International Journal of Medical Microbiology 300 (2010) 449–456

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.de/ijmm

Enterobacterial tumor colonization in mice depends on bacterial metabolism and macrophages but is independent of chemotaxis and motility

Jochen Stritzker[a,b,c], Stephanie Weibel[b,c], Carolin Seubert[b,c], Andreas Götz[b], Achim Tresch[d], Nico van Rooijen[e], Tobias A. Oelschlaeger[c], Philip J. Hill[f], Ivaylo Gentschev[a,b,c], Aladar A. Szalay[a,b,c,g,*]

[a] Genelux Corporation, 3030 Bunker Hill Street, San Diego, CA 92109-5754, USA
[b] Department of Microbiology and Institute of Biochemistry, Biocenter, University Würzburg, Würzburg, Germany
[c] Institute for Molecular Infection Biology, University Würzburg, Würzburg, Germany
[d] Gene Center, Department of Chemistry and Biochemistry, Ludwig-Maximilians-University, Munich, Germany
[e] Department of Cell Biology and Immunology, Vrije Universiteit of Amsterdam, Amsterdam, The Netherlands
[f] University of Nottingham, School of Biosciences, Sutton Bonington Campus, UK
[g] Department of Radiation Oncology, Moores Cancer Center, University of California, San Diego, CA 92093-0843, USA

ARTICLE INFO

Article history:
Received 2 October 2009
Received in revised form 14 April 2010
Accepted 14 April 2010

Keywords:
Cancer
Enterobacteria
Chemotaxis
Macrophage
Metabolism
Mechanism of action

ABSTRACT

Despite promising results and increasing attention in bacterial cancer therapy, surprisingly little is known about initial tumor colonization and the interaction between bacteria and surrounding tumor tissue. Here, we analyzed the role of chemotaxis, motility, and metabolism both in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium strains upon intravenous injection into tumor-bearing mice. In contrast to previous models, we found that chemotaxis and motility do not play a significant role in tumor colonization and bacterial distribution within the tumor. Rather, the whole colonization and intratumoral migration process seems to be a passive mechanism that is influenced by the reticuloendothelial system of the host, by the tumor microenvironment and by the bacterial metabolism. These conclusions were supported by experimental data demonstrating that disruption of the basic branch of the aromatic amino acid biosynthetic pathway and depletion of macrophages, in contrast to flagellar mutations, led to significant changes in bacterial accumulation in tumors of live mice.

Introduction

In the past several years, cancer therapy with live bacteria has regained attention and significant advances have been achieved (Loeffler et al., 2007; Mengesha et al., 2007; Royo et al., 2007; Wei et al., 2007; Zhang et al., 2007).

Initial studies using intentional bacterial injection into human cancer patients had already been carried out in 1866 by W. Busch. At the end of the same century, F. Fehleisen and, later on, W. Coley used live *Streptococcus pyogenes* and *Serratia marcescens* as tumor-therapy agents (Kienle and Kiene, 2003). W. Coley, who is now credited as the founder of cancer immunotherapy, then changed his therapeutic approach to cell extracts of the same bacteria. However, due to the success of radio- and chemotherapies, and a better understanding of the principles behind these therapies, the medical community has preferred them to bacterium-mediated therapies. In the middle of the 20th century, scientists again became interested in using bacteria, especially *Clostridium* spp., as therapeutic agents and, meanwhile, several studies were performed using genetically engineered bacterial species, including virulence-attenuated mutants of pathogenic species that could be injected into mouse tumor models without causing disease, and/or expressing newly added heterologous therapeutic genes (for review see Wei et al., 2007). The use of *Salmonella* spp. has been particularly successful in many murine tumor models (Pawelek et al., 2003) and the VNP20009 strain has even been tested in clinical trials in human cancer patients (Toso et al., 2002).

Despite the regained attention in bacterial cancer therapy, surprisingly little is known about initial tumor colonization and the interaction between bacteria of the *Enterobacteriaceae* family and surrounding tumor tissue. Here, we tried to shed light on these events by analyzing the role of chemotaxis, motility, and metabolism, both in *E. coli* and *S. typhimurium* strains, as well as the effects of systemic macrophage depletion. In contrast to previous models, our data described in this manuscript indicate that active chemotaxis and motility did not play a significant role in

* Corresponding author at: Genelux Corporation, 3030 Bunker Hill Street, San Diego, CA 92109-5754, USA. Tel.: +1 909 307 9300; fax: +1 909 307 2251.
E-mail addresses: aaszalay@genelux.com, aladar.szalay@virchow.uni-wuerzburg.de (A.A. Szalay).

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doi:10.1016/j.ijmm.2010.02.004

Table 1
Bacterial strains used in this study.

Strain	Relevant characteristics	Reference or source
E. coli Nissle 1917	Probiotic *E. coli*	Altenhoefer et al. (2004)
E. coli Nissle 1917 Δ*fliA*	*fliA*::*cat*	Schlee et al. (2007)
E. coli Nissle 1917 Δ*fliC*	*fliC*::*cat*	Schlee et al. (2007)
E. coli Nissle 1917 Δ*flgE*	*flgE*::*cat*	Schlee et al. (2007)
E. coli Nissle 1917 Δ*fliC* pDB2	*fliC*::*cat*, *fliC* expressed in trans from pDB2	Schlee et al. (2007)
E. coli Nissle 1917 Δ*flgE* pDB3	*flgE*::*cat*, *flgE* expressed in trans from pDB3	Schlee et al. (2007)
E. coli Nissle 1917 Δ*aroA* (pENTR221-GFP)	*aroA*::*cat*	This study
E. coli Nissle 1917 Δ*aroA* pENTR221-aroA	*aroA*::*cat*, *aroA* expressed in trans from pENTR221-aroA	This study
E. coli 4608-58	Enteroinvasive *E. coli*	Hale et al. (1983)
E. coli 4608-58 Δ*aroA* (pENTR221-GFP)	*aroA* deletion	This study
E. coli 4608-58 Δ*aroA* (pENTR221-aroA)	*aroA* deletion, *aroA* expressed in trans from pENTR221-aroA	This study
S. typhimurium SL1344	Virulent *S. enterica* serovar Typhimurium 'wild-type', histidine auxotroph	B.A.D. Stocker
S. typhimurium M913	*fliGHI*::Tn*10*, derived from SL1344	Stecher et al. (2004)
S. typhimurium M935	*cheY*::Tn*10*, derived from SL1344	Stecher et al. (2004)
S. typhimurium SL7207	*aroA*::Tn*10*, derived from SL1344	Hoiseth and Stocker (1981)

tumor colonization or intratumoral migration, whereas disruption of the aromatic amino acid biosynthetic pathway and depletion of macrophages significantly altered the bacterial accumulation within tumors of mice.

Materials and methods

Bacteria

All bacterial strains used in this study are listed in Table 1.

For injection of tumor-bearing animals, bacteria were grown in LB broth at 37 °C and 190 rpm until reaching an $OD_{600\,nm}$ of 0.4, which corresponds to about 2×10^8 CFU/ml (Stritzker et al., 2007). Bacteria were harvested by centrifugation and washed twice in endotoxin-free phosphate buffered saline (PBS) (PAA, Pasching, Austria). The bacteria were then diluted accordingly and 100 μl of the suspension (containing approximately 5×10^6 CFU if not otherwise indicated) was injected into the lateral tail vein of tumor-bearing mice.

The number of bacteria as CFU/g was obtained after homogenization of respective tissue in 1.0 ml ice-cold PBS and plating 0.1 ml of serial dilutions of the suspension on LB agar plates. The limit of detection was about 100 CFU/g tissue analyzed.

Determination of bacterial load in tumors was done at 6 h (macrophage depletion experiments, confocal microscopy studies) or 48 h (chemotaxis and motility studies, aromatic amino acid biosynthesis mutants) post-injection.

aroA deletion and complementation

Strains *E. coli* 4608-58 (EIEC) and *E. coli* Nissle 1917 (*EcN*) were used for construction of the respective *aroA* mutants using the method developed by Datsenko and Wanner (2000). Primer pairs (5′-TTT TAT TTC TGT TGT AGA GAG TTG AGT TCA TGG AAT CCC TGA CGG TGT AGG CTG GAG CTG CTT C-3′)/(5′-AGA TTT GGC TAT TTA TTG CCC GTT GTT CAT TCA GGC TGC CTG GCT CAT ATG AAT ATC CTC CTT A-3′) for EIEC and (5′-TGG GGT TTT TAT TTC TGT TGT AGA GAG TTG AGT TCG TGT AGG CTG GAG CTG CTT-3′)/(5′-AGA AAG GAT TGT CTA TGT TAT CGC CCG TTA TTC ACA TAT GAA TAT CCT CCT TAG TTC CTA-3′) for *EcN*, respectively, were used to amplify the chloramphenicol resistance cassette (*cat*) from pKD3. After electroporation of the PCR product, transformants were cultured for 1 day in LB supplemented with 10 μg/ml vitamin K_2 and 0.2% L-arabinose and then plated on LB agar plates supplemented with 0.2% (w/v) L-arabinose, 30 μg/ml chloramphenicol and 10 μg/ml vitamin K_2. Deletion of *aroA* was confirmed by PCR using the primer pairs (5′-TTA TAC GCA AGG CGA CAA GG-3′/5′-CAG TTG GCG GAC AGT G-3′) for EIEC 4608-58 or (5′-CAG CAT AAT CCC CAC AGC CA-3′) (5′-CAC AAG GTC CGA AAA AAA ACG C-3′) for *EcN*. In EIEC, the resistance cassette was additionally removed by transforming the *aroA*::*cat* mutant with pCP20 (Cherepanov and Wackernagel, 1995). Deletion of the chloramphenicol resistance gene was confirmed by PCR.

For in trans complementation of *aroA* mutants, the *aroA* gene including its promoter region was PCR amplified from wild-type *EcN* using the primer pair 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA AGG AGG AAT AAA AAG CCA TGC CGC TGG AAG GTG T-3′/5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CGG CAA TGT GCC GAC GTC TT-3′ which also contained attB1 and attB2 sites. The resulting PCR fragment was cloned into pDONR^{TM}221 (Invitrogen, Carlsbad, CA, USA) using BP-clonase according to the manufacturer's instructions. The resulting pENTR221-aroA was then electroporated into the *aroA* mutants of *EcN* and EIEC 4608-58. Controls were transformed with pENTR221-GFP.

Soft agar motility assay

Testing of bacterial motility was performed in a soft agar motility assay. Bacteria were stabbed into semisolid LB agar plates with 0.25% agar and incubated at 37 °C for 6 (*S. typhimurium*) or 8 (*EcN*) hours. The diameter of the bacteria-containing turbid region around the inoculation site was determined using an electronic caliper and 1 mm was subtracted (diameter of the tip used to stab the bacteria into the agar). Data were given as % motility of each wild-type bacterial strain (note that *S. typhimurium* has about 3-fold higher motility than *EcN* in the soft agar motility assay).

Histology and fluorescence microscopy

Histology and fluorescence microscopy were performed as described previously (Stritzker et al., 2007; Weibel et al., 2008). Briefly, snap-frozen tumors were fixed in 4% formaldehyde, and 100-μm vibratome tissue sections were permeabilized in PBS containing 0.3% Triton X-100. Sections were then incubated with FITC-labeled phalloidin or rat anti-mouse CD68 antibodies (Serotec, Düsseldorf, Germany), Hoechst 33342 (optional) and biotinylated polyclonal antibodies against *E. coli* or *Salmonella* spp. (ViroStat, Portland, USA) for 12–15 h, followed by washing and incubation with Cy3-conjugated streptavidin (Sigma, Taufkirchen, Germany) and, when rat anti-mouse CD68 primary antibodies were used, Cy3-conjugated donkey anti-rat IgG antibodies. After several rinses in PBS, tissue sections were incubated in PBS containing 60% (v/v) glycerol and then mounted in PBS containing 80% (v/v) glycerol.

Confocal microscopy was performed using a Leica TCS SP2 AOBS equipped with an argon, helium–neon and UV laser. Digital images were processed with Photoshop 5.0 (Adobe Systems, Mountain View, USA) and merged to yield pseudo-colored images.