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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

prepared at a concentration of 5×10^6 cells/mL in RPMI medium (Gibco, USA) containing polymixin (1 μg/mL) and were plated in 24-well plates. Cells were left unstimulated or were stimulated for 48 h with extracts of Smeg, BCG, purified BfpA, purified intimin or ConA (Sigma, USA) at a concentration of 5 μg/mL at 37 °C in 5% CO_2. Cytokine secretion was evaluated using the Cytometric Bead Array Th1/Th2 Kit (CBA; BD Bioscience, USA) and samples were read on a FACS Calibur flow cytometer (BD Biosciences, USA). Each experiment was repeated three times.

2.9. Inhibition of EPEC adherence to Hep-2 cells

To evaluate the ability of the anti-recombinant BfpA and intimin antibodies to interfere with the adhesion of EPEC to host cells, a standard assay using HEp-2 target cells was used. Hep-2 cells were maintained in DMEM supplemented with 10% SFB in a humidified atmosphere containing 5% CO_2 at 37 °C. To evaluate the inhibitory action of the specific antibodies, serum or fecal samples were incubated at a ratio of 1:4 with 10^7 EPEC bacteria for 1 h at 37 °C before being added to the Hep-2 cultures. After the incorporation of the bacteria, the HEp-2 monolayers were kept at 37 °C for 3 h. The HEp-2 monolayers were washed with PBS, fixed with methanol and stained with Giemsa solution to visualize the adherent bacteria by light microscopy. The percentage of HEp-2 cells with bound bacteria was determined for each tested antibody.

2.10. Statistical analysis

Student's *t*-test was employed to determine the significance of differences between the studied groups. p values <0.05 (*) were considered to be significant.

3. Results

3.1. Expression of BfpA and intimin by BCG and Smeg

DNA fragments encoding *bfpA* (600 bp) and *intimin* ($eae_{388-667}$) (840 bp), were amplified by PCR from EPEC (E2348/69) and ligated into the *Kpn*I and *BamH*I sites of the pMIP12 vector under the control of the pblaF* promoter (Supplementary Figure); the constructs were named pMH12-*bfp*A and pMH12-*intimin*, respectively. The plasmids were electroporated into BCG and Smeg, and the resulting strains were examined for BfpA and intimin expression. Expression of both *bfpA* and *intimin* (*eae*) was confirmed by immunoblotting bacterial whole-cell extracts using anti-BfpA or anti-intimin antisera. As observed in Fig. 1A and B, the antisera specifically recognized bands of approximately 19.5 and 34 kDa, corresponding to BfpA and intimin, respectively, from both rBCG and rSmeg strains. No proteins were recognized by the antisera in whole-cell lysates from BCG or Smeg controls without the plasmid vectors (Fig. 1A and B).

3.2. Induction of antibodies by immunizing mice with recombinant BCG and Smeg

C57BL/6 mice were immunized by oral gavage or intraperitoneal injection with 4 doses of 1×10^8 CFU in 200 μL of rBCG-b*fp*A, rSmeg-*bfp*A, rBCG-*intimin* or rSmeg-*intimin* at two-week intervals. As a mucosal adjuvant, SBA-15 silica was used. Control mice were immunized with non-recombinant BCG or Smeg or with PBS following the same immunization schedule. A significantly higher level of anti-BfpA and anti-intimin IgA or IgG antibodies was observed in both the feces and serum of mice immunized with rBCG or rSmeg as compared with that of serum collected in the groups that received non-recombinant BCG or Smeg or PBS ($p < 0.001$) (Fig. 2A and B). Pre-immune sera and feces that were collected and pooled were evaluated, and presented no reactivity to BfpA or intimin (data not shown), suggesting the absence of anti-BfpA or anti-intimin antibodies prior to immunization. Our analysis of serum IgG subclass responses also revealed that mice subjected to intraperitoneal immunization predominantly developed an IgG2a response, indicating a Th1-type cell response (Fig. 2C).

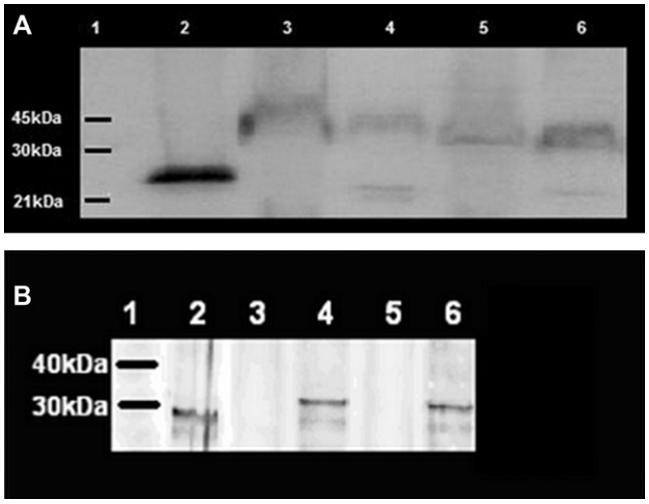

Fig. 1. Expression of BfpA by recombinant Smeg and BCG vaccine strains. (A) Whole extracts (30 μg) of recombinant bacteria strains were resolved on SDS-PAGE and BfpA protein identified by Immunoblotting using specific polyclonal mouse IgG antibody as the first antibody. Lane 1, MW; Lane 2, purified BfpA; Lane 3, native Smeg; Lane 4, Smeg-BfpA; Lane 5, native BCG; and Lane 6, BCG-BfpA. (B) Expression of intimin by recombinant Smeg and BCG vaccine strains. Whole extracts (30 μg) of recombinant bacteria strains were resolved on SDS-PAGE and intimin protein identified by Immunoblotting using specific polyclonal mouse IgG antibody as the first antibody. Lane 1, MW; Lane 2, purified intimin; Lane 3, native Smeg; Lane 4, Smeg-intimin; Lane 5, native BCG; and Lane 6, BCG-intimin.

3.3. Development of a prevailing Th1-type cell response pattern

To evaluate the involvement of Th1-type cells on the immune responses induced by recombinant BCG-bfpA, BCG-intimin, Smeg-bfpA and Smeg-intimin, spleen cells were recovered 15 days after the final immunization and treated in vitro with the corresponding recombinant protein expressed in the vaccine used. We assayed the supernatants for the presence of the cytokines TNF-α, IFN-γ, IL-4 and IL-5. As is shown in Fig. 2A–C, anti-BfpA and anti-intimin, respectively, IgA and IgG antibodies were detected in feces and serum. Immunization with recombinant vaccine expressing BfpA induced higher production of IFN-γ, in vitro, by spleen cells (Fig. 3). The vaccination of mice with recombinant BCG or Smeg expressing intimin induced higher levels of TNF-α and IFN-γ production, in vitro, by spleen cells (Fig. 4).

3.4. Immunization with recombinant BCG and Smeg induces antibodies that inhibit EPEC (E2348/69) binding to Hep-2 cells in vitro

EPEC samples (E2348/69) pre-treated for 3 h with dilutions of serum or fecal extracts obtained from mice immunized with BCG-*bfp*A, BCG-*intimin*, Smeg-*bfp*A or Smeg-*intimin*, were added to HEp-2 monolayers cultivated on coverslips. As a negative control, EPEC (E2348/69) samples were similarly pre-treated with dilutions of serum or feces collected prior to the immunizations. After incubation for 3 h at 37 °C, the coverslips were stained and examined by light microscopy. Untreated EPEC (E2348/69) typically displays a localized pattern of adhesion, generating tight microcolonies of bacteria on the epithelial cell surface. As shown in Fig. 5A–C,

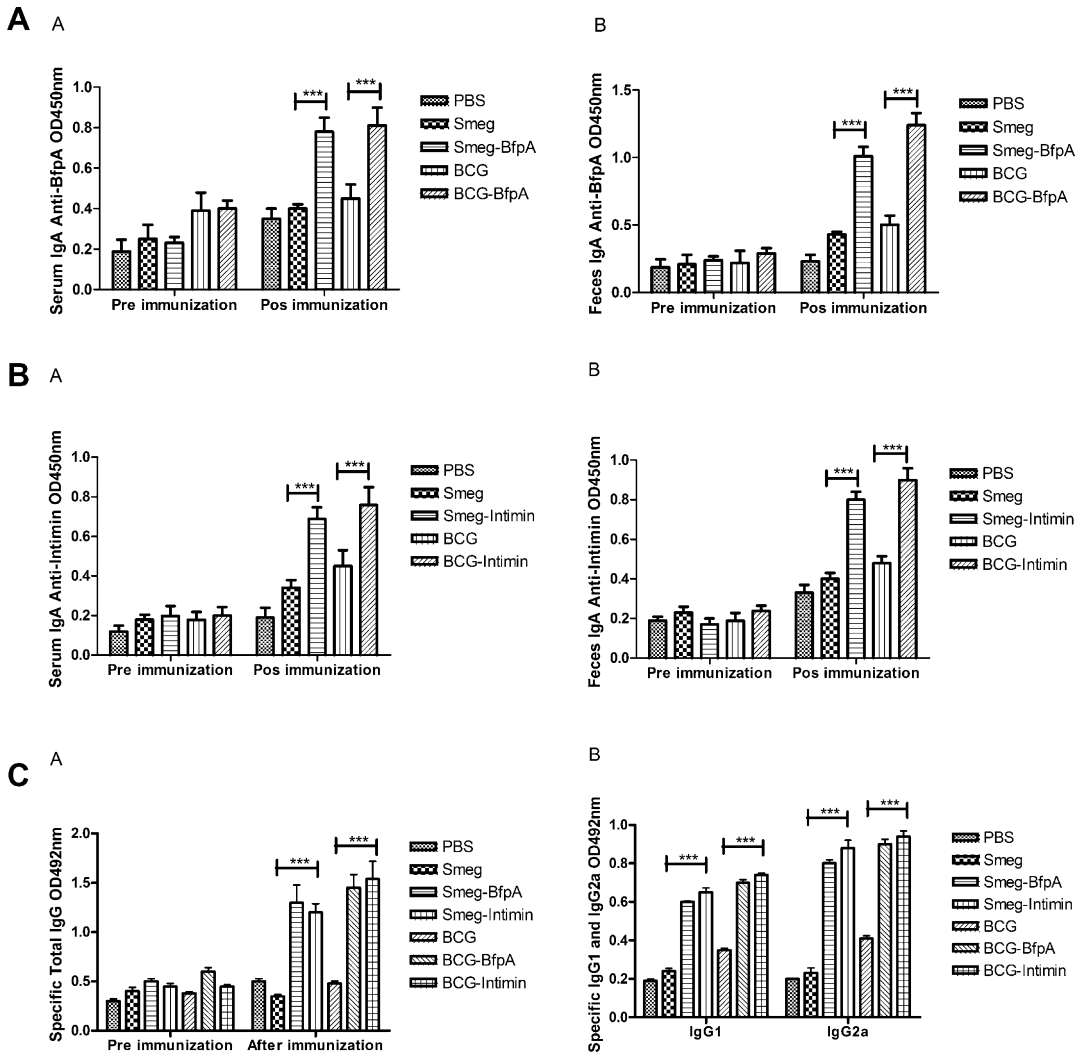

Fig. 2. Detection of serum and feces antibodies IgA before and after oral immunization. (A) Serum (A) and feces (B) from immunized mice with four p.o. doses containing 108 CFU/200 μL of Smeg-BfpA or BCG-BfpA or control group (PBS, native Smeg and native BCG) with SBA-15 adjuvant were collected on days 0 and 60 and the specific IgA antibody anti-BfpA was analyzed by ELISA. (B) Detection of serum and feces antibodies IgA before and after oral immunization. Serum (A) and feces (B) from immunizaded mice with four p.o. doses containing 108 CFU/200 μL of Smeg-intimin or BCG-intimin or control group (PBS, native Smeg and native BCG) with SBA-15 adjuvant, were collected on days 0 and 60 and the specific IgA antibody anti-intimin was analyzed by ELISA. (C) Detection of serum total specific antibodies IgG and isotypes IgG1 and IgG2a after intraperitoneal immunization. Serum antibodies total IgG (A) and isotypes (B) anti-BfpA and anti-intimin were determined by ELISA from mice immunized four times with recombinant vaccine Smeg-BfpA or Smeg-intimin (1×108 ufc/200 μL) or BCG-BfpA or BCG-intimin (1×108 ufc/200 μL) without SBA-15. Mice immunized with PBS or Smeg or BCG was used as negative controls. *t*-Student $^{***}p < 0.001$.

adherence of EPEC (E2348/69) cells pre-treated with dilutions of immune serum or fecal extracts was blocked by over 90%. In contrast, in EPEC (E2348/69) cells pre-treated with dilutions of serum or feces collected before immunization, adherence was blocked by less than 5%.

4. Discussion

Attenuated *M. bovis* BCG vaccine strains have been intensively investigated as a vehicle for delivering heterologous antigens and allowing the induction of both humoral (mucosal and systemic) and cell-mediated immune responses [21].

In this study, we used recombinant BCG that expressed BfpA or intimin as vaccines against EPEC. As an alternative, *M. smegmatis* was also used to present the BfpA and intimin antigens to the host. It is interesting to note that the recombinant strains of both species were able to induce systemic and mucosal BfpA and intimin-specific antibody responses with adherence-neutralizing activity following oral administration to mice. This evidence demonstrates that the different rBCG-EPEC or Smeg-EPEC vaccine strains are potential live vectors for the generation of strategies to prevent EPEC.

Three important qualities for a recombinant vaccine were positively evaluated in our study. First, a live attenuated vaccine was constructed with the ability to express two important proteins, BfpA and intimin, involved in the pathogenesis of EPEC. Second, the expression of the recombinant proteins induced specific and long lasting immune response in immunized mice, characterized by serum and mucosal IgG and IgA antibodies. The third important property of our recombinants is that the induced antibodies were able to prevent, in in vitro EPEC adherence to HEp-2 cells. IgA production was probably enhanced by the adjuvant effects of mesoporous silica SBA-15 [20].

SBA-15 is a nontoxic positive modulator of the mucosal immune response even in low immune responsive mice and is a natural candidate to be included as an adjuvant in an anti-EPEC vaccine. The anti-EPEC antibodies specifically recognize recombinant and native BfpA and intimin proteins free in solution and naturally fixed on the bacterial cell surfaces (Fig. 1A and B). The significant production of TNF-α and IFN-γ identifies BfpA and intimin as

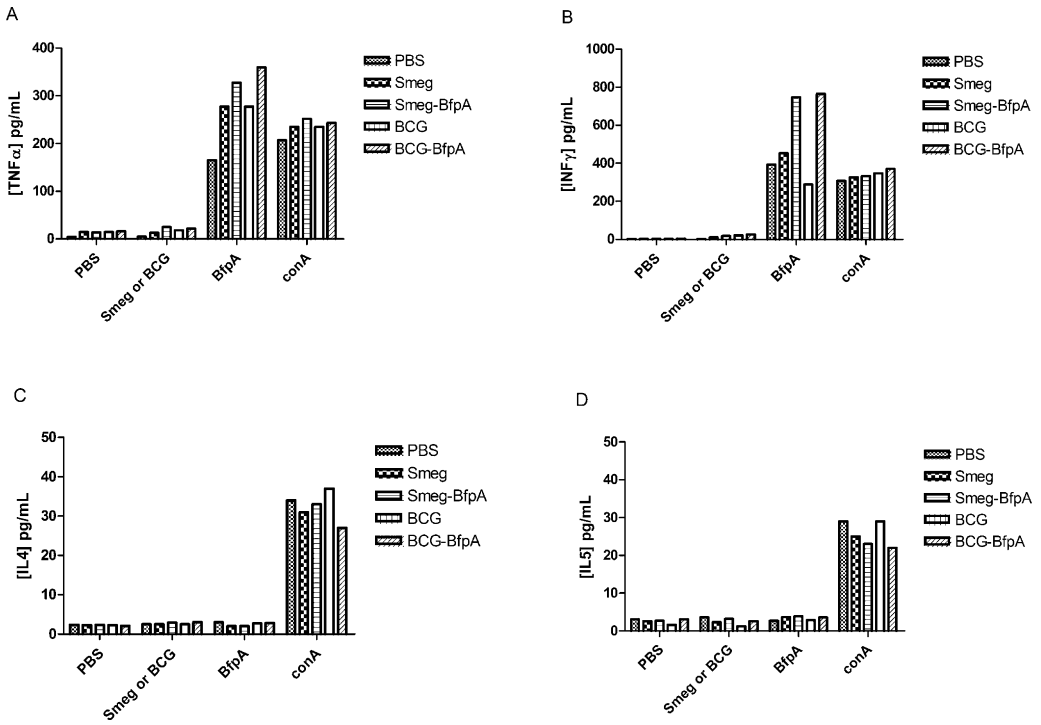

Fig. 3. Detection of cytokine production after in vitro stimulation of spleen cells from mice orally immunized with recombinant vaccines expressing recombinant BfpA. Antigen specific production of TNF-α (A); IFN-γ (B); IL-4 (C); and IL-5 (D) by spleen cells from mice immunized by the oral route with Smeg-BfpA or BCG-BfpA, was measured after stimulus with total extract soluble proteins of Smeg or BCG (25 μg/mL); purified recombinant protein BfpA (25 μg/mL) and ConA (5 μg/mL), as positive control. Presented results are representative of 3 independent experiments.

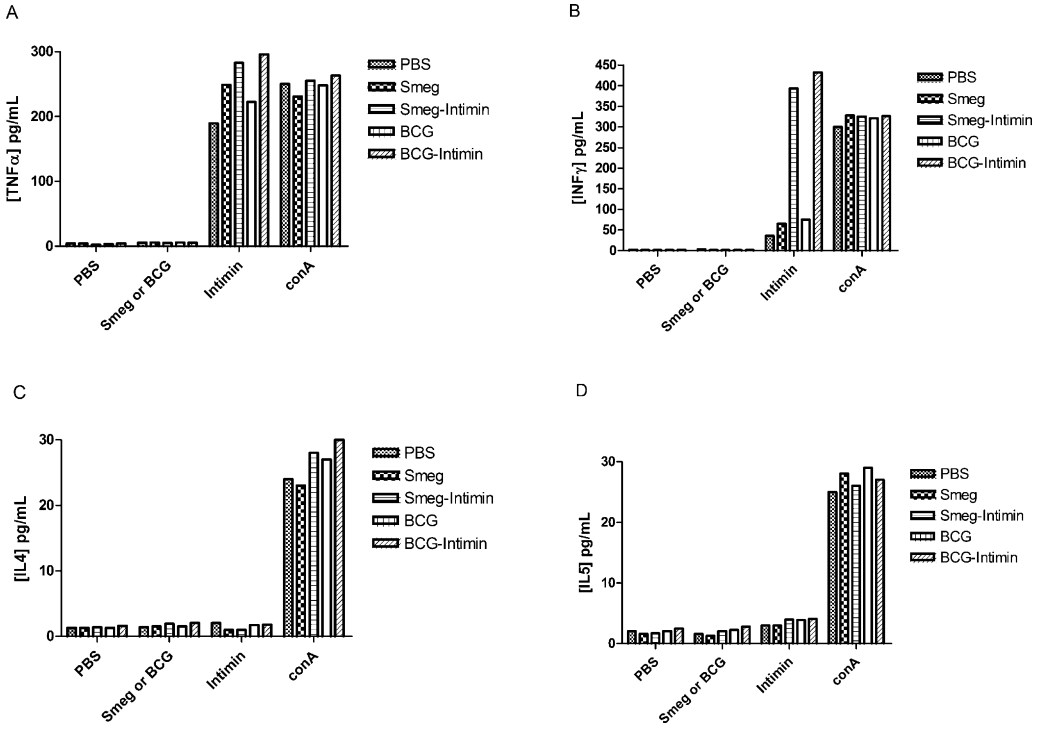

Fig. 4. Detection of cytokines production after stimulation of spleen cells in vitro from mice orally immunized with recombinant vaccines expressing protein intimin. Antigen specific production of TNF-α (A); IFN-γ (B); IL-4 (C); and IL-5 (D) by spleen cells from mice immunized by the oral route with Smeg-intimin or BCG-intimin, was measured after stimulus with total extract soluble proteins of Smeg or BCG (25 μg/mL); purified recombinant protein intimin (25 μg/mL) and ConA (5 μg/mL), as positive control. Presented results are representative of 3 independent experiments.

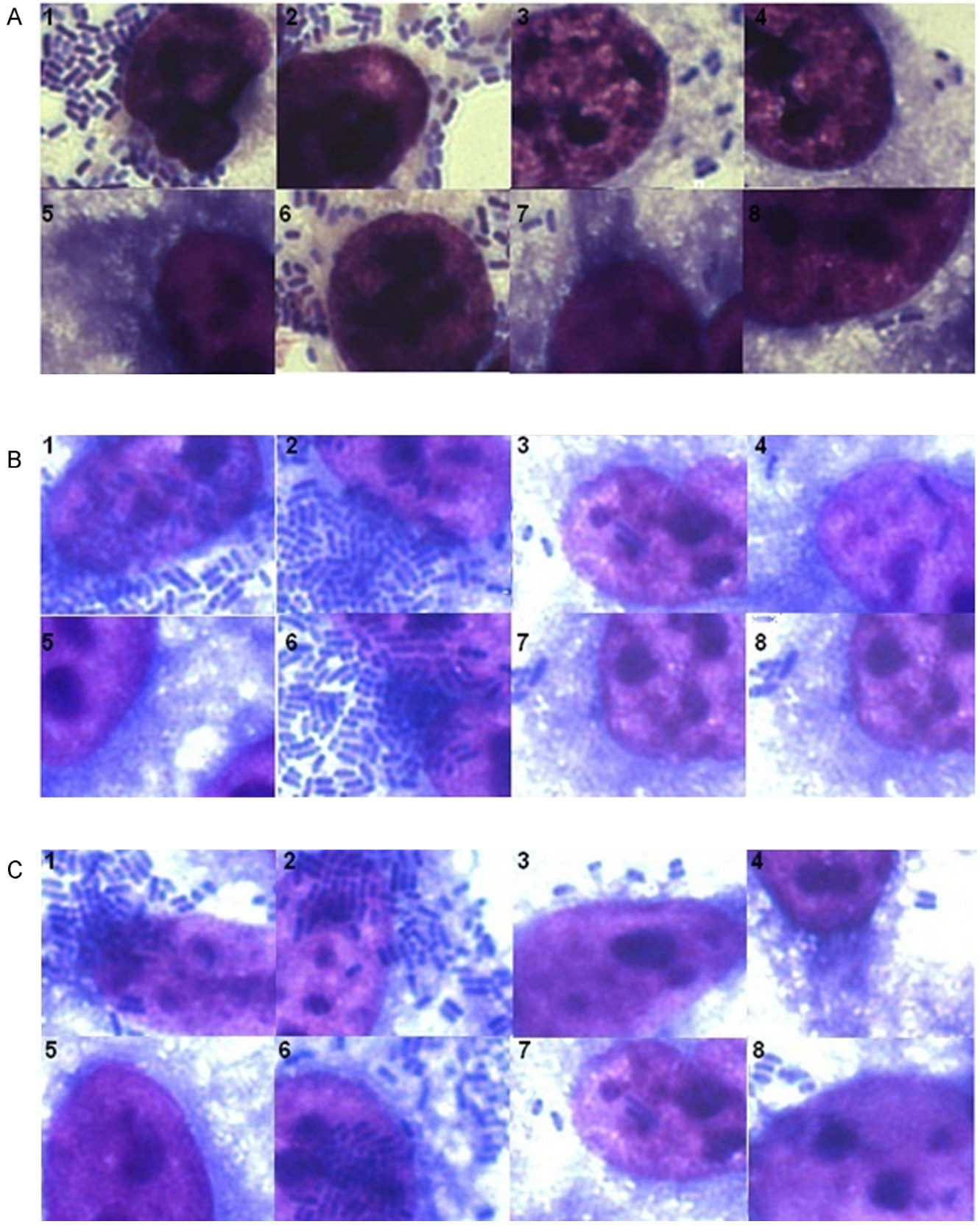

Fig. 5. Inhibition of binding properties of virulence factors BfpA and intimin by serum and feces samples collected from mice 15 days after last orally immunization. The EPEC E2348/69 strain was incubated with different serum or feces samples for 1 h at 37 °C, at a final dilution of 1:4 with 10^7 EPEC, before being added to HEp-2 cell cultures. The inhibition of bacterial binding was evaluated by assays using feces (A1) or serum pools (B1 and C1) from PBS immunized mice, feces (A2) or serum (B2 and C2) pools from mice orally immunized with Smeg, feces (A3) or serum (B3 and C3) pools from mice orally immunized with Smeg-*bfpA*, feces (A4) or serum pools from mice orally immunized Smeg-intimin (B4 and C4), Hep-2 cells culture (A5, B5 and C5), feces (A6) or serum (B6 and C6) pools from mice orally immunized with BCG, feces (A7) or serum (B7 and C7) pools from mice orally immunized with BCG-bfpA, and feces (A8) or serum (B8 and C8) pools from mice orally immunized with BCG-intimin. The results are representative of three independent experiments with the same observed results.

inducers of cellular immunity [22,23]. Although TNF participates in the immune destruction of several infectious microbes [22], TNF was first identified in the plasma of LPS-treated animals exhibiting the Shwartzman reaction [23]. The positive and negative effects of TNF must be taken into account when its production is induced by candidates for protective vaccines. Although in vitro assays cannot entirely be used as substitutes for in vivo methods, the effective and specific blockage of bacterial attachment to HEp-2 cells strongly indicates that the antibodies induced by the recombinant Smeg and BCG generated to express BfpA and/or intimin may be active in vivo.

In a previous study, we demonstrated that an IgY antibody raised against recombinant BfpA identifies *E. coli* that express BfpA, blocks colonization of HeLa cells by EPEC-EAF$^{(+)}$ in vitro and inhibits the in vitro growth of EPEC-EAF$^{(+)}$ but not of EPEC-EAF$^{(-)}$ (the BfpA-cured counterpart bacteria) [24]. More recently, we also showed that EPEC-EAF$^{(+)}$-expressing BfpA, but not EPEC-EAF$^{(-)}$, induced apoptosis in HeLa cells. This effect was blocked by prior neutralization of BfpA with an IgY anti-BFP antibody [25]. These data agree with previous observations indicating that induction of epithelial cell death by *E. coli* depends on the expression of bundle-forming pili by the bacterium [26]. Therefore, BfpA is an important virulence factor expressed by EPEC and is significantly involved in bacterial cell adhesion and induction of host cell death, either by necrosis or apoptosis. Intimin is a 94–97 kDa outer membrane protein [4] that mediates intimate contact between the bacteria and the target cell upon interaction with its translocated intimin receptor (Tir) [27]. Recent observations indicate that *Lactobacillus casei* expressing intimin-β fragments and containing the immunodominant epitopes of Int_{280} induced both humoral and cellular immune responses in mice. The antibodies were able to bind to EPEC and inhibit bacterial adhesion to the epithelial cell surface in vitro. C57BL/6 mice immunized with this recombinant strain became partially protected against intestinal colonization by *Citrobacter rodention*, a mouse intestinal pathogen that also expresses intimin-β [28]. BfpA and intimin are therefore significant immunogens to be used in vaccines.

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Conflict of interest statement: The authors have no financial conflict of interest. This research is under the scope of the International Patents WO 07030901, IN248654, ZA2008/02277, KR 1089400 and MX297263.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.05.083.

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