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Research Paper

Inflammatory response of *Haemophilus influenzae* biotype *aegyptius* causing Brazilian Purpuric Fever

Gisele Cristiane Gentile Cury, Rafaella Fabiana Carneiro Pereira, Luciana Maria de Hollanda, Marcelo Lancellotti*

Biotechnology Laboratory, Department of Biochemistry, Institute of Biology, State University of Campinas, Campinas, SP, Brazil.

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Abstract

The Brazilian Purpuric Fever (BPF) is a systemic disease with many clinical features of meningococcal sepsis and is usually preceded by purulent conjunctivitis. The illness is caused by *Haemophilus influenza* biogroup *aegyptius,* which was associated exclusively with conjunctivitis. In this work construction of the *las* gene, hypothetically responsible for this virulence, were fusioned with *ermAM* cassette in *Neisseria meningitidis* virulent strains and had its DNA transfer to non BPF *H. influenzae s*trains. The effect of the *las* transfer was capable to increase the cytokines TNFα and IL10 expression in Hec-1B cells line infected with these transformed mutants (in eight log scale of folding change RNA expression). This is the first molecular study involving the *las* transfer to search an elucidation of the pathogenic factors by horizontal intergeneric transfer from meningococci to *H. influenzae.*

Key words: Brazilian purpuric fever, *Haemophilus influenzae* biogroup *aegyptius,* genetic transfer, *Neisseria meningitidis*, *Haemophilus influenzae*.

Introduction

The Brazilian Purpuric Fever (BPF) is a fulminant pedriac disease caused by *Haemophilus influenzae* biogroup *aegyptius* (Hae). BPF was first described just over a decade ago when an outbreak emerged in several locations in the Sao Paulo State, Brazil. The illness has many clinical features of meningococcal sepsis as high fever, vomiting, abdominal pain, rapid progression of petechiae and vascular collapse. These symptons generally manifest between 7-10 days after an episode of purulent conjunctivitis (1985; 1986; 1987a; 1987b).

Major outbreaks of BPF occurred from 1984 through 1990, and sporadic cases also have been reported in Australia (McIntyre and others 1987), USA (Virata and others 1998) and recently in 2007 in Amazonas region, Brazil (Santana-Porto and others 2009). However, the disease may be more common than expected. Since the clinical picture is very similar to the meningoccocal septicemia, possible cases of BPF could be misreported. Currently, BPF is a disease requiring mandatory reporting in Brazil, because BPF agents may potentially lead to new outbreaks.

Before the emerging of BPF, Hae was a bacterium only associated with conjunctivitis, pro,ducing seasonal and epidemic infection in hot climates (Pittman and Davis 1950). Little is known of the determinants of Hae virulence or the pathogenesis of infection in BPF. Potential virulence factors such as pilus proteins and lipopolysaccharide have been investigated in the infant rat model (Rubin and St Geme 1993) and *in vitro* with endothelial cells (Quinn and others 1995; Weyant and others 1994), but these factors have not been consistently associated with the pathogenesis of BPF. The invasive unique phenotype in Hae makes evident that this bacterium has virulence factors absent in the rest of *Haemophilus* strains.

The horizontal transfer of virulence genes has a major role in the evolution of bacterial pathogens and since the natural genetic exchange between *Haemophilus influenzae* and *Neisseria meningitidis* was already described (Kroll and others 1998), the highly virulent ``meningococcal''

Send correspondence to M. Lancellotti. Department of Biochemistry, Institute of Biology, Rua Monteiro Lobato s/n, Caixa Postal 6109, 13083-970 State University of Campinas, Campinas, SP, Brazil. E-mail: mlancell@unicamp.br.

phenotype in Hae may be result of the genetic transfer from the meningococcus to Hae-BPF. One possible explanation for the emergence of the invasive Hae strains is an occurrence of a genetic exchange of invasiveness genes between those two bacteria. Meningococcal conserved sequences were identified in the Hae strains associated with BPF determined as NMB0419 in *N. meningitidis* and *bfp001* in Hae (Li and others 2003).

The *lav* gene is another proof of the lateral transfer between *Haemophilus* and *Neisseria.* This gene is predicted to encode a virulence-associated autotransporter and to be transferred from *H. influenzae* to *Neisseria*. Through homology and base sequences analysis, it was observed that a novel type of this autotransporter had emerged in Hae, which was described as *las* (Davis and others 2001).

In the present work, we aimed to analyze the role of the *las* gene as a virulence determinant in BPF. Constructions of the *las* gene from Hae were transfer to non BPF *H. influenzae s*trains and the inflammatory effects were analyzed and measured in an endothelial cellular system *in vitro*. This work is the first molecular study involving the *las* transfer to search an elucidation of the pathogenic factors by horizontal intergeneric transfer between meningococci and *H. influenzae.*

Material and Methods

Bacterial conditions

The *Neisseria meningitidis and Haemophilus influenzae* strains used in this work (Table 1) were grown in chocolate agar at 37 °C with 5% CO_2. When needed, culture media were supplemented with erythromycin at 2 mg/mL for *N. meningitidis* receptor strains or 1 μg/mL for *H. influenzae* receptor strains.

DNA techniques

Recombinant DNA protocols and transformation were performed as described previously (Hollanda and others; Lancellotti and others 2008). The oligonucleotides used are listed in Table 2. All the mutants obtained by homologous recombination were verified by PCR analysis using an oligonucleotide harboring the target gene and another harboring the cassette.

Construction of fusion mutation in *las::ermAM*

The *las* gene was amplified from Hae11116 strain using the template sequence from GenBank GI: 14994100. Initially, the upstream region of the start codon was amplified using the primers lasiF and lasiR (Table 2). The downstream region to start codon of *las* was also amplified using lasfF and lasfR primers (Table 2). The lasi and lasf amplicons were cloned in pGEMT Easy (Promega), originating the plasmids pLAN 75 and pLAN76, respectively. Both plasmids had one *BamHI* site, which was necessary to future insertions. In the *BamHI* site of pLAN75 was inserted the *ermAM* cassette to generate the pLAN77. A new amplification reaction was performed using the ERAM3 and lasiF primers using pLAN77 as template. The amplicon obtained was inserted in *BamHI* site of pLAN76 generating the transcriptional fusion vector pLAN78. Then, this vector was transformed in *N. meningitidis* B4 strain, originating the LG2 strain (Figure 1).

Genetic transfer assays of *las* gene from *N. meningitidis* to *H. influezae*

The fusioned mutant LG2 was used as DNA donor to performed *in vitro* transformation of *H. influenzae* RdKw20 and *H. influenzae* βlac strains. The transformation process was performed according specifications already

Table 1 - Bacterial Strains used in this work.

Strain	Characteristics	Origin (Reference)
DH5μ	*Escherichia coli* F-, *end*A1, *hsd*R17 c, *sup*E44, *thi*-1, *gir* A96, *rel*A1	[23]
pLAN75	pGEM TEasy pGEM TEasy containing the amplicon of lasiF and lasiR, the initial portion of *las* gene	This work
pLAN76	pGEM TEasy containing the amplicon of lasfF and lasfR, the final portion of *las* gene	This work
pLAN77	pLAN 76 with amplicom containing the upstream region of *las* gene and *ermAM* cassette from pLAN 75	This work
C2135	*Neisseria meningitidis* serogroup C, BIOMERIEUX	INCQS - FIOCRUZ
B4	*Neisseria meningitidis* B4:P1-16 serogroup B	IAL – SP
Rd	*Haemophilus influenzae* strain serotype d Rd KW20 ATCC 51907	INCQS – FIOCRUZ
βlac	*Haemophilus influenzae* strain serotype b ATCC 33533	INCQS – FIOCRUZ
Hae 254/86	*Haemophilus influenzae* biotipo *aegyptius* BPF	IAL-SP[1]
Hae 258/86	*Haemophilus influenzae* biotipo *aegyptius* BPF	IAL – SP[1]
Hae 284/86	*Haemophilus influenzae* biotipo *aegyptius* BPF	IAL – SP[1]
LG2	*Neisseria meningitidis* B4 transformed with pLAN77 with a transcriptional fusion of *las::ermAM*	This work
RdLG2	*H. influenzae* Rd strain transformed with genomic DNA from LG2 *las::ermAM*	This work
βlacLG2	*H. influenzae* βlac strain transformed with genomic DNA from LG2 *las::ermAM*	This work

Table 2 - Oligonucleotides used in this work.

Oligonucleotide	Sequence 5'– 3'	Reference
IL10	FW GTGATGCCCCAAGCTGAGA	(Overbergh and others 2005)
	RV CACGGCCTTGCTCTTGTTT	
TNF-α	FW TCTTCTCGAACCCCGAGTGA	(Overbergh and others 2005)
	RV CCTCTGATGGCACCACCAG	
GAPDH	FW TGCACCACCAACTGCTTAGC	(Overbergh and others 2005)
	RV GGCATGGACTGTGGTCATGAG	
lasi F	GAACCAAATGGCACTTTTTGTTCAGTTTTATGC	This work
lasi R	GCGGATCCATCTTTTAATGAATAGAATACGGAAGCAC	This work
lasf F	GCGGATCCATGCTCGATCGAAATTCTG	This work
lasf R	CTCGACGTTCATTGTCTATTTCCACACCG	This work
ERAM1	GCAAACTTAAGAGTGTGTTGATAG	(Hollanda and others)
ERAM3	AAGCTTGCCGTCTGAATGGGACCTCTTTA GCTTCTTGG	(HOLLANDA AND OTHERS)

*The underlined sequences in italic are the insertion of the *BamHI* site into original sequence.

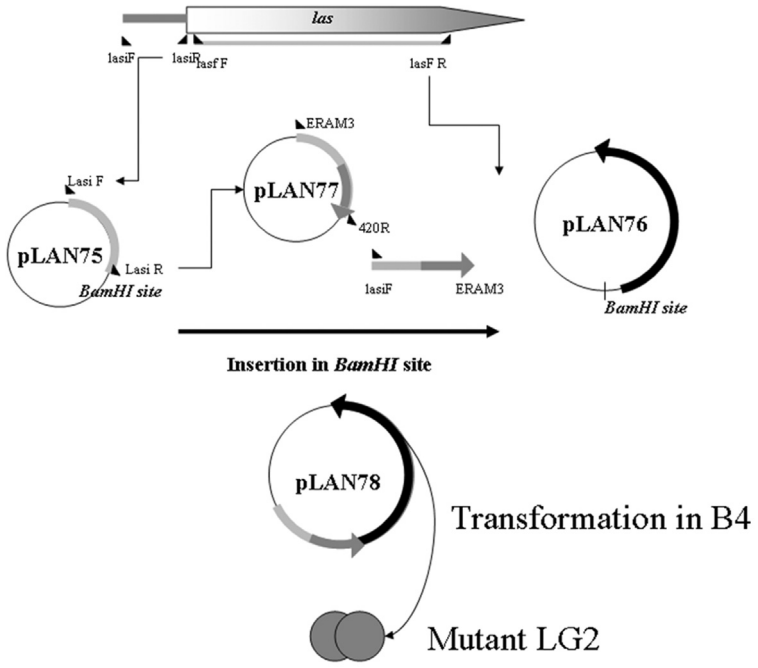

Figure 1 - The gene *las* was amplified from Hae11116 strain using the template sequence from GeneBank GI: 14994100. Initially, the upstream region of start codon of *las* gene was amplified using the primers lasiF and lasiR (Table 2). Also the downstream region of *las* was amplified with the primers lasfF and lasfR. Both amplicons were cloned in pGEMT Easy (Promega), originating pLAN 75 and pLAN76. In *BamHI* site of pLAN75, was inserted the *ermAM* cassette to generate the pLAN77. A new amplification reaction was performed using the ERAM3 and lasiF primers with the pLAN77 as template. The amplicon obtained was inserted in *BamHI* site of pLAN76 generating the transcriptional fusion vector pLAN78. This vector was then transformed in B4 *N. meningitidis* strain, originating the LG2 strain.

described (Taha and others 1998) adapted for *H. influenzae* using the BHI liquid medium (supplemented with haemin and NAD at 10 μg/mL). The *H. influenzae* strains were growth in chocolate agar and suspensions of DO 600 in supplemented BHI were made. The 5 μg of DNA from LG2 strain was added in the bacterial suspensions and then incubated at 37 °C in atmosphere of 5% of CO_2 by 5 hours. The suspensions were plated in chocolate agar with erythromycin.

Hec1b cell culture and adhesion assay

Hec1B cells line were grown in plastic flasks (25 cm^2) with RPMI 1640 medium (Cultilab, Campinas, SP Brazil), supplemented with 2% L-glutamine, 120 μg/mL of garamycin and 10% inactivated fetal bovine serum (com-

plete medium). The tests for adhesion in cells followed specifications already described (Pereira RFC 2011).

Production of inflammatory cytokines by real time PCR

For those strains that promoted morphological alterations in Hec1b cells were performed analysis of the cytokines (IL10 and TNFα) production. Total RNA was extracted using the Trizol Reagent (Invitrogen, Calsbag, CA, USA). RNA yield was estimated by Nanodrop (Thermo Scientific). A minimum amount of 0.2 ng of the RNA was submitted to reverse transcription followed by qRT PCR. Real-time PCR primers are listed in Table 2 and the reaction was performed using the StepONE Plus thermocycler (Applied Biosystems). Each 10 μL reaction contained 400 nM of each primer, 5 μL Master Mix and 1/60 000 Fast EVA Green Master Mix (both from Biotium), 0.25 uL of Super Script III Platinum One-Step qRT-PCR System (Invitrogen Calsbad, CA, USA) and the recommendations for cDNA production described in the kit were followed. Reactions were repeated on three different biological replicates using as endogenous control the GAPDH gene and fold expression changes were averaged. The data was analyzed by the comparative Ct method revised in (Schmittgen and Livak 2008).

Statistical analysis

The data from each assay were statistically analyzed using Turkey's test compared with a control sample and $p < 0.05$ was considered significant. All experiments were performed in triplicate and the data shown in the graphs and in the table represent the means ± standard errors.

Results

For the production of the *las::ermAM* gene aiming the detection of the *las* transfer was used the template strain Hae11116 (GenBank GI: 14994100). The construction of pLAN78 and the correct insertion of each construction component were verified using *ermAM'*s and *las'* primers. This construction was used in the transformation of B4 strain for the achievement of the mutant B4 LG2.

The artificial horizontal transfer from *N. meningitidis* to *H. influenzae* strains was performed using the DNA extracted from strain LG2 and the strains Rd and βlac were used as receptors, originating the mutants Rd LG2 and βlac LG2. The transference process was verified by erythromycin resistant transformants at 1μg/mL. Also, analyses for *las::ermAM* insertion were made using the primer lasi and ERAM1 (data not shown).

H. influenzae and *N. meningitidis* transformants and their wild types, three Hae-BPF strains (Hae 254/86, Hae 284/86 and Hae 258/86) and one typical *H. influenzae* (Hi 46) strain were analyzed in inducing expression of cytokines TNFα and IL10 in Hec-1-B cells. The *in vitro* assays showed that the insertion of the foreign construction in the *N. meningitidis* B4 donor strain did not influence the cytokines production when the B4 mutant LG2 infected Hec-1B cells. There was an increase in the expression of these inflammatory cytokines in Hec-1B cells when infected with the transformed strains βlac and Rd. Still, the levels of IL10 expression in the infection with the mutants were higher than in the presence of the Hae-BPF strains (Figure 2).

Discussion

The use of the increase of cytokines in septic shock characterization is normally done in *N. meningitidis* pathologies. The implication of TNFα and interleukines, such as IL10, in meningococci sepsis is well described by many authors (Bjerre and others 2004; Brandtzaeg and others 2001; Jacobs and Tabor 1990). In this work the use the Hec-1-B cells line, which had already used as model of TNFα verification in meningococcal infection (Pereira RFC 2011; Taha 2000), for the immunological analysis in the transfer process of the *las* gene from meningoccci strains to *H. influenzae* not associated with the *purpura fulminans* process. Nevertheless, the study of the immune response of *H. influenzae* biotype *aegyptius* causing the Brazilian

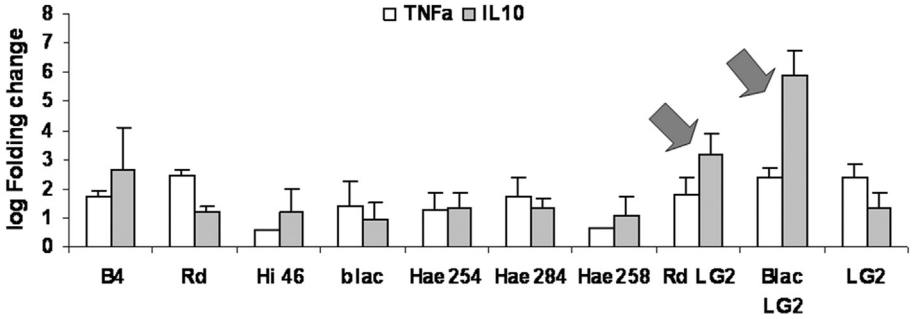

Figure 2 - Expression of tumor necrose factor alpha – TNFα (white bars) and interleukin 10 – IL10 (gray bars). The Y axis shows the log folding change of RNA expression. The mutants RdLG2 and βlac LG2 demonstrated significant increase of TNFα and IL10 expression when compared with receptor strains Rd and βlac LG2 (black arrows). $p > 0.05$.

purpuric fever had never been described before *in vitro*. The strains involved in the first BPF outbreak Hae254, Hae258 and Hae284 were able to activate the expression of IL10 and TNFα in Hec-1B cells. Though, the expression profile was lower than that observed in the transformants (Figure 2). The differential cytokines production is an important result still in study by our group. Whereas the invasive phenotype is unique in Hae strains within the *Haemophilus* genre, the use of a transcriptions fusion, as mimicking models of bacterial genetic transfer, is a useful and neglected tool in the search of Hae's virulence factors.

Nevertheless, the effect of the genetic transfer of the intergeneric of *las* gene open a new discussion about its implication in BPF physiopathology. Since the gene *las* was proved to have differences between *lav* from *H. influenzae* (Davis and others 2001), it is possible that this gene is associated with Hae virulence. The present work do not touch the details of the ancestrality of *las* gene described (Davis and others 2001), However, the role of *las* in the transfer of pathogenic characteristics in NTHi strains was evidenced with production of IL10 and TNFα in cells infected with the βlacLG2 and RdLG2 mutants (Figure 2).

Still, it is very important to reinforce that the gene acquisition was mediated by an environmental action. The BPF outbreak occurred in the Southest Brazilian region where the primitive agriculture was performed with a great emission of carbon dust from sugar cane burn. The atmosphere is similar to the one described to *N. meningitidis* using nanostructures mimicking the action of dust and particles formed by silica and carbon in atmosphere (Hollanda and others; Mattos and others). Thereby, the involvement of environment mimicked through the use of nanoparticles is one of the focus in our group research.

Recently the whole genome of Hae254/86 (also described as F3031) was sequenced and deposited in GenBank (accession no. FQ670178). The genomes of 254/86, F3047 a nontybeable *H. influenzae* that causes otitis and other five *H. influenzae* strains also non-invasive were compared. It was observed that more than 75% of the Hae 254/86 genome is similar among this *H. influenzae* strains (Strouts and others). The genome of the strain Hae 258/86 was sequenced as well as it's assembling and annotation is in process currently by our research group. Despite the fact that 254/86, 258/86 and all the other strains isolated from the Brazilian outbreak belong to the same clonal group (Brenner and others 1988), the strains are genetically distinct and the complete genome sequence of more than one strain of this clone will be a very helpful tool to better define the virulence determinants in the unique BPF clinical picture.

Also, new strategies to study this interesting bacterium must be considered and the search for new virulence variants. The emergence of a new outbreak in Brazil (Santana-Porto and others 2009) might be an indicative of omitted cases, reinforcing the importance of new studies about its pathogenicity and prophylaxis.

In conclusion, the *las* gene could be a possible predominant virulence gene involved in the pathology caused by *H. influenzae* biogroup *aegyptius* – BPF. This is the first molecular study involving the *las* transfer to search an elucidation of the pathogenic factors by horizontal intergeneric transfer from meningococci to *H. influenzae.* Also, the effect of the *las* transfer was capable to increase the cytokines TNFα and IL10 expression in Hec-1B cells line infected with these transformed mutants. These results reinforced the hypothesis of the real involving of *las* gene in *H. influenzae* biotype *aegyptius* causing the brazilian purpuric fever.

Acknowledgments

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