Bacteriolytic therapy can generate a potent immune response against experimental tumors


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When spores of the anaerobic bacterium Clostridium novyi-NT are systemically injected into animals, they germinate exclusively within the hypoxic regions of cancers. The germinated bacteria destroy adjacent tumor cells but spare a rim of well-oxygenated tumor cells that subsequently expand. Surprisingly, we found that ~30% of mice treated with such spores were cured of their cancers despite the viable tumor rim initially remaining after spore germination. The mechanism underlying this effect was shown to be immune-mediated, because cured animals rejected a subsequent challenge of the same tumor. Similar effects were observed in rabbits with intrahepatic tumors. It was particularly notable that the induced immune response, when combined with the bacteriolytic effects of C. novyi-NT, could eradicate large established tumors.

In the 1890s, William B. Coley (1) observed that a fraction of cancer patients who developed postoperative bacterial infections went into remission and were cured of their tumors. Although the mechanisms underlying Coley’s observations were unclear, it was known even then that bacteria are immunostimulatory. Coley’s work was thereby a major stimulus to the burgeoning field of tumor immunology. Coley’s toxin and bacillus Calmette–Guérin are two offshoots of this stimulus; bacillus Calmette–Guérin is still used today for the treatment of human bladder cancers (2).

The studies described herein are based on serendipitous observations in cancer-bearing animals that are reminiscent of Coley’s observations in patients. For the past few years, our group has been attempting to treat experimental tumors in mice with spores of a bacterial strain called Clostridium novyi-NT (3–5). The rationale for this treatment is that strict anaerobes can proliferate only in severely hypoxic tissues of live mammals; the only regions in healthy nontraumatized animals that are sufficiently hypoxic to allow anaerobic bacterial growth and accessible to the bacteria are those within tumors (3). Although tumors are angiogenic (6, 7), blood vessel growth does not keep pace with tumor cell growth in the majority of human cancers, leading to pronounced hypoxia often accompanied by necrosis (8). The capacity of anaerobic bacteria to target tumors was recognized ~50 years ago and led to early clinical trials with one anaerobic strain (Clostridium sporogenes) (9). Interest in microbe-based approaches to cancer therapy has recently reemerged with the development of methods to genetically engineer bacteria, reducing their toxicity and arming them with genes encoding prodrug-metabolizing enzymes (10–13).

C. novyi was chosen for research in our laboratory on the basis of an extensive screen of anaerobic bacterial species for those with optimal properties (3). C. novyi proved to be particularly oxygen-sensitive as well as highly mobile within tumors due, in part, to their numerous peritrichous flagellae (4). This mobility endowed them with the capacity to rapidly spread to hypoxic areas within tumors once they had germinated within a small necrotic focus. By removing the major systemic toxin (α-toxin) gene from C. novyi, an attenuated derivative named C. novyi-NT was created that could be more safely administered to mice (3).

When C. novyi-NT spores are injected intravenously into immunodeficient mice bearing human xenografts, the spores quickly germinate within necrotic regions of the tumors. Hypoxic and necrotic regions are generally localized within the central parts of tumors, with well perfused tumor cells occupying the rim. Because of the exquisite sensitivity of C. novyi-NT to oxygen, bacterial germination and spread halt when the bacteria reach the well-oxygenated rim. It was shown that conventional chemotherapy and radiation therapy could be used to destroy the well-oxygenated cells in this rim, and that the combination of C. novyi-NT with these therapies [combination bacteriolytic therapy (COBALT)] provided substantial antitumor activity in several xenograft models (3–5).

As described below, we have now attempted to more carefully assess the effects of C. novyi-NT in immunocompetent mice, using syngeneic murine tumors rather than human xenografts. We were particularly interested in whether an immune response to the bacteria might diminish the effects of combination bacteriolytic therapy (COBALT). Surprisingly, we observed that a significant fraction of immunocompetent animals treated with C. novyi-NT spores exhibited complete tumor regression in the absence of any additional chemotherapy or radiation, resulting in long-term cures. Because such cures had been observed only rarely in nude mice treated with C. novyi-NT spores alone, we sought to investigate the basis and generality of this phenomenon.

Methods

Animals. All animal experiments were overseen and approved by the Animal Welfare Committee of Johns Hopkins University and were in compliance with university standards. Six- to 8-wk-old BALB/c mice, purchased from Harlan Breeders (Indianapolis), were used for tumor implantation of CT26 and RENCA. Eight- to nine-pound New Zealand White rabbits, purchased from Myrtle’s Rabbitry (Thompson Station, TN), were used for VX2 tumor implantation.

Cell Lines. CT26 and RENCA were purchased from American Type Culture Collection. Both cell lines were grown in McCoy’s 5A medium (Invitrogen) supplemented with 5% FCS at 37°C in the presence of 5% carbon dioxide. VX2 was provided by J. A. Hilton at the Johns Hopkins Medical Institutions and passaged intramuscularly in the hind leg of donor rabbits.

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Abbreviations: CT, computed tomography; PET, positron emission tomography; FDG, [18F]-fluorodeoxyglucose.

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**Results**

**Tumor Inoculation.** Five hundred thousand to 5 million cells of CT26 or RENCA were injected subcutaneously into the flank of each mouse. Tumor volume was calculated as length × width² × 0.5. Tumors were allowed to grow for ~14 days, at which time the animals were treated either surgically or with *C. novyi-NT* spores. The average size of tumors when treatment was initiated was ~350 mm³. VX2 tumors were implanted into New Zealand White rabbits after a midline subxiphoid incision. Minced tumor was injected into the left lobe of the liver by using a 16-gauge angiocatheter. The abdomen was closed in two layers by using vicryl. The tumors were allowed to grow for 14–22 days, at which time computed tomography (CT) imaging was performed and treatment was initiated. The average diameter of tumors when treatment was initiated was ~2 cm. Tumor sections were processed through routine histopathology or stained with 2% Pico Green (a specific stain for DNA; Stratagene) in 10 mM Tris-HCl/1 mM EDTA, pH 8.0.

**Treatment.** *C. novyi-NT* spores were prepared as described (3). Mice were injected with 300 million spores suspended in 200 µl of PBS (Invitrogen) via the tail vein. Rabbits received 2 billion spores of *C. novyi-NT* suspended in 3 ml of PBS via the ear vein.

**Cytokine Assays.** The RayBio Mouse Cytokine Array II was purchased from RayBiotech (Norcross, GA) and used according to the manufacturer’s instructions. Briefly, after blocking, membranes were incubated for 2 h with 10-fold diluted sera. The membranes were washed and then incubated with biotin-conjugated antibodies for 1 h. The membranes were washed again and incubated with horseradish peroxidase-conjugated streptavidin for 30 min, washed, and then developed.

**Adoptive Transfer.** Splenocytes and lymphocytes were isolated from naive or cured mice that had rejected a subsequent tumor challenge. CD4⁺ and CD8⁺ cells were purified by magnetic separation (Miltenyi Biotec, Auburn, CA). The purity of cells ranged from 82% to 96%. Twenty million total cells, purified CD4⁺, or purified CD8⁺ cells were adoptively transferred into BALB/c mice. Two days later, the mice were challenged with 5 × 10⁵ tumor cells. The χ² test was used to compare the responses to tumor challenge among the various groups.

**Imaging.** CT and positron emission tomography (PET/CT) (14) images were obtained with a clinical scanner [Siemens Volume Zoom (Siemens Medical, Malvern, PA) and Discovery LS (GE Healthcare, Waukesha, WI), respectively]. Three-millimeter CT slices were obtained in the following sequence: noncontrast, arterial dominant contrast phase with 10 ml of Omnipaque administered at a rate of 1 ml/sec, and portal venous delayed phase. PET images were obtained 1 h after administration of ~37 MBq (1 mCi) of [18F]-fluorodeoxyglucose (FDG). A whole-body emission PET scan was performed in the two-dimensional mode with 10 min of acquisition.

**Results**

**C. novyi-NT Treatment of CT26 Tumors.** The first model tested involved subcutaneous tumors of CT26 colorectal cancer cells in BALB/c mice. This model is representative of minimally to moderately immunogenic tumors in that tumors are formed when relatively small numbers of cells are injected into syngeneic mice but do not form in allogeneic mice even when large numbers of cells are injected (15). When *C. novyi-NT* spores were intravenously injected into mice with CT26 tumors, bacteria germinated exclusively within the tumors. By 24 h after treatment, hemorrhagic necrosis could be observed at the centers of most of the tumors. Three to four weeks later, the tumors had regrown from the small nonnecrotic region at the periphery in 66% (31 of 47) of the animals. In the other 34% of the mice, the tumors had completely regressed, and the mice remained tumor free until the end of the experiment (>60 days).

Morphological analysis of the tumors showed a striking accumulation of inflammatory cells at the periphery, already present at 24 h after spore injection (Fig. 1). The inflammatory cells appeared to be restraining the bacterial infection, because a ring of bacteria was surrounded by a dense concentric ring of inflammation (Fig. 1). Standard histopathological analysis (Fig. 1) demonstrated that the infiltrate was largely composed of neutrophils. Measurements of serum cytokines showed the presence of polypeptides associated with neutrophil chemotaxis and activation, such as IL-6, MIP-2, G-CSF, TIMP-1, and KC.
naive mice injected with 5
10^5 tumor cells. The responses were compared to those in mice that had not previously been injected with tumor cells ("Control"). (b) Lymphocytes were purified from naive mice or from mice that were cured of their CT26 tumors by C. novyi-NT treatment. Forty-eight hours after i.v. injection of the lymphocytes, the mice were challenged with a s.c. injection of 5 × 10^6 tumor cells. The y axes in both a and b represent the fraction of animals that formed tumors after tumor challenge. The number (n) of mice used in each group is indicated.

Fig. 3. Rechallenge experiments. (a) Mice with the indicated tumors cured by treatment with C. novyi-NT or by surgical excision were challenged with a s.c. injection of 5 × 10^6 tumor cells. The responses were compared to those in mice that had not previously been injected with tumor cells ("Control"). (b) Lymphocytes were purified from naive mice or from mice that were cured of their CT26 tumors by C. novyi-NT treatment. Forty-eight hours after i.v. injection of the lymphocytes, the mice were challenged with a s.c. injection of 5 × 10^6 tumor cells. The y axes in both a and b represent the fraction of animals that formed tumors after tumor challenge. The number (n) of mice used in each group is indicated.

(Fig. 2). Seventy-two hours after spore injection, the cellular characteristics of the inflammatory ring had changed to include more monocytes and lymphocytes (data not shown).

To determine whether this inflammatory response to C. novyi-NT germination had stimulated an immune response against the tumors, mice that had been cured of their tumors were challenged with 5 × 10^5 CT26 cells in the opposite flank. Eight of ten such mice were resistant to this relatively large challenge (Figs. 3a and 4). As expected, tumors formed in all 10 naive mice injected with 5 × 10^5 cells (Fig. 3a), as well as in all naive mice injected with 10-fold fewer cells (data not shown).

The immune responses that developed in these mice could theoretically be explained in two ways. First, it was possible that CT26 cells naturally elicit an immune response during tumorigenesis that was not strong enough to eradicate all tumor cells. The bacteriolytic effects of C. novyi-NT could simply have reduced the tumor load, thereby permitting the normal immune response to deal with the few remaining tumor cells and curing the animals (the permissive model). Second, it was possible that CT26 tumor cells could not initiate a robust immune response on their own, and that the infection with C. novyi-NT acted as an adjuvant to stimulate the response (the stimulant model). To distinguish between these two models, tumor-bearing mice were treated either with C. novyi-NT or with surgical excision. One to three months later, when it was clear that the animals were cured, they were rechallenged with 5 × 10^6 CT26 cells. In these experiments, 83% of the surgically cured mice but only 20% of the C. novyi-NT-treated mice developed tumors, supporting the stimulant model (P < 0.01, Fig. 3a).

Treatment of RENCA Tumors in Mice. To determine whether these results could be reproduced in another murine system, we repeated the experiment using RENCA cells, derived from a murine renal cell carcinoma that is also minimally to moderately immunogenic (16). Results obtained with this model were very similar to those observed with CT26. C. novyi-NT treatment of RENCA tumors routinely resulted in large regions of central necrosis, generally leaving a small viable rim. Of 15 treated mice, five (33%) were cured. Cured mice were completely resistant to challenge with 5 × 10^5 RENCA cells (Fig. 3a). The stimulant vs. permissive model for immunity was tested by comparing these results with surgically cured mice: none of 10 surgically cured mice were resistant to challenge with 5 × 10^6 RENCA tumor cells.

To demonstrate the specificity of the immune response, mice that were cured of RENCA tumors by C. novyi-NT treatment were rechallenged with both RENCA cells (on the right flank) and CT26 cells (on the left flank). These mice developed CT26 tumors at the same rate and of the same size as naive mice but did not develop RENCA tumors (Fig. 4).

Adoptive Transfer. We next sought to determine what cells were responsible for the immune response in C. novyi-NT-treated mice. Lymphocytes were isolated from the lymph nodes and spleens of mice cured with C. novyi-NT and were used to purify CD4+ or CD8+ cells, which were then injected into naive BALB/c mice. Two days later, all of the mice were inoculated s.c. with 5 × 10^5 live CT26 tumor cells. Tumors grew in all mice that had not been preinjected with lymphocytes (Fig. 3b). Similarly, tumors grew in all mice that had been injected with lymphocytes from naive control mice. In contrast, lymphocytes from mice cured with C. novyi-NT prevented tumor growth in each of six mice tested. The cells responsible were CD8+, because adoptive transfer of these cells, but not CD4+ cells, completely prevented tumor growth (Fig. 3b).

Treatment of Tumors in Rabbits. To determine whether these results were species-specific, as well as to assess the capacity of C. novyi-NT to germinate within internal tumors, we used the
VX2 tumor model in rabbits. These tumors are weakly (if at all) immunogenic, as documented by their ability to cause tumors in outbred strains of rabbits. When inoculated intrahepatically, VX2 tumors are highly aggressive, spreading rapidly throughout the liver, abdominal cavity, and pleural space, killing the animals within 6–8 weeks. Fig. 9, which is published as supporting information on the PNAS web site, illustrates the typical appearance of the abdomen at necropsy, showing widespread invasion and metastases in the liver and adjacent anatomical structures.

To determine whether the inflammatory responses observed in s.c. tumors in mice were also induced in liver tumors in rabbits, animals were euthanized 4 days after administering C. novyi-NT. Fig. 5 shows a section of part of a representative tumor in a control rabbit, with large numbers of viable tumor cells surrounding central regions of necrosis. Four days after treatment with C. novyi-NT, the necrotic areas expanded, and the tumor cells were largely replaced with inflammatory infiltrates. Pico green staining showed that the necrotic regions of the treated lesions were filled with vegetative C. novyi-NT.

A total of 23 rabbits were treated with a single i.v. injection of C. novyi-NT spores after their liver tumors became visible in pretreatment imaging studies. Tumor growth was assessed through serial CT with arterial-dominant phase-contrast agent enhancement. As shown in Fig. 6, untreated tumor nodules had hyperattenuated peripheries reflecting the high blood flow to actively proliferating tumor cells. If no treatment was administered, the tumors progressively grew, replacing much of the liver within 5–6 weeks. In contrast, the appearance of tumors decided changed 12 days after i.v. injection of C. novyi-NT spores (Fig. 6, day 34). Although the tumors had swelled, there was little or no peripheral hyperattenuation. Moreover, a gas pocket could sometimes be observed in the central necrotic region of the tumors, a classic feature of Clostridial infections. The gas deposits gradually disappeared, and in some cases no tumor could subsequently be detected radiologically (Fig. 6, day 77).

Necropsies revealed C. novyi-NT germination and extensive necrosis of tumors in every rabbit examined, leading to substantial increases in survival ($P < 0.005$; Fig. 10, which is published as supporting information on the PNAS web site). No germinated bacteria or necrosis was evident in any of the normal tissues of necropsied rabbits.

Of 23 rabbits treated with C. novyi-NT, 7 were apparently cured, because they exhibited no clinical or radiologic evidence of tumors at the end of the experiment (Fig. 10). Two observations suggested that these cures were in part due to an induced immune response. First, one rabbit that underwent PET in addition to CT scanning was particularly informative. There was a single liver tumor present on day 14, 1 day before treatment with C. novyi-NT (Fig. 7, day 14). Treatment with C. novyi-NT destroyed this lesion, as is evident by the lack of FDG uptake 1 week later (Fig. 7, day 22). But by that time, a second tumor not visible on day 14 had become apparent as a result of local extension or metastasis. This second lesion presumably arose after the initial germination event and was not effectively colonized by the bacteria, as is evident by its robust PET signal (Fig. 7, day 22). A third lesion, an abdominal wall metastasis that...
elsewhere. was substantially decreased by day 86 (red arrow). On day 455, the animal was the abdominal wall (red arrow). The FDG uptake in the abdominal wall lesion (yellow and blue arrows), but a new FDG-incorporating lesion was evident in arrows). Seventeen days later (day 51), the first two lesions were resolving Twelve days later (day 34), both lesions had less FDG uptake (yellow and blue...treated with C. novyi-NT. An i.v. injection of C. novyi-NT spores was administered on day 15. Seven days later (day 22), two lesions were visible. One had considerably decreased uptake of FDG, suggesting a necrotic focus. Continued tumor growth and progressive FDG uptake were evident on the images at days 34 and 51, and the animal died on day 52 with widespread FDG-positive metastatic disease in the mediastinum. (Right) Analogous images of a rabbit treated with C. novyi-NT. There was intense uptake of FDG 14 days after tumor implantation, similar to that in the untreated rabbit. An i.v. injection of C. novyi-NT spores was administered on day 15. Seven days later (day 22), two lesions were visible. One had considerably decreased uptake of FDG, suggesting C. novyi-NT germination (blue arrow), and the other, which appeared to be budding from the first, exhibited intense FDG uptake (yellow arrow). Twelve days later (day 34), both lesions had less FDG uptake (yellow and blue arrows). Seventeen days later (day 51), the first two lesions were resolving (yellow and blue arrows), but a new FDG-incorporating lesion was evident in the abdominal wall (red arrow). The FDG uptake in the abdominal wall lesion was substantially decreased by day 86 (red arrow). On day 455, the animal was alive and healthy, and there was no abnormal FDG uptake in the liver or elsewhere.

Fig. 7. PET/CT images of lesions after C. novyi-NT therapy. (Left) Fused PET/CT images of an untreated rabbit. Fourteen days after implantation of VX2 cells, there was intense uptake of FDG in the tumor. Twenty-two days after implantation, the region incorporating FDG was much larger and contained a central area of decreased uptake, suggesting a necrotic focus. Continued tumor growth and progressive FDG uptake were evident on the images at days 34 and 51, and the animal died on day 52 with widespread FDG-positive metastatic disease in the mediastinum. (Right) Analogous images of a rabbit treated with C. novyi-NT. There was intense uptake of FDG 14 days after tumor implantation, similar to that in the untreated rabbit. An i.v. injection of C. novyi-NT spores was administered on day 15. Seven days later (day 22), two lesions were visible. One had considerably decreased uptake of FDG, suggesting C. novyi-NT germination (blue arrow), and the other, which appeared to be budding from the first, exhibited intense FDG uptake (yellow arrow). Twelve days later (day 34), both lesions had less FDG uptake (yellow and blue arrows). Seventeen days later (day 51), the first two lesions were resolving (yellow and blue arrows), but a new FDG-incorporating lesion was evident in the abdominal wall (red arrow). The FDG uptake in the abdominal wall lesion was substantially decreased by day 86 (red arrow). On day 455, the animal was alive and healthy, and there was no abnormal FDG uptake in the liver or elsewhere.

also contained viable tumor cells evident on PET, was observed several weeks later (Fig. 7, day 51). Yet all lesions eventually disappeared in the absence of any further treatment with C. novyi-NT (Fig. 7, day 455), suggesting (but not proving) that they might have been eradicated by an immune response that occurred after the initial germination.

To obtain more direct evidence of the development of tumor immunity in these rabbits, we selected three that had been cured of their hepatic tumors by C. novyi-NT and challenged them with an intramuscular (quadriiceps) inoculation of 1 × 10⁶ tumor cells. In 12 naive animals, such inoculations always led to rapid tumor growth that destroyed the muscle and surrounding cutaneous tissues within a few weeks. However, no tumor growth occurred in C. novyi-NT-cured rabbits after identical intramuscular injections, even though the injection sites were far from the site of the original hepatic tumors (data not shown). This systemic immunity was apparently long-lasting, because the three rabbits were challenged 3, 5, or 11 months, respectively, after their initial treatment with C. novyi-NT.

**Discussion**

The results described above are notable for at least three reasons. They demonstrate that a purely bacteriolytic approach, in the absence of any additional forms of therapy, can cure experimental tumors. Second, they demonstrate that even large tumors are susceptible. Immunotherapeutic approaches have generally been found to be effective only against relatively small tumors, both experimentally and in patients (17–20). And third, the data show that both internal (hepatic) and external (s.c.) tumors in different species are responsive.

Our working hypothesis to explain these results is depicted in Fig. 8. Systemically administered C. novyi-NT spores are distributed throughout the body, but due to their strict anaerobic growth requirements, germinate only within anoxic or markedly hypoxic regions of tumors. Once germinated, the bacteria destroy adjacent cancer cells through the secretion of lipases, proteases, and other degradative enzymes. At the same time, the host reacts to this localized infection, producing cytokines such as IL-6, MIP-2, G-CSF, TIMP-1, and KC (Fig. 2) that attract a massive influx of inflammatory cells, initiated largely by neutrophils and followed within a few days by monocyte and lymphocyte infiltration. The inflammatory reaction restrains the spread of the bacterial infection, providing a second layer of control in addition to that provided by the requisite anaerobic environment. The inflammation may also directly contribute to the destruction of tumor cells through the production of reactive oxygen species, proteases, and other degradative enzymes. Moreover, it stimulates a potent cellular immune response that can subsequently destroy residual tumor cells not lysed by the bacteria. The cure rate is determined by the balance between bacteriolysis, angiogenesis, regrowth of residual tumor cells, and the rate of development of the immune response. In the experiments reported here, the host “wins” in ~30% of cases, in either mice or rabbits, and the tumor is victorious in the remainder.

![Working model](image-url)
Several new and previously recorded observations support this model. *C. novyi* is well known for its capacity to induce massive leukocytosis and inflammation, whereas many other species of *Clostridia* do not induce this level of response (21). The inflammatory reaction is classic in many ways, including the observed increase in neutrophil-directed cytokines in serum and the cellular nature and time course of the infiltrate (22). The antitumor effects of inflammation are well documented (23). At the molecular level, bacterial infections like those of *C. novyi* are associated with the release of pathogen-associated molecular patterns (PAMPs) from bacteria and Hsp70 from necrotic cells (26). Hsp70 induces maturation of dendritic cells, professional antigen-presenting cells that are essential for the development of adaptive immunity (25).

At the cellular level, inflammatory reactions are directed to the tumor-specific nature of the response (Fig. 3). *C. novyi* germinates within human tumor xenografts in the nude mouse host (although complete regressions and cures are not generally observed as there is minimal T cell-mediated immunity). And third, there are many case reports of *C. novyi* germination and gangrene developing in penetrating wounds or after illicit drug injection (28). These reports demonstrate that the parental strain of *C. novyi*, differing from *C. novyi*-NT only in that the latter is devoid of the lethal α-toxin gene, can proliferate within hypoxic regions in humans.

Whether *C. novyi*-NT infection of cancers in humans will induce tumor immunity is more difficult to predict. There are many studies indicating that human tumors are immunogenic, as assessed by the presence of specific antibodies or reactive T cells in untreated patients (29, 30). Furthermore, it has been shown that stronger immune responses can be elicited through the administration of various vaccines in several clinical trials (31). But there are also many studies indicating that human tumor cells can protect themselves against potent immune responses through a variety of direct and indirect mechanisms (20, 32). As similar observations, both with respect to the potential of tumors to elicit an immune response and their ability to evade such responses, have been recorded in animals, there is reason to hope that the immune therapeutic effects stimulated by *C. novyi*-NT germination might be obtainable in carefully selected patients.

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