The patient failed to respond to reinitiation of VNP20009 therapy and was euthanized shortly thereafter. Brain and lung metastases were detected on postmortem evaluations, but the previously affected lymph node consisted of significant perinodal fibrosis and hemosiderosis without histologic evidence of melanoma. A second dog was diagnosed with digital melanoma metastatic to lymph node and was euthanized as a result of complications of vegetative endocarditis presumed secondary to Proteus infection 14 days following its first VNP20009 treatment at $1 \times 10^8$ cfu/kg. There was no histologic evidence of melanoma in the affected lymph node at the time of postmortem evaluations. A dog with STS of the lateral thoracic wall experienced clinical PR following seven infusions at $5 \times 10^6$ cfu/kg (2.5 months following initiation). His disease became clinically undetectable at the time of the 17th infusion, 8.5 months following initiation of treatment. He died 321 days following treatment initiation from ruptured splenic hemangiosarcoma with hepatic metastasis, histologically distinct from the previous STS. The previously documented thoracic wall mass consisted histologically of dense collagen with interspersed normal fibroblasts, aggregates of lymphocytes and plasma cells, and variably sized pockets of necrotic debris (Fig. 2). A dog with epitheliotropic lymphoma experienced PR following two VNP20009 infusions at $5 \times 10^6$ cfu/kg. There was no measurable disease following the fifth infusion, at which time treatment was discontinued (Fig. 3). The patient was disease free at 142 days following treatment initiation, at which time the dog was lost to follow-up.

PRs were documented in two additional dogs with metastatic malignant melanoma and metastatic osteosarcoma for 257 and 68 days, respectively. Meaningful disease stabilization was seen in one dog with bronchogenic carcinoma (80 days) and three dogs with STS (137, 126, and 56 days).

The median PFS was 28 days (range, 14 to >321 days), and the median OS of the entire population was 87 days (range, 0 to >617 days). The median OS was significantly longer for dogs with STS (137, 126, and 56 days).

In this study, the acute and short-term toxicities associated with the administration of genetically modified Salmonella to dogs with a variety of tumors were evaluated in a dose escalating fashion. Pyrexia was the most common adverse effect reported, which became dose limiting, despite premedication with antipyretics and prolongation of the infusion interval, at a dose of $1 \times 10^5$ cfu/kg. Although left-shifted neutrophilia and hepatic transaminasemia were seen in many patients, these were not dose limiting or dose related and were never associated with clinical signs of illness. Whereas pharmacokinetic studies were not carried out as part of this trial, no bacteria could be identified in circulation 1 week following treatment in the first 13 dogs entered into the study. Information from rodent studies suggests that despite a short circulating half-life, VNP20009 organisms can often be cultured from tumor tissue for several weeks following i.v. infusion (20).

There was one fatality reported in the immediate period following VNP20009 administration. Clinical, radiographic and postmortem findings suggested a systemic inflammatory response syndrome–like reaction. There were no obvious characteristics with regard to signalment, disease stage, tumor type or performance status that separated this patient from the other 40 dogs. Two other dogs were euthanized as a result of infectious complications. In one case, tumor abscessation resulted in severe local tissue destruction. In the other case, vegetative endocarditis led to congestive heart failure. Although a pure culture of Proteus was isolated from this dog’s urine, cardiac tissue was not cultured at the time of postmortem evaluations and thus a VNP20009-related septic event cannot be ruled out. All 3 of the dogs experiencing infectious complications leading to death or euthanasia were treated at the highest dose, $1 \times 10^6$ cfu/kg.

Discussion

In addition to the legendary work of Coley at the turn of the century (1–3), several studies done more recently have indicated an association between bacterial colonization and tumor regression, reduction of recurrence, or outcomes in the majority of these studies, however, whereas the mechanisms responsible for these findings have not been elucidated, these studies support the notion that bacterial infection can be associated with clinically relevant antitumor activity.

In this study, the acute and short-term toxicities associated with the administration of genetically modified Salmonella to dogs with a variety of tumors were evaluated in a dose escalating fashion. Pyrexia was the most common adverse effect reported, which became dose limiting, despite premedication with antipyretics and prolongation of the infusion interval, at a dose of $1 \times 10^5$ cfu/kg. Although left-shifted neutrophilia and hepatic transaminasemia were seen in many patients, these were not dose limiting or dose related and were never associated with clinical signs of illness. Whereas pharmacokinetic studies were not carried out as part of this trial, no bacteria could be identified in circulation 1 week following treatment in the first 13 dogs entered into the study. Information from rodent studies suggests that despite a short circulating half-life, VNP20009 organisms can often be cultured from tumor tissue for several weeks following i.v. infusion (20).
Dogs with a wide variety of tumor histotypes were treated in this study. These included carcinomas, sarcomas, and round cell tumors and represent a more diverse gamut of tumor types than are often encountered in human phase I clinical trials. Although the primary focus of this study was to characterize the short-term toxicoses associated with VNP20009 administration, preliminary information regarding antitumor activity was also generated. The overall response rate of 15% is noteworthy in patients such as these with heavy disease burdens. Including disease stabilization, minor/transient responses, and responses in sentinel lesions only, evidence of VNP20009 antitumor activity was seen in 37%. Antitumor activity was observed over the entire range of dose levels, and there seemed to be no correlation between dose and the detection of tumor colonization. Thus, it is possible that the maximum tolerated VNP20009 dose may not necessarily be the optimum therapeutic dose for use in future studies. Although the LPS moiety in VNP20009 is attenuated, it is conceivable that a component of the antitumor activity observed could be as a result of nonspecific LPS effects (e.g., tumor necrosis factor-α induction).

Whereas the median PFS and OS in this group of treated patients are short, this is not unexpected given the generally advanced stage of disease present at the time of enrollment in most dogs and the biologically aggressive histotypes involved. Additionally, the option to euthanize at a time of perceived diminished quality of life may often result in shorter survival times than might be reported in a similar study in humans.

Five dogs with vascular neoplasia (three with biopsy-documented hemangiosarcoma and two with probable occult hemangiosarcoma based on presentation and clinical findings) were treated as part of this study. In four of the five cases, acute tumor rupture or transient tumor regression was observed. The interesting clinical activity seen in dogs with endothelial-derived tumors, plus the shown proapoptotic effects of bacterial LPS on endothelial cells (37) suggests that despite LPS attenuation, VNP20009 could be exerting some of its antineoplastic effects via targeting of the endothelial compartment. An antiangiogenic mechanism has been shown in murine models of toxoplasmosis-associated tumor regression (38, 39). Indeed, we have shown that VNP20009 or cell-free supernatants thereof are capable of inducing apoptosis in cultured endothelial cells, and that much of this proapoptotic activity can be inhibited by inactivation of LPS by polymyxin B.

It is interesting to note that no objective responses have been reported to VNP20009 in human melanoma patients treated to date (24, 25), and that in rodents, unmodified VNP20009 seems associated primarily with tumor stabilization rather than regression (20). It is possible that dogs may differ from humans and rodents in some critical way that allows VNP20009 to exert a more substantial antitumor effect. Possibilities could include a more robust or more cytotoxic immune reaction occurring at the site of VNP20009 colonization, heightened sensitivity of canine tumor or endothelial cells to VNP20009’s cytotoxic effects, enhanced cytokine (e.g., tumor necrosis factor-

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Hepatic and renal changes were noted in some patients at necropsy. These were subclinical in all patients except one with clinical protein-losing nephropathy and were heterogeneous with regard to histologic description, severity, and time of detection relative to VNP20009 administration. In some cases, they were associated with other disease processes or medications that could have contributed to their occurrence. The heterogeneity of findings and lack of effect of acute or cumulative dose on incidence make interpretation of their significance difficult; however, it is possible that VNP20009 may have exerted subclinical hepatic or renal toxicity in some patients. This possibility will need to be addressed carefully in subsequent studies.

We were unable to document significant histologic changes in tumor tissue obtained 1 week following VNP200009 treatment, and VNP20009 organisms were only detected in 42% of cases. The percentage of cases in which colonization was detected is higher than that reported previously in human clinical trials of VNP20009 (24); however, no correlation could be made between colonization or VNP20009 dose and biological activity. The majority of samples obtained for histopathology and culture following treatment were small needle core biopsies obtained from large tumors. Thus, it is possible that these samples may have not adequately represented the nature of the reaction occurring within the tumor on a global level. Similarly, heterogeneous tumor colonization could have resulted in false negative culture results in some cases. Future studies are planned to resect and evaluate entire canine tumor specimens at varying time points following treatment for presence, kinetics, and pattern of VNP20009 colonization and gene expression.

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production in dogs in response to VNP20009, enhanced sensitivity of canine tumor/endothelial cells to those cytokines, or a difference in the magnitude or pattern of tumor colonization better facilitating tumor destruction.

The demonstration of local disease regression in the face of progression of metastatic disease in several patients is likewise notable. It is possible that differences in the biology of metastatic tumor clones with regard to apoptosis or hypoxia resistance and angiogenesis, or differences in the tumor interstitium or microenvironment (e.g., cytokine/growth factor milieu and interstitial fluid pressure) between primary and metastatic tumors could account for this finding.

In conclusion, we have shown safety, tumor colonization, and evidence of antitumor activity of VNP20009 genetically modified \textit{S. typhimurium} in dogs with spontaneous cancer. Future studies should further characterize possible toxicity in a uniform group of patients treated with a generally well-tolerated dose and evaluate the kinetics and pattern of bacterial distribution within tumor tissue, the antitumor effect of genetically modified \textit{Salmonella} carrying therapeutic transgenes, and the efficacy of \textit{Salmonella} when combined with standard cytotoxic therapy.

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