RESEARCH ARTICLE

Influence of infection route and virulence factors on colonization of solid tumors by *Salmonella enterica* serovar Typhimurium

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Received 19 October 2010; revised 31 January 2011; accepted 1 February 2011.
Final version published online 22 March 2011.

DOI:10.1111/j.1574-695X.2011.00790.x

Editor: Eric Oswald

Keywords
Salmonella typhimurium; tumor colonization; virulence factors; mutants.

Abstract

Administration of facultative anaerobic bacteria such as *Salmonella enterica* serovar Typhimurium as anticancer treatment holds a great therapeutic potential. Here, we tested different routes of application of *S. typhimurium* with regard to tumor colonization and therapeutic efficacy. No differences between intravenous and intraperitoneal infection were observed, often leading to a complete tumor clearance. In contrast, after oral application, tumor colonization was inefficient and delayed. No therapeutic effect was observed under such conditions. We also showed that tumor invasion and colonization were independent of functional *Salmonella* pathogenicity island (SPI) 1 and SPI 2. Furthermore, tumor invasion and colonization did not require bacterial motility or chemotactic responsiveness. The distribution of the bacteria within the tumor was independent of such functions.

Introduction

For almost 200 years it has been known that bacteria have the ability to colonize solid tumors and induce tumor shrinkage. Despite some success, the employment of bacteria or bacterial components was only anecdotal due to the severe side effects of such therapies (Coley, 1893). However, the dramatic improvements of molecular genetics of bacteria within the last decades have made feasible the application of appropriately attenuated pathogenic bacteria to cancer patients. Consequently, this possibility is at present under intense investigation (Leschner & Weiss, 2010).

Many obligate and facultative anaerobic bacteria are able to colonize solid tumors, among them *Salmonella enterica* serovar Typhimurium. Thus far, *S. typhimurium* has been shown to exert strong therapeutic effects on tumors upon intravenous administration (Zhao *et al.*, 2005). In the tumor, *Salmonella* mainly resides in the inner necrotic part of neoplasias (Pawelek *et al.*, 1997a; Avogadri *et al.*, 2005; Westphal *et al.*, 2008). Obviously, conditions like low oxygen tension, protection from phagocytic immune cells and probably also the high nutrient supply from dying tumor cells support the survival and proliferation of the bacteria within the tumor. In contrast, a strain, auxotrophic for leucine and arginine, targets tumor tissue specifically, including the complete viable malignant tissue (Zhao *et al.*, 2006; Hayashi *et al.*, 2009; Kimura *et al.*, 2010).

Normally, *S. typhimurium* uses an intestinal port of entry via ingestion of contaminated food and water (Jones *et al.*, 1992). After breaching the epithelial barrier, the bacteria colonize Peyer's patches, mesenteric lymph nodes and subsequently spread to the deep organs spleen and liver (Finlay & Brumell, 2000; Barthel *et al.*, 2003). Different components of *Salmonella* are involved in the invasion and infection process. For instance, the cell envelope component lipopolysaccharide is important for survival in the host (Gunn, 2008). In addition, particular virulence factors are found, most of which are encoded in particular genetic elements including *Salmonella* pathogenicity islands 1 and 2 (SPI 1 and SPI 2). Their prominent features are the so-called type three secretion systems, which allow the injection of bacterial effector proteins into the cytosol of the host cell (Waterman & Holden, 2003; Stecher *et al.*, 2004; Bueno *et al.*, 2005; Jones *et al.*, 2007). Here, SPI 1 is important for the invasion of host epithelial cells (Main-Hester *et al.*, 2008), while SPI 2 is essential for intracellular survival in the *Salmonella*-containing vacuoles after invasion (Cirillo *et al.*, 1998).

For successful *Salmonella*-mediated tumor therapy, bacteria are generally administered intravenously. However, there are some reports described an application via the natural oral route that successfully inhibited tumor growth (Medina *et al.*, 1999; Panthel *et al.*, 2008; Fest *et al.*, 2009). Nevertheless, comparison of the different requirements of bacterial administration for tumor therapy has only been approached to a small extent.

In addition, the tumor invasion process of the systemically applied *Salmonella* is not yet clear, although this knowledge will be required for optimization of the bacterial targeting of cancerous tissue. An active scenario that involves several bacterial chemotactic systems has been suggested using cylindroids of tumor cells *in vitro* (Forbes *et al.*, 2003). From our studies, we suggest a rather passive mechanism by which the cytokines that are elicited after intravenous application of the *Salmonella* open the blood vessels in the tumors and allow the entry of the bacteria. Similar results published by the Szalay group also support a passive tumor mechanism (Stritzker *et al.*, 2010). However, both scenarios are not mutually exclusive and need to be investigated in more detail.

In addition, a controversy exists with regard to the involvement of SPI 1 or SPI 2 in tumor targeting and survival. As the bacteria are normally applied intravenously, factors encoded in SPI 1 should not be necessary. However, products of SPI 2 were described to be essential for tumor targeting (Pawelek *et al.*, 2002). This would suggest that bacteria might exist intracellularly in the tumors. Thus far, when examining infected tumors we hardly ever found bacteria residing within cells. Therefore, we wanted to re-examine the requirement of intact SPI 1 and SPI 2 for systemic tumor therapy.

Materials and methods

Bacterial strains and growth conditions

Salmonella typhimurium strain SL7207 (Δ*hisG*, Δ*aroA*) was kindly provided by Bruce Stocker (Hoiseth & Stocker, 1981). For *in vivo* imaging, *lux* was integrated into the chromosome (Loessner *et al.*, 2007). *Salmonella typhimurium* SL1344 and the mutants Δ*cheY*, Δ*fliGHI*, Δ*invG*, Δ*phoP*, Δ*sseD*, Δ*ssrB*, Δ*aroA* and Δ*purA* (Table 1) were provided by Wolf-Dietrich Hardt (Stecher *et al.*, 2004a; Hapfelmeier *et al.*, 2005). The strains were prepared via deletion of the described genes (Datsenko & Wanner, 2000). The bacteria were grown on Luria–Bertani (LB) agar plates supplemented with 30 μg mL ^{1} streptomycin at 37 °C.

Cell lines and animals

Six-week-old female BALB/c mice were purchased from Janvier (France). CT26 colon carcinoma cells (ATCC CRL-2638) were grown as monolayers in IMDM medium (Gibco BRL, Germany) supplemented with 10% v/v heat-inactivated fetal calf serum (Integro, the Netherlands), 250 μmol L ^{1} β-mercaptoethanol (Serva) and 1% v/v penicillin/streptomycin (Sigma, Germany).

Infection of tumor-bearing mice and recovery of bacteria from tissue

Six-week-old female BALB/c mice were subcutaneously injected with 5 10^5 CT26 cells in the abdomen. When the tumors had reached a size of 5–8 mm in diameter, mice were injected intravenously or intraperitoneally with 5 10^6 CFU of *S. typhimurium* in phosphate-buffered saline (PBS). For oral infection 5 10^8 CFU were used. At different time points postinfection, mice were sacrificed and tumor, spleen and liver were removed for further analysis. The organs were transferred into 1 mL (2 mL for liver) ice-cold PBS containing 0.1% v/v Triton X-100. The tissue was homogenized with a Polytron PT3000 homogenizer. To determine CFU g ^{1} of organ, homogenates were serial diluted in PBS and plated on LB plates containing streptomycin (30 μg mL ^{1}).

All animal experiments have been performed with the permission of the local authorities (LAVES) according to the Animal Welfare act.

Table 1 Mutant strains of *Salmonella typhimurium*

SL1344	Affecting	Function
Δ*cheY*	Chemotaxis	Regulator of agella rotation; mutants are unable to respond to chemotactic signals
Δ*fliGHI*	Flagella	Assembly of agella structural proteins
Δ*invG*	SPI 1	Component of SPI 1 TTSS response regulator of two-component transcriptional regulator
Δ*phoP*	SPI 1/2	phoP/phoQ
Δ*sseD*	SPI 2	Translocon protein of SPI 2 TTSS; part of two-component regulatory system ssrA/ssrB of SPI 2
Δ*ssrB*	SPI 2	TTSS
Δ*aroA*	Metabolism	Biosynthesis of aromatic amino acids
Δ*purA*	Metabolism	Biosynthesis of adenine and guanine

TTSS, type three secretion systems.

Tumor studies

To follow the development of tumor size, 6-week-old female BALB/c mice were injected subcutaneously with 5 10^5 CT26 cells. As soon as small tumors become visible, tumor size was monitored every other day using calipers. The volume of the tumor was calculated using the formula: $V = 4/3 \quad \pi \quad (h \quad w^2)/8$, where h is the height and w the width. It was assumed that the depth and width of the tumor were equal.

In vivo imaging

For the visualization of early time points of bacterial spreading after infection, the IVIS system (Xenogen) was used. The *lux* operon was used as reporter system for the distribution of bacteria. BALB/c mice were infected intravenously, intraperitoneally or orally with SL7207*lux* and immediately anesthetized using isoflurane for *in vivo* imaging. Pictures were taken immediately, 10 min, 2 h and 24 h after infection using a comparable intensity scale.

Histology

Tumors were taken from sacrificed mice and frozen in Tissue-Tek OCT Compound (Sakura Finetek). Sections of 10 µm were cut using a microtome-cryostat (Cryo-Star HM560V, Microm). Slides were air dried at room temperature overnight and fixed in 20 °C acetone for 3 min. Slides were rehydrated in PBS and blocked with 1 µg mL ^{1} FcR blocker (rat-α-mouse CD16/CD32). Staining was done with the following reagents: polyclonal rabbit anti-*S. typhimurium* (US Biological), polyclonal goat anti-rabbit Alexa 488 (Sigma-Aldrich), Phalloidin Alexa 594 (Molecular Probes) and DRAQ5 (Biostatus). Afterwards, the slides were washed and dried and covered with Neomount (Merck) and coverslips. The samples were analyzed using a laser scanning confocal microscope (LSM 510 META, Zeiss). Images were processed with LSM5 IMAGE BROWSER (Zeiss) and ADOBE PHOTOSHOP 7.0.

Results

Comparison of application routes for bacterial tumor colonization

The natural port of entry for *S. typhimurium* is the intestine. However, the tumor targeting experiments are usually carried out via the intravenous route, although some reports describe oral application. We therefore wanted to systematically compare the various infection routes for their efficiency with respect to tumor colonization. Within these experiments we employed SL1344Δ*aroA*. This strain is auxotrophic for histidine (Wray & Sojka, 1978) as well as aromatic amino acids. Mice were injected subcutaneously with the colon carcinoma cell line CT26. After the tumor had reached a size of 5 mm in diameter, the tumor-bearing mice were infected by different routes with the attenuated *S. typhimurium* strain. After intravenous and intraperitoneal infection, mice were sacrificed on days 1, 3, 10 and 17 after infection and the number of bacteria was determined for tumor, spleen and liver (Fig. 1). As expected, tumors were preferentially colonized and showed up to 100 times higher colonization than spleen and liver. Bacterial tumor loads

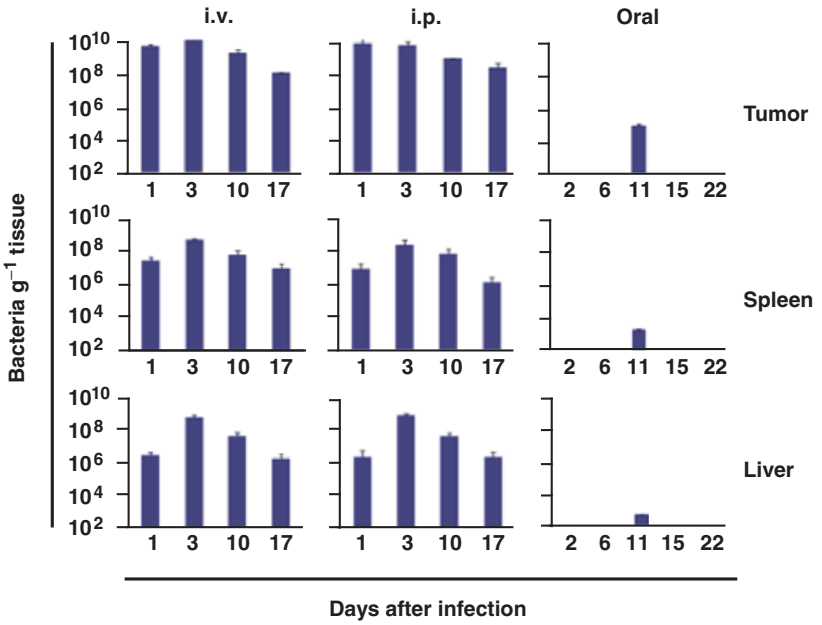

Fig. 1. Colonization of tumor, spleen and liver after intravenous, intraperitoneal and oral application of SL1344Δ*aroA*. Bars represent the mean of five mice per group ± SD.

decreased slightly till day 17. In contrast, in normal target organs, spleen and liver bacteria increased till day 3 and then slowly subsided.

Because of delayed tissue colonization in the case of oral infection, mice were sacrificed at days 2, 6, 11, 15 and 22 postinfection and tissues were plated. Bacteria could only be detected at day 11. The tumors were preferentially colonized but to a much lower degree than after systemic application. In general, the number of bacteria in the tissues was about 100 times lower compared with intravenous or intraperitoneal infection.

The development of tumor size was also monitored under these conditions. After intravenous and intraperitoneal infection, a dramatic reduction of tumor volume was observed (Fig. 2). The tumor colonization of SL1344Δ*aroA* was reflected in a complete clearance of tumors. Consistently, after oral infection, which had led to a transient low colonization of the tumor, no reduction of tumor size was detectable.

The colonization of organs and tumor was also followed via *in vivo* imaging (Fig. 3). Recombinant bacteria of the strain SL7207*lux*, carrying an integrated *lux* operon, were used to visualize bacterial distribution during early infection. Bacteria of the strain SL7207 contain the same attenuations and were shown to behave comparable to SL1344Δ*aroA*. The amount of CFU was lower in different organs but the pattern of infection and survival of mice was comparable (Supporting Information, Figs S1 and S2). After intravenous infection, bacteria immediately spread out through the whole organism. A strong signal from liver and spleen was already detectable after 10 min. After 24 h, an additional very strong signal from the tumor appeared. After intraperitoneal infection, the signal from liver and spleen was not that obvious due to a long-lasting strong signal from the peritoneum. Nevertheless, after 24 h the tumor showed strong bioluminescence.

In contrast, a 100 times weaker signal was observed directly after oral infection. Bacteria could be detected in the gut up to a few hours after infection. After 24 h the signal was too weak for detection.

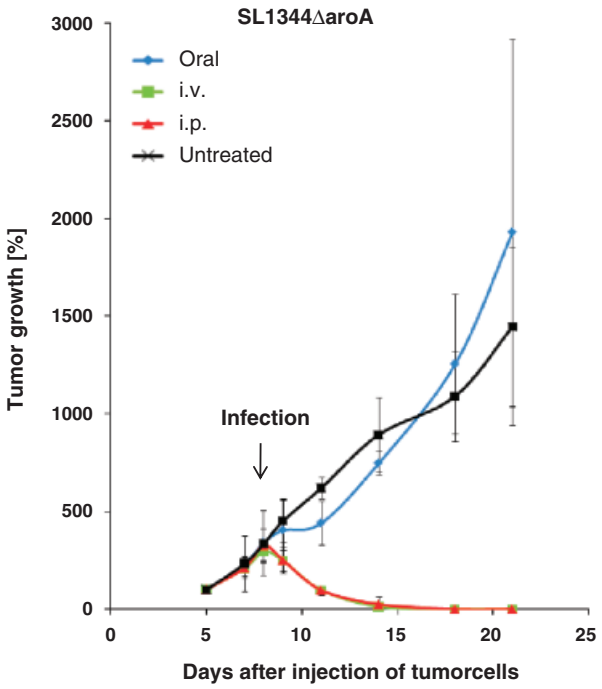

Fig. 2. Therapeutic effect of the colonization of tumor-bearing mice by SL1344Δ*aroA*. The infection was carried out 9 days after the administration of tumor cells and the development is given as percent of tumor size at day 5. The values shown represent the mean values of five mice per group ± SD.

Tumor colonization by metabolically attenuated mutants

The employment of SL1344 allowed the comparison of wild-type (WT) bacteria with mutant bacteria (Table 1) that were mutated in metabolic pathways, commonly used for attenuation of vaccine carrier strains (Fig. 4). Therefore, we compared WT bacteria with Δ*aroA* and Δ*purA*, which are unable to synthesize either aromatic amino acids or purines such as adenine and guanine. Interestingly, at 12-h postinfection, WT bacteria were able to colonize tumors as well as spleen to a higher degree the attenuated strains could. By 24 h, bacteria of the Δ*aroA* strain had colonized tumor and spleen to the same extent as the WT. The Δ*purA* strain, however, never grew to such numbers and appeared to be overattenuated. After 2 days all mice had to be killed due to a severe health status after intravenous infection with WT SL1344, while most of the mice infected with the mutant strains recovered from the side effects of the infection.

Tumor colonization by mutants in SPI 1 and SPI 2

The SPI 1 and SPI 2 are essential for bacterial invasion and intracellular survival under natural conditions, respectively. However, in the case of tumor targeting such functions might be dispensable, as the *Salmonella* are applied intravenously and appear to reside extracellularly in the tumor tissue (Loessner *et al.*, 2007a). We therefore tested the bacterial mutants Δ*invG* and Δ*phoP*. These are essential for the function of SPI 1, Δ*phoP* is influencing SPI 2, or Δ*sseD* and Δ*ssrB* mutants, which abolish the function of SPI 2, for tumor invasion and colonization. The efficiency of tumor invasion and survival was obviously not affected by such mutations (Fig. 4). The mutants invaded the tumor even slightly better than the WT bacteria as seen at 12-h postinfection. By 24 h, no difference between bacterial counts was observable anymore. Thus, virulence factors of *S. typhimurium* encoded in SPI 1 and SPI 2 are not essential for tumor colonization under our conditions.

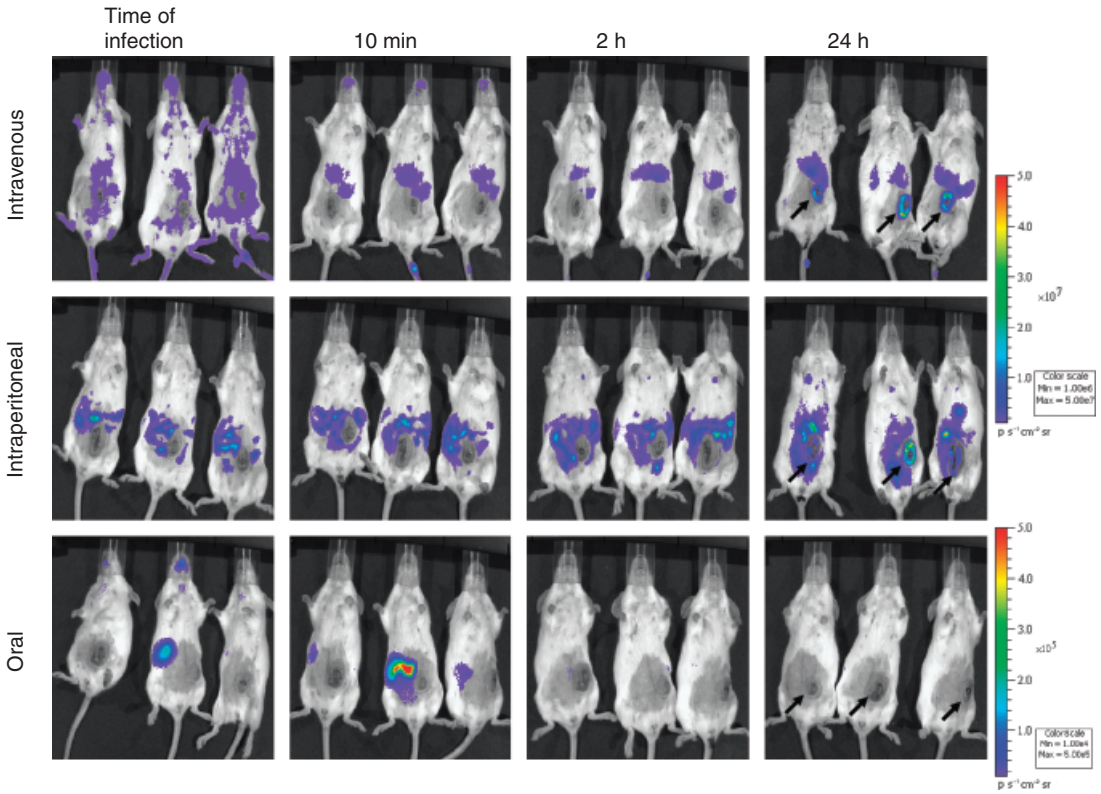

Fig. 3. Colonization of tumor-bearing mice infected with SL7207/*lux*, carrying chromosomally integrated *lux*, after intravenous, intraperitoneal or oral application. Note that the scale is different for intravenous/intraperitoneal and oral infection. Positions of tumors are indicated by black arrows.

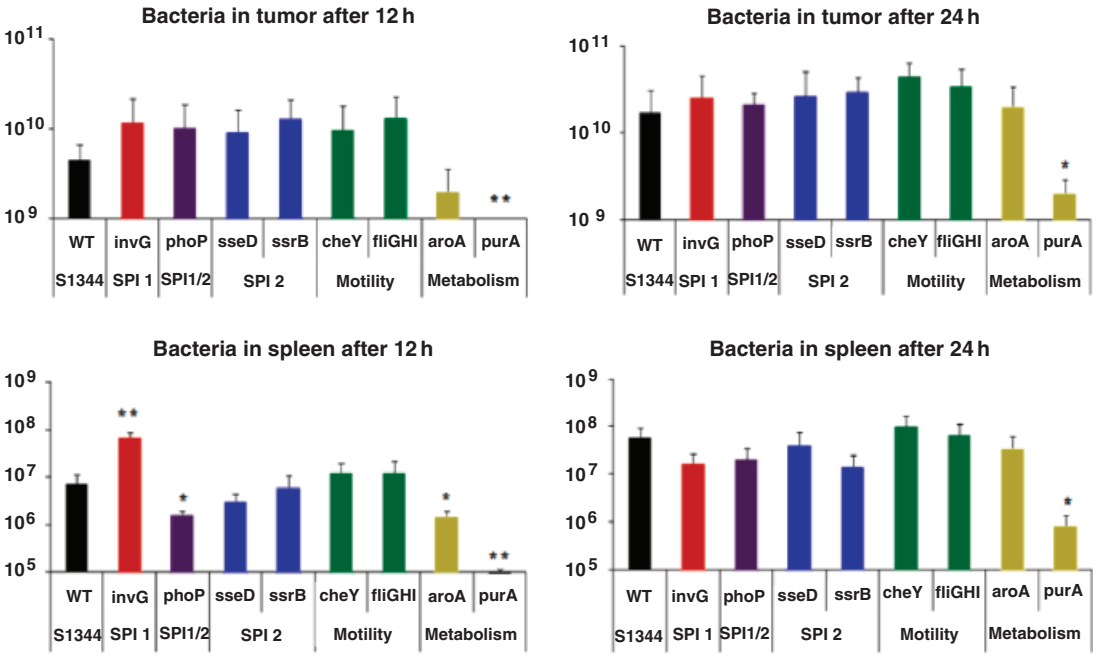

Fig. 4. Colonization of tumor-bearing mice by mutants of SL1344 12 and 24 h after intravenous infection. Tumor and spleen were tested for the number of bacteria per gram of tissue. Bars represent the mean value of five mice per group ± SD. Statistical significance, calculated via Student's *t*-test, is indicated by asterisks: $^{*}P < 0.05$; $^{**}P < 0.01$.

Tumor colonization by immobile variants and variants with chemotaxis defects

Conflicting ideas exist as to how bacteria enter the tumor tissue. An active process was suggested that required motility and chemotaxis by the bacteria (Kasinskas & Forbes, 2006, 2007a). These experiments were carried out *in vitro* using tumor cell cylindroids. Hence, we wanted to test the requirement of motility and chemotaxis under our conditions *in vivo*. Two variant strains were tested: mutant Δ*fliGHI* is immobile due to an inability to assemble flagella and Δ*cheY* is unable to respond to chemotactic gradients, as it can no longer regulate the rotation of the flagella.

Importantly, both mutant strains invaded the tumors slightly better than the WT bacteria, as can be seen at 12-h postinfection. No difference could be observed at 24 h anymore, indicating that tumor invasion and colonization are independent of bacterial motility and chemotaxis. No difference between such strains could be observed for the colonization of the spleen at either time point.

Tumor distribution of nonmotile variant bacteria

Under our conditions, tumor-colonizing *Salmonella* reside in the necrotic areas and in the areas between the necrotic and the viable region, where high numbers of host neutrophils are normally also found (Rosenberg *et al.*, 2002; Westphal *et al.*, 2008a). It is possible that the deletion of the motile or chemotactic capabilities of the variant strains might influence their distribution in the tumor. Therefore, tumors colonized by WT, Δ*cheY* or Δ*fliGHI* bacteria were analyzed by immunohistology 24-h postinfection. Bacteria of the different strains were similarly distributed within the tumor (Fig. 5). All of them colonized the necrotic areas and accumulated to some extent in the interface between necrotic and viable regions. Thus, motility and chemotaxis do not appear to be required for tumor invasion and colonization under our conditions.

Discussion

In industrialized countries, cancer is the second most frequent cause of death, with an increasing incidence. This is due to the improved life expectancy of the human population, as cancer is essentially a disease of old age. In the face of these facts, the development of novel therapies or intervention strategies against this devastating disease is an obligatory task for current biomedical research. Despite the drawbacks of the first clinical trials (Toso *et al.*, 2002), the employment of bacteria for the treatment of solid tumors holds great promises. However, it also became clear that the understanding of the invasion and colonization process is essential for a further development of efficacious strains that can be safely administered to tumor patients. We investigated the role of some bacterial properties in tumor invasion and colonization, for which conflicting ideas exist.

Tumor-targeting bacteria are usually applied systemically. However, a few reports describe the successful application via the oral route. Obviously, an oral delivery of therapeutic bacteria would be advantageous as it would not require any special equipment or any particular skills for delivery. In addition, the risk of an over-reaction of the immune system of the patient would be reduced. However, comparison of the different routes of application revealed that under our conditions, systemic administration of the bacteria is much superior to the oral route. Oral application of SL1344Δ*aroA* only led to a transient colonization of the tumors late after infection. In contrast, systemic application of bacteria led to an immediate and robust colonization of the tumor. In accordance, tumor growth was unaltered when the bacteria were applied orally, whereas systemic application led to a strong shrinkage of the tumors and resulted in complete clearance. Thus, *Salmonella* when applied orally are able to reach the tumor tissue but their present therapeutic capabilities render the effects negligible. Nevertheless, appropriately engineered strains might eventually allow oral application for therapeutic purposes.

During these experiments, we noticed that the therapeutic effect was very much dependent on the size of the tumor. Our standard conditions, to apply the bacteria when the tumor had reached 0.5–0.8 cm in diameter, allow complete tumor clearance by the systemically applied *Salmonella*. In contrast, less therapeutic efficacy was noticed when the tumor had reached larger sizes. This might be one of the reasons why the bacteria after oral application to tumors 0.5 cm in diameter had no effect on tumor growth. Despite the lower number of colonizing bacteria, a retardation of tumor growth was expected. However, by the time the orally applied bacteria reached the tumor it might have been larger than the critical size for a therapeutic effect.

The mutant strains employed in this study were generated on the SL1344 genetic background, but for *in vivo* imaging of early time points of infection, SL7207*lux* was used. The colonization pattern and therapeutic effect on tumor-bearing mice of SL1344Δ*aroA* and SL7207 were compared (Figs S1 and S2). Only slight differences could be observed. SL7207 colonized various target tissues less efficiently compared with SL1344Δ*aroA*. Both strains also differed slightly in their capacity to induce tumor shrinkage. Whereas infection with SL1344Δ*aroA* induced complete tumor clearance, treatment with SL7207 only eliminated of three of five tumors. This might be due to differences in the genetic make-up of the two strains. Nevertheless, the numbers of bacteria during colonization and especially the kinetics were comparable.

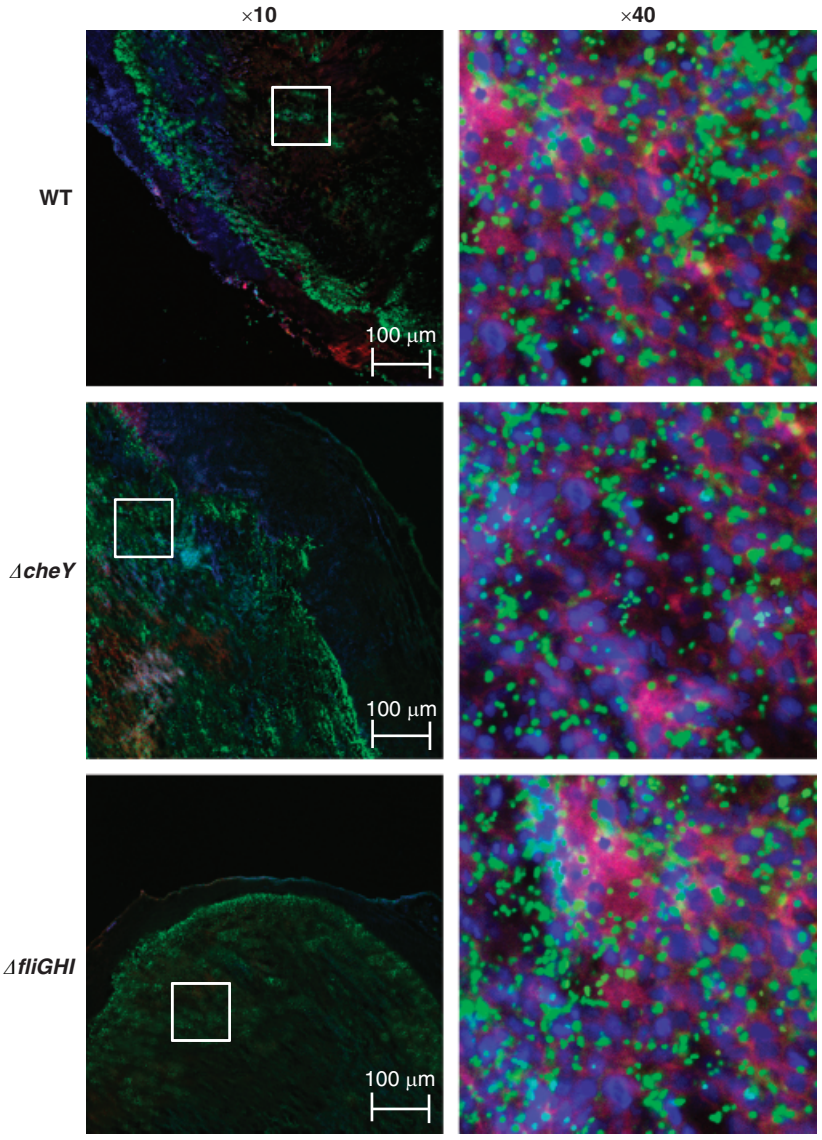

Fig. 5. Immune histology of tumors colonized by WT SL1344, immobile mutants (Δ*fliGHI*) or a mutant strain, unable to react to chemotactic gradients (Δ*cheY*). Cryosections of 10 μm were stained for nuclei (blue), actin (red) and *Salmonella typhimurium* (green). Confocal images show overviews (10) and 40 magnifications of the area in boxes.

Metabolic attenuation in the purine synthesis pathway, like Δ*purI*, is often used in studies concerning tumor targeting of *Salmonella* (Pawelek *et al.*, 1997; Clairmont *et al.*, 2000). Such strains usually exhibit good performance. In contrast, the SL1344Δ*purA*, used in our study, appeared overattenuated.

Under our conditions, we found that *Salmonella* usually resided extracellularly after systemic application. This suggested that proteins encoded in the SPI 1 and SPI 2 are not essential for tumor invasion and colonization. This was indeed the case. Mutants, in which essential functions of SPI 1 or SPI 2 were deleted, were efficient tumor colonizers. Thus, the original report that SPI 2 functions are required for tumor colonization (Pawelek *et al.*, 2002a) could not be confirmed. Instead of our CT26 colon carcinoma model, those previous experiments were carried out in a melanoma model, in which the bacteria might behave in another way. Different strain backgrounds were also used and might explain the different experimental outcomes.

Similarly, an active process was suggested from *in vitro* data for tumor invasion (Kasinskas & Forbes, 2007b). This would require motility and chemotactic responsiveness of the bacteria. However, in our system, variant *Salmonella* that do not have such properties invaded and colonized the tumors just as efficiently as bacteria exhibiting these properties. Therefore, we feel that our original hypothesis is correct, namely, that *Salmonella* after systemic application via induction of tumor necrosis factor-α and other cytokines creates a tumor microenvironment in which the bacteria are passively flushed in (Leschner *et al.*, 2009). Even the distribution of the *Salmonella* within the tumor is independent of motility and chemotactic responsiveness. To

generalize these findings, more and independent tumor systems will have to be studied.

In conclusion, we have tested several properties of tumor-targeting bacteria. Our results will help to better understand the tumor invasion and colonization process for this novel and highly potential therapeutic strategy.

Acknowledgements

This work was supported in part by the Bundesministerium für Bildung und Forschung (BMBF), Deutsche Forschungsgemeinschaft (DFG), the Deutsche Krebshilfe and the Helmholtz Graduate School for Infection Research. We would like to thank Susanne zur Lage, Regina Lesch and Martina Krey for expert technical assistance.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Comparison of the colonization of tumor, spleen and liver after intravenous, intraperitoneal and oral application.

Fig. S2. Comparison of the therapeutic effect of SL7207 and SL1344Δ*aroA* to tumor-bearing mice.

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