Secretory production of biologically active rat interleukin-2 by Clostridium acetobutylicum DSM792 as tool for anti-tumor treatment

ELSEVIER

FEMS Microbiology Letters 246 (2005) 67–73

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Secretory production of biologically active rat interleukin-2 by *Clostridium acetobutylicum* DSM792 as a tool for anti-tumor treatment

Sofie Barbé [a], Lieve Van Mellaert [a], Jan Theys [b], Nick Geukens [a], Elke Lammertyn [a], Philippe Lambin [b], Jozef Anné [a,*]

[a] *Laboratory of Bacteriology, Rega Institute for Medical Research, K.U. Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*
[b] *MAASTRO Lab/GROW, University Maastricht, P.O. Box 616, 6200 MD Maastricht, The Netherlands*

Received 4 January 2005; received in revised form 18 March 2005; accepted 21 March 2005

First published online 1 April 2005

Edited by R.Y.C. Lo

Abstract

The search for effective means of selectively delivering high therapeutic doses of anti-cancer agents to tumors has explored a variety of systems in the last decade. The ability of intravenously injected clostridial spores to infiltrate and thence selectively germinate in the hypoxic regions of solid tumors is exquisitely specific, making this system an interesting addition to the anti-cancer therapy arsenal. To increase the number of therapeutic proteins potentially useful for cancer treatment we have tested the possibility of *Clostridium acetobutylicum* to secrete rat interleukin-2 (rIL2). Therefore, rIL2 cDNA was placed under the control of the endo-β-1,4-glucanase promoter and signal sequence of *C. saccharobutylicum*. Recombinant *C. acetobutylicum* containing the relevant construct secreted up to 800 $\mu g\, l^{-1}$ biologically active rIL2. The obtained yield should be sufficient to provoke in vivo effects.

Keywords: *Clostridium acetobutylicum*; Protein secretion; Interleukin-2; Anti-cancer treatment

1. Introduction

During the last decade, several bacterial species have been tested for cancer treatment purposes. The selected bacteria specifically colonize tumors and can be used to explicitly deliver prodrug converting enzymes and other therapeutic proteins to tumors of selected animal models [1]. For strictly anaerobic bacteria such as *Clostridium* and *Bifidobacterium* this specificity is based on the fact that solid tumors are characterized by the presence of hypoxic/necrotic regions [2,3]. An auxotrophic, attenuated *Salmonella enferica* serovar Typhimurium strain (TAPET VNP20009) specifically colonizes tumor tissue because of its requirement of nutrients, abundantly present in necrotic tumor regions [4].

In recent years, some non-apathogenic *Clostridium* strains were transformed with genes coding for the prodrug-converting enzyme cytosine deaminase [5] and the cytokine murine tumor necrosis factor alpha (TNF-α) [6] giving rise to biologically functional proteins. This system could be used to deliver prodrug converting enzymes to tumors of selected animal models [5], and has been shown in many instances to result in tumor regression following the systemic administration of prodrug. In the case of TNF-α, a problem remains to obtain sufficient amounts of the biological active

* Corresponding author. Tel.: +32 16 337371; fax: +32 16 337140.
E-mail address: jozef.anne@rega.kuleuven.ac.be (J. Anné).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.03.037

compound at the tumor site. This could be overcome either by finding ways to increase the amount of TNF-α produced at the tumor site or by taking advantage of the synergistic effect of interleukin-2 (IL2) and TNF-α [7]. Moreover, IL2 has more general anti-tumor effects. IL2 is a pluripotent cytokine that enhances both non-specific immune responses such as the activation of natural killer and lymphokine-activated killer cells, and major histocompatibility complex restricted T-cell responses resulting in neoplastic cell killing [8].

In 1985, Rosenberg et al. [9] published their first successful results on the systemic treatment of metastatic melanoma and renal cell cancer patients with lymphokine-activated killer cells and high-dose recombinant IL2. Unfortunately, this systemic high-dose IL2 therapy is associated with severe, toxic side effects including vascular leak syndrome, oedema, anemia, nausea and hypotension [10]. Nevertheless, high-dose IL2 therapy is currently clinically applied to patients with metastatic melanoma and renal carcinoma and mediates tumor regression in a number of patients [11,12].

The toxic side effects of this therapy can be avoided by local administration of the cytokine at the tumor site to stimulate the activity of the tumor-infiltrating lymphocytes. This was accomplished in multiple murine tumor models by direct intratumoral injection [13] and various gene therapy strategies [14–16] resulting in significant anti-tumor efficacy. These animal studies support the applicability of local IL2 administration.

An alternative approach to provide IL2 locally at the tumor site is making use of the exquisite specificity of *Clostridium* to colonize tumors. When *Clostridium* can secrete enough IL2, an additional, specific IL2 delivery system would become available. A first step to investigate the applicability of this approach is to examine if *Clostridium* can produce sufficient amounts of biologically active IL2. To investigate this, we have cloned rat IL2 (rIL2) cDNA in the *Escherichia coli–Clostridium* shuttle vector pIMP1. In this paper we present data on rIL2 production by recombinant *C. acetobutylicum* DSM792, which holds new perspectives for the clinical application of the *Clostridium*-mediated anti-cancer system.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

C. acetobutylicum DSM792 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH (Braunschweig, Germany). This strain was grown at 37 °C in 2 × yeast-tryptone (2 × YT) medium [17], buffered with 50 mM 2-[*N*-morpholino]ethanesulfonic acid pH 6.3, in a MACS anaerobic chamber (Don Whitley Scientific Ltd., West Yorkshire, UK) containing 90% N_2 and 10% H_2 and palladium as a catalyst.

For primary vector constructions, *E. coli* TG1 [18] was used. *E. coli* ER2275 was used for in vivo methylation of plasmid DNA prior to electroporation of *Clostridium* [19]. These strains were grown in Luria–Bertani broth at 37 °C (300 rpm). For solid media, 15 g of agar was added per liter. Media were supplemented, when applicable, with erythromycin (25 μg ml^{-1}), ampicillin (50 μg ml^{-1}), chloramphenicol (35 μg ml^{-1}), isopropyl-β-thiogalactopyranoside (120 μg ml^{-1}) or 5-bromo-4-chloro-3-indolyl-β-galactoside (25 μg ml^{-1}).

Table 1 lists the plasmids used in the study. For cloning purposes the *E. coli–Clostridium* shuttle plasmid pIMP1 [20] was used. Plasmid pHZ117 served as source for the *eglA* gene of *C. saccharobutylicum* P262 [21].

2.2. Transformation procedures and DNA manipulations

Standard techniques for DNA manipulations were carried out as described by Sambrook et al. [18]. Restriction endonucleases and other DNA-modifying enzymes were purchased from Invitrogen Life Technologies (Paisley, UK), New England BioLabs (Beverly, USA) or TaKaRa Bio Inc. (Shiga, Japan) and used as indicated by the suppliers. Oligonucleotides were obtained from Eurogentec (Seraing, Belgium).

DNA fragments were isolated from 1% agarose gels with a Wizard SV gel and PCR clean-up system (Promega Inc., Madison, WI). Plasmid DNA from *E. coli* was isolated with a Wizard Plus SV miniprep kit (Promega Inc., Madison, WI), while plasmid DNA from *C. acetobutylicum* was isolated by an alkaline lysis procedure [22]. Prior to electroporation of *C. acetobutylicum* DSM792, plasmid DNA preparations were desalted by two spins in Microcon 100 microconcentrators (Amicon Inc., Beverly, MA) as recommended by the manufacturer.

DNA sequence analysis was carried out according to the dideoxy chain termination method with an automated laser fluorescence ALFexpress sequencer (Amersham Biosciences, Uppsala, Sweden). The Cy5-labeled primers used for sequencing were the M13 universal forward and reverse primer and the EGLAP oligonucleotide (CCATGAAGGGAGGAAAAAACTATC) complementary to a fragment at the 3′ end of the *eglA* promoter.

2.3. Isolation of rIL2 cDNA

To obtain rIL2 cDNA, the spleen of a WAG/Rij rat was removed and homogenized. After isolation of the mononuclear cells with the Accuspin System-Histopaque-1077 (Sigma–Aldrich, St. Louis, USA) and stimulation of these cells with 5 μg ml^{-1} phytohaemag-

Table 1
Plasmids used in this study

Plasmid	Description[a]	Source or reference
E. coli plasmids		
pBluescriptIIKS (pBS)	MCS, Ap^r, LacZα	Stratagene
pGEM®-T Easy	MCS, Ap^r, LacZα	Promega
pGEMrIL2	pGEM-T Easy derivative with rIL-2 cDNA	This work
pBSeglA	pBS derivative containing the *eglA* promoter and signal sequence	This work
pBSeglArIL2	pBS derivative with rIL-2 cDNA fused to the *eglA* promoter and signal sequence	This work
pAN1	MCS, Cm^r, *B. subtilis* phage phi 3TI methyltransferase gene	[19]
pHZ117	Contains the *C. saccharobutylicum* P262 *eglA* gene	[21]
E. coli–C. acetobutylicum shuttle plasmids		
pIMP1	MCS, Ap^r, Em^r	[20]
pIMP1eglArIL2	pIMP1 derivative with rIL-2 cDNA fused to the *eglA* promoter and signal sequence	This work

[a] Ap^r, ampicillin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant; MCS, multiple cloning site.

glutinin for 90 h, RNA was isolated with TRIzol Reagent (Invitrogen Life Technologies, Paisley, UK). Subsequently, cDNAs were prepared using random hexanucleotides and the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences, Vilnius, Lithuania). rIL2 cDNA (GenBank Accession No. NM1053836) was PCR-amplified from the obtained rat spleen cDNA mixture using the oligonucleotides RIL2FW (TATATGCGCACCCACTTCAAGCCCTGC, *Mst*I site underlined, complementary to nucleotides 78–94) and RIL2REV (TAGAATTCTTACTGAGTCATTGTTGAGATG, *Eco*RI site underlined, complementary to nucleotides 461–482) and ExTaq DNA polymerase following the protocol provided by the manufacturer. An *Mst*I and an *Eco*RI restriction site were introduced at the 5′ and 3′ end of the rIL2 cDNA, respectively. Subsequently, the 0.4-kb PCR fragment was cloned in pGEM®-T Easy (Promega, Madison, WI), resulting in pGEMrIL2. The rIL2 cDNA was cloned in such orientation that a unique *Sal*I restriction site was located downstream of the rIL2 stop codon. The sequence of rIL2 cDNA was determined and appeared to contain a silent mutation: a substitution (A > G) had occurred at the third base position of codon 38 without changing the encoded Leu residue.

2.4. Vector construction

A 0.4-kb *Eco*RI/*Hin*dIII fragment containing the *eglA* promoter, the predicted ribosome binding site and the signal sequence [6] was cloned in pBluescriptIIKS (pBS), resulting in pBSeglA. Next, pBSeglA was digested with *Hin*dIII of which the recognition site is situated at the 3′ end of the *eglA* signal sequence and the 5′ protruding ends were blunted in a Klenow fragment polymerase reaction. Following *Sal*I digestion, this vector was used for ligation from the 0.4-kb *Mst*I/*Sal*I rIL2 fragment isolated from pGEMrIL2. The resulting plasmid was named pBSeglArIL2. The direct in-frame fusion of the *eglA* signal sequence with rIL2 cDNA was confirmed by DNA sequence analysis. Finally, the expression/secretion cassette was transferred to the *E. coli–Clostridium* shuttle plasmid pIMP1 as a 0.8-kb *Eco*RI fragment, resulting in pIMP1eglArIL2 (Fig. 1).

2.5. Immunoblot analysis

For rIL2 detection, cells were harvested at various time periods during growth of *C. acetobutylicum* (pIMP1eglArIL2). At each interval, 10 ml *Clostridium* culture was centrifuged (12,000*g*, 10 min) and the cell pellet was resuspended in 1 ml 100 mM Tris/HCl pH 7.4. Next, the cells were lysed by sonication at 0 °C with pulses of 30 s. Finally, the cell remnants were removed by centrifugation (13,000*g*, 10 min). Proteins present in 5 ml culture filtrate were precipitated with trichloroacetic acid at a final concentration of 20%. Subsequently, proteins present in lysate and culture supernatant samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [23]. Transfer of proteins onto a nitrocellulose Porablot membrane (Macherey-Nagel, Düren, Germany) was performed using a BioRad Transblot semidry transfer cell (BioRad, Hercules, CA, USA) according to the manufacturer's recommendations. For immunodetection of rIL2, polyclonal rabbit anti-rIL2 (BioSource International, Camarillo, CA, USA) and alkaline phosphatase-conjugated anti-rabbit antibodies (Sigma–Aldrich, St. Louis, USA) were used. Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche, Mannheim, Germany) were used as substrate for the alkaline phosphatase reaction and the colour development was carried out as described by the manufacturer.

2.6. ELISA and biological activity assessment

At various time intervals during growth of *C. acetobutylicum* (pIMP1eglArIL2), 1 ml culture was centrifuged (13,000*g*, 3 min) and the culture supernatant was collected and stored at −80 °C. The cell pellet was resuspended in 1 ml 100 mM Tris/HCl pH 7.4 and

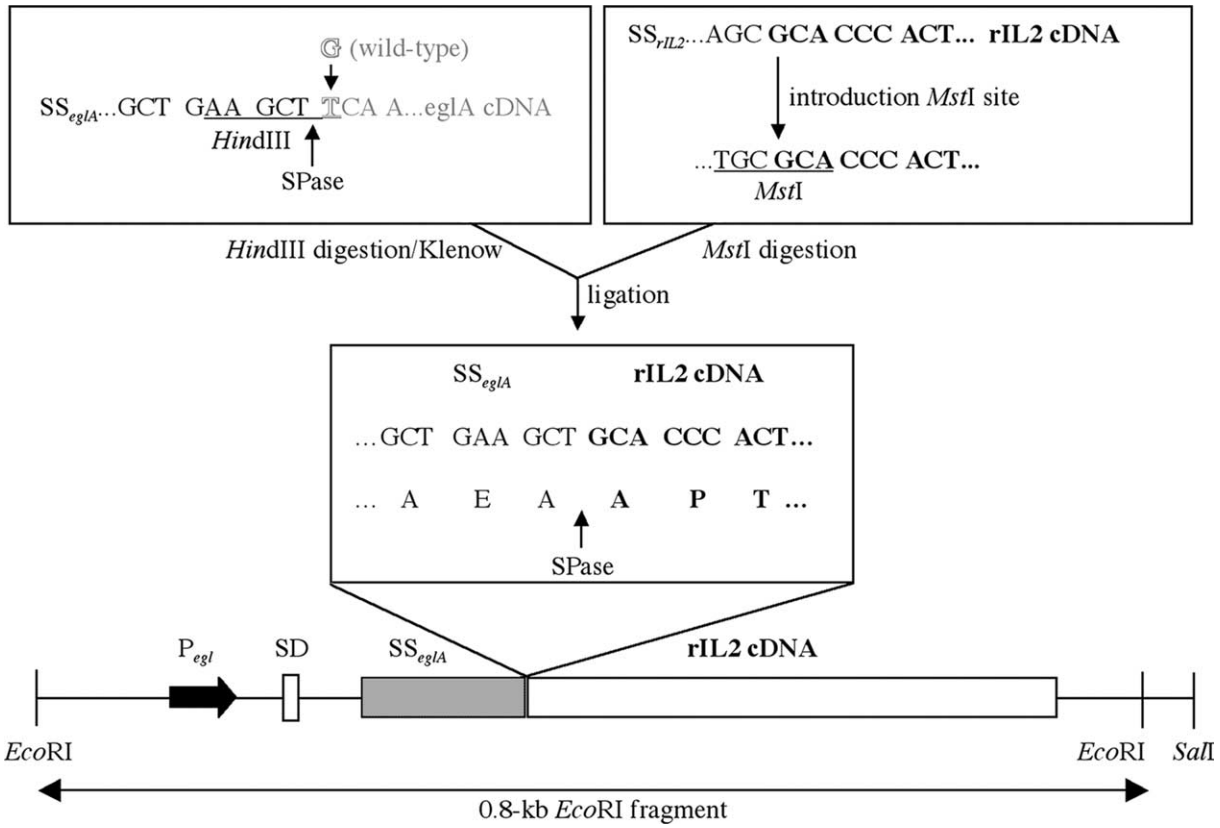


Fig. 1. Schematic representation of the construction of the *eglA*-rIL2 cDNA expression/secretion cassette cloned in pIMP1eglArIL2. P_{eglA}, promoter region of *eglA*; SS_{eglA}, signal sequence of *eglA*; SD, Shine–Dalgarno sequence; SPase, signal peptidase cleavage site.

the cells were broken by ultrasonic treatment with pulses of 30 s. The cellular debris was removed by centrifugation for 10 min at 13,000*g* and the cleared lysate was stored at −80 °C. rIL2 present in lysates and extracellular fractions was quantified with an Endogen rat IL2 ELISA Kit (PerBio, Rockford, USA) according to the manufacturer's protocol. Lysates and culture supernatants were diluted 100- and 1000-fold, respectively.

The biological activity of the rIL2 produced by *C. acetobutylicum* (pIMP1eglArIL2) was determined using a proliferation assay with an IL2-dependent murine cytotoxic T-lymphocyte cell line [24]. The IL2 concentration was proportional to the amount of [^{3}H]thymidine incorporated by proliferating T-cells. The international standard recombinant murine IL2 (National Institute for Biological Standards and Controls, Hertfordshire, UK) was used as internal control.

3. Results

3.1. Construction of the rIL2 expression/secretion vector

To establish secretory production of rIL2 under the control of the promoter, ribosome binding site, and signal sequence of the *eglA* gene, the mature part of the rIL2 cDNA was translationally fused to the *eglA* signal sequence. To obtain this in-frame fusion between the *eglA* signal sequence and the rIL2 cDNA coding sequence, restriction sites were created at the 3′ end of the *eglA* signal sequence [6] and at the 5′ end of the rIL2 cDNA. The direct in-frame fusion was confirmed by DNA-sequence analysis. The complete expression/secretion cassette was finally inserted in the *E. coli–Clostridium* shuttle vector pIMP1, resulting in pIMP1eglA-rIL2 (Fig. 1).

Coincidentally, the first codon of the rIL2 cDNA, GCA (alanine), is the same as the first codon of the mature eglA protein. As such, the +1 position amino acid residue of the signal peptidase recognition site in the fusion protein was the same as in the EglA protein. The conservation of the native signal peptidase cleavage site has previously been shown to have a positive effect on yield, probably as a result of enhanced processing of the heterologous preprotein [25].

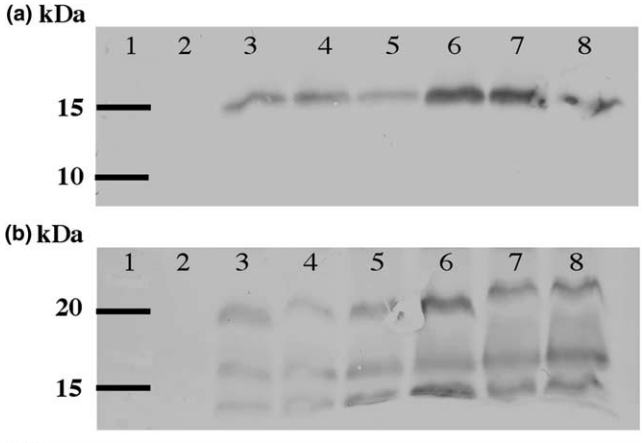
Expression of the fusion gene could already be observed in *E. coli* TG1 (pIMP1eglArIL2) by immunodetection of rIL2, since the *eglA* promoter is functional in *E. coli*. The results (data not shown) clearly showed the production of rIL2 in the *E. coli* transformant.

3.2. rIL2 production by C. acetobutylicum

The pIMP1eglArIL2 plasmid was methylated in vivo [19] and then transferred to *C. acetobutylicum* DSM792 by electroporation [22]. The presence of the correct plasmid within the obtained *C. acetobutylicum* recombinants was verified by plasmid isolation, restriction enzyme analysis and appropriate PCR amplifications.

Selected recombinants were cultured and sampled at different time intervals. rIL2 production was monitored in culture filtrates and cell lysates. Immunoblot analysis of precipitated proteins present in culture supernatants revealed a single immunoreactive band of 15 kDa, which correlates to the molecular mass of rIL2 (Fig. 2(a)). Immunoblot analysis of lysates of *C. acetobutylicum* (pIMP1eglArIL2) showed two strong immunoreactive bands of about 15 and 19.2 kDa, corresponding to the calculated mass for mature rIL2 and unprocessed precursor protein, respectively. A third, smaller band represents presumably a partially degraded protein (Fig. 2(b)). As expected no rIL2 could be detected in samples of the negative control containing *C. acetobutylicum* with the empty vector. Correlating the results of rIL2 immunodetection with the growth curve (Fig. 2(c)) reveals that the production of rIL2 predominantly accumulates during the exponential growth phase. It was shown that the growth rate of *C. acetobutylicum* was not affected by rIL2 production compared to the wild-type strain.

To quantify the amounts of rIL2 expressed and secreted by recombinant *C. acetobutylicum*, an ELISA was performed with lysates and extracellular fractions taken during the exponential growth phase. Levels of extracellular rIL2 reached from 85 up to 800 ng ml^{-1} culture in the mid-exponential growth phase (OD value of 2.7) while intracellular amounts of 3–133 ng ml^{-1} culture were obtained in the late exponential phase (OD value of 4.0). Secretion efficiencies were calculated as the ratio of secreted rIL2 to the total amount of rIL2 produced. These were 97% initially and decreased to 77% in the late exponential phase.



(c)

Lane	2	3	4	5	6	7	8
OD	1.00	0.52	0.84	1.00	2.08	3.48	3.91

Fig. 2. Immunoblot analysis with rIL2-specific antibodies to detect rIL2 in (a) extracellular and (b) intracellular fractions of *C. acetobutylicum* (pIMP1eglArIL2). Lanes: 1, molecular mass; 2, lysate *C. acetobutylicum* DSM792 (negative control); 3–8, samples taken at different time intervals during growth. The OD values of the *Clostridium* culture at the time of sampling are shown in the table (c).

To evaluate the biological activity of the produced IL2, the T-cell proliferation assay was used. Results indicated that IL2 levels reached up to 1×10^5 IU IL2 per ml in the supernatant, while intracellularly 12×10^3 IU ml^{-1} was measured.

4. Discussion

The experiments described here aimed to enlarge the therapeutic toolbox of the *Clostridium*-mediated approach of anti-tumor therapy. By showing that *Clostridium*, besides the already tested proteins including the prodrug converting enzyme cytosine deaminase [5] and the cytokine TNF-α [6], can efficiently produce IL2 in therapeutically relevant concentrations and as a biologically active compound, makes this protein transfer system a more versatile system that can be deployed in anti-tumor therapy. The selection to test IL2 was based on several criteria discussed below.

Immunotherapy with products that enhance the immune system's natural anti-cancer functions such as IL2, is a promising new addition to the family of cancer treatments that includes surgery, chemotherapy and radiation therapy. The anti-tumor response of IL2 in cancer immunotherapy can be attributed to a number of actions: the activation and expansion of tumor-specific T-cells recognizing tumor antigens, the activation of cytotoxic activity of natural killer cells and macrophages, the release of secondary cytokines by activated lymphocytes and the offset/overcoming of immunosuppression mechanisms in the tumor microenvironment [8,11].

The obtained rIL2 levels are likely to be of therapeutically relevant amounts as significant reduction in tumor growth has been established following administration of IL2-secreting tumor cells, in vitro capable of secreting 300 ng IL2 (10^6 tumor cells)$^{-1}$ 24 h^{-1}, to pancreatic tumor-bearing mice [26].

Systemic administration of high doses of IL2 (maximum tolerable dose: 18×10^6 IU kg^{-1} for up to 5 days [9]) to patients with metastatic melanoma or renal cell cancer resulted in response rates of approximately 15% and 21%, respectively [12,27]. However, the application of this therapy is limited by the occurrence of undesirable systemic toxicity, which can be circumvented by tumor-directed cytokine approaches such as intratumoral

IL2 injection [13] and various gene therapy strategies [14–16]. However, the utility of IL2 as a single agent is limited. Enhanced or synergistic anti-tumor activities were obtained in various rodent tumor models treated with a combination of IL2 therapy with other cytokines, prodrug-converting enzymes, radiotherapy or chemotherapeutic compounds [28,29,15,16].

The combination of IL2 recombinant *Clostridium* spores with vascular targeting agents, which induce severe vascular shutdown followed by necrosis [30,31], could possibly enhance the therapeutic efficacy of IL2 secreting clostridia. The combination of both agents will not only enhance the colonization of IL2 secreting *Clostridium*, but will also increase the release of tumor antigens from cells that have become necrotic. This release can improve the anti-tumor response by IL2-activated immune cells.

Because small metastatic lesions cannot be colonized by *Clostridium*, even after administration of vascular targeting compounds, it was agreed that the *Clostridium* system was not suitable for the effective treatment of metastised cancer. However, the clinical trials carried out by Rosenberg et al. [11] were evidence that manipulation of the immune system could result in regression of metastatic lesions growing in distant, visceral organs of cancer patients. Possibly the effector cells which become activated at the primary tumor site by locally secreted IL2 can exert their augmented anti-tumor effects in the metastatic lesions.

The ability of *C. acetobutylicum* to secrete biologically active IL2 in therapeutically sufficient amounts reinforces the *Clostridium*-mediated anti-cancer system. Taking advantage of the tumor-specific targeting of the recombinant clostridia, IL2 can now be delivered intratumorally avoiding systemic toxicity. The availability of rIL2-producing *Clostridium* opens the way to investigate its efficacy in tumor growth delay in animal tumor models. This will be investigated in the near future. In addition, therapeutic effects emanating from combination of IL2 recombinant *Clostridium* with *Clostridium*-delivered cytokines such as TNF-α or prodrug-converting enzymes and with radiotherapy or chemotherapy will be investigated.

Acknowledgements

We acknowledge financial support from Het Fonds voor Wetenschappelijk Onderzoek Vlaanderen (grant G.0375.02) and the European Union (grant QLRT – 2000 – 01737). Sofie Barbé is a research fellow of IWT-Vlaanderen (Institute for the Promotion of Innovation through Science and Technology in Flanders). We would like to thank Oda Steenhoudt (DiaMed Eurogen, Turnhout, Belgium) for providing rat spleen cDNA. We are grateful to Pieter Rottiers and Wilma Burm (VIB, Ghent, Belgium) for biological activity measurements of produced rIL2. Plasmid pHZ117 was a kind gift from H. Zappe.

References

[1] Theys, J., Barbé, S., Landuyt, W., Nuyts, S., Van Mellaert, L., Wouters, B., Anné, J. and Lambin, P. (2003) Tumor-specific gene delivery using genetically engineered bacteria. Curr. Gene Ther. 3, 207–221.

[2] Lambin, P., Theys, J., Landuyt, W., Rijken, P., van der Kogel, A., van der Schueren, E., Hodgkiss, R., Fowler, J., Nuyts, S., de Bruijn, E., Van Mellaert, L. and Anné, J. (1998) Colonisation of *Clostridium* in the body is restricted to hypoxic and necrotic areas of tumors. Anaerobe 4, 183–188.

[3] Fujimori, M., Amano, J. and Taniguchi, S. (2002) The genus *Bifidobacterium* for cancer gene therapy. Curr. Opin. Drug. Discov. Devel. 5, 200–203.

[4] Clairmont, C., Lee, K.C., Pike, J., Ittensohn, M., Low, K.B., Pawelek, J., Bermudes, D., Brecher, S.M., Margitich, D., Turnier, J., Li, Z., Luo, X., King, I. and Zheng, L.M. (2000) Biodistribution and genetic stability of the novel antitumor agent VNP20009, a genetically modified strain of *Salmonella typhimurium*. J. Infect. Dis. 181, 1996–2002.

[5] Liu, S.C., Minton, N.P., Giaccia, A.J. and Brown, J.M. (2002) Anticancer efficacy of systemically delivered anaerobic bacteria as gene therapy vectors targeting tumor hypoxia/necrosis. Gene Ther. 9, 291–296.

[6] Theys, J., Nuyts, S., Landuyt, W., Van Mellaert, L., Dillen, C., Bohringer, M., Dürre, P., Lambin, P. and Anné, J. (1999) Stable *Escherichia coli–Clostridium acetobutylicum* shuttle vector for secretion of murine tumor necrosis factor α. Appl. Environ. Microbiol. 65, 4295–4300.

[7] McIntosh, J.K., Mulé, J.J., Krosnick, J.A. and Rosenberg, S.A. (1989) Combination cytokine immunotherapy with tumor necrosis factor α, interleukin 2 and α-interferon and its synergistic antitumor effects in mice. Cancer Res. 49, 1408–1414.

[8] Parmiani, G., Rivoltini, L., Andreola, G. and Carrabba, M. (2000) Cytokines in cancer therapy. Immunol. Lett. 74, 41–44.

[9] Rosenberg, S.A., Lotze, M.T., Muul, L.M., Leitman, S., Chang, A.E., Ettinghausen, S.E., Matory, Y.L., Skibber, J.M., Shiloni, E., Vetto, J.T., Seipp, C.A., Simpson, C. and Reichert, C.M. (1985) Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. Eng. J. Med. 313, 1485–1492.

[10] Siegel, J.P. and Puri, R.K. (1991) Interleukin-2 toxicity. J. Clin. Oncol. 9, 694–704.

[11] Rosenberg, S.A. (2001) Progress in human tumour immunology and immunotherapy. Nature 411, 380–384.

[12] Baaten, G., Voogd, A.C. and Wagstaff, J. (2004) A systematic review of the relation between interleukin-2 schedule and outcome in patients with metastatic renal cell cancer. Eur. J. Cancer 40, 1127–1144.

[13] Den Otter, W., Cadée, J., Gavhumende, R., De Groot, C.J., Hennink, W.E. and Stewart, R. (1999) Effective cancer therapy with a single injection of interleukin-2 at the site of the tumour. Cancer Immunol. Immunother. 48, 419–420.

[14] Li, D., Ronson, B., Guo, M., Liu, S., Bishop, J.S., Van Echo, D.A. and O'Malley, B.W. (2002) Interleukin 2 gene transfer prevents NKG2D suppression and enhances antitumor efficacy in combination with cisplatin for head and neck squamous cell cancer. Cancer Res. 62, 4023–4028.

[15] Hillman, G.G., Slos, P., Wang, Y., Wright, J.L., Layer, A., De Meyer, M., Yudelev, M., Che, M. and Forman, J.D. (2004) Tumor irradiation followed by intratumoral cytokine gene

therapy for murine renal adenocarcinoma. Cancer Gene Ther. 11, 61–72.
[16] Lichtor, T. and Glick, R.P. (2003) Cytokine immuno-gene therapy for treatment of brain tumors. J. Neurooncol. 65, 247–259.
[17] Oultram, J.M., Loughlin, M., Swinfield, T.J., Brehm, J.K., Thompson, D.E. and Minton, N.P. (1988) Introduction of plasmids into whole cells of *Clostridium* by electroporation. FEMS Microbiol. Lett. 56, 83–88.
[18] Sambrook, J.E., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[19] Mermelstein, L.D. and Papoutsakis, E.T. (1993) In vivo methylation in *Escherichia coli* by the *Bacillus subtilis* phage phi 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC824. Appl. Environ. Microbiol. 59, 1077–1081.
[20] Mermelstein, L.D., Welker, N.E., Bennett, G.E. and Papoutsakis, E.T. (1992) Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC824. Bio/Technology 10, 190–195.
[21] Zappe, H., Jones, W.A., Jones, D.T. and Woods, D.R. (1988) Structure of an endo-β-1,4-glucanase gene from *Clostridium acetobutylicum* P262 showing homology with endoglucanase genes from *Bacillus* spp. Appl. Environ. Microbiol. 54, 1289–1292.
[22] Nakotte, S., Schaffer, S., Böhringer, M. and Dürre, P. (1998) Electroporation of, plasmid isolation from and plasmid conservation in *C. acetobutylicum* DSM792. Appl. Microbiol. Biotechnol. 50, 564–567.
[23] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
[24] Gillis, S., Fern, M.M., Ou, W. and Smith, K.A. (1978) T cell growth factor: parameters of production and quantitative microassay for activity. J. Immunol. 120, 2027–2032.
[25] Lammertyn, E., Van Mellaert, L., Schacht, S., Dillen, C., Sablon, E., Van Broekhoven, A. and Anné, J. (1997) Evaluation of a novel subtilisin inhibitor gene and mutant derivatives for the expression and secretion of mouse tumor necrosis factor alpha by *Streptomyces lividans*. Appl. Environ. Microbiol. 63, 1808–1813.
[26] Clary, B.M., Coveney, E.C., Philip, R., Blazer III, D.G., Morse, M., Gilboa, E. and Lyerly, H.K. (1997) Inhibition of established pancreatic cancers following specific active immunotherapy with interleukin-2 gene-transduced tumor cells. Cancer Gene Ther. 4, 97–104.
[27] Keilholz, U. and Eggermont, A.M.M. (2000) The emerging role of cytokines in the treatment of advanced melanoma. Oncology 58, 89–95.
[28] Burke, F. (1999) Cytokines (IFNs, TNF-α, IL-2 and IL-12) and animal models of cancer. Cytokines Cell. Mol. Ther. 5, 51–61.
[29] Zhang, R. and DeGroot, L.J. (2001) An adenoviral vector expressing functional heterogeneous proteins herpes simplex viral thymidine kinase and human interleukin-2 has enhanced in vivo antitumor activity against medullary thyroid carcinoma. Endocrin. Relat. Cancer 8, 315–325.
[30] Dang, L.H., Bettegowda, C., Huso, D.L., Kinzler, K.W. and Vogelstein, B. (2001) Combination bacteriolytic therapy for the treatment of experimental tumors. Proc. Natl. Acad. Sci. USA 98, 15155–15160.
[31] Theys, J., Landuyt, W., Nuyts, S., Van Mellaert, L., Bosmans, E., Rijnders, A., Van Den Bogaert, W., van Oosterom, A., Anné, J. and Lambin, P. (2001) Improvement of *Clostridium* tumor targeting vectors evaluated in rat rhabdomyosarcomas. FEMS Immunol. Med. Microbiol. 30, 37–41.