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RAPID COMMUNICATION

Mycobacterium avium subspecies *paratuberculosis* infects and multiplies in enteric glial cells

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Abstract

AIM: To establish the role of enteric glial cells during infection with *Mycobacterium avium* subspecies paratuberculosis (MAP) in Crohn's disease.

METHODS: In order to establish the role of enteric glial cells during infection with *M. avium* subspecies *paratuberculosis* (MAP) in Crohn's disease, Map adhesion experiments on enteric glial cells were performed as well as expression analysis of Map sigma factors during infection.

RESULTS: In this study, for the first time, we found a high affinity of MAP to enteric glial cells and we analyzed the expression of MAP sigma factors under different conditions of growth.

CONCLUSION: The fact that Map showed a high affinity to the glial cells raises concerns about the complicated etiology of the Crohn's disease. Elucidation of the mechanisms whereby inflammation alters enteric neural control of gut functions may lead to novel treatments for Crohn's disease.

Key words: *Mycobacterium avium* subspecies *Paratuberculosis*; Enteric glial cells; Inflammatory bowel diseases; Crohn's disease; Sigma factors expression

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INTRODUCTION

Several cell lines of the gut have been extensively studied such as intestinal epithelial cells, immune cells, smooth muscle cells and enteric neurons[1-4]. However, very little is known about enteric glial cells which belong to the enteric nervous system (ENS) along with the neurons[5]. The ENS, comprised of several plexuses, is located alongside the intestinal wall. The enteric glia are localised within the mucosal plexuses and the glial processes make close contact with the epithelial cell layers. The epithelial crypt bases, in particular, are surrounded by a dense network of glial cells[6]. These glial cells are small, star shaped with different processes of various length and shape[6]; they are morphologically and immunohistochemically different from microglia of the central nervous system and from all other peripheral glia and appear more closely related to astrocytes although functionally different[6]. Evidence for functional heterogeneicity of enteric glia has recently been described[7]. The enteric glial cells (EGC) could participate in neurotransmitter synthesis/inactivaction or in synaptic transmission. Moreover, they may also interact with intestinal capillaries to modulate endothelial permeability[8].

It has been shown that the enteric nervous system regulates intestinal barrier functions by regulating the zonula occludens-tight junctions[9]. Regarding EGC, it has been shown in a mouse model that EGC ablation results in fulminant intestinal inflammation due to disregulation and rupture of the epithelial intestinal barrier[8,10]. It has been suggested that there is a direct interaction between EGC (releasing soluble factors) and epithelial cells to enhance the intestinal epithelial cells[5]. Moreover they could have a role in the prevention of epithelial barrier disorganization and hyperproliferation during bacterial infection, inflammatory processes and neoplasia[11-13]. All these findings indicate that glial cells may be an important component of the intestinal mucosal defense system.

On the other hand, *Mycobacterium avium* subspecies *paratuberculosis* is the causative agent of Johne's disease, a chronic and incurable disease affecting ruminants and other animals[14]. Moreover, there is increasing evidence of its involvement in the enteric granulomatous syndrome of

Table 1 PCR primers, targets, position on the sequences

Target	Primer name	5'-3' sequence	Position[1]
16S	MAP16Sfor	ATCATGCCCCTTATGTCCAG	1179-1198
	MAP16Srev	TGAGACCGGCTTTAAAAGGA	1259-1278
sigA	sigAfor	GTACGCCACCCAGCTGATGTCG	714-735
	sigArev	CGTCGCGGCAGATCCACAT	787-805
sigB	sigBfor	GACCTGCTCGAGCACAGC	646-663
	sigBrev	CAGCACGCTGCGGATGTCGGTG	783-804
sigC	sigCfor	ACATCCGTCACCTGCAGTC	1088-1106
	sigCrev	GTCACCTCGACCAGATCCTC	1177-1195
sigD	sigDfor	CTTCCTGGCTTTCCTGTACG	249-268
	sigDrev	GATGGACTCGGTCGGGTAG	324-242
sigE	sigEfor	CACCCAGGAGACCTTCATCC	327-346
	sigErev	GACCATGTCCAGGAACAG	418-435
sigF	sigFfor	GGCAGCTCCTACAACACCTT	493-512
	sigFrev	ACTCCTGGTCCTCGATCCGG	597-616
sigF-like	sigF-likefor	ATGACCAACGCAATCGCTCC	1-20
	sigF-likerev	GGCATCCGGCGCAGTTCCA	88-107
sigG	sigGfor	GCGTTCGAAAGCTACGACAT	622-641
	sigGrev	CTGATACCACCCGGTGTACG	692-711
sigH	sigHfor	AATCTCAAGGCGTGGCTCTA	385-404
	sigHrev	TGATTTCCTCGGTCGGATAC	465-484
sigI	sigIfor	GGGCGACATCGACGACGTGC	150-169
	sigIrev	GACATCGCCCGGACGTTCGTG	235-255
sigJ	sigJfor	GCATCTACACGGCGGGCCTG	740-759
	sigJrev	GGCGAACCGGTGAACTTGT	857-875
sigL	sigLfor	CGTGATCGAACGGTCCTACT	399-418
	sigLrev	CCGCACCGCATAGTGTAGT	491-504
sigM	sigMfor	TGGCTGCACCGCATCGTG	238-255
	sigMrev	TCGGCGACCGGATAGTAGTCT	315-335
Other ECF-1	sigECF1for	GTTCTCCGCCGAGTCGATTT	310-328
	sigECF1rev	GTCGAATCCGAACACCTCAC	492-411
Other ECF-2	sigECF2for	GCATCCACACGATCGACAT	839-857
	sigECF2rev	GGTTGTGATGTTCCTGAACC	914-933
Other ECF-3	sigECF3for	GTCGGTCATGGGTTTCGT	780-797
	sigECF3rev	GCACCCAGCTCCAGTTTC	855-873
Other ECF-4	sigECF4for	GATCTCGTCGGCATCTCG	502-519
	sigECF4rev	TCCAATTCGTTTCGGAGATT	592-611
Other ECF-5	sigECF5for	GCAATTGACCCGTTCACC	945-962
	sigECF5rev	CTCCTCCAAAGCGGCTAAG	1020-1038
Other ECF-6	sigECF6for	TGCAAGGTAATTCGATCAAGG	419-439
	sigECF6rev	TCCCTCGTTGACCTGTGC	511-528

[1]longest sequence available (primary and TIGR annotation). ECF: Extracitoplasmatic function; TIGR: The Institute for Genomic Research.

humans called Crohn's disease[15-17]. For all these reasons we analyzed the interactions between EGC and M. paratuberculosis *in vitro*.

Sigma factors are part of the transcriptional regulators family and are responsible for binding to the RNA polymerase complex (composed of four distinct subunits); to recognize promoters and separate DNA strands[18]. In fact, every sigma factor has its own specificity, allowing the initiation of transcription of different subset of genes[18,19]. Some sigma factors (sigD, sigE, sigC, sigH and sigL) play a role in the virulence of *M. tuberculosis*[20,21]. The number and abundance of sigma factors reflect the ability of the bacterium to cope with various environmental conditions, stresses and insults[18,22].

In this study the expression of all 19 sigma factors of M. paratuberculosis were also tested during EGC infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions

M. paratuberculosis ATCC 43015, of human source was obtained from The RIVM, Bilthoven, The Netherlands. *M. paratuberculosis* for DNA extraction was grown in Mycobacteria Growth Indicator Tube (MGITl) medium supplemented with Mycobactin J and egg yolk. Murine enteric glial cells were previously isolated and characterized by Dr. Anne Ruhel[23].

Identification of M. avium subsp. paratuberculosis putative sigma factors

Identification of individual sigma factors in *M. avium* subspecies. paratuberculosis was obtained by text annotation searches and by BlastP similarity searches using *M. smegmatis* and *M. tuberculosis* H37Rv Sigma ORF as queries. Primers used in real time PCR are described in Table 1.

DNA extraction

DNA extraction was performed using the Ribolyser system (HYBAID, USA) and purified as previously described[17].

PCR conditions

Oligonucleotide sequences and position are reported in

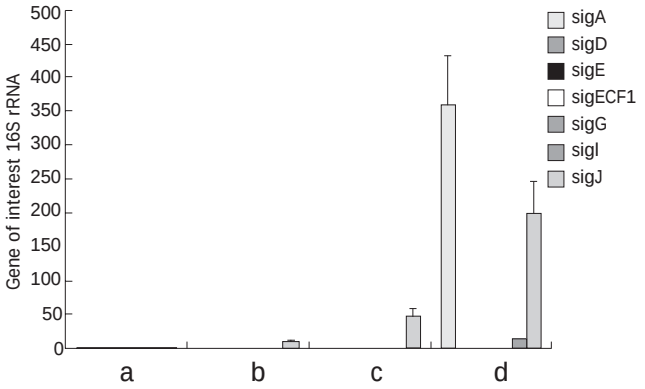

Figure 1 Relative quantification of expression of MAP sigma factors relative to the expression of the 16S rRNA MAP gene (calculated by the Biorad software) by Real Time PCR after different conditions of growth. a: After growth in 7H9 medium plus mycobactin J; b: Infection of EGC after 6 h of incubation; c: Infection of EGC after 48 h of incubation; d: Infection of EGC cells after 7 d of incubation.

Table 1. Specificity of primer pairs was ascertained with PCR with the following conditions: $MgCl_2$ 1.5 mmol/L, deossi Nucleotide Triphosphate (dNTPS) 150 μmol/L, primers 0.2 μmol/L each, Taq 0.025 U/μL reaction volume. After an initial denaturation of 3 min at 94℃, 30 cycles of 1 min at 94℃, 1 min at 58℃, 1 min at 72℃ and a final extension at 72℃ for 5 min were performed. Only the amplification products of the expected length were obtained.

RNA extraction

RNA was extracted using the Ribolyser instrument[17]. RNA was extracted from MAP cells after different conditions: (1) growth in 7H9 medium plus Mycobactin J, (2) after addition of lysozyme at 24 h and 7 d; (3) after infection of EGC line, after 6 h, 24 h, 48 h and 7 d. RNA was quantified for further experiments and samples were stored at -80℃ until further use. cDNA was prepared by an initial incubation at 65℃ for 10 min of the reaction mixture containing 100 μmol/L oligo-dT and 10 μL of extracted RNA; this first step was followed by a second incubation of 1 h at 42℃ of the mixture containing 50 U/reaction of reverse transcriptase (Mooloney-Murine Leukemia Virus M-MLV 200 U/μL), 0.1 mol/L buffer 5 × Tris HCl pH 8.3 (Life Technologies) with 150 mmol/L KCl, 7 mmol/L $MgCl_2$ and 20 mmol/L dithiothreitol (DTT); 0.1 mol/L DTT, 10 mmol/L dNTPs, 40 U/μL RNase inhibitor.

Real time PCR analyses

Real Time PCR was performed using the iCycler Detection System (Biorad) with the SyberGreen assay (Applied Biosystems). Gene expression of all the Sigma factors (except Extracitoplasmatic function3 ECF3) was determined using quantitative Real-Time PCR by comparing the fluorescence produced by test samples to that of the 16S rRNA gene expression (house-keeping gene). Five micro liters of cDNA was subjected to PCR (1 cycle at 94℃ for 1 min; 50 cycles at 94℃ for 30 s and at 60℃ for 40 s) with primers that amplified 16S rRNA, all 19 sigma factor genes. PCR was carried out with the following reaction-mixture in a total volume of 30 μL: 1 × of 10 × buffer, $MgCl_2$ 1.5 mmol/L, dNTPs 0.2 mmol/L, primers 0.5 μmol/L each, DNA polymerase 0.5 U/sample. PCR was performed in a 96 well plate, for each sample 2 wells were utilized to guarantee uniformity in results. The quantitative analysis of the data obtained was performed by Biorad's method of relative quantitation of gene expression (Bio-Rad Laboratories, USA).

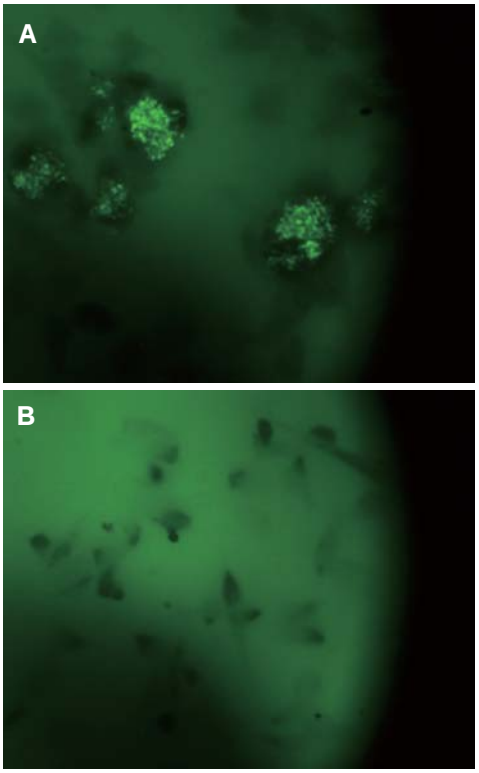

Figure 2 MAP cells stained with auramine rodhamine at 6 h after infection of EGC (**A**) and without infection (**B**) and visualized under a fluorescence microscope (× 100).

Infection experiments

Glial Enteric cell lines were cultivated in 24 well plastic dishes. The EGCs were cultivated in Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum and 4 mmol of L-glutamine per liter. All tissue culture reagents were obtained from Sigma (Sigma Chemical Co.). Cells were seeded at 2×10^4 cells per well and incubated at 37℃ in 5% CO_2. EGC cells were used when they were semiconfluent. MAP grown over night in 7H9 broth (with mycobactin J) was diluted in cell medium. One milliliter containing 10^6 bacteria was added to each well containing EGCs (cells were infected at a ratio of bacteria/cells of 10:1). After two hours infected EGCs were washed with cell medium containing Kanamycin 100 μg/mL and incubated with the same medium. Cells were stained with auramine rodhamine at the different incubation times (6 h, 48 h and 7 d at 37℃) to visualize MAP, and counted as previously described[17].

RESULTS

Expression of sigma factors in different conditions of Growth

7H9 broth plus mycobactin J: MAP during growth in

broth 7H9 supplemented with Mycobactine J, expressed several sigma factors as expected (Figure 1A). SigA, Sig D, SigE, Sig G and Sig I were among the most expressed ones as compared to the 16S rRNA expression taken as house keeping gene whereas ECF1 and sig J were expressed in lower quantity (Figure 1A).

Infection of EGC: The expression of the 19 sigma factors of MAP was observed and quantified after growth infection of EGC at 6 h, 48 h and 7 d (Figure 1 B-D). Soon after infection, expression of all sigma factors shut down, except for sig J which was expressed in high quantity (Figure 1B). The expression of sig J increased after 48 h (Figure 1C) and 7 d. SigA was overexpressed after seven days (Figure 1D), at the same time an expression of sig I was also detected. Figure 2 reveals an example of EGC infection by MAP where bacteria were visualized within the intestinal glial cells after staining with auramine rodamine as compared to control cells not infected.

DISCUSSION

Enteric glias are distinct from all other glial cell types[5,7]. No gastrointestinal disorder has been reported linked to glial defect yet, most probably because subtle changes in glial function might be involved in the etiopathogenesis of enteric disorders. Crohn's disease with neuroinflammation and neurodegeneration components may be associated with EGC alteration[23-25]. Indeed EGC interact with enteric neurons, endothelial cells, immune cells and the intestinal epithelium; all these factors can contribute to the pathogenesis of Crohn's disease. In our experiments, we found that MAP has a high affinity to EGCs. Experiments "*in vitro*" show a high adhesion and intracellular multiplication as confirmed by the active expression of sigA (the housekeeping sigma factor) after seven days of infection along with the expression of sigJ and sigI (expressed in different conditions of cellular stress).

The fact that this dangerous intestinal pathogen has a demonstrated affinity to the glial cells and that MAP has recently been reported in substantial percentages of Crohn's patients[15-17] raises concerns about the complicated etiology of the Crohn's disease.

MAP expression of sigma factors in EGC is very similar to sigma factor expression after infection of the Caco2 intestinal epithelial cell line (manuscript in preparation) and shows how there is a rapid change in gene expression after cell infection.

EGCs are an active part of an intestinal network system essential for a healthy and functional gut[26-28]. The role of EGC in Crohn's and other enteric diseases is not well studied and this is the first report that attempts to unravel interaction between an intestinal pathogen and EGC. Future work is certainly needed to elucidate this complex interaction.

COMMENTS

Background

Enteric glia might play a role in regulating barrier functions in mucosal epithelia. *Mycobacterium avium subspecies paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic and incurable disease affecting ruminants and other animals. Moreover, evidence of its involvement in Crohn's disease is accumulating. MAP enter the host through Peyer's patches and intestinal epithelium and are sampled by intestinal dendritic cells; they survives inside macrophages. Nothing is known about the role of enteric glial cells during MAP infection thus we performed this study to establish the role of enteric glial cells during infection with *Mycobacterium avium subspecies paratuberculosis* (MAP) in Crohn's disease.

Research frontiers

It is firmly established that intestinal inflammation is accompanied by functional and structural alterations of the Enteric Nervous System to which belong Enteric Glial Cells. In Crohn's disease patients, a compromised glial network that responds poorly to inflammatory stimuli has been suggested. Our report will open a new frontier on the interaction of MAP and enteric glia cells supporting the idea that Crohn's disease is associated with the invasion and persistence of a multi host pathogen as it is MAP.

Innovations and breakthroughs

This is the first research that highlights the interaction of a true enteric pathogen such as Map with Enteric glial cells. This study will open the research field in this matter.

Applications

Enteric glial cells may be the target of a series of cytokine modulators or drugs. Map will be recognized as a pathogen associated with intestinal disorders.

Terminology

EGCs: enteric glial cells; Map: Mycobacterium avium subs. Paratuberculosis; ENS: Enteric nervous system; Johne's disease: Enteric granulomatous infection of ruminants caused by Map; Crohn's disease: Enteric granulomatous chronic inflammation in humans with ulcerative colitis (UC) forms inflammatory bowel disease (IBD).

Peer review

The manuscript by Sechi *et al* describes the expression of MAP sigma factors during infection of enteric glial cells. The first findings that sigma factors are differentially expressed during infection are very promising.

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