Murine solid tumours as a novel model to study bacterial biofilm formation *in vivo*

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Abstract

Bacteria of many species are able to invade and colonize solid tumours in mice. We have focused on *Salmonella enterica* serovar Typhimurium. Detailed analysis revealed that such tumour-invading *Salmonella* form biofilms, thus providing a versatile *in vivo* test system for studying bacterial phenotypes and host–pathogen interactions. It appears that biofilm formation by *S. Typhimurium* is induced as a defence against the immune system of the host, and in particular against neutrophils. Further we extended our work to the clinically more relevant biofilm infection by *Pseudomonas aeruginosa*. The induction of *P. aeruginosa* biofilms in neoplastic tissue appears to be elicited as a reaction against the immune system. Reconstitution experiments reveal that T cells are responsible for biofilm induction. Isogenic mutants that are no longer able to form biofilms can be used for comparison studies to determine antimicrobial resistance, especially therapeutic efficacy against *P. aeruginosa* located in biofilms.

Keywords: biofilms, mice, *Pseudomonas aeruginosa*, *Salmonella* typhimurium, *Shigella flexneri*, solid tumours.

Introduction

Bacteria of many species demonstrate the surprising ability to invade and colonize solid tumours. This often results in growth retardation of the neoplasm and in some cases in complete tumour clearance [1–4]. This phenomenon was discovered about two centuries ago, when a French physician made the observation that tumours of patients who were also suffering from gas gangrene started to shrink. We know now that gas gangrene is caused by the bacterium *Clostridium perfringens* which highlights a role of bacteria in cancer therapy [5]. Later, attempts were made to intentionally infect cancer patients with bacteria [6] or treat them with bacterial components. The best known example in this context is still Coley's toxin, which comprises of a mixture of heat-killed or extracts of *Streptococcus pyogenes* and *Serratia marcescens* [7]. Although remarkable success was observed at that time [8], the severe side effects that were encountered during treatment precluded the routine clinical use of this therapeutic strategy. Thus, the application of bacteria in cancer therapy was abandoned. Nevertheless, the Bacillus Calmette-Guérin (BCG) vaccine strain was introduced into clinical practice in the meantime as an adjuvant therapy for bladder cancer. It is highly effective in preventing relapse of this neoplasm after surgical removal [9].

Approximately two decades ago, the idea of using bacteria as anti-cancer agents was revived [1]. This was strengthened by the accumulating knowledge of host-bacterial interactions as well as the advanced molecular genetics of many bacteria that would allow the tailoring of particular, effective bacterial strains. Such bacteria should retain their full therapeutic potential but lack the toxic properties that caused the problems in earlier clinical attempts [5, 6]. In the meantime, several bacterial species have been shown to be able to invade tumours and cause growth retardation after systemic application (Table 1). These include facultative anaerobic bacteria, such as *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) [3, 10, 11], *S*. cholerasuis [12] or *Escherichia coli*, as well as obligate anaerobic bacteria, such as *Bifidobacterium longum* [13] or *Clostridium novyi* [14, 15]. In addition to a direct effect, tumour-targeting bacteria might also be used as carriers for therapeutic molecules. Accordingly, *Listeria monocytogenes* coated with radioactively labelled antibodies showed high efficacy against metastases of pancreatic carcinoma after repeated systemic application [16]. In addition, direct injection of *Salmonella* into the tumour has also proved effective [17, 18].

We, as well as others, have focused on *S. Typhimurium* for systemic cancer therapy [3, 10, 12, 18, 19]. *Salmonella* exhibit several advantages over other types of bacteria. As facultative anaerobe, *Salmonella* strains are able to grow in oxygenated and also in hypoxic regions of tumours [20]. The established genome sequences of several *Salmonella* strains [21] and the well-known biology favour their use in cancer therapy. Finally, the close relation

to *E. coli* allows the construction of expression plasmids in laboratory *E. coli* strains which can be transferred to *Salmonella*. In addition, *Salmonella* are pathogens [21, 22] and we noted that certain bacterial virulence traits are required to exhibit strong therapeutic properties [23].

An obvious question that should be addressed is: how can bacteria such as *Salmonella* selectively invade and colonize solid tumours after intravenous application? We observed that shortly after the systemic administration of *Salmonella*, there was severe haemorrhaging within the tumour. We obtained evidence to suggest that the influx of blood is due to cytokine 'expression', especially of tumour necrosis factor (TNF)-α, that is elicited when the bacteria are administered intravenously to tumour-bearing mice. Under normal circumstances, TNF-α is known to interact with blood vessel endothelium in a manner that allows blood cells and plasma proteins to reach massive infection in the tissue. In the tumour setting, with pathological angiogenesis, blood vessels are already leaky. Additional TNF-α in the circulation will particularly affect the blood vessels in the tumour. Thus, upon haemorrhae, bacteria might be flushed into the cancer tissue. Consequently we observed large necrotic regions in the tumours. Individual bacteria that have reached the tumour can extensively proliferate in these hypoxic areas because they represent immune privileged sites and provide ample nutrition through scavenging of dying cells [3]. This idea is in agreement with the type of tumour-specific *Salmonella* promoters that we have defined recently [24].

The consequence of bacterial tumour colonization and the formation of a large necrotic area is the attraction of innate immune cells of the host. In particular, neutrophilic granulocytes migrate into the tumour containing bacteria and arrange themselves as a ring between the necrotic and the viable regions (Fig. 1a, 1b). Immunohistology revealed that bacteria reside in the necrotic areas (Fig. 1a, 1b). This was not unexpected as the bacteria can grow under hypoxic or anoxic conditions. In addition, bacteria were close to neutrophils, which are found in the quiescent zone of the tumour [25] where they form dense clusters (Fig. 1b). To distinguish between extracellular and intracellular (i.e. phagocytosed) bacteria, electron microscopic analysis was conducted. *Salmonella* were found extracellularly and were surrounded by extracellular matrix which resembles that found in biofilms (Fig. 1c,d) [26, 27].

Biofilm formation by *S*. Typhimurium strain SL7207 in murine CT26 tumours

We carried out initial experiments using subcutaneously placed murine colon CT26 tumours and the *Salmonella* strain SL7207. This strain is generally considered to be a safe variant that can be used for human vaccination [28, 29]. It is metabolically attenuated through inactivation of the genes *aroA* and *hisG.* Thus, the bacteria are auxotrophic for aromatic amino acids and histidine. Nevertheless, the bacteria are able to survive for a considerable

length of time in the wild- type (WT) murine host without harming the animals although immunocompromised mice might succumb to infections with this *Salmonella* strain SL7207. This residual virulence might be advantageous for efficient colonization in tumour tissue which is essential for bacteria-mediated cancer therapy (unpublished data).

As described, the combination of bacterial colonization as well as the formation of large necrotic areas in the tumour centre led to considerable migration of neutrophilic granulocytes into the tumours (Fig. 1a). Such cells became establihed between the necrotic areas and the viable part of the tumour [25]. Although they are highly phagocytic under normal conditions, we did not observe intracellular bacteria in tumours colonized by SL7207 (unpublished data). This might be explained by the following reasoning: (i) hypoxia might interfere with the function of neutrophils [30] and/or (ii) bacterial defence mechanisms might obstruct phagocytosis [27]. Nevertheless, the neutrophils might interfere with the bacteria as evidenced by the fact that extensive plasmolysis can be observed in tumour-colonizing *Salmonella* (Fig. 1d, arrow heads). This can be considered as a sign of stress imposed on the bacteria most probably by the neutrophils [31, 32].

The neutrophils appear to form a physical barrier resulting in the containment of bacteria within the hypoxic regions. To increase the therapeutic potential of the *Salmonella* strain, we aimed to remove this barrier to allow more extensive spreading of the bacteria into viable parts of the tumour [25]. Accordingly, neutrophils were depleted with antibodies. Indeed, the necrotic areas were enlarged under such circumstances and the therapeutic potency of *Salmonella* was also increased. It is interesting that bacterial biofilm formation was greatly reduced under such conditions as observed by electron microscopy (Fig. 1e). This correlated with the absence of severe plasmolysis that was observed in the previous setting when neutrophils were still present [27].

To extend these findings, we also tested *Salmonella* isolated from tumours of neutrophil-depleted mice for expression of genes that are involved in biofilm formation. We chose to investigate the genes *csgD* and *adrA*, which directly or indirectly regulate synthesis of the biofilm constituent cellulose as well as the adhesion molecules curli and type I fimbria [33]. In contrast to *bscA,* which is a structural gene known to be constitutively expressed, *csgD* and *adrA* were found to be downregulated in the absence of neutrophils. Hence, as a response to host neutrophils, it is likely that the bacteria form biofilms as a defence mechanism. This could be shown independently by electron microscopy and by gene expression analysis [27].

To evaluate this hypothesis, we deleted the genes *csgD* and *adrA* in SL7207. As expected, such variants were no longer able to form the pattern characteristic for biofilm formation on plates. In addition, the colonies displayed limited calcofluor staining (which is indicative of

cellulose production). Of importance, such variants were no longer able to form biofilms in the *in vivo* tumour model as observed by electron microscopy. However, we observed for the first time intracellularly localized bacteria. Thus, it appears that components involved in biofilm formation that were characterized by *in vitro* experiments on bacterial plates, in cultures or via attachment to host cells might also be required in the *in vivo* situation in our tumour model. Here, the biofilms act as part of the protective system to avoid host defence mechanisms such as phagocytosis or antimicrobial peptides. The latter might also act as the trigger for the bacterial protection system as similar activities of antimicrobial peptides have been described as triggers for the two-component system (phoP/phoQ) of *Salmonella* [34, 35].

In order to determine whether biofilm formation in murine solid tumours could be generally observed using *Salmonella* species, we tested the commonly used *S*. Typhimurium strains SL1344 and ATCC14028 using electron microscopy. While SL1344 showed only limited amounts of electron-dense material surrounding the bacteria indicative of weak biofilm activity of this strain *in vivo*, no such structures could be found using ATCC14028 (data not shown) [23]. It is known that the *fimH* gene encoding a cell adhesion molecule of the *Salmonella* strain SL1344 carries two point mutations that result in inability to adhere to host cells and block biofilm formation *in vitro* [23, 36]. This could explain the reduced biofilm formation by SL1344 in the tumour and suggests that additional components such as type I fimbria [19, 37] are required for biofilm formation in our *in vivo* model. But on the other hand, SL7207 derived from SL1344 shows strong biofilm formation in tumors.. Thus, more clarification is required to explain the difference between these two strains. Nevertheless, the inability to form biofilms does not hamper tumour colonization by these bacteria. Of note, this indicates that the genetic profile of the bacteria observed *in vitro* allows prediction of their *in vivo* behaviour in the murine tumour system and, thereby, of their general behaviour in the mammalian host [23, 27].

In vivo biofilm formation by bacteria closely related to *S*. Typhimurium

Next we questioned how closely related bacterial species would behave in our *in vivo* biofilm system. We ourselvesfirst tested laboratory strains of *E. coli* such as Top10 [25]. Because these strains originated from a K12 WT strain which is known to exhibit reduced proficiency in biofilm formation [38], it was not surprising that electron microscopic analysis showed that such bacteria were not surrounded by the extracellular matrix that had been observed for *Salmonella* SL7207. Similarly, probiotic WT strains of *E. coli* such as *E. coli* Nissle 1917 or Symbioflor® strains were not found to be surrounded by an extracellular polymer. Thus, under the present conditions, it is not clear whether *E. coli* are generally unable to form biofilms in murine solid tumours or whether we have by chance selected strains that are no

longer able to do so. Of interest, despite their inability to form distinct biofilms the bacteria were never observed in an intracellular location (data not shown). Thus, these bacteria must make use of protection mechanisms against immune cells other than biofilm formation. Such mechanisms might, however, also depend on functional *csgD* and *adrA* genes.

We also tested another closely related bacterium, *Shigella flexneri* strain M09T (*Δdap*). As *S. flexneri* is not a mouse pathogen, we could not apply the bacteria intravenously. Rather, the microorganisms were applied directly into the tumour. The gross appearance of the colonized tumour with regard to haemorrhage and necrosis formation was similar to that of a tumour colonized by *Salmonella* [25]. Again, well-organized structures were observed under these circumstances by electron microscopy. The bacterial clusters were surrounded by extracellular matrix suggesting extensive biofilm formation (Fig. 2). The appearance of the biofilms formed by *Shigella* is clearly different from that of extracellular matrices formed by *Salmonella*. Because *Shigella* are very closely related to *E. coli*, it is reasonable to assume that E*. coli* in general are also capable of forming biofilms in our tumour model.

Biofilm formation by *Pseudomonas aeruginosa*

Clinically, one of the most important bacterial pathogens that produces biofilms is *Pseudomonas aeruginosa*. This pathogen colonizes the lungs of patients with the inherited disease cystic fibrosis (CF) [39, 40]. Due to biofilm formation, the bacteria are protected from exogenous attack, for example by host defence mechanisms or the administration of antibiotics. In CF, pulmonary colonization by *P. aeruginosa* will eventually lead to death of the patient due to complete destruction of the lungs [41, 42]. Progress in understanding biofilm formation in the CF lung is seriously hindered by the absence of a suitable and versatile small animal model. Such a model should take into account most characteristics observed for biofilm infection in the human patient. Several animal models have been described to date; for instance, tracheal injection of *P. aeruginosa* embedded in agar or seaweed alginate beads in mice. In this example, matrix embedment is belived to avoid rapid bacterial clearance from the mouse lungs [43]. Similarly, a chronic bronchopulmonary infection model was proposed on the basis of a stable mucoid CF sputum isolate to overcome artificial embedding issues [43]. An alternative mammalian model of chronic otitis media infections was established in the chinchilla to evaluate bacterial persistence and biofilm formation in the middle ear [44]. However, this infection model is very inefficient and genetic manipulation is not possible in these animals. Furthermore, *Drosophila melanogaster* has been suggested as a potential model for studying biofilm formation *in vivo* [45]. This insect model suffers from the lack of an adequate immune system which might be important for the induction of biofilm formation by *P. aeruginosa*. Thus, none of the small animal models reported to date offers the versatility that is currently required for investigations of the

physiology and development of *in vivo* biofilms as well as the bacterial factors involved. We therefore sought to determine whether the murine CT26 solid tumour model, which has already been successfully employed to investigate biofilm formation by *S*. Typhimurium, could be used in this situation.

When *P. aeruginosa* was adminsteredintravenously to CT26-bearing mice, the tumours were colonized with kinetics similar to those observed for *Salmonella*. Haemorrhage of the tumour was observed shortly after bacterial administration and a large necrotic area was subsequently formed. Although the bacteria are not truly anaerobic, individual bacteria could be detected in the necrotic areas which are most likely to be completely anoxic. As observed previously, the bacteria and probably also the necrotic tissue attracted a substantial number of neutrophils that accumulated between the viable and the necrotic part of the tumour [3, 46]. It is interesting that the bacteria were located on both sides of the neutrophil accumulation and intermingled with these cells. Thus, for as yet unknown reasons and in contrast to *Salmonella*, this bacterial species was found in viable parts of the tumour [25, 27].

Histology revealed that the WT bacteria formed dense clusters that would be a precondition for biofilm formation (Fig. 3a). Indeed, there is electron microscopic evidence that biofilms are formed by *P. aeruginosa* (Fig. 3a), and the bacteria are surrounded by extracellular matrix. It is noteworthy that cluster and biofilm formation was dependent on an intact quorum-sensing system. *P. aeruginosa* carries two independent quorum-sensing systems using N-acylhomoserine lactone and 4-quinolones as ligands. Therefore we tested an isogenic mutant of PA14 carrying a defect in *pqsA,* a structural gene involved in the synthesis of 4-quinolone. Although able to colonize tumours in the same manner as WT bacteria, the *pqsA* mutant was unable to form clusters and biofilm-like structures as observed by electron microscopy (Fig. 3). Similar data were obtained using a variant that was defective in the pel system which is essential for forming the polycarbonate extracellular matrix (unpublished data).

Next we addressed whether biofilm formation by *P. aeruginosa* is also a bacterial defence reaction against the immune system of the host, as shown for *Salmonella*. Therefore, we depleted neutrophils as described previously [27]. All neutrophil-depleted mice died shortly after infection with WT strain PA14. Similar observations were made following complement depletion. Thus, mechanisms of innate immune defence are apparently more important for the mice to survive *P. aeruginosa* infections than *Salmonella* infections. Nevertheless, we obtained evidence that biofilm formation by *P. aeruginosa* in murine tumours is also a protective response against the immune system of the host. When similar experiments were carried out in $Rag1^{-/-}$ mice that lack the adaptive immune system, i.e. T and B cells, bacteria no longer formed biofilms. However this ability was regained after the mice had been

reconstituted with splenic cells from normal mice or mice lacking B cells but containing T cells [45]. Therefore we do not believe that T cells directly influence the bacteria as at present no efficient harmful effector T cell mechanism is known. Instead we propose that T cells activate myeloid cells such as macrophages or neutrophils which then act on the bacteria to elicit biofilm formation for protection [46]. However the details of this hypothesis require further investigation.

The availability of isogenic bacterial strains capable of colonizing tumours to a similar degree, but selectively able or unable to form biofilms, provides the possibility to screen the efficiency of antibiotics for treatment of *P. aeruginosa* infections. Among others, we tested ciprofloxacin – an antibiotic that is commonly used in clinical practice – which is also used to treat *Pseudomonas* in CF patients. A clear distinct phenotype was observed between bacteria able and unable to form biofilms (Fig. 3b). Whereas the biofilm-forming bacteria were resistant to a single dose of antibiotics, the variants that were unable to form biofilms showed significant sensitivity to treatment. By contrast, when localized in organs outside the tumour (e.g. liver), bacteria were equally sensitive, as they are unable to form biofilms in these tissues. Thus, the murine tumour model of biofilm formation by *P. aeruginosa* has great potential as a system in which (i) physiological parameters involved in triggering biofilm formation by these bacteria can be determined, (ii) co-infection experiments can be conducted with bacteria that are known to simultaneously colonize the CF lung and, most importantly, (iii) the activity of (new) antibiotics can be dissected as a prerequisite for the development of novel anti-infective agents able to act against biofilm-forming *P. aeruginosa* [46].

Conclusion

Investigation of biofilm formation by pathogenic bacteria *in vivo* is of utmost importance for biomedical research. However, model systems to carry out experiments in this context are often limited and reflect the precise clinical situations only to a limited extent. Here we have presented a simple murine model system in which such studies can be conducted. A subcutaneous transplantable tumour is used as the bacterial niche. Several strains, including *S*. Typhimurium SL7207 and *P. aeruginosa* PA14, invade and colonize these tumours efficiently and initiate biofilm formation within a short period of time. This *in vivo* biofilm model originated from our studies to develop strategies against cancer using bacteria, especially *S*. Typhimurium. At present, our main aim is to attenuate *S*. Typhimurium in order to generate bacterial strains that will be suitable for treatment of patients. Nevertheless, with regard to clinical applications, there is still speculation that attenuated safe strains might be

therapeutically inferior to virulent strains. Therefore, it may be a considerable time before the systemic application of therapeutic bacteria becomes a reality. The use of humanized mice might eventually resolve this problem. On the other hand, more than 100 years ago Coley applied his bacterial mixture directly into the tumour or nearby sites; his attempts were restricted mainly to superficial tumours. With appropriate safety constraints, such an approach could currently be feasible. In addition, ultrasound-guided application of bacteria to deep tumours is an option that might prove successful. Thus, bacteria-mediated cancer therapy represents a future option for oncologists.

Furthermore this model is useful to decipher the complexities within *in vivo* bacterial gene expression. For example comparison of the genetic profile of *P. aeruginosa* isolated from burn wounds and the same strains isolated from murine tumours demonstrated that these environments strongly resemble each other [47]. Thus, murine tumours represent a unique and versatile option to study biofilm formation *in vivo*. Employing a mouse model provides several additional benefits. For instance the physiology, especially of the immune system, is well characterized in the mouse. Thus, appropriate reagents as well as recombinant mouse strains are available that allow the characterization of effector cells and molecules responsible for induction of biofilm formation. Furthermore, this model provides a simple assay system for evaluating anti-biofilm strategies and compounds. Thus, in the case of *P. aeruginosa,* this system provides great promise for understanding and treating biofilms in patients.

Conflict of interest statement

None of the authors has any conflicts of interest to declare.

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Figure legends

Fig. 1 Immune histology and electron micrographs of a CT26 tumour colonized by *Salmonella enterica* serovar Typhimurium strain SL7207 for 48 h**.** a and b, Mice received subcutaneous administration of the murine colon carcinoma CT26, and subsequently bacteria ($5x10^6$) were injected intravenously. After 48 h the tumour was harvested, frozen at -70°C and prepared for immunohistological analysis. Antibodies against GR1 staining neutrophilic granulocytes (red), CD11b staining viable tumour cells (blue) and *Salmonella* (green) were used for detection. Samples were examined using a Zeiss confocal microscope LSM510. N indicates necrotic regions and V denotes viable regions. Magnifications: a**:10X** overview, b**:40X** enlargement. c**,** Scanning electron microscopic freeze-fracture image of a CT26 tumour colonized by *S*. Typhimurium SL7207 for 48 h. The bacteria and the extracellular matrix are clearly visible. d**,** Transmission electron microscopic image of a colonized tumour. Electron-dense material (*) is apparent between the bacteria. The white arrows point to bacteria in which severe plasmolysis has taken place. **e:** Transmission electron micrograph of *S*. Typhimurium SL7207-colonized CT26 tumour from mice in which neutrophils have been depleted. It appears that after depletion of neutrophils very low levels of extracellular matrix and adhesion molecules are produced by the bacteria; note that plasmolysis can only be observed occasionally.

Fig. 2 Electron micrograph of a CT26 tumour colonized by *Shigella flexneri.* A scanning electron microscopic freeze-fracture image is shown. Fibrous material probably representing adhesion molecules and extracellular material (arrows) enveloping the bacteria can be observed.

Fig. 3 Comparison of *Pseduomonas aeruginosa* wild-type (WT) and pqsA mutant. a. Immunhistology and electron micrgraphs of CT26 tumours colonized by *P. aeruginosa* PA14 WT or a *pqsA* variant. Upper panels show WT bacteria. Cluster formation is apparent. White bar represents 10 µm. Electron-dense material can be detected between bacteria probably representing biofilms. Asterisks denote bacteria in which severe plasmolysis has taken place, indicative of bacterial stress. Lower panels show the quorum-sensing mutant *pqsA*. The absence of clusters or of electron-dense material around individual bacteria (asterisks) is apparent.

b. Differential *in vivo* sensitivity to ciprofloxacin of *P. aeruginosa* strains that do or do not form biofilms in CT26 tumours. CT26 tumour-bearing mice were infected with WT or ΔpqsA bacteria. After 2 days the mice were treated with a single dose of 5 mg/kg ciprofloxacin. After 18 h the tumours were harvested and the bacterial counts determined. WT bacteria were completely insensitive to the antibiotic whereas the ΔpqsA strain, which is not able to

efficiently form biofilms, was significantly reduced in number (antibiotics versus saline, asterisks p>0.05, t-test)).

Table 1 The main bacteria known to colonize tumour tissue

Genus	**Bacterial species/strains**	**Anti-tumour effect**	***In vivo* biofilm formation**	**References**
Bifidobacterium	*B. longum*	+	n.d.	[13]
	B. adolescentis	+	n.d.	[48]
Clostridium	*C. histolyticus*	+	n.d.	[49]
	C. butyricum	+	n.d.	[50]
	C. novyi	+	n.d.	[14, 15]
	C. beijerincki	+	n.d.	[51]
Escherichia	*E. coli* K-12	+	n.d.	[52]
	E. coli Nissle 1917	-	n.d.	[53]
Listeria	*L. monocytogenes*	+	n.d.	[16]
Salmonella	*S. Typhimurium* VNP 20009	+	n.d.	[4]
	S. Typhimurium A1R	+	n.d.	[54]
	S. Typhimurium SL1344	+	–	[24]
	S. Typhimurium SL7207	+	+	[3, 10, 11, 27]
	S. Cholerasuis	+	n.d.	[12]
Pseudomonas	*P. aeruginosa PA14*	+	+	[55]
	P. aeruginosa PAO1	+	+	[55]

+**,** present; –, absent; n.d., not determined.