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Generation of recombinant bacillus Calmette–Guérin and *Mycobacterium smegmatis* expressing BfpA and intimin as vaccine vectors against enteropathogenic *Escherichia coli*

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ABSTRACT

Enteropathogenic Escherichia coli (EPEC) is an important cause of diarrhea in children. EPEC adheres to the intestinal epithelium and causes attaching and effacing (A/E) lesions. Recombinant *Mycobacterium smegmatis* (Smeg) and *Mycobacterium bovis* BCG strains were constructed to express either BfpA or intimin. The entire bfpA gene and a portion of the intimin gene were amplified by PCR from EPEC genomic DNA and inserted into the pMIP12 vector at the *BamHI/KpnI* sites. The pMIP_bfpA and pMIP_intimin vectors were introduced separately into Smeg and BCG. Recombinant clones were selected based on kanamycin resistance and designated rSmeg_pMIP_(bfpA or intimin) and rBCG_pMIP_(bfpA or intimin). The expression of bfpA and intimin was detected by Immunoblotting using polyclonal anti-BfpA and anti-intimin antibodies. The immunogenicity of these proteins was assessed in C57BL/6 mice by assaying the feces and serum for the presence of anti-BfpA and anti-intimin IgA and IgG antibodies. TNF-α and INF-γ were produced in vitro by spleen cells from mice immunized with recombinant BfpA, whereas TNF-γ was produced in mice immunized with recombinant intimin. The adhesion of EPEC (E2348/69) to HEp-2 target cells was blocked by IgA or IgG antibodies from mice immunized with recombinant BfpA or intimin but not by antibodies from non-immunized mice. Immunogenic non-infectious vectors containing relevant EPEC virulence genes may be promising vaccine candidates.

1. Introduction

Diarrhea remains one of the top causes of death in low- and middle-income countries, in children under 5 years of age. A wide range can be responsible for this illness. *Enteropathogenic Escherichia coli* (EPEC) strains are among the main bacterial causes of this disease [1,2]. EPEC adheres to the host cells and induces attaching and effacing (A/E) lesions, culminating with induction of diarrhea [3]. The formation of A/E lesions involves a type III secretion system encoded on pathogenicity island locus of enterocyte effacement (LEE), which is responsible for delivering several pathogenic factors into host cells [4].

Intimin is a 94–97 kDa protein expressed on the EPEC surface that mediates adhesion of EPEC to the epithelial gut cells [4] that mediates intimate contact with the bacterial translocated intimin receptor (Tir) [5]. The N-terminal region is conserved among the different intimin subtypes, while the C-terminal regions are highly variable. The 29 intimin subtypes are identified according to their C-terminal amino acid sequences [6–8]. Intimin-β is the most common subtype expressed in EPEC isolates [9–11].

Bundle-forming pilus (BfpA) is another virulence factor, which mediates the initial contact between EPEC and the host cell [12]. BfpA is encoded by a gene localized on a plasmid 50–70 MDa in size and is designated as EPEC adherence factor (EAF) [3,13–15]. Within adherent micro-colonies of EPEC, BfpA organizes a meshwork that allows bacteria to attach to each other and to tether themselves to the host cell surface [3]. Therefore, BfpA and intimin are two important virulence factors and are considered to be strategic target candidates for the design of a new vaccine against EPEC.

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The generation of stable vectors expressing the desired immunogens is the goal of modern vaccine technology. The inclusion of genes encoding relevant epitopes into living, non-infective vectors that constitutively express immunological adjuvant components would be ideal. Attenuated bacteria have been used as vectors to express and deliver heterologous antigens. This type of vaccine vector is an attractive system because it can elicit mucosal, humoral and cellular host immune responses to foreign antigens [16]. These live vectors have been used extensively to express antigens of different types of pathogens, including viruses, bacteria and parasites, some of which have demonstrated positive results [17]. However, each vector has its unique features that should be considered before it is used. In this study, the genes encoding BfpA and intimin were investigated using two different live vectors: *Mycobacterium bovis* BCG Moreau (BCG) and *Mycobacterium smegmatis* mc^2155 (Smeg) to generate the recombinant strains.

2. Materials and methods

2.1. Animals

C57BL/6 female mice, 4 weeks old, 18–22 g were supplied by Isogenic Mouse Breeding Facility of the Butantan Institute. All animals were cared under ethical conditions according to the Brazilian code for the use of laboratory animals [18]. All protocols were approved by the Animal Care and Ethics Committees at the Butantan Institute, São Paulo, Brazil.

2.2. Bacterial strains and growth conditions

All cloning steps were performed in DH5-α *E. coli* strain grown in Luria–Bertani broth (LB) supplemented with kanamycin (20 μg/mL) or ampicillin (100 μg/mL). Liquid cultures of BCG and Smeg were grown in Middlebrook 7H9 media (MB7H9; Difco, MI, USA) supplemented with oleic-albumin-dextrose-catalase (OADC) (MB7H9/OADC) with or without 20 μg/mL kanamycin with gentle shaking at 37 °C. Eletrocompetent BCG and Smeg cells were prepared and transformed by electroporation as previously described [19]. Transformed cultures were plated onto Middlebrook 7H10 agar plates supplemented with OADC (MB7H10/OADC) containing 20 μg/mL kanamycin. The plates were incubated at 37 °C for 3 weeks, and the transformants were expanded in liquid MB7H9/OADC media containing appropriate antibiotics.

2.3. Plasmid construction

The *bfp*A and *intimin* (*eae*) genes were amplified by polymerase chain reaction (PCR). The EPEC E2348/69 prototype genomic DNA was used as a template, and the constructed oligonucleotide primers were as follows: *bfpA* forward primer (FP) 5′-TAG GGA TCC CTG TCT TTG ATT GAA TCT GCA ATG GTG CTT-3′ and reverse primer (RP) 5′-TAG GGT ACC TTA CTT CAT AAA ATA TGT AAC TTT ATT GGT-3′; *intimin* FP 5′-TAG GGA TCC GGG ATC GAT TAC C-3′ and RP 5′-TAG GGT ACC TTT ATC AGC CTT AAT CTC A-3′. The underlined regions indicate *KpnI* and *BamHI* sites. Briefly, the amplified BfpA and *intimin* (eae) PCR products were purified and sub-cloned into the pGEM-T Easy vector (Promega, USA). Both genes were digested with *BamH*I and *Kpn*I and sub-cloned into the mycobacterial vector pMIP12 (kindly provided by Brigitte Gicquel, Pasteur Institute, France). The resulting plasmids were identified as *pMH12-bfpA* and *pMH12-intimin*. The plasmids were validated by successive analyses with restriction endonucleases and DNA sequencing using the primer 5′-TTC AAA CTA TCG CCG GCT GA-3′.

2.4. Detection of BfpA and intimin in recombinant BCG and Smeg by immunoblotting

Whole-cell protein extracts of the recombinant BCG and Smeg strains were resolved by SDS-PAGE (15%) and subsequently transferred onto a nitrocellulose membrane. After the transfer, nitrocellulose sheets were probed with mouse anti-BfpA or anti-intimin polyclonal sera followed by anti-mouse IgG conjugated with horseradish peroxidase as the secondary antibody. Purified BfpA (19.5 kDa) and intimin (34 kDa) were used as positive controls. The membranes were developed with a chemiluminescent kit (MilliPore, USA) and were exposed on an Image Quant LAS 4000 (GE, USA).

2.5. Mice immunization

Recombinant bacterial strains and their respective controls (empty BCG or Smeg) were grown for 2 weeks until the late stationary phase ($O.D._{600\,nm} = 1.0$), collected by centrifugation ($2000 \times g$ at 4 °C for 10 min), washed twice and resuspended in PBS. Mice were immunized on days 0, 15, 30 and 45 with 10^8 CFU in 200 μL PBS by oral gavage or by intraperitoneal injection. Control groups received 200 μL PBS or empty BCG and Smeg. Pre-immune sera and feces were collected and analyzed for the presence of anti-BfpA and anti-intimin antibodies prior to immunization.

2.6. Adjuvants

Recombinant BCG or Smeg expressing BfpA or intimin were mixed with nanostructured silica adjuvant (SBA-15) according to a previously described method [20]. SBA-15 silica was kindly provided by Osvaldo Augusto SantĭAnna, Butantan Institute, Brazil.

2.7. Measurement of antibodies by ELISA

Fifteen days after the final immunization, blood and feces were collected. Blood was collected by retro-orbital bleeding and incubated overnight at 4 °C before centrifuged at $1000 \times g$ for 10 min at room temperature and sera was transferred to new tubes and stored at −20 °C. Feces were collected, weighed and re-suspended in PBS containing 1 mM phenylmethylsulphonyl fluoride (PMSF) (Boehringer Mannheim Co., USA) and 1% Bovine Serum Albumin (BSA) (Fisher Scientific Co., USA) at a ratio of 1 g feces per 5 mL inhibitory solution. After 15 min on ice, the samples were shaken and then centrifuged at $22{,}000 \times g$ for 10 min, and the supernatants were stored at −80 °C until use. Total immunoglobulin G (IgG) and A (IgA) isotypes and the IgG1 or IgG2a antibody subclasses specific for BfpA and intimin were evaluated by ELISA. Briefly, microtiter plates were coated overnight at 4 °C with 5 μg/mL recombinant BfpA or intimin (purified in our laboratory) in 100 μL PBS. The plates were then blocked with 10% BSA in PBS for 1 h at room temperature. After each incubation, the plates were washed three times with PBS containing 0.05% Tween-20 (PBST). Aliquots of serum and fecal extracts were added to individual wells (100 μL), and the plates were incubated for 1 h at room temperature. After washing, the plates were incubated with 100 μL peroxidase-conjugated goat anti-mouse IgG or anti-mouse IgA or anti-mouse IgG1 and IgG2a (Southern Biotechnologies, USA) at a dilution of 1:1000 in the same diluent pursued by 1 h incubation at room temperature. The peroxidase activity was measured using the o-phenylenediamine (OPD) substrate and read at a wavelength of 450 nm.

2.8. Detection of cytokines in spleen cell cultures

Spleens were recovered from immunized mice (5 animals per group) 15 days after the final immunization. Cell suspensions were