Anti-Cancer Drugs 2002, **13,** pp. 115–125

Minireview paper

Clostridium spores for tumor-specific drug delivery

S Nuyts,[1,2] L Van Mellaert,[1] J Theys,[1] W Landuyt,[2] P Lambin[3] and J Anné[1]
[1]Laboratories of Bacteriology, Rega Institute, Katholieke Universiteit Leuven, 3000 Leuven, Belgium. [2]Experimental Radiobiology, University Hospital Gasthuisberg, 3000 Leuven, Belgium. [3]Department of Radiation Oncology, RTIL, Academic Hospital Maastricht, 6229 HX Maastricht, The Netherlands.

Insufficient blood supply of rapidly growing tumors leads to the presence of hypoxia, a well-known feature in solid tumors. Hypoxia is known to decrease the efficiency of currently used anti-cancer modalities like surgery, chemotherapy and radiotherapy. Therefore, hypoxia seems to be a major limitation in current anti-cancer therapy. The use of non-pathogenic clostridia to deliver toxic agents to the tumor cells takes advantage of this unique physiology. These strictly anerobic, Gram-positive, spore-forming bacteria give, after systemic administration, a selective colonization of hypoxic/necrotic areas within the tumor. Moreover, they can be genetically modified to secrete therapeutic proteins like cytosine deaminase or tumor necrosis factor-α. The specificity of this protein delivery system can be further increased when expression is controlled by the use of a radio-inducible promoter, leading to increased spatial and temporal regulation of protein expression. This approach of bacterial vector systems to target protein expression to the tumor can be considered very safe since bacteria can be eliminated at any moment by the addition of proper antibiotics. The *Clostridium*-based delivery system thus presents an alternative therapeutic modality to deliver anti-tumor agents specifically to the tumor site. This high selectivity offers a major advantage in comparison with the classical gene therapy systems. [© 2002 Lippincott Williams & Wilkins.]

Key words: Anti-cancer therapy, *Clostridium*, gene transfer, hypoxia, radio-induction, review, tumor.

Introduction

The occurrence of systemic side effects remains one of the major problems in the application of anti-cancer drugs today, limiting their use. It is widely recognized that if therapeutic agents could be delivered at an appropriate high dose to the tumor or its microenvironment, a complete tumor eradication could be obtained. A variety of strategies and vector systems have therefore been investigated in attempts to deliver appropriate high doses of therapeutic agents to the tumor vicinity, whilst limiting the exposure of normal tissues to the drug. However, the efficiency and accuracy of the delivery of a drug still remains one of the most significant barriers to success. So far, all strategies have failed in delivering sufficient levels of therapeutic agents to target cells, in order to generate acceptable cell killing.

Several of the current difficulties might be overcome through the use of a delivery system based on clostridia (Figure 1). Solid tumors are characterized by the presence of hypoxia/necrosis due to irregular blood perfusion, insufficient angiogenesis and lack of nutrients. Systemically administered clostridial spores will exclusively germinate in the hypoxic/necrotic region of solid tumors. Here we discuss how this novel property may be exploited in cancer therapy.

Tumor-specific targeting system due to hypoxia

Normal tissues are characterized by a well-defined and structured vascular tree, which enables a spatial and temporal homeostatic flow and oxygen equilibrium. Most normal tissues have an average oxygen pressure of about 40 mmHg. In contrast, numerous investigations of experimental tumors have documented severe disturbances of the microcirculation, which occur already at an early stage of tumor growth.[1] This consequently leads to inefficient

Financial support from 'Het Fonds voor Wetenschappelijk Onderzoek-Vlaanderen', 'De Vlaamse Kankerliga', 'Verkennende Internationale Samenwerking', 'Het KU Leuven Onderzoeksfonds' and 'Sportvereniging tegen Kanker' of Belgium is acknowledged.

Correspondence to S Nuyts, Department of Radiation Oncology, University Hospital Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium.
Tel: (+32) 16 347600; Fax: (+32) 16 347623;
E-mail: Sandra.Nuyts@uz.kuleuven.ac.be

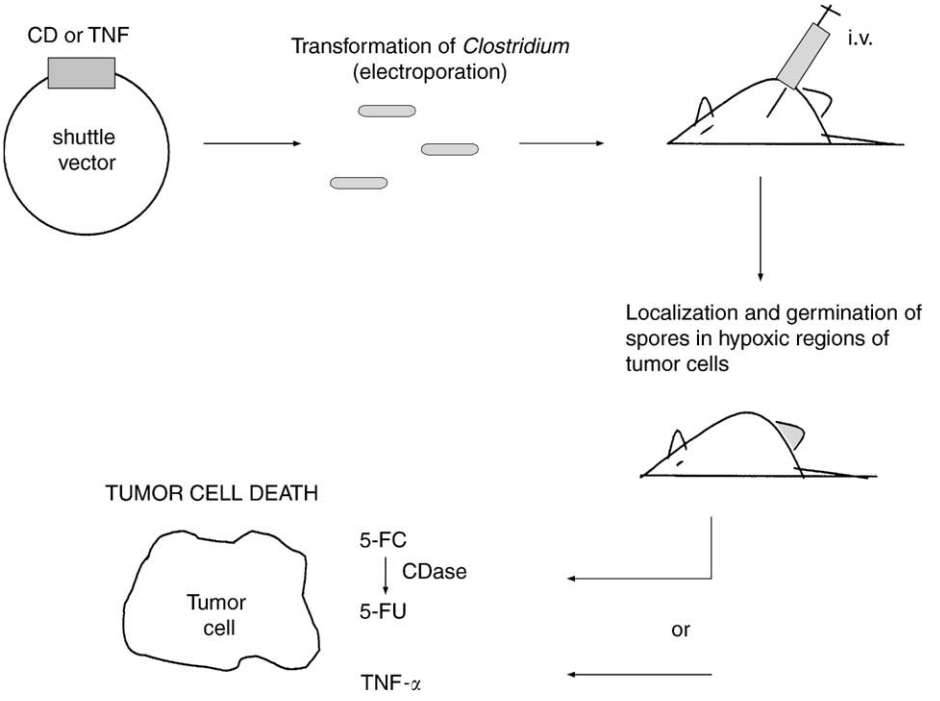

Figure 1. Schematic overview of the *Clostridium*-mediated gene delivery system for transfer of therapeutic proteins to hypoxic/necrotic regions of solid tumors. (Reproduced with permission of Wiley & Sons, New York.)

delivery of oxygen and other nutrients to many cells in tumors. Blood perfusion in tumors is thus markedly inferior to that in most normal tissues, leading to regions of poorly oxygenated (hypoxic) viable cells. Cellular respiration depletes oxygen as it diffuses from blood vessels through packed layers of cells, with chronic hypoxia occurring at distances of 150–200 μm from capillaries, where almost all the oxygen has been used. Next to chronic or diffusion-limited hypoxia, acute or perfusion-limited hypoxia can also be present. Transient opening and closing of blood vessels in tumors will lead to acutely hypoxic cells for short periods of time, followed by re-oxygenation as the vessels resume their normal function. Heterogeneities in the metabolic milieu have been demonstrated not only for experimental tumors but also for malignant disease in patients. Cells in this aberrant environment can remain viable and are often resistant to any kind of therapy.[2] Hypoxic cells are 3 times more resistant to radiotherapy than well-oxygenated cells, due to the fact that oxygen normally interacts with radicals formed by radiation, resulting in complexes which are more difficult for the cell to repair. Hypoxic cells intend to be more resistant to anti-cancer drugs, since most anti-cancer agents are only active against rapidly dividing cells. Under hypoxic conditions cells divide less rapidly than if well oxygenated. In addition, drug delivery is less adequate to hypoxic cells. It has been shown that hypoxia induces DNA over-replication and enhances metastatic potential of murine tumor cells.[3] Moreover, several findings provide evidence that hypoxia, by selecting for mutant p53, might predispose tumors to a more malignant, pro-angiogenic phenotype.[4,5] Clinical data support this observation.[6]

Hypoxia is thus generally considered a major hindrance in cancer therapy. However, hypoxia is also a tumor characteristic which is potentially exploitable using bioreductive drugs or gene therapy. Hypoxia-activated drugs, like mitomycin C, tirapazamine and AQ4N, are, due to their metabolism, only toxic to hypoxic cells.[2] In the same context, a strategy to exploit hypoxia in solid tumors was the identification of a promoter, highly responsive to the so-called 'hypoxia inducible factor' (HIF-1) that could drive the expression of a therapeutic gene such as the cytosine deaminase enzyme (CDase) specifically in the tumor.[7] Another approach involving the presence of hypoxia/necrosis is the specific targeting of anerobic bacteria such as *Clostridium* to solid tumors.

Clostridium and tumor colonization

Clostridium spp. are obligate anerobic, spore-forming Gram-positive rod-shaped bacteria. More than 80 species are described, but only a few are human pathogens. All require anerobic conditions to grow but do vary in their oxygen tolerance and their biochemical profile. Their normal habitat is in the soil, in aquatic sediments, and in the intestinal tract of both animals and humans. The pathogenic species such as *C. tetani, C. botulinum, C. perfringens* and *C. difficile* cause diseases as a consequence of toxin production. Germination of clostridial spores will only occur when they encounter the requisite anerobic conditions. Their ability to germinate in hypoxic/necrotic tissue plays a central role in the pathogenesis of tetanus and gas gangrene, which are caused by the direct colonization of necrotic tissues by *C. tetani* and *C. perfringens*, respectively. Spontaneous colonization of tumors in cancer patients was already noticed in the clinic very soon after the description of the detection of bacteria. In addition, the apparent selectivity of clostridia for tumors has already attracted significant attention for more than 50 years. The first experiments to prove this selectivity were carried out by Malmgren and Flanigan in 1955[8] who injected mice i.v. with spores of *C. tetani*, the causative agent of tetanus. The animals remained healthy unless they had tumors, in which case death by tetanus occurred within 48 h. This resulted from the fact that the bacteria were able to germinate in the tumors, thereby releasing toxins systemically. Furthermore, they did not find vegetative organisms in the healthy tissues of mice carrying colonized tumors.

Later on, Möse and Möse isolated a non-pathogenic strain of *C. butyricum* M-55 (later renamed *C. oncolyticum*) and used this strain to show its oncolytic effect with Ehrlich carcinoma bearing mice following i.v. infection of spores, resulting in the destruction of large parts of the tumor, but animals rarely survived past this stage. On the other hand, Möse and Möse established the benign nature of this species by administration of the spores to themselves. Thereafter, cancer patients were i.v. injected with up to 10^{10} *C. oncolyticum* spores. These injections were well tolerated and patients only showed a low-grade fever. The presence of the bacteria resulted in partial lysis of the tumor.[9–12] A number of publications have been reported to use clostridial spores in the treatment of malignant brain tumors.[13–15]

The combined outcome of both the animal and human experiments showed that clostridial spore treatment was remarkably well tolerated and that growth of the organism frequently led to the destruction of large parts of the tumor. Invariably, however, an outer viable rim remained from which tumor regrowth frequently occurred. From these observations it may be concluded, that spore treatment of wild-type clostridia is not sufficient to effect complete tumor regression.

Whilst the strain (M-55) used in the early studies of tumor spore treatment was originally classified as *C. butyricum*, and later as *C. oncolyticum*, taxonomic studies have now clearly established that it is a *C. sporogenes* strain (ATCC13732). This is a proteolytic species explaining the occurrence of liquefaction of colonized tumors. However, it is debatable whether such a species may be categorized as 'non-pathogenic'. From a safety perspective, therefore, it would be far more acceptable to utilize a host which has no known association with disease. It may also be advantageous to use a host which is less aggressive with regard to destruction of the tumor mass, as extensive lysis all too often results in toxemia.

In recent years, strains which are considered as non-pathogenic have been examined as potential delivery system for therapeutic agents. Especially saccharolytic strains of clostridia have been tested such as *C. acetobutylicum* and *C. beijerinckii*, that produce acetone and butanol as end products of the acetone–butanol–ethanol (ABE) fermentation process.[16,17] These organisms have no known association with human disease. In addition, numerous vector systems have been developed for the introduction of heterologous DNA into a number of strains.[18,19]

More recently, the ability of four different clostridial strains, *C. beijerinckii* ATCC17778, *C. limosum* DSM1400, and *C. acetobutylicum* strains ATCC824 and NI4082 (now re-classified as *C. saccharoperbutylacetonicum*), to colonize tumors was compared to that of *C. oncolyticum.*[20] WAG/Rij rats with syngeneic rhabdomyosarcomas were used as tumor model. In general, it was found that sufficient infiltration into tumors required the systemic administration of at least 10^7 spores. The efficiency with which *C. acetobutylicum* colonized tumors was found to be significantly greater than that of the other three saccharolytic strains and achieved comparable populations to *C. oncolyticum*. Analysis of tumor tissues 4–5 days after injection showed that up to 10^9 c.f.u./g tissue were present compared to only 10^4 to 10^6 in normal tissues (Figure 2). The c.f.u. present in tumor tissue was reduced some two orders of magnitude by heating (73°C for 20 min), a treatment that kills vegetative cells but not spores. A

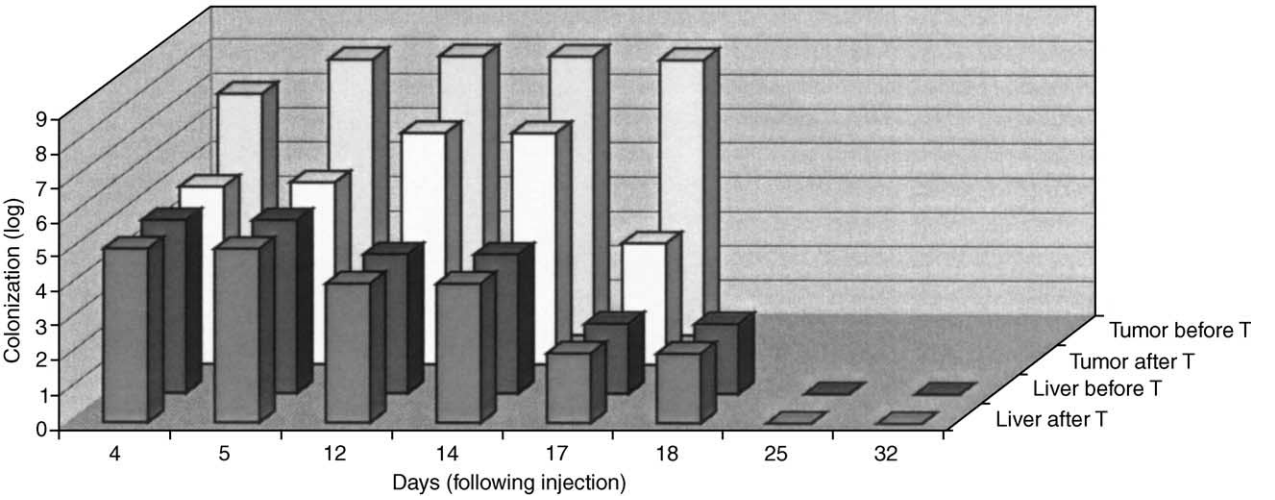

Figure 2. The c.f.u. counts in tumors and liver as a function of time (up to 32 days) following systemic injection of *C. acetobutylicum*. Longer periods could not be investigated due to the fact that tumors grew to the maximum size permitted. Heat treatment at 73°C for 20 min (T) kills vegetative cells but not spores. Before T, the c.f.u. represent the combined amount of spores and vegetative cells. After T, only spores are present. (Reproduced with permission of Wiley & Sons, New York.)

similar treatment had no effect on normal tissue samples, indicating that whereas significant number of vegetative cells are present in the tumor, the c.f.u.s in normal tissues are due to the presence of spores. The results were quantitatively confirmed: the number of c.f.u. in samples of liver, spleen, brain or eye was not changed after heating. Moreover, the number of spores present in normal tissues decreased as a function of time. After 32 days, in none of the investigated normal tissues, spores could be detected. There was no evidence of clostridia in the urine at either 4 or 8 days following administration of spores. This was not surprising since healthy tissues are well oxygenated and clostridial spores do not germinate under these circumstances. Consistent with this conclusion was the finding that no vegetative cells could be microscopically detected in healthy tissues following Gram staining. Histochemical staining confirmed that vegetative cells were confined to the hypoxic and acellular necrotic areas of the tumor, but could not be detected in the oxygenated regions of the tumor. These results showed that *C. acetobutylicum* has excellent tumor-specific colonization properties.

Lemmon *et al*. investigated the ability of *C. beijerinckii* NCIMB 8052 (formerly *C. acetobutylicum*) to colonize tumors.[21] EMT6 tumor-bearing mice were injected i.v. with 10^8 spores, killed 24 h later and the presence of vegetative bacteria was examined by Gram staining, showing high numbers of bacterial cells in the necrotic regions of tumors and the surrounding region. Occasionally, isolated rods were also found in the more oxygenated regions of the tumor, but no evidence for the presence of bacterial cells was obtained in other tissues, such as heart, kidney, liver, lung and spleen.

The obtained results regarding the tumor colonization properties of *Clostridium*, revealed that to obtain efficient colonization after systemic administration, an inoculum of at least 10^7 spores and a tumor volume threshold of 4 cm^3 was necessary. Tumors smaller than 4 cm^3 were not or only poorly colonized.

The degree of hypoxia in tumors can be increased by treatment with vascular targeting agents, which may improve the tumor colonization and thus allowing small tumors to become colonized by *Clostridium* after i.v. injection. The tumor specific anti-vascular activity of vascular targeting drugs is based on the presence of morphologically and functionally abnormal blood vessels in solid tumors, as a prerequisite for continuous tumoral expansion.[22] Combretastatin A4-phosphate (CombreAp) (OXiGENE, Lund, Sweden) is the first in a new class of tumor vascular targeting drugs that are intended to selectively attack and destroy tumor-specific blood vessels formed by angiogenesis.[23] It interferes with tubulin polymerization, resulting in a massive, rapid and irreversible shutdown of these blood vessels, while leaving normal vasculature unharmed. Treatment of tumor bearing rats with Combretastatin resulted in increased colonization of tumors, espe-

cially of small tumors. This was demonstrated by administration of 10^8 spores of *C. acetobutylicum* NI4082 to WAG/Rij rats with rhabdomyosarcomas of different sizes ranging between 0.2 and 10 cm^3, followed 4 h later by an injection of a single non-toxic dose of Combretastatin (25 mg/kg). *Clostridium* spores were given prior to CombreAp, to allow their distribution throughout the vascular network and in the tumors, before any possible interfering blood vessel change. It was shown that treatment with Combretastatin resulted in a significant improvement of colonization both of small (1–3 cm^3) and very small ($< 1\ cm^3$) tumors. Whereas in Combretastatin-untreated animals, tumors smaller than 4 cm^3 were only poorly colonized ($10^{0.5}$–10^3 c.f.u./g tissue), a dramatic increase in the number of c.f.u. was noticed with a colonization of 10^7–10^8 c.f.u./g tumor tissue for Combretastatin-treated animals. This was obviously a consequence of the massive shutdown of tumor blood vessels in the treated rats, which successfully induced hypoxia within tumors of varying size and allowed anerobic bacteria to proliferate within the tumors. The result of the histopathological examinations and the gross appearance of the tumor at the time of transsection, led to the conclusion that a strong relationship exists between the necrosis induced by CombreAp and the increased presence of *Clostridium*.[24]

Safety of and immune response towards *Clostridium*

To demonstrate that systemically applied clostridia (a class I hazardous agent according to the European list of infectious agents) could be removed from the tumor if desired, the effectiveness of antibiotic treatment with metronidazole (Flagyl®) was evaluated. This antibiotic is used routinely in the clinic and it appears to reach the poorly perfused hypoxic areas, making it ideal for treating anerobic infections. Metronidazole was given twice a day starting at day 5 following spore administration. The number of c.f.u. decreased as a function of time and after 9 days of treatment no bacterial growth could be detected, while colonization in non-treated control animals remained high.[25]

Another important factor to be considered is the eventual induction of an immune response after a single or repeated administration of *Clostridium* and its consequences on tumor colonization. It was shown that a severe host immune response following repeated administration of clostridial spores is not induced. Neither fever nor any loss of body weight was observed when 10^8–10^{10} *Clostridium* spores were systemically administered to rhabdomyosarcoma-bearing WAG/Rij rats, regardless of the injected bacterial load.

Experiments were also designed to investigate whether *Clostridium* could still colonize tumors following a second administration of spores, after eradication of previously administered clostridia with antibiotics. Interestingly, regardless of the immune response status of the host, colonization efficiency of tumors was not affected following repeated spore administration and always occurred to the same extent compared to animals treated only once.[25] This could have important implications since it implies that long-term colonization of *Clostridium* and hence long-term expression of the therapeutic genes introduced into *Clostridium* is possible.

Recombinant clostridia for specific drug delivery in tumors

A new impetus to use clostridia in tumor treatment was the advent of recombinant technology. It provides a possible solution to overcome the deficiency of using wild-type strains for tumor treatment. *Clostridium* can now be genetically modified to produce anti-tumor agents. The production of such therapeutic molecules locally in the tumor, in combination with oncolysis, has the potential to effect a complete cure. This strategy was first realized by Schlechte and Elbe, who attempted to introduce into *C. butyricum* M-55 the gene encoding Colicin E3, an *Escherchia coli*-derived bacteriocin reported to have canceriostatic properties.[26] However, the evidence presented to support the creation of the desired recombinant clostridial strains was not convincing.

Two types of therapeutic agents have, so far, been chosen for delivery to tumors: drugs that exert a direct cytotoxic effect, such as the cytokine tumor necrosis factor (TNF)-α and proteins such as cytosine deaminase or nitroreductase, encoded by so-called 'suicide genes' that convert a non-toxic prodrug into a toxic therapeutic drug. In directed-enzyme prodrug therapy (DEPT), tumor cells are transduced with a gene encoding a protein that converts a relative non-toxic prodrug to a toxic metabolite, thereby creating artificial metabolite differences between normal and malignant cells. The generation of the active drug, therefore, occurs only within the vicinity of the

tumor. Whilst the benefits of selectively eradicating tumor cells are obvious, an important limitation associated with this kind of molecular chemotherapy is the inability to modify 100% of the tumor cells with the toxic gene. However, this limitation has proved not to be as severe as initially thought due to the phenomenon known as 'bystander effect' whereby the eradication of transduced cells elicits a killing effect upon the surrounding non-transduced cells. A number of enzymes and prodrug/drug combinations have been proposed for use in DEPT strategies. So far, targeting of the enzyme to the tumor cell has been done using viral vectors [gene-directed enzyme prodrug therapy (GDEPT)] or antibody-directed enzyme prodrug therapy (ADEPT). However, these methods show low tumor specificity, a limitation which does not apply for a delivering system based on clostridia [clostridial-directed enzyme prodrug therapy (CDEPT)], since the germination of spores is merely reliant on the presence of hypoxic/necrotic regions within the tumor.

Suicide genes such as nitroreductase and cytosine deaminase have been tested already in *Clostridium*. Nitroreductase converts the monofunctional alkylating agent CB1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) to a cytotoxic DNA interstrand cross-linking agent by reduction of its 4-nitro group to the corresponding hydroxylamino species.[27,28] The *E. coli* cytosine deaminase (CDase) converts the non-toxic 5-fluorocytosine (5-FC) to its toxic anabolite 5-fluorouracil (5-FU), which is further metabolized and ultimately interferes with the synthesis of DNA and RNA. In the CDase/5-FC system, the effectiveness of 5-FC in killing tumor cells transfected with the CDase gene has been shown both *in vitro* and *in vivo*. Anti-cancer effects of the CDase/5-FC system have been observed for a wide variety of solid tumors, including colorectal,[29] gastric,[30] hepatocellular,[31] breast cancers [32] and glioma.[33] The action of these suicide genes is associated with a so-called 'bystander effect': eradication of tumor cells transduced with the suicide gene elicits a killing effect upon the surrounding non-transduced tumor cells. In the CDase/5-FC system, less than 4% of transfected cells were proven to be sufficient to achieve a 60% cure rate.[32] This particular enzyme/prodrug system has a number of advantages. As with nitroreductase and CB1954, the enzyme is not found in human tissue, and the differential toxicity between prodrug (5-FC) and drug (5-FU) is large (10^4). An additional attraction of this system is, however, that both 5-FU and 5-FC are currently approved for clinical applications, the former in the treatment of breast and gastrointestinal cancers.

To establish that clostridia may be modified such that they produce anti-cancer enzymes, use has been made of the clostridial expression vectors pIMP1, pKNT19 and pMTL500E. Sequences from the clostripain gene (coding for a cysteine endopeptidase) of *C. histolyticum*[34] were used for expression and secretion of the therapeutic genes. To obtain in-frame fusions between the selected clostridial signal sequence and the coding sequence of CDase, appropriate mutations were introduced at the 3 end of the signal sequence and at the 5 end of the coding sequence of the *E. coli* CDase gene (*codA*). Following transformation of *C. acetobutylicum* using strain-specific electroporation protocols, CDase expression was monitored in lysates and supernatants of early logarithmic growth phase cultures of recombinant *C. acetobutylicum* (pKNT19closcodA). In all samples, a protein of 52 kDa could be observed following SDS–PAGE and Western blotting using the 16D8F2 monoclonal antibody for detection of the *E. coli* CDase protein. No immunoreactive proteins were detected in lysates or supernatants of plasmid-free or *C. acetobutylicum* cultures carrying the control plasmid. These results clearly show the functionality of both the clostripain promoter and signal sequence preceding the CDase DNA. Moreover, a considerable amount of heterologous protein was expressed and efficiently secreted by *Clostridium,* notwithstanding the large size of the *E. coli* CDase protein. Recombinant bacteria containing the pKNT19closcodA construct were cultured and sampled at various stages of growth. CDase activity measurements in lysates and supernatants showed an increase in enzyme activity in both lysates and supernatants until early stationary growth phase (optical density at 600 nm of 1.2). The level of activity in lysates of stationary phase cultures remained high within the subsequent 20 h follow-up period (e.g. maximum enzyme activity = 1084.5 ± 189.5 pmol 5-FC converted to 5-FU/min/ml cell lysate for recombinant *C. acetobutylicum* cultures).[24] In supernatants, CDase activity decreased after this time point. Maximum levels obtained in supernatants were slightly lower as compared to CDase enzymatic activity in lysates (e.g. maximum enzyme activity = 701.9 ± 104.3 pmol 5-FC converted to 5-FU/min/ml supernatant for recombinant *C. acetobutylicum*).

Also, *C. acetobutylicum* strains NI4082 and DSM792 engineered to produce cytosine deaminase are able to express and secrete this enzyme at the tumor site.[25] Functional cytosine deaminase enzyme was detected in the tumor of rhabdomyosarcoma-bearing WAG/Rij rats that were injected with the

recombinant *C. acetobutylicum*, but not in control animals. More importantly, no CDase was found in the normal tissue investigated.

Animals, concomitantly treated with CombreAp, showed higher incidence of CDase-positive tumors (100 versus 58%). Moreover, the level of active CDase in these tumor specimens was considerably higher (mean conversion efficiency of 5-FC to 5-FU ~11%) as compared to tumors not treated with the vascular targeting drug (mean conversion efficiency of 5-FC to 5-FU ~3%). These results illustrate that combining the administration of clostridia and CombreAp treatment increases the therapeutic dose intensity. Obviously, this can also be expected in combination with any other strategy that induces tumor necrosis, such as radiotherapy. In that context, 5-FU has been reported to be an effective radiosensitizer. Based on published data,[35] it was calculated that a 1–3% conversion of 5-FC to 5-FU would be sufficient to achieve clinically significant radiosensitization. Based on the obtained *in vitro* and *in vivo* results, it is reasonable to believe that this is achievable with recombinant clostridia.

An alternative strategy to the use of prodrug converting enzymes is to deliver a therapeutic agent that is anti-tumorogenic on its own, such as TNF-α, a trimeric protein consisting of 17 kDa monomers. This polypeptide was initially identified as a protein released by endotoxin-stimulated macrophages, although several other types of cells are able to synthesize small amounts.[36,37] TNF-α has pleiotropic effects, including selective action on the neovasculature of tumors, stimulation of T cell-mediated immunity and direct cytotoxicity to tumor cells. It can induce both necrotic and apoptotic forms of cell death. Preclinical *in vitro* and *in vivo* studies have demonstrated the anti-tumor capacities of TNF. However, its clinical use is limited by systemic toxicity when administered at an appropriate therapeutic dose. These side effects would be minimized if TNF-α production could be confined to the tumor.

To prove that clostridia are capable to produce biologically active TNF-α, the gene encoding mouse TNF-α was fused to the signal peptide sequence of the *eglA* gene (encoding endo-β1,4-glucanase) of *C. acetobutylicum* P262,[38] together with the *eglA* promoter region. The derivatized TNF-α gene was then cloned into pIM13-derived vectors pIMP1 and pKNT19.[39] Following transformation of *C. acetobutylicum* using strain-specific electroporation protocols, mTNF-α expression in *C. acetobutylicum* recombinant cultures was monitored by Western blot analysis.[41] In lysates of the recombinant cultures, mTNF-α was detected both as a preprotein (21 kDa) and as the mature form (17 kDa). Only the latter was present in the supernatant. mTNF-α could not be detected in lysates or supernatants of the control *Clostridium* cultures. Biologically active mTNF-α was quantified in lysates and supernatants of recombinant *C. acetobutylicum* cultures grown for different time periods, using a cytotoxicity test towards WEHI164 clone 13 cells. In this test, the amount of mTNF-α was spectrophotometrically determined by measuring the *in situ* reduction of the yellow colored MTT to a blue formazan by mitochondrial dehydrogenases of metabolically active cells, thus measuring essentially the percentage of non-viable cells. The high sensitivity of WEHI164 clone 13 cells for TNF makes it possible to detect very low TNF-α concentrations. The mTNF-α concentration in lysates and supernatants of recombinant clostridia increased as the cells grew exponentially to an OD_{600} of approximately 0.6 (mid-log phase). In lysates and supernatants, a maximum of 10^3–10^5 U/ml was found, depending on the recombinant plasmid and strain that was used. The amount of mTNF-α present in the supernatant decreased below the detection limit (3.1 U/ml) after 12 h, whereas in lysates biologically active mTNF-α was still detectable during the 20 h follow-up period. mTNF-α activity was not detected in supernatants or lysates of cultures that were not transformed or transformed with a control plasmid that does not contain the mTNF-α gene. ELISA experiments could detect TNF-α in tumor homogenates of animals treated with the recombinant bacteria, but no TNF-α was detected in the liver of the animals.

Temporal and increased spatial control of gene expression: use of radio-inducible promoters in *Clostridium*

To further increase the specificity of the use of *Clostridium* as a tumor-directed delivery system, the therapeutic gene of interest may be placed under the control of a radio-induced promoter. This will result in an activation of the promoter after irradiation of the tumor, leading to spatial and temporal control of gene expression (i.e. expression of the therapeutic genes will be limited to the irradiated tissues only). More than 75% of all cancer patients are treated with radiotherapy during the progress of their disease. In spite of the enormous progress made over the last decennia, radiotherapy still encounters some limitations. One of the most important problems is hypoxia, a known feature of solid tumors. Since

oxygen fixates DNA damage after ionizing radiation, the absence of oxygen leads to radio-resistance. Therefore, it seems promising to combine radiotherapy with an additional therapeutic modality, such as recombinant anerobic *Clostridium*, which will specifically target these hypoxic cells.

Radio-inducible promoters are being used in many viral vector systems to achieve spatial and temporal control of gene expression.[42–44] It was investigated if radiation-induced gene expression could also be applied for a bacterial vector system using anerobic non-pathogenic clostridia.

Bacteria are known to be very radio-resistant, as a consequence of efficient DNA repair mechanisms. One of these mechanisms is the SOS repair system,[45] which consists of more than 20 genes that are all activated by the occurrence of single-strand DNA breaks. One of the central genes is the *recA* gene. Northern blot hybridizations using RNA extracted from irradiated and non-irradiated clostridia confirmed radio-induction of the *recA* gene, which was evident already at the clinically relevant dose of 2 Gy.[46,47] Quantification of the degree of induction of the *recA* promoter using a reporter system, demonstrated an overall 30% significant increase in activity.[47]

As a consequence, it was further investigated if the secretion yield of a therapeutic protein under the control of this radio-inducible promoter could be increased. Therefore, an *E. coli/Clostridium* shuttle vector was constructed which contained the *recA* upstream the signal sequence that was fused to the 5′ end of the mTNF coding sequence to obtain secretion of TNF-α. *Clostridium* was transformed with the construct via electroporation and subsequently irradiated with a dose of 2 Gy. At different time intervals, samples were taken to quantify the amount of secreted TNF-α using an ELISA. After a single dose of 2 Gy, a significant 44% increase in TNF-α secretion was measured 3.5 h after irradiation as compared to non-irradiated controls[48] (Figure 3). However, in clinical practice, patients are not treated with single doses, but generally with daily doses of 2 Gy. Therefore, it was tested if the promoter activity could be reactivated by a second dose of irradiation. From these experiments, it could be concluded that gene activation indeed could be repeated and that the increase in TNF-α secretion was in the same range as after the first dose.[48] This proved that the radio-inducible promoter could be reactivated after each irradiation when using fractionated radiotherapy. Consequently, cell killing will be increased by an exponent equal to the number of treatments.

However, the *recA* promoter has some basal expression and this will result in some expression of TNF-α under non-irradiated conditions. This is not optimal if temporal control is to be achieved. Therefore, it was examined if basal transcription could be suppressed by adding an extra repressor-binding site, or Cheo box, to the promoter region. Under basal conditions, the repressor DinR binds to this repressor-binding site, limiting transcription of the SOS genes. After activation by radiotherapy, both

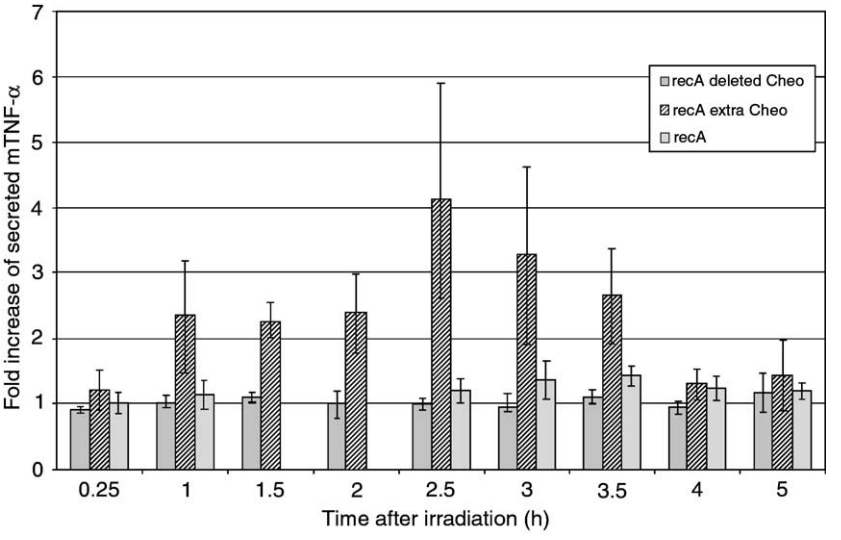

Figure 3. Fold increase of mTNF-α secretion in *Clostridium acetobutylicum* DSM792 pIMP-*recA-mTNF-α* (dotted bars), pIMP-*recA deleted Cheo-mTNF-α* (grey bars) and pIMP-*recA extraCheobox-mTNF-α* (hatched bars) 15 min, and 1, 1.5, 2, 2.5, 3, 3.5, 4 and 5 h after a single dose of 2 Gy. The bars represent data from three independent experiments. Vertical bars represent standard deviations. (Reproduced with permission of Wiley & Sons, New York.)

binding sites would become free and repression would be relieved. This would lead to an increase in transcription of the SOS genes, including *recA*, after radiotherapy. To verify that the Cheo box sequence from the *recA* promoter is indeed the repressor-binding site responsible for the increase in promoter activity after radiotherapy, this sequence was deleted. Bacteria containing this construct, did not show increased TNF-α secretion after irradiation (Figure 3). On the other hand, addition of an extra Cheo box to the *recA* promoter resulted in a 412% increase of secreted TNF-α after irradiation, while only 44% yield increase was obtained using the wild-type promoter. As confirmed by RT-PCR, the increase in secretion after irradiation was evidently the consequence of an increased promoter activity. Hence, the Cheo box sequence is the radio-responsive element and can be used to decrease basal transcription or to increase transcription upon induction.[49] It was also investigated if the Cheo box sequence could be used to bring a constitutive promoter under the control of irradiation. To this end, the Cheo box sequence was incorporated in the promoter region of the constitutive *eglA* promoter. This led to a 242% increase in TNF-α secretion after irradiation with 2 Gy, while the wild-type promoter did not exhibit an increase in TNF-α secretion under the same conditions. These data prove that the Cheo box is functional outside its natural sequence and can be used to bring other promoters under the control of ionizing irradiation.[49]

Taken together, these data show the proof-of-principle that radio-induced promoters can be used to control expression of therapeutic proteins by recombinant clostridia. In contrast to eukaryotic promoters tested so far, the *recA* promoter is activated at the clinically relevant dose of 2 Gy. This promoter gave rise to a significant yield increase of secreted TNF-α after irradiation. Moreover, radio-responsiveness of the promoter could be enhanced by the insertion of an additional repressor-binding site to the promoter sequence. We also proved that strong, constitutive promoters can be brought under the control of ionizing irradiation if repressor-binding sites are incorporated into their promoter region.

Concluding remarks

Conventional cancer therapies showed considerable progress over the last decades thanks to an increasing understanding of the cellular processes underlying oncogenesis and metastasis. They have shown their usefulness in a number of patients, but for some patients current therapies fall short. Therefore, extensive efforts are being made in developing new therapeutic approaches, one of which is gene therapy. However, gene therapy will be only a successful alternative in the case certain technical problems can be overcome. Most importantly, the therapeutic gene or its product must be efficiently and specifically delivered to the tumor cell, while sparing the normal tissue. *Clostridium* seems to fulfill this requirement since they give a selective colonization of solid tumors due to the presence of hypoxia in such tumors. Whereas in the beginning it was hoped that hydrolytic enzymes of the bacteria would defeat the tumor, and as such would cause a complete cure, a major breakthrough came with the possibility to engineer *Clostridium* spp. to produce a variety of heterologous proteins that cause cytotoxic effects. A protein delivery system based on *Clostridium* has, next to its high specificity, several advantages compared to the classical approach using viruses or liposomes. There is no need to transduce the heterologous gene into the genome of the tumor cell, since the therapeutic protein will be secreted from the bacteria and targeted gene expression can be stopped at any time by administration of suitable antibiotics. This is obviously not the case when a gene is inserted within the genome of mammalian cells, as with traditional gene therapeutic approaches. Specificity and safety can even be further increased using radio-inducible promoters to drive gene expression. In *Clostridium*, a radio-induced promoter is present which is already activated at a clinically relevant dose of irradiation and which can be reactivated with a second dose of radiation. The combination of radiotherapy, which preferentially kills well-oxygenated cells, with *Clostridium*-mediated protein delivery to target the hypoxic fraction, opens new perspectives for the future of cancer therapy.

The use of bacterial vectors in the battle against cancer is not limited to *Clostridium*. Besides *Clostridium*, an attenuated *Salmonella typhimurium* auxotroph[50,51] and *Bifidobacterium longum*[52] are being investigated as systems to deliver anti-tumor agents specifically to the tumor site. Selectivity of *Salmonella* for tumors is not based on hypoxia, but on its auxotrophic nature. Therefore, *Salmonella* is supposed to be less tumor specific than *Clostridium*. On the other hand, selectivity of *Bifidobacterium* for tumors is also based on hypoxia as is the case for *Clostridium*, since these bacteria are also obligate anerobic. However, their possibility to produce

therapeutic proteins locally in the tumors remains to be proven.

In conclusion, the presented data show the proof-of-principle that *Clostridium* is an interesting host to specifically deliver therapeutic proteins to the tumor. If further improvement can be obtained with respect to the secretion of proteins to therapeutically relevant doses, an objective which certainly can be obtained when using stronger promoters and improved signal sequences, this system will without doubt be an invaluable alternative to the current classical gene therapy approaches tested in the clinic.

References

1. Horsman MR. Hypoxia in tumors: its relevance, identification and modification. In: Beck-Bornholdt HP, ed. *Current topics in clinical radiobiology*. Berlin: Springer 1993: 99–112.
2. Brown JM, Giacca AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 1998; **58**: 1408–16.
3. Young M, Marshall RS, Hill RP. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. *Proc Natl Acad Sci USA* 1988; **85**: 9533–7.
4. Giacca AJ. Hypoxic stress proteins: survival of the fittest. *Semin Radiat Oncol* 1996; **6**: 46–58.
5. Graeber TG, Osmanian C, Jacks T, *et al.* Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* 1996; **379**: 88–91.
6. Brizel DM, Scully SP, Harrelson JM, *et al.* Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. *Cancer Res* 1996; **56**: 941–3.
7. Dachs GU, Patterson AV, Firth JD, *et al.* Targeting gene expression to hypoxic tumor cells. *Nat Med* 1997; **3**: 515–20.
8. Malmgren RA, Flanigan CC. Localization of the vegetative form of *Clostridium tetani* in mouse tumours following intravenous spore administration. *Cancer Res* 1955; **15**: 473–8.
9. Möse JR, Möse GZ. Onkolyseversuche mit apathogen, anaerogen sporenbildern am Erhlich-Tumor der maus. *Z Krebsforsch* 1959; **63**: 63–74.
10. Möse JR. Zur Beeinflussbarkeit verschiedener tiertumoren durch einen apathogenen Clostridienstamm. *Z Krebsforsch* 1960; **63**: 447–55.
11. Möse JR, Möse G. Oncolysis by clostridia. I. Activity of *Clostridium butyricum* (M-55) and other nonpathogenic clostridia against the Ehrlich carcinoma. *Cancer Res* 1964; **24**: 212–6.
12. Möse JR. Versuche zur verbesserung der onkolyse mit dem clostridienstamm. *Zbl Bakt Hyg I Abt Orig A* 1979; **244**: 541–5.
13. Kretschmer H. Treatment of malignant brain tumours by *Clostridium butyricum* M55. In: *Proc VIIth Int Congr of Chemotherapy* 1972: 721–723.
14. Heppner F, Möse JR. The liquefaction (oncolysis) of malignant gliomas by a non pathogenic. *Clostridium. Acta Neurochir* 1978; **42**: 123–5.
15. Heppner F, Möse JR, Ascher PW, Walter G. Oncolysis of malignant glioma of the brain. In: *Proc VIIIth Int Congr of Chemotherapy* 1983: 38–44.
16. Minton NP, Clarke DJ, eds. *Clostridia biotechnology handbooks*. New York: Plenum 1989; **3**.
17. Woods DR. *The Clostridia and biotechnology*. Boston, MA: Butterworth-Heinemann 1993
18. Minton NP, Brehm, JK, Swinfield, TJ, *et al*. Clostridial cloning vectors. In: Woods DR, ed. *The Clostridia and biotechnology*. Boston, MA: Butterworth-Heinemann 1993: 119–50
19. Mauchline ML, Davis TO, Minton NP. Genetics of clostridia. In: Demain AL, Davies JE, eds. *Manual of industrial microbiology and biotechnology*, 2nd edn. Washington DC: ASM Press 1999: 475–90.
20. Lambin P, Theys J, Landuyt W, *et al.* Colonisation of the *Clostridium* in the body is restricted to hypoxic and necrotic areas of tumours. *Anaerobe* 1998; **4**: 183–8.
21. Lemmon MJ, van Zijl P, Fox ME, *et al.* Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment. *Gene Ther* 1997; **4**: 791–6.
22. Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992; **3**: 65–71.
23. Horsman MR, Murata R, Breidahl T, *et al.* Combretastatins novel vascular targeting drugs for improving anti-cancer therapy. Combretastatins and conventional therapy. *Adv Exp Med Biol* 2000; **476**: 311–23.
24. Theys J, Landuyt W, Nuyts S, *et al.* Specific targeting of cytosine deaminase to solid tumors by engineered *Clostridium acetobutylicum*. *Cancer Gene Ther* 2001; **8**: 294–7.
25. Theys J, ,Landuyt W, Nuyts S, *et al.* Improvement of *Clostridium* tumour targeting vectors evaluated in rat rhabdomyosarcomas. *FEMS Immunol Med Microbiol* 2001; **30**: 37–41.
26. Schlechte H, Elbe B. Recombinant plasmid DNA variation of *Clostridium oncolyticum*—model experiments of cancerostatic gene transfer. *Zbl Bakt Hyg A* 1988; **268**: 347–56.
27. Knox RJ, Friedlos F, Jarman M, Roberts JJ. A new cytotoxic DNA interstrand crosslinking agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. *Biochem Pharmacol* 1988; **37**: 4661–9.
28. Knox RJ, Boland MP, Friedlos F, Coles B, Southan C, Roberts JJ. The nitroreductase in Walker cells that activates 5-(aziridin-1-y1)-4-hydroxylamino-2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone) (EC 1.6.99.2). *Biochem Pharmacol* 1988; **37**: 4671–7.
29. Nanni P, De Giovanni C, Nicoletti G, *et al.* The immune response elicited by mammary adenocarcinoma cells transduced with interferon-gamma and cytosine deaminase genes cures lung metastases by parental cells. *Hum Gene Ther* 1998; **9**: 217–24.

30. Ge K, Xu L, Zheng Z, *et al.* Transduction of cytosine deaminase gene makes rat glioma cells highly sensitive to 5-fluorocytosine. *Int J Cancer* 1997; **71**: 675–9.
31. Ichikawa T, Tamiya T, Adachi Y, *et al. In vivo* efficacy and toxicity of 5-fluorocytosine/cytosine deaminase gene therapy for malignant gliomas mediated by adenovirus. *Cancer Gene Ther* 2000; **7**: 74–82.
32. Trinh QT, Austin EA, Murray DM, Knick VC, Huber BE. Enzyme/prodrug gene therapy: comparison of cytosine deaminase/5-fluorocytosine versus thymidine kinase/ganciclovir enzyme/prodrug systems in human colorectal carcinoma cell line. *Cancer Res* 1995; **55**: 4808–12.
33. Aghi M, Hochberg F, Breakefield XO. Prodrug activation enzymes in cancer gene therapy. *J Gene Med* 2000; **2**: 148–64.
34. Dargatz H, Diefenthal T, Witte V, Reipen G, von Wettstein D. The heterodimeric protease clostripain from *Clostridium histolyticum* is encoded by a single gene. *Mol Gen Genet* 1993; **240**: 140–5.
35. Lambin P, Nuyts S, Landuyt W, *et al.* The potential therapeutic gain of radiation-associated gene therapy with the suicide gene cytosine deaminase. *Int J Radiat Biol* 2000; **76**: 285–93.
36. Seung LP, Mauceri HJ, Beckett MA, Hallahan DE, Hellman S, Weichselbaum RR. Genetic radiotherapy overcomes tumor resistance to cytotoxic agents. *Cancer Res* 1995; **55**: 5561–5.
37. Weichselbaum RR, Hallahan DE, Beckett MA, *et al.* Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. *Cancer Res* 1994; **54**: 4266–9.
38. Zappe H, Jones WA, Jones DT, Woods DR. Structure of an *endo-β-1,4-glucanase* gene from *Clostridium acetobutylicum* P262 showing homology with endoglucanase genes from *Bacillus* spp. *Appl Environ Microbiol* 1988; **54**: 1289–92.
39. Azzedoug HJ, Hubert J, Reysset G. Stable inheritance of shuttle vectors based on plasmid p1M13 in a mutant strain of *Clostridium acetobutylicum*. *J Gen Microbiol* 1992; **138**: 1371–8.
40. Mermelstein LD, Welker HE, Bennett GN, Papotsakis ET. Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. *BioTechnology* 1992; **10**: 190–5.
41. Theys J, Nuyts S, Landuyt W, *et al.* Stable *Escherichia coli–Clostridium acetobutylicum* shuttle vector for secretion of murine tumor necrosis factor alpha. *Appl Environ Microbiol* 1999; **65**: 4295–300.
42. Datta R, Rubin E, Sukhatme V, *et al.* Ionizing radiation activates transcription of the EGR1 gene via CarG elements. *Proc Natl Acad Sci USA* 1992; **89**: 10149–53.
43. Hallahan DE, Maucero HJ, Seung LP, *et al.* Spatial and temporal control of gene therapy using ionizing irradiation. *Nat Med* 1995; **1**: 786–91.
44. Marples B, Scott D, Hendry JH, *et al.* Development of synthetic promoters for radiation-mediated gene therapy. *Gene Ther* 2000; **7**: 511–7.
45. Miller RV, Kokjohn TA. General microbiology of *recA*: environmental and evolutionary significance. *Annu Rev Microbiol* 1990; **44**: 365–94.
46. Nuyts S, Theys J, Landuyt W, Van Mellaert L, Anné J, Lambin P. Increasing specificity of anti-tumor therapy: cytotoxic protein delivery by non-pathogenic clostridia under regulation of radio-induced promoters. *Anticancer Res* 2001; **21**: 857–62.
47. Nuyts S, Van Mellaert L, Theys J, Landuyt W, Lambin P, Anné J. The use of radio-induced bacterial promoters in anaerobic conditions: a means to control gene expression in *Clostridium* mediated therapy for cancer. *Radiat Res* 2001; **155**: 716–23.
48. Nuyts S, Van Mellaert L, Theys J, *et al.* Radioresponsive *recA* promoter significantly increases TNFα production in recombinant clostridia after 2 Gy irradiation. *Gene Ther* 2001; **8**: 1197–201.
49. Nuyts S, Van Mellaert L, Barbé S, *et al.* Insertion or deletion of the Cheo box modifies radio-inducibility of *Clostridium* promoters. *Appl Environ Microbiol* 2001; **67**: 4464–70.
50. Pawelek JM, Low KB, Bermudes D. Tumor-targeted Salmonella as a novel anticancer vector. *Cancer Res* 1997; **57**: 4537–44.
51. Low KB, Ittensohn M, Le T, *et al.* Lipid A mutant *Salmonella* with suppressed virulence and TNFalpha induction retain tumor-targeting *in vivo*. *Nat Biotechnol* 1999; **17**: 37–41.
52. Yazawa K, Fujimori M, Amano J, Kano Y, Taniguchi S. *Bifidobacterium longum* as a delivery system for cancer gene therapy: selective localization and growth in hypoxic tumors. *Cancer Gene Ther* 2000; **7**: 269–74.

(Received 5 November 2001; accepted 13 November 2001)