Potential and limitations of bacterial-mediated cancer therapy

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1. ABSTRACT

Bacterial-based tumor-targeted therapy is an area of growing interest and holds promise for the treatment of solid tumors. Upon systemic administration, various types of non-pathogenic obligate anaerobes and facultative anaerobes have been shown to infiltrate and selectively replicate within solid tumors. The tumor specificity is based upon the unique physiology of solid tumors, which is often characterized by regions of hypoxia and necrosis. Prokaryotic vectors can be safely administered and their potential to deliver therapeutic proteins has been demonstrated in a variety of preclinical models. Although the amount of clinical experience with bacterial vectors is limited to date, the available data clearly demonstrated the feasibility of bacterial-mediated therapy in humans. There are several issues however that are still unknown and remain major challenges. In this review, using Clostridium and modified Salmonella as prototypical agents, we will discuss the major advantages, challenges and shortcomings of bacterial systems for tumor-specific therapy. In addition, we will highlight the requirements needed to advance the approach into clinical trials.

2. BACTERIAL DELIVERY SYSTEMS

Major advances have been made in the understanding of the genetic basis of cancer and this knowledge drives intensive activity worldwide to develop alternative treatment approaches. One of these is the use of gene therapy to selectively target and destroy tumor cells (1, 2). Over the past decade, many strategies have been devised, and even more vehicles to deliver therapeutic genes have been constructed (3-5). However, one of the major drawbacks of most of these vectors is still the lack of tumor specificity. Therefore, when making choices regarding suitable vectors for gene therapy for cancer, it is important to recognize both the factors that distinguish a tumor from its surrounding normal tissue as well as the factors that limit successful therapy with currently available treatments. A good example of this is tumor hypoxia. The majority of solid tumors investigated to date have been shown to contain hypoxic and/or necrotic regions (6). This microenvironmental condition arises due to the chaotic organization and irregularity of blood vessels preventing sufficient delivery of oxygen, nutrients and consequently also therapeutic agents (i.e. gene delivery vectors) to all
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Figure 1. Timeline – advance in bacterial-mediated cancer therapy.

cells within the tumor (7). Although hypoxia causes resistance to radiotherapy and chemotherapy, it also represents a unique environment not found elsewhere in the body (8, 9). This environment supports the growth of anaerobic bacteria and provides the nutrients that allow the growth of attenuated facultative anaerobes (10, 11). The concept of using anaerobic bacteria as a single agent to treat tumors has been around for a long time (Figure 1). Live bacteria were first associated with cancers almost two centuries ago when tumor regression was observed in patients who suffered gas gangrene (12). Today, interest in prokaryote-based approaches to cancer therapy has reemerged with the discovery of non-pathogenic strains that specifically and preferentially target solid tumors. This method has drawn further attention with the development of techniques to genetically modify the bacteria, thereby enabling the expression of anti-cancer therapeutic genes in these hosts (13, 14). Numerous publications provide convincing evidence that several genera of bacteria, including Clostridium, Bifidobacterium, and attenuated Salmonella, have potential in cancer therapy (15-19). Their therapeutic efficacy is due not only to the high selectivity of the bacteria for hypoxic tumor areas, but also because these hypoxic areas are considered one of the most important barriers to current cancer therapy (20). Interestingly, the bacteria can be removed simply by using suitable antibiotics, thus allowing the control of bacterial presence and therapeutic gene expression levels in the tumor (21). In addition, the bacteria can be easily stored and handled, making implementation straightforward (10).

During the last decade, research has focused on determining the best tumor colonizing bacterial host, testing different therapeutic proteins and assessing the therapeutic efficacy of different strategies either as a single treatment or in combination with other therapies (22-27). So far, clinical experience with prokaryotic vectors is limited to early studies with Clostridium and non-extensive Phase I clinical trials with attenuated Salmonella vectors (28, 29). Although the information gathered from these studies is restricted, these trials showed that bacterial treatment was safe and well tolerated by patients.

Here, we discuss the achievements made in this research area during the last decade and highlight the limitations and hence areas that need further investigation in order to move on to extended clinical evaluation.

3. WHAT LESSONS HAVE WE LEARNED?

3.1. Which vehicle should we choose as a host?

3.1.1. Clostridium

The concept of using bacteria as tumor vectors has been most vigorously pursued using several Clostridium species. The genus Clostridium is one of the largest prokaryotic genera consisting of anaerobic, Gram-positive rods that are unified by their ability to form spores. Although they have probably achieved greatest prominence as a consequence of pathogenic representatives such as C. botulinum or C. tetani, most of the members are non-pathogenic.

As early as 1813, it was reported that tumors from patients who suffered gas gangrene regressed following clostridial infection (10) (Figure 1). In 1935, Connell used sterile filtrates from C. histolyticum to treat advanced cancers (30). The observed tumor regression was attributed to the production of proteolytic enzymes and thus Parker and colleagues (31) were the first to deliberately infect tumor-bearing mice with clostridial spores. These studies indicated the potential of using clostridial spores for inducing tumor lysis. The exquisite tumor selectivity of the system was further demonstrated by Malmgren and Flanagan (32) who intravenously administered C. tetani spores to tumor-bearing mice resulting in death from tetanus poisoning within 48 hours while being entirely
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benign to healthy control animals. This indicated that the spores germinated exclusively within the tumors, thereby causing release of the tetanus toxins that resulted in death. Möse and Möse demonstrated the nondetrimental effect of this group of bacteria using C. butyricum M55 (later named C. oncolyticum, and now classified as C. sporogenes ATCC 13732) by self injecting a spore suspension (33). Intravenous administration of these bacteria into mice bearing Ehrlich ascites tumors resulted in tumor colonization and extensive oncolysis. These results were confirmed in other studies using a variety of rodent tumor models and non-pathogenic strains. Overall, growth of wild-type clostridial species was well tolerated and frequently resulted in destruction of a significant portion of the tumor. Invariably however, regrowth occurred from a remaining outer viable rim. This occurred even if done in combination with other therapies, such as the administration of drugs or decreasing the oxygen level in respiratory air of animals (11).

Although the development of gene transfer methods for Clostridium in the 90’s allowed the generation of recombinant strains (see below), the use of unmodified clostridia for treatment of tumors has gained new interest due to the recent work performed in the laboratory of Dr. Vogelstein (34). Screening a number of anaerobic bacterial species (bifidobacteria, lactobacilli and pathogenic clostridia) for their ability to accumulate in experimental tumors in animals, has led to the isolation of a superior tumor colonizing strain, C. novyi. Upon removal of a lethal toxin expressed by this strain, a non-toxic variant named C. novyi-NT (in which NT stands for non-toxic) is produced. Dang et al demonstrated that C. novyi-NT can efficiently infiltrate and extensively spread throughout the necrotic tumor regions. Similar to the observations initially made with C. butyricum M55, germination of the spores led to enlargement of the necrotic regions and to tumor growth delay. The therapeutic effects increased further when a combination bacteriolytic therapy (COBAL-1T) was set up in order to increase the therapeutic effect (see below). Although these results are certainly very promising, it should also be noted that the observations made were tumor-type dependent and that some combinations led to severe toxicity as a consequence of the so-called ‘tumor lysis’ syndrome (34).

Alternatively, one can choose to use less aggressive tumor colonizing strains, but instead enhance their therapeutic properties through genotypic changes. In this concept, put forward a decade ago by Brown and colleagues (35), the Clostridium host is used as a tumor specific gene delivery system. Advances made in the development of clostridial gene transfer systems in the early 90’s have allowed for the reproducible generation of recombinant strains (36). As these gene systems were only applicable for saccharolytic strains, the initial experiments were undertaken with C. acetobutylicum and C. beijerinckii (37). Unfortunately, these strains have been shown to have limited suboptimal tumor colonization properties. Indeed, upon systemic administration of spores, colonization levels of the saccharolytic C. acetobutylicum and C. beijerinckii were shown to be 1000-fold lower compared to proteolytic C. sporogenes strains (38). Despite their suboptimal tumor colonization properties, studies with recombinant saccharolytic strains have provided invaluable information with regards to heterologous gene expression in Clostridium (see below). The use of saccharolytic strains instead of a proteolytic host may even be beneficial if the secretion of the desired therapeutic gene is required, as degradation of extracellular therapeutic protein might be enhanced when using the latter (39). The superior tumor colonizing strains have very long been refractory to genetic engineering. Although Liu et al (15) described a protocol for their transformation in 2002, this protocol has been shown to be highly inefficient. Fortunately, the very recent development of a gene transfer protocol based on conjugation now allows the construction of recombinant C. sporogenes strains. Consequently, it is now possible to use the strain with the highest tumor colonization (i.e. C. sporogenes) and thus the highest therapeutic gene expression levels. Not surprisingly, preclinical experiments with recombinant C. sporogenes have shown increased anti-tumor efficacy in comparison with C. acetobutylicum or C. beijerinckii (see below). Besides Clostridium, other anaerobic bacterial species such as Bifidobacterium can be used to deliver a variety of effector genes to tumors (19). However, the rather low colonization efficiency and the tendency to clump rather than distribute within necrotic areas (34), would appear to make Bifidobacterium inferior to the optimum strain of clostridia.

3.1.2. Salmonella

Genetically engineered strains of Salmonella have also been proposed for tumor selective therapy (40). In contrast to clostridia, Salmonella are Gram-negative motile bacteria that grow well in both oxygenated and hypoxic tumor areas. To overcome its pathogenicity, S. typhimurium was attenuated by genetically stable chromosomal deletion of the pur1 and mshB genes (25, 40-42). The mshB deletion alters the lipid A component of lipopolysaccharide (LPS), resulting in a strongly reduced induction of proinflammatory cytokines, thereby reducing the risk for a subsequent septic shock. The deletion of pur1 created a requirement for an external source of adenine. The low toxicity profile of the pur1, mshB strain (which was designated VNP20009) has been demonstrated in rodents, pigs and monkeys (43-45). The potential of this strain to function as anti-tumor vector was reported in 1997, in a study where VNP20009 injected into tumor-bearing animals were shown to replicate preferentially in tumors (40). Tumor-to-normal tissue ranged from 250:1 to 10.000:1 at 2 days post-injection. In addition to this selective tumor accumulation, VNP20009 have been shown to have inherent anti-tumor activity in a number of tumor models (46). The exact mechanism for this innate anti-tumor effect is unknown, although it has been suggested that the major Salmonella virulence regulon, SPI-1, might play a role (47). Another double auxotroph mutant strain of Salmonella (S. typhimurium AI), which has a requirement for amino acids Leu and Arg, has also been shown to be successful at tumor inhibition and regression (48). Recently, Lee and colleagues (49) reported the use of attenuated S. choleraesuis as an anti-tumor agent, capable of preferentially accumulating and amplifying within tumors. This vaccine strain of S. choleraesuis is capable of
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delaying tumor growth and enhancing survival in both subcutaneous tumor and experimental metastasis models. Similar to VNP20009, tumor-to-normal tissue ratios ranged from 1000:1 to 10,000:1.

Despite the preferential tumor colonization following the administration of attenuated Salmonella to tumor-bearing animals, also the normal tissues are colonized, albeit transiently and to a lesser extent. Obviously this biodistribution pattern can cause undesired side effects and negatively influence the specificity of this gene transfer system. As the main potential of attenuated Salmonella in anti-cancer therapy does not lie within the use of the vector as such but ‘armed’ with a therapeutic protein, this is especially true for therapeutic genes controlled by strong constitutive promoters. One way to address this issue and to obtain controlled gene expression is to use inducible promoter systems (see below).

3.2. Which therapeutic protein should be delivered?
The main advantage of therapeutic proteins produced in situ by prokaryotic vectors directly in the tumor is that high local levels can be achieved, while concurrently avoiding the toxicity that occurs when delivery of these agents would be systemic (17, 50). To date, the introduction of two classes of recombinant protein has been explored: (1) proteins that have a direct cytotoxic effect, such as toxins and cytokines (51, 52) and (2) enzymes that convert a non-toxic prodrug into a toxic agent, the so-called prodrug converting enzymes (13, 17). An overview is depicted in Figure 2.

Both Clostridium and attenuated Salmonella strains have been genetically engineered to express cytotoxic agents and cytokines. C. acetobutylicum DSM792 has been genetically engineered to express and secrete murine tumor necrosis factor-alpha (TNF-alpha) and rat interleukin-2 (IL-2) (Figure 2A). Although both cytokines could be efficiently secreted and were shown to be biologically active, the therapeutic benefit has yet to be demonstrated. It can be anticipated that this might be difficult, as levels of recombinant C. acetobutylicum in tumors are low. TNF has also been expressed in VNP20009 (53). In vivo experiments with TNF-recombinant VNP20009 showed promising anti-tumor responses in mice bearing colon C38 carcinomas and M27 lung carcinomas.

A slightly different approach has been used with attenuated S. choleraesuis as this strain was engineered to carry a eukaryotic expression plasmid, coding for the anti-angiogenic agent endostatin, and administered to C3H/HeN mice bearing MTB-2 tumors (49). Upon tumor colonization, the expression plasmid is transferred from S. choleraesuis to the tumor cells where the cytotoxic protein is expressed. This strategy resulted in tumor growth inhibition and prolonged survival of tumor-bearing mice. Importantly, transduction of the expression vector to normal tissue did also occur, thereby highlighting the need for additional levels of control. Similarly, S. choleraesuis transformed with a eukaryotic expression vector encoding thrombospondin-1 (TSP-1) was administered to C57BL/6 tumor-bearing mice. The recombinant S. choleraesuis inhibited the growth of melanomas and experimental lung metastasis by more than 40\% compared with injection of S. choleraesuis carrying an empty vector. Survival rates of the tumor-bearing mice increased, but complete regression was never observed (54).

The principle focus with bacterial vectors has been the delivery of prodrug converting enzymes. The considerable advantage of using these enzymes lies within their amplifying effect as each individual enzyme can convert large quantities of innocuous prodrug into toxic therapeutic agents, a phenomenon known as the ‘bystander’ effect. Moreover, as the (pro)drug can diffuse in and out the bacterial cell, there is no need for secretion of the therapeutic protein, thereby avoiding potential problems with enzymatic breakdown in the extracellular environment. In the mid-nineties, C. beijerinckii and C. acetobutylicum were the first to be transformed with plasmids carrying either the E. coli enzyme cytosine deaminase (CD) (13) or nitroreductase (NTR) (55). Upon in vitro confirmation of their biological activity, spores of the recombinant strains were injected into tumor-bearing animals. The NTR and CD protein were demonstrated to be produced solely in the tumors, thereby providing the proof-of-principle that a foreign protein delivered by Clostridium can be expressed exclusively in the tumors of different animal models (Figure 2A). Due to the insufficient levels of viable clostridia in the tumor, studies failed to produce anti-tumor activity.

In 2002, the development of an electroporation procedure by Liu et al (15) enabled for the first time the transformation of C. sporogenes. Intravenous spore injection of C. sporogenes transformed using this protocol with an expression plasmid for CD, combined with 5-fluorocytosine (5-FC) prodrug administration, resulted in significant anti-tumor activity (Figure 2A). The reported development of this transformation system for C. sporogenes was certainly encouraging, however, the reported transformation turned out not to be reproducible. As exogenous endonucleases might represent a major impediment to DNA transfer, we therefore devised a procedure based on conjugal transfer from E.coli donors, initially developed for the introduction of plasmids into C. difficile (56). Using this procedure, we have introduced a novel prodrug converting NTR enzyme with superior prodrug turnover characteristics into C. sporogenes and demonstrated the in vivo efficacy of the approach (57). Both sets of data clearly show that use of the superior tumor colonizing C. sporogenes as a host instead of C. acetobutylicum or C. beijerinckii, results in increased in vivo anti-tumor efficacy.

In attenuated Salmonella, most experimental work using a prodrug converting enzyme has been done using TAPET-CD, i.e. VNP20009 with CD stably integrated into the chromosome (50). Extensive preclinical toxicology was carried out in rodents and monkeys while preclinical data also demonstrated very similar biological characteristics for TAPET-CD as compared to VNP20009 (43). High conversion capacity of 5-FU to 5-fluouracil (5-FU) was observed following the use of a single TAPET-CD injection combined with 5-FC administration. Although
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Figure 2. Scheme summarizing bacterial-mediated cancer therapy. (A) Intravenous administration of either \textit{C. novyi}-NT or recombinant \textit{Clostridium} spores into a tumor-bearing animal. The spores are dispersed throughout the body, but only those that encounter the hypoxic/necrotic regions of the tumor are able to germinate and multiply as vegetative cells. Administration of \textit{C. novyi}-NT results in the secretion of hydrolytic enzymes that lyse the surrounding tumor cells. The recombinant \textit{Clostridium} is generated by transforming the bacteria with clostridial expression plasmids encoding either a prodrug-converting enzyme (CD, NTR) or a cytokine (TNF-alpha, IL-2). Depending on the type of therapeutic protein employed, use of these recombinant strains results in direct or indirect cytotoxic effects. Recombinant Salmonella spread throughout the body and colonize normal tissues (albeit transiently and to a lesser extent) but preferentially replicate in the tumor. Its tumor localization is not restricted only to the hypoxic/necrotic region of solid tumors. When a therapeutic gene is driven of a constitutive promoter, undesired damage to colonized normal tissues may occur. (B) Combination therapy with radiotherapy or vascular targeting agents and use of spatial control of gene expression in recombinant Salmonella using HIP. Radiotherapy leads to cell killing of the well oxygenated cells, while the \textit{Clostridium} specifically target the hypoxic/necrotic areas leading to an enhanced or supra-additive effect on tumor growth inhibition and/or cure. Vascular targeting agents lead to rapid tumor vascular shutdown thereby promoting hypoxia and necrosis resulting in increased tumor colonization by \textit{Clostridium}. Administration of recombinant Salmonella, with the therapeutic gene controlled by HIP restricts expression of cytotoxic proteins and cell killing to the hypoxic region of solid tumors. CD: cytosine deaminase, NTR: nitroreductase, TNF: tumor necrosis factor, IL: interleukin, HIP: hypoxia-inducible promoter.
5-FU was detectable in normal tissues, the levels were significantly lower. *In vivo* efficacy was further demonstrated in 3 different mouse tumor models, showing a 90% tumor growth inhibition at 6 weeks after the start of the treatment (28). Based on these data, a protocol for the use of TAPET-CD in the clinic has been proposed (see below).

Although the obtained preclinical *in vivo* data are encouraging, the specificity of the Salmonella-mediated gene transfer system is negatively influenced by the additional colonization of normal tissues (46). Obviously, this biodistribution pattern can cause undesired side-effects (58). Therefore, to limit therapeutic gene expression specifically to the tumor, we recently developed a hypoxia-inducible promoter (HIP-1) system and showed that gene expression was driven of HIP-1 under both acute and chronic hypoxia, with induction factors up to ~200-fold following hypoxia treatment. Most interestingly, use of HIP-1 confined gene expression strictly to the tumor (Figure 2B). Thus, HIP-1 has the potential to increase the specificity of the Salmonella-mediated gene delivery system by establishing spatial control of gene expression (59). Spatial and temporal control of therapeutic protein expression has previously also been demonstrated in *Clostridium*, that carried a plasmid in which the cDNA encoding TNF was ligated downstream of the radiation-inducible recA promoter (RIP) (60). Genetic engineering of the wild-type recA promoter resulted in 412% increase in TNF activity following irradiation with a clinically relevant 2 Gray (Gy) dose. Moreover, reactivation of the promoter was possible by giving a second dose of 2 Gy (61). The application of RIP thus holds promise for use in a clinical setting, as patients generally receive small 2 Gy daily fractions during fractionated radiotherapy.

### 3.3. Should we combine bacterial-mediated therapy with other treatment options?

Although wild-type strains of *C. novyi*-NT and VNP20009 have been successfully used as a single treatment with some anti-tumor effects, concurrent treatments have been explored in an attempt to enhance their therapeutic efficacy. The two most extensively investigated strategies include the combination with radiation or vascular targeting agents (Figure 2B).

#### 3.3.1. Radiotherapy

Radiotherapy (RT) is used in more than 75% of patients with malignant tumors (62). While radiation is very efficient in killing well oxygenated cells, it is much less effective in killing hypoxic cells. Since the bacterial-mediated approach specifically targets these radiation-resistant hypoxic cells, it is anticipated that the combination of these two therapies can increase the therapeutic ratio.

The combination of radiotherapy with both *C. novyi*-NT and VNP20009 has been investigated in the past. Treatment of athymic nu/nu mice bearing HCT116 xenographs with *C. novyi*-NT spores and fractionated RT (2 Gy/day for 5 days) resulted in significant tumor shrinkage as compared to either treatments alone or to control tumors (63). Importantly, this combination therapy was shown to be tumor-type specific and, in all cases, a small number of residual cells eventually caused tumor recurrence. In addition to external beam radiation, *C. novyi*-NT was also combined with high dose rate brachytherapy leading to a complete cure of two tumor types (63). Recent experiments have suggested that damage to microvascular endothelial cells might be an important component of the radiation effects. Such microvascular damage is predicted to increase the niche for *C. novyi*-NT growth by creating more hypoxic areas within tumors, thereby increasing tumor colonization. Interestingly, the efficacy of the combination treatment was shown to be independent of the tumor volumes suggesting that a wide range of tumor volumes may be treated with this strategy (63). Furthermore, the addition of radiosensitizing drugs such as 5-FU may increase the efficacy of this combined treatment. For example, it is conceivable to design a treatment regime combining *C. novyi*-NT with systemic 5-FU administration and RT. Perhaps the most interesting combination would be to combine CD-recombinant *C. sporogenes* with radiation and 5-FC as the 5-FU would be produced locally in the tumor via the CD activity of the recombinant bugs, thereby avoiding the toxicity of systemic 5-FU administration. We have calculated that 1-3% conversion of 5-FC to 5-FU would be sufficient to achieve clinically significant radiosensitization and importantly, reported conversion efficiencies of CD recombinant *Clostridium* strains exceed this value, making this combination particularly promising (62).

Analogous to *C. novyi*-NT, attenuated Salmonella have been shown to be effective when combined with radiotherapy. Platt and coworkers (64) applied doses ranging from 5 to 15 Gy in two melanoma tumor models. Although the combination treatment resulted in prolonged survival for both tumor models, the doses used were above clinically relevant levels. Therefore, further investigation using lower radiation doses is required to obtain more applicable information.

#### 3.3.2. Vascular Targeting Agents

The use of vascular targeting agents is an attractive strategy that extended the use of clostridia to very small tumors which have yet to develop necrotic areas. These agents specifically target the dividing endothelial cells and cause rapid vascular shutdown in tumors, thereby promoting necrosis and hypoxia (65). Our group was the first to show the benefit of such vascular targeting agent such as Combretastatin A4-phosphate (CombretAp) to improve *Clostridium* tumor colonization. Our data demonstrated that systemic administration of CombretAp resulted in severe vascular shutdown specifically in the tumor within 3-4 hours with subsequent development of necrosis. This phenomenon led to consistent and high levels of clostridial colonization of (very) small tumors that were very inefficiently colonized in the absence of CombretAp (see Figure 2). Similarly, in animals treated with CD-recombinant *C. acetobutylicum* spores, the incidence of CD-positive tumors increased from 58% to 100% when treated concomitantly with CombretAp (17). Dang *et al.* (34) recently reported notable results using a comparable regime (anti-vascular agent dolastatin 10 in
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combination with other chemotherapeutics and C. novyi-NT). Although most treated mice showed tumor regression, and complete cures were observed in a considerable proportion of mice, this treatment strategy was also associated with significant toxicity due to the combination of vascular targeting and chemotherapeutic agents. Nevertheless, the available data certainly indicate the potential benefit of this combination strategy and therefore merits further investigation.

3.4. Clinical experience so far

Based on encouraging initial animal studies in the 60's that demonstrated the ability of clostridia to target solid tumors, clinical trials were soon thereafter initiated as spores of C. butyricum M-55 were injected intravenously to cancer patients. As with the animal tumors, most of the human malignancies experienced partial tumor lysis with no evidence of clostridial germination or tissue destruction in the surrounding normal tissues. Although the patients suffered no adverse side effects from the injection of these organisms with the exception of mild to moderate fever, most patients showed no evidence of measurable tumor regression (66, 67). The clinical trials were discontinued due to the lack of clinical benefit, as well as tumor regrowth from an outer rim of viable tumor cells that usually remained despite the destruction of large parts of the tumor. Although these studies are difficult to repeat and would not live up to current standards for clinical trials, they clearly demonstrate that administration of spores from non-pathogenic Clostridium strains is nonetheless safe, and that the spores specifically germinate in the necrotic regions of tumor.

The use of attenuated Salmonella as tumor targeting agents in the clinic is much more recent. The first Phase I clinical trial included 26 patients with either metastatic melanomas or metastatic renal cell carcinoma (68). This study showed that the maximum tolerated dose of VNP20009 was $3 \times 10^8$ cfu/m² as doses above this range resulted in dose-dependent elevated levels of circulating pro-inflammatory cytokines such as IL1-beta, TNF-alpha, and IL6. Unfortunately and rather unexpected based on preclinical data, no patients exhibited tumor regression and only three patients had tumors containing viable attenuated bacteria (68). Despite the disappointing results with the parental VNP20009, another pilot clinical trial, this time using TAPET-CD, was conducted in 2003 (29). In this study, the effects of a local intratumoral injection of TAPET-CD in combination with 5-FC was tested in three refractory cancer patients. Two patients had evidence of bacterial colonization of the tumor that persisted for at least 15 days following the initial injection. Conversion of 5-FC to 5-FU as a result of CD expression was demonstrated in these two patients. Although no significant adverse events related to the treatment were reported, this clinical study also failed to demonstrate significant clinical efficacy, presumably because of insufficient bacterial colonization in the tumors. On the other hand, these human clinical trials do highlight the need for the generation of strains with reduced toxicities and improved tumor colonization properties.

4. WHAT LESSONS ARE YET TO BE LEARNED?

Despite significant progresses achieved so far, there are still several issues that need to be addressed before prokaryote-based therapy becomes a standard practice in the clinic. The significance of such issues varies among each therapeutic strain.

4.1 Clostridium

The vast majority of preclinical studies with clostridial vectors were conducted in only a limited number of murine experimental models. Consequently, detailed toxicologic, pharmacologic and pharmacokinetic information with clostridial vectors in a wider range of animal models is still absent. Therefore, extensive pharmaceutical studies need to be put in place before a clostridial vector can be routinely used in a clinical setting. One of the most important outstanding issues for recombinant Clostridium vectors is undoubtedly the development of bacterial strains that carry no antibiotic resistance genes along with the therapeutic gene. Furthermore, the development of a clinically relevant delivery vector requires the construction of a strain in which the introduced genes will be integrated into the Clostridium chromosome so that consistent gene expression is maintained over time and to prevent the occurrence of horizontal gene transfer. Recent advances in the field of clostridial engineering have suggested that these challenging issues can be resolved in the near future. The latter tasks are not relevant for wild-type C. novyi-NT, at least as long as this strain does not carry a therapeutic gene (34). However, if this strain is to be used in the clinic, more detailed information on its genomic and transcriptomic profile will be required to overcome the observed toxicity during the preclinical studies. For example, if the specific toxins should be identified, they could subsequently be confronted. Furthermore, since use of C. novyi-NT has been shown to induce the so-called ‘tumor lysis’ syndrome, a means to counteract this issue must be achieved (34).

Although for therapeutic efficacy of clostridial vectors, the nature of the best therapeutic gene can be argued (see above), independent of the gene that will be expressed, the levels of biologically active compound at the tumor site should be as high as possible. Optimizing the production of the therapeutic agent may require interference of the protein production process at different levels (transcription, translation). This may involve the use of strong promoters with optimized ribosomal binding sites and adaptation of the codon usage within the cDNA of the gene to the codon preferences in the Clostridium host. Indeed, since the G+C% content in Clostridium is extremely low (~30%), insertion of heterologous DNA that does not follow the typical clostridial codon usage will probably lead to instability. Initial studies with the prodrug-converting NTR enzyme in which the codons were adapted for use in Clostridium appear to be useful (57). In addition, benefit is
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anticipated from the use of more effective enzyme/prodrug combinations, more soluble prodrug derivatives and enzymes with improved catalytic properties. Several recent studies have highlighted the importance and the potential impact of these improvements, and experimental activity within these areas should be encouraged in order to fully exploit the clostridial-mediated approach.

Whether secretion of the therapeutic compound from the prokaryotic cell is advantageous, remains unclear. Although more extensive spreading of the compound within the tumor consequently increasing the therapeutic effect is expected, an increase in the likelihood of therapeutic protein leakage out of the tumor, and subsequent specific immune response against the protein may be seen. Moreover, since optimal tumor colonization requires the application of proteolytic enzymes such as C. sporogenes, it can be anticipated that proteolysis will decrease the levels of secreted therapeutic proteins.

4.2. Salmonella

During their clinical development, the attenuated Salmonella vectors have already been extensively tested using many different animal models ranging from mice to non-human primates. While preclinical studies have yielded sufficient information for approval of the conducted clinical trials, the disappointing results of the clinical trials clearly indicated that there are still some lessons to be learned.

The key issue that remains before widely using the Salmonella-mediated approach is understanding the exact mechanisms that account for the preferential bacterial tumor colonization and that give rise to the observed innate anti-tumor effects. Hypoxic and necrotic areas within the tumors are speculated to give optimal tumor microenvironment that a nutrient-rich accumulation of attenuated Salmonella remain poorly understood to date. Yet, this information is likely critical to understand why tumors in patients were so inefficiently colonized (68). A possible explanation is that in patients a more robust immune reaction is induced towards the attenuated Salmonella thereby counteracting bacterial accumulation. Other factors such as the time during which the bacteria are present in the peripheral blood should also be considered as rapid clearance of VNP2009 from the blood was observed during the Phase I clinical trial. In contrast, during preclinical studies in a variety of experimental animal models, high level of administered bacteria could be maintained in the blood stream for longer duration. Extending the infusion times of the bacteria in humans might thus help to improve tumor colonization. Overall, a better understanding of the mechanism of preferential tumor accumulation and of host-vector interactions is essential when designing rational strategies to develop a better and more effective therapy for the future.

To date, a number of studies have reported therapeutic efficacy within the context of using Clostridium or attenuated Salmonella as gene therapy vectors (11, 13, 50, 71). Importantly, tumor cure was not tested in any of the aforementioned reports of therapeutic efficacy. To go beyond the point of 'proof-of-principle', a large scale assessment of tumor cure such as TCD50 experiments must be performed. This type of experiment which measures the required radiation dose to cure 50% of the tumors, has never been performed before in the context of prokaryote-mediated therapy and is the strongest proof of the potential of this system for improving tumor cure.

5. CONCLUDING REMARKS

Notwithstanding the lessons that yet have to be learned and despite some barriers that need to be torn down, the application of live prokaryote vectors hold considerable promise for treating solid tumors. Recent progress made in the field of genetic engineering of Clostridium will certainly allow the required progress to develop a relevant vector for clinical testing and without doubt, continuous investigations will eventually reveal the mechanisms that explain the preferential tumor colonization of attenuated Salmonella vectors. The arguments nonetheless remain as to which strain would be the best to use, which therapeutic gene should be integrated or which treatment combination would be most preferred.

In this review, we have discussed the various options with their advantages and shortcomings, and almost certainly the choice of bacteria and treatment regime will be different for each situation and tumor type. Currently, the most pressing need to establish the potential of prokaryote-mediated therapy in humans is for well designed clinical trials. These critical evaluations will facilitate the transfer of bacterial-based treatment to routine clinical practice by relieving the fears and concerns that some clinicians may have to deliberately infect patients with live bacteria.

6. ACKNOWLEDGMENTS

L.D. is a predoctoral fellow supported by the LSCH-CT-2003- 502932/EUROXY Grant.
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Bacterial-mediated cancer therapy


**Key Words:** Clostridia, Bifidobacteria, Salmonella, Cancer Therapy, Tumor, Bacteria, Review

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