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(54) Titre : PROTEINE IPAD DE SHIGELLA ET SON UTILISATION COMME UN VACCIN POSSIBLE CONTRE L'INFECTION A SHIGELLA

(54) Title: SHIGELLA IPAD PROTEIN AND ITS USE AS A POTENTIAL VACCINE AGAINST SHIGELLA INFECTION

(57) Abrégé/Abstract:
The present invention relates to a composition and a method for eliciting an immunogenic response in animals, including responses that provide protection against, or reduce the severity of shigella infections. More particularly it relates to the use of the IpaD protein obtained from natural sources and/or through synthesis or recombinant technology, and conjugates thereof to induce neutralizing antibodies having protective activity against several serotypes of Shigella, in particular S. flexneri. The composition of the invention is useful to prevent and/or treat shigellosis caused by a bacterium of the Shigella family.

ABSTRACT

The present invention relates to a composition and a method for eliciting an immunogenic response in animals, including responses that provide protection against, or reduce the severity of shigella infections. More particularly it relates to the use of the IpaD protein obtained from natural sources and/or through synthesis or recombinant technology, and conjugates thereof to induce neutralizing antibodies having protective activity against several serotypes of *Shigella*, in particular *S. flexneri*. The composition of the invention is useful to prevent and/or treat shigellosis caused by a bacterium of the Shigella family.

SHIGELLA IPAD PROTEIN AND ITS USE AS A POTENTIAL VACCINE AGAINST SHIGELLA INFECTION

FIELD OF THE INVENTION

The present invention relates to a composition and a method for eliciting an immunogenic response in animals, including responses that provide protection against, or reduce the severity of shigella infections. More particularly it relates to the use of the IpaD protein obtained from natural sources and/or through synthesis or recombinant technology, and conjugates thereof to induce neutralizing antibodies having protective activity against several serotypes of *Shigella*, in particular *S. flexneri*. The composition of the invention is useful to prevent and/or treat shigellosis caused by a bacterium of the Shigella family.

BACKGROUND OF THE INVENTION

Many Gram-negative pathogenic bacteria use a type three secretion (T3S) system to interact with cells of their host. Each T3S system consists of a secretion apparatus (T3SA) that spans the bacterial envelope and extends on the bacterial surface, translocators that transit through the T3SA and insert into the membrane of the host cell where they form a pore, effectors that transit through the T3SA and the translocator pore to reach the cell cytoplasm, specific chaperones that associate with translocators and effectors in the bacterial cytoplasm and transcriptional regulators. Approximately 15 proteins are required for assembly of the T3SA.

Bacteria belonging to the *Shigella* family are the causative agents of bacillary dysentery in humans [1]. Genes required for entry of bacteria into epithelial cells and inducing apoptosis in macrophages are clustered in a 30-kb region, designated the entry region, of a 220-kb virulence plasmid. The entry region contains 20 *mxi* and *spa* genes encoding components of the T3SA, the *ipaA*, *B*, *C* and *D*, *ipgB1*, *ipgD* and *icsB* genes encoding proteins that transit through the T3SA, the *ipgA*, *ipgC*, *ipgE* and

spa15 genes encoding chaperones, and the *virB* and *mxiE* genes encoding transcriptional regulators [2].

The T3SA, which is weakly active in bacteria growing in broth, is activated upon contact of bacteria with epithelial cells [3]. Inactivation of *ipaB*, *ipaC* or *ipaD*, as well as most *mxi* and *spa* genes, abolishes the ability of bacteria to enter epithelial cells, induce apoptosis in macrophages and express contact hemolytic activity. IpaB and IpaC contain hydrophobic segments and remained associated with the membrane of lyzed erythrocytes, suggesting that these two proteins are components of the *S. flexneri* translocator. In addition, effector functions have been proposed for IpaB and IpaC [4, 5,6,7,8]. Inactivation of *ipaB* and *ipaD*, but not *ipaC*, leads to a deregulated, i. e. constitutively active, T3SA, suggesting that IpaB and IpaD play a role in maintaining the T3SA inactive in the absence of inducers [9, 10]. A small proportion of IpaD is associated with the bacterial envelope [9, 11]. Picking and collaborators [12] reported that the role of IpaD in the control of the T3SA activity can be separated from its role in entry of bacteria into epithelial cells.

To get further insights on the structure of the needle complex, the inventors performed an immuno-electron microscopic analysis on bacteria treated with the cross-linking agent BS^3, both on entire bacteria and on the mildly purified needle complex (NC). The inventors present evidence that IpaD is a component of the NC localized at the tip of the needle and that antibodies raised against IpaD have an inhibitory effect on entry of *S. flexneri* into epithelial cells.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Electron micrographs of negatively stained bacteria treated with BS^3. Arrows indicate distinct densities at the tip of needles protruding from the bacterial surface. The bar represents 100 nm.

Fig. 2. Projection maps (class-sums) of the needle part of NCs obtained by single particle analysis. (A-B) maps of NCs isolated from wild-type, treated with BS^3 (A-C); from wild-type without BS^3 treatment (D-F) and from an *ipaD* mutant strain with BS^3 treatment (G-I). The arrows point to remnant densities attached to needle parts

prepared without BS^3-treatment. The bar represents 10 nm.

Fig. 3. **(A)** Immunobloting analysis of purified NCs. Whole cell extracts (WCE) and cross linked (+CR) and non-cross linked (-CR) NCs purified from wild-type and IpaD deficient strains were analyzed by SDS-PAGE and immunoblotting using antibodies specific to MxiJ, MxiN, and IpaD. IpaD was only enriched to WCE level in NCs prepared from cross-linked wild-type bacteria. MxiJ is a positive control to demonstrate intact T3SA. MxiN, a cytoplasmic component of T3S, is a positive control to demonstrate contamination by non-bound cytoplasmic proteins.

Fig. 4. IpaD localization by immuno-electron microscopy. NCs purified from BS^3-treated wild-type bacteria were incubated with anti-IpaD antibodies and negatively stained (A-D). The average image of 250 NCs purified from bacteria not treated with BS^3 is shown in E for comparison. The bar represents 10 nm.

Fig. 5. Invasion assay of epithelial cells by wild-type *S. flexneri*. Bacteria were incubated with serial dilutions of the anti-IpaD or anti-IpaB polyclonal antibodies and intracellular, gentamycin-resistant bacteria were counted by plating cell lysates. The efficiency of entry in each condition is expressed with respect to that of the wild-type strain treated with PBS. The values are the means of at least three independent experiments, and the error bars indicate standard deviations.

DESCRIPTION OF THE INVENTION

The inventors have surprisingly found that IpaD-specific antibodies can block the entry of Shigella into epithelial cells and that this neutralizing effect is observed on different Shigella serotypes. In this connection, the present invention specifically relates to the use of the IpaD protein or the polynucleotide encoding same in the preparation of compositions and elaboration of methods to elicit a cross protection against Shigella infection.

Definitions

The term "animal" refers to any animal susceptible to be infected by a *Shigella* strain, such as *S. flexneri*. Specifically, the animal consists of a human.

The term "treating" refers to a process by which the symptoms of an infection or a disease associated with a *Shigella* strain are alleviated or completely eliminated. As used herein, the term "preventing" refers to a process by which symptoms of an infection or a disease associated with a *Shigella* strain are obstructed or delayed.

The term "protective response" means prevention of onset of a *Shigella* - associated disease or an infection caused by a *S. species* or lessening the severity of such a disease existing in an animal.

The expression "an acceptable carrier" means a vehicle for containing the compounds obtained by the method of the invention that can be administered to an animal host without adverse effects. Suitable carriers known in the art include, but are not limited to, gold particles, sterile water, saline, glucose, dextrose, or buffered solutions. Carriers may include auxiliary agents including, but not limited to, diluents, stabilizers (i.e., sugars and amino acids), preservatives, wetting agents, emulsifying agents, pH buffering agents, viscosity enhancing additives, colors and the like.

A “functional derivative”, as is generally understood and used herein, refers to a protein/peptide sequence that possesses a functional biological activity that is substantially similar to the biological activity of the whole IpaD protein/peptide sequence. In other words, it preferably refers to a polypeptide or fragment(s) thereof that substantially retain(s) the capacity of eliciting an immune response, such as a protective response against a *S.* strain infection when said functional derivative is administered to an animal.

A “functional fragment”, as is generally understood and used herein, refers to a nucleic acid sequence that encodes for a functional biological activity that is substantially similar to the biological activity of the whole IpaD nucleic acid sequence. In other words, and within the context of the present invention, it preferably refers to a nucleic acid or fragment(s) thereof that substantially retains the capacity of encoding a IpaD polypeptide/protein which elicits an immune response, and more preferably a protective response, to a *Shigella* strain infection when administered to an animal.

Composition of the invention

It is therefore an embodiment of the invention to provide a composition for the treatment and/or the prevention of a Shigella infection. The composition of the invention comprises at least one of the following material:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide encoding an IpaD polypeptide or a functional fragment thereof;
- an antibody that specifically binds to an IpaD polypeptide. More specifically, the antibody is a purified polyclonal or monoclonal antibody that specifically binds to the IpaD polypeptide.

The antibodies contemplated by the invention may be prepared by a variety of methods known to one skilled in the art. For example, the IpaD polypeptide may be administered to an animal in order to induce the production of polyclonal antibodies. Alternatively, and as mentioned above, antibodies used as described herein may be monoclonal antibodies, which are prepared using known hybridoma technologies (see, e.g., Hammerling *et al.*, In Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, NY, 1981).

In a preferred embodiment, the composition of the invention further comprises an adjuvant. As used herein, the term "adjuvant" means a substance added to the composition of the invention to increase the composition's immunogenicity. The mechanism of how an adjuvant operates is not entirely known. Some adjuvants are believed to enhance the immune response (humoral and/or cellular response) by slowly releasing the antigen, while other adjuvants are strongly immunogenic in their own right and are believed to function synergistically. Known adjuvants include, but are not limited to, oil and water emulsions (for example, complete Freund's adjuvant and incomplete Freund's adjuvant), Corytzebactei-ium parvuin, Quil A, cytokines such as IL12, Emulsigen-Plus®, Bacillus Calmette Guerin, aluminum hydroxide, glucan, dextran sulfate, iron oxide, sodium alginate, Bacto Adjuvant, certain synthetic polymers such as poly amino acids and co-polymers of amino acids, saponin,

paraffin oil, and muramyl dipeptide. Adjuvants also encompass genetic adjuvants such as immunomodulatory molecules encoded in a co-inoculated DNA, or as CpG oligonucleotides. The coinoculated DNA can be in the same plasmid construct as the plasmid immunogen or in a separate DNA vector.

According a preferred embodiment of the invention, the composition may further comprise a polyosidic antigen, such as those selected derived from the group consisting of *S. flexneri* 2a, *S. flexneri* 1b, *S. flexneri* 6, *S sonnei* and *S. dysenteriae*.

With respect to contemplated antibodies of the present invention, the term "specifically binds to" refers to antibodies that bind with a relatively high affinity to one or more epitopes of the IpaD polypeptide, but which do not substantially recognize and bind molecules other than the IpaD polypeptide. As used herein, the term "relatively high affinity" means a binding affinity between the antibody and the IpaD polypeptide of at least 10^6 M^{-1}, and preferably of at least about 10^7 M^{-1} and even more preferably 10^8 M^{-1} to 10^{10} M^{-1}. Determination of such affinity is preferably conducted under standard competitive binding immunoassay conditions which are common knowledge to one skilled in the art.

Methods of treatment and prevention

The IpaD polypeptide, the polynucleotide encoding same and antibodies contemplated by the invention may be used in many ways in the treatment and/or prevention of *Shigella* infection.

For instance, and according to an aspect of the invention, the IpaD polypeptide may be used as immunogens for the production of specific antibodies for the treatment and/or prevention of *Shigella* infection. Suitable antibodies may be determined using appropriate screening methods, for example by measuring the ability of a particular antibody to passively protect against *Shigella* infection in a test model.

According to another aspect, the polynucleotides encoding an IpaD polypeptide or derivatives thereof may be used in a DNA immunization method. That is, they can be incorporated into a vector which is replicable and expressible upon

injection thereby producing the antigenic polypeptide *in vivo*. For example polynucleotides may be incorporated into a plasmid vector under the control of the CMV promoter which is functional in eukaryotic cells. Preferably the vector is injected intramuscularly.

The use of a polynucleotide of the invention in genetic immunization will preferably employ a suitable delivery method or system such as direct injection of plasmid DNA into muscles [Wolf et al. H M G (1992) 1: 363, Turnes et al., Vaccine (1999), 17 : 2089, Le et al. , Vaccine (2000) 18 : 1893, Alves et al. , Vaccine (2001)19 : 788], injection of plasmid DNA with or without adjuvants [Ulmer et al. , Vaccine (1999) 18: 18, MacLaughlin et al. , J. Control Release (1998) 56: 259, Hartikka et al. , Gene Ther. (2000) 7: 1171-82, Benvenisty and Reshef, PNAS USA (1986) 83: 9551, Singh et al. , PNAS USA (2000) 97: 811], targeting cells by delivery of DNA complexed with specific carriers [Wa et al. , J Biol Chem (1989) 264: 16985, Chaplin et al., Infect. Immun. (1999) 67:6434], injection of plasmid complexed or encapsulated in various forms of liposomes [Ishii et al. , AIDS Research and Human Retroviruses (1997) 13: 142, Perrie et al., Vaccine (2001) 19:3301], administration of DNA with different methods of bombardment [Tang et al., Nature (1992) 356: 152, Eisenbraun et al. , DNA Cell Biol (1993) 12: 791, Chen et al. , Vaccine (2001) 19:2908], and administration of DNA with lived vectors[Tubulekas et al. , Gene (1997) 190: 191, Pushko et al., Virology (1997) 239: 389, Spreng et al. FEMS (2000) 27: 299, Dietrich et al., Vaccine (2001) 19: 2506].

A further aspect of the invention is the use of the IpaD-specific antibodies for passive immunization.

Yet, a further embodiment of the present invention is to provide a method for treating and/or preventing a *Shigella* infection in an animal. The method of the invention comprises the step of administering to the animal a composition according to the invention.

The amount of the components or the elements of the composition of the invention is preferably a therapeutically effective amount. A therapeutically effective amount of the contemplated component is the amount necessary to allow the same

to perform their immunological role without causing overly negative effects in the host to which the composition is administered. The exact amount of the components to be used and the composition to be administered will vary according to factors such as the type of condition being treated, the type and age of the animal to be treated, the mode of administration, as well as the other ingredients in the composition.

The composition of the invention may be given to an animal through various routes of administration. For instance, the composition may be administered in the form of sterile injectable preparations, such as sterile injectable aqueous or oleaginous suspensions. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparations may also be sterile injectable solutions or suspensions in non-toxic parenterally-acceptable diluents or solvents. They may be given parenterally, for example intravenously, intramuscularly or subcutaneously by injection, by infusion or *per os*. Suitable dosages will vary, depending upon factors such as the amount of each of the components in the composition, the desired effect (short or long term), the route of administration, the age and the weight of the animal to be treated. Any other methods well known in the art may be used for administering the composition of the invention.

The present invention will be more readily understood by referring to the following examples. These examples are illustrative of the wide range of applicability of the present invention and are not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, preferred methods and materials are described hereinafter.

EXAMPLE

IPAD IS LOCALIZED AT THE TIP OF THE *SHIGELLA FLEXNERI* TYPE III SECRETION APPARATUS

Type III secretion (T3S) systems are used by numerous Gram-negative pathogenic bacteria to inject virulence proteins into animal and plant host cells. The core of the T3S apparatus, known as the needle complex, is composed of a basal body transversing both bacterial membranes and a needle protruding above the bacterial surface. In *Shigella flexneri*, IpaD is required to inhibit the activity of the T3S apparatus prior to contact of bacteria with host and has been proposed to assist translocation of bacterial proteins into host cells. We investigated the localization of IpaD by electron microscopy analysis of cross-linked bacteria and mildly purified needle complexes. This analysis revealed the presence of a distinct density at the needle tip. A combination of single particle analysis, immuno-labeling and biochemical analysis, demonstrated that IpaD forms part of the structure at the needle tip. Anti-IpaD antibodies were shown to block entry of bacteria into epithelial cells.

Materials and methods

Bacterial strains and growth media

Strains used in this study are the wild-type *S. flexneri* 5 strain M90T-Sm [13], its *ipaD* derivative SF622 [14]. Bacteria were grown in tryptic casein soy broth (TSB) (Sigma) at 37°C.

Purification of NC

NCs were purified as described [15]. Bacteria in the exponential phase of growth in 1 l of TSB at 37°C were collected by centrifugation, resuspended in 25 ml of phosphate-buffered saline and incubated in the presence of 1 mM Bis (Sulfosuccinimidyl)suberate (BS^3) for 30 min at 37°C. The mixture was supplemented with 100 mM Tris-HCl and incubated for 15 min at 37°C. BS^3-treated cultures were harvested and resuspended in an ice-cold lysis buffer (0.5 M sucrose, 20 mM Tris-

HCl [pH 7.5], 2 mM EDTA, 0.5 mg/ml lysozyme) supplemented with 1 mM phenylmethylsulfonyl fluoride and incubated for 45 min at 4°C and for 15 min at 37°C. Resulting spheroplasts were incubated with 0.01% Triton X-100 for 30 min and treated with 4 mM $MgCl_2$ and 80 µg/ml DNAse (Sigma) for 20 min at 30°C. Debris were removed by centrifugation (20,000 *g* for 20 min at 4°C) and the membrane fraction was pelleted by centrifugation (110,000 *g* for 30 min at 4°C) and resuspended in TET buffer (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.01% Triton X-100). Immunoblotting analysis was performed with antibodies raised against MxiJ, MxiN and IpaD as described [16].

Electron microscopy and image analysis

Whole cells and samples of purified NCs were negatively stained with 2% uranyl acetate on glow discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120FEG equipped with a field emission gun operated at 120 kV. Images were recorded with a 4000 SP 4K slow-scan CCD camera at 80,000 x magnification at a pixel size (after binning the images) of 3.75 Å at the specimen level, with "GRACE" software for semi-automated specimen selection and data acquisition [17]. Single particle analysis including multi-reference and non-reference procedures, multivariate statistical analysis and classification was performed as described [15]. For immuno-labeling, purified NCs were incubated with affinity purified IpaD polyclonal antibodies (pAbs) at a final concentration of 0.132 ng/µl) for 1 hr at 20°C. Samples were stained with 2% uranyl acetate and observed as above.

Invasion assay

Two ml of cultures of wild-type or *mxiD* strains in the exponential phase of growth (OD_{600nm} of 0.4) were incubated in the presence of anti-IpaD (dilution 1/2000 to 1/50) or anti-IpaB (1/50) antibodies for 1 h at 37°C and bacteria were centrifuged on plates containing 2 10^5 Hela cells for 10 min at 2000 g. After 1 h incubation at 37°C, cells were washed three times with 2 ml EBSS and incubated during 1 h with 2 ml MEM milieu containing 50 µg/ml gentamycin. After three washes with 2 ml EBSS, plates were incubated with a solution of deoxycholate 0.5% for 15 min at 20°Cand cell lysates were diluted and plated on agar plates for colony counting.

Results

A distinctive structure at the tip of the T3SA needle

Protein purification procedures tend to select for most stable complexes that might not contain weakly associated subunits. We recently showed that a Triton-X100 detergent concentration as low as 0.01% was sufficient to induce the release of NCs from the membrane (Sani *et al.*, 2006). To detect potentially labile subunits attached to the needle, we performed a cross-linking step with BS^3 on bacteria prior to any purification. Electron microscopy analysis indicated that, following BS^3 treatment, most bacteria exhibited needle appendages with an additional density at the extremity of the tip (Fig. 1).

NCs were purified from BS^3-treated bacteria after detergent solubilization of membranes as described [18]. Preparations contained a sufficient number of NCs with the additional densities at the needle tip to perform a structural analysis. To calculate two-dimensional projection maps of isolated NCs, electron microscopy images were analysed by single particle analysis. We selected several hundred images of NCs with a relatively straight and short needle appendage and a length close to 45 nm. The averaged NCs clearly showed the presence of a density around the needle tip, as well as the upper part of the basal body (Fig. 2A and 2B; see also Fig. 4E for a total view of a NC). However, NCs appeared a bit blurred after averaging as a result of variations in the needle length. Sharper features at the tip of the needle portion were obtained when projections were aligned and classified after masking the basal part (Fig. 2C). Striking features of the average map of cross-linked particles are the presence of densities at either side of the needle tip. In contrast, average maps of particles prepared from the wild-type strain without cross-linking showed needles lacking most of these densities (Fig. 2D-F). Faint densities are visible in these samples at the same position where the strong densities were present in cross-linked preparations (arrows, Fig. 2E and 2F). These results suggest that, in the absence of cross-linking, most purified NCs lost the additional molecule(s) forming the density observed after cross-linking. To identify molecule(s) forming the density at the tip of the T3SA, we performed similar experiments with an *ipaD* mutant lacking the IpaD expression. IpaD is required, together with IpaB, to maintain the

T3SA inactive in the absence of inducers and a small proportion of IpaD is membrane associated [9]. NCs purified from the BS^3-treated *ipaD* mutant did not exhibit densities at either side of the needle tip (Fig. 2G-I), suggesting that IpaD is part of or required for assembly of this structural element.

IpaD is present at the tip of the T3SA needle

To test whether IpaD constitutes the observed density, NCs purified from BS^3-treated wild-type bacteria were analyzed by SDS-PAGE and immunoblotting (Fig. 3). IpaD was enriched in NCs prepared from cross-linked wild-type bacteria, as compared to NCs prepared from non-cross-linked bacteria (Fig. 3, right panel, right lane), though small amounts also co-purifiy in non-cross linked preparation and thus corroborate the faint densities at the needle tip for averages of non cross linked NCs. MxiJ that is a major NC component is present in similar amounts in all preparations (Fig. 3). Control experiments using antibodies recognizing cytoplasmic components of the T3S system, such as MxiN, did not reveal any contamination of NCs by intracellular components (Fig. 3), indicating that the presence of IpaD in the preparations was not due to a contamination by cytoplasmic proteins.

To confirm that densities detected at the tip of NCs contained IpaD, we performed immuno-staining using an anti-IpaD serum. Antibodies specifically bound to the tip of the needle in NCs prepared from BS^3 treated wild-type bacteria (Fig. 4 A-D). Some needles were also observed to be associated by their tip, presumably as a result of the interaction to divalent antibodies with two needles (lower left frame, Fig. 4D). In control experiments, no antibodies were found to bind the needle of NCs isolated from the *ipaD* mutant (data not shown).

Anti-IpaD antibodies blocks entry of bacteria into epithelial cells

Since IpaD is required for entry of bacteria into epithelial cells [14] and since, as shown here, it is localized at the tip of the T3SA, we investigated whether anti-IpaD antibodies might interfere with entry of bacteria into HeLa cells. Bacteria incubated with different concentrations of the anti-IpaD serum, or an anti-IpaB serum as a control, were used to infect HeLa cells. Exposure of bacteria to the anti-IpaD serum,

but not to the anti-IpaB serum, inhibited bacterial entry in a dose-dependent manner (Fig. 5). Treatment with the anti-IpaD antibodies also inhibited entry of a *S. flexneri 2a* strain (data not shown).

Discussion

The *S. flexneri* T3SA is activated upon contact of bacteria with epithelial cells and is deregulated by inactivation of *ipaB* or *ipaD*. It was proposed that these proteins are required to form a complex pluging the T3SA. Here, we presented evidence that IpaD is present at the tip of the needle. Transmission electron micrograph of surface exposed needles from cross-linked bacteria showed a distinctive structure present at the tip of the needle and immunoblot analysis of mildly purified NCs indicated that IpaD is copurified with the cross-linked NCs.

Calculated averages of NCs isolated from cross-linked wild-type bacteria showed distinct densities at either sides of the needle tip. This feature was not observed in NCs isolated from both the wild-type strain that had not been treated with the cross-linker and from the *ipaD* mutant treated with the cross-linker. Results of immunoelectron microscopy indicate that the observed density at the tip of the needle contains IpaD molecules. The exact configuration of the additional density, however, cannot be retrieved from 2D projections maps. The two additional masses have dimensions of about 7 x 7 nm. Since the size of IpaD is 37-kDa, several copies of IpaD are probably present in these structures. Indeed, IpaD has been proposed to form oligomers [19] IpaD presents some functional analogies with LcrV of *Yersinia enterocolitica*, inasmuch as the two proteins have similar sizes and are both required for insertion of the proposed translocators, IpaB and IpaC in S. flexneri and YopB and UopD in *Y. enterocolitica*, in the membrane of host cells [9, 20, 21]. Recent data indicated that LcrV is localized at the tip of the T3SA needle [20]. The structure in *Yersinia* appears to be slightly different to that in *Shigella* with smaller protruding densities at the side and with a different tip.

The identification of a structural element containing IpaD at the tip of the T3SA needle provides further insights on the composition and structure of the *S. flexneri* T3SA. Very recently it was also demonstrated with biochemical methods that IpaD

localizes to the T3SA needle tip, where it functions to control the secretion and proper insertion of translocators into host cell membranes [22]. Our single particle analysis, however, directly demonstrates the position of IpaD at the tip of the needle and adds credence to the hypothesis that IpaD acts as a plug to the T3SA prior to contact of bacteria with cells. As proposed for LcrV in *Yersinia*, IpaD might also facilitate insertion of components of the translocators within the cell membrane. The inhibition of entry of bacteria into HeLa cells by treatment with anti-IpaD antibodies indicates that binding of antibodies to IpaD interferes with the function of the protein. LcrV has also been shown to be a protective antigen for plague disease in animal studies [23, 24]. Accordingly, IpaD represent an interesting target for the preparation of vaccines that would be effective against several serotypes of *Shigella*.

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WHAT IS CLAIMED IS:

1. A composition for the treatment and/or the prevention of a Shigella infection, comprising at least one of the following material:

- an IpaD polypeptide or functional derivative thereof;
- a polynucleotide or functional fragment thereof encoding an IpaD polypeptide;
- an IpaD specific antibody.

2. The composition of claim 1, further comprising a polyosidic antigen.

3. The composition of claim 2, wherein said polyosidic antigen is selected from the group consisting of S. flexneri 2a, S. flexneri 1b, S. flexneri 6, S sonnei and S. dysenteriae.

4. A method for the treatment and/or the prevention of a Shigella infection in an animal, comprising the step of administering to said animal a composition as defined in any one of claims 1 to 3.

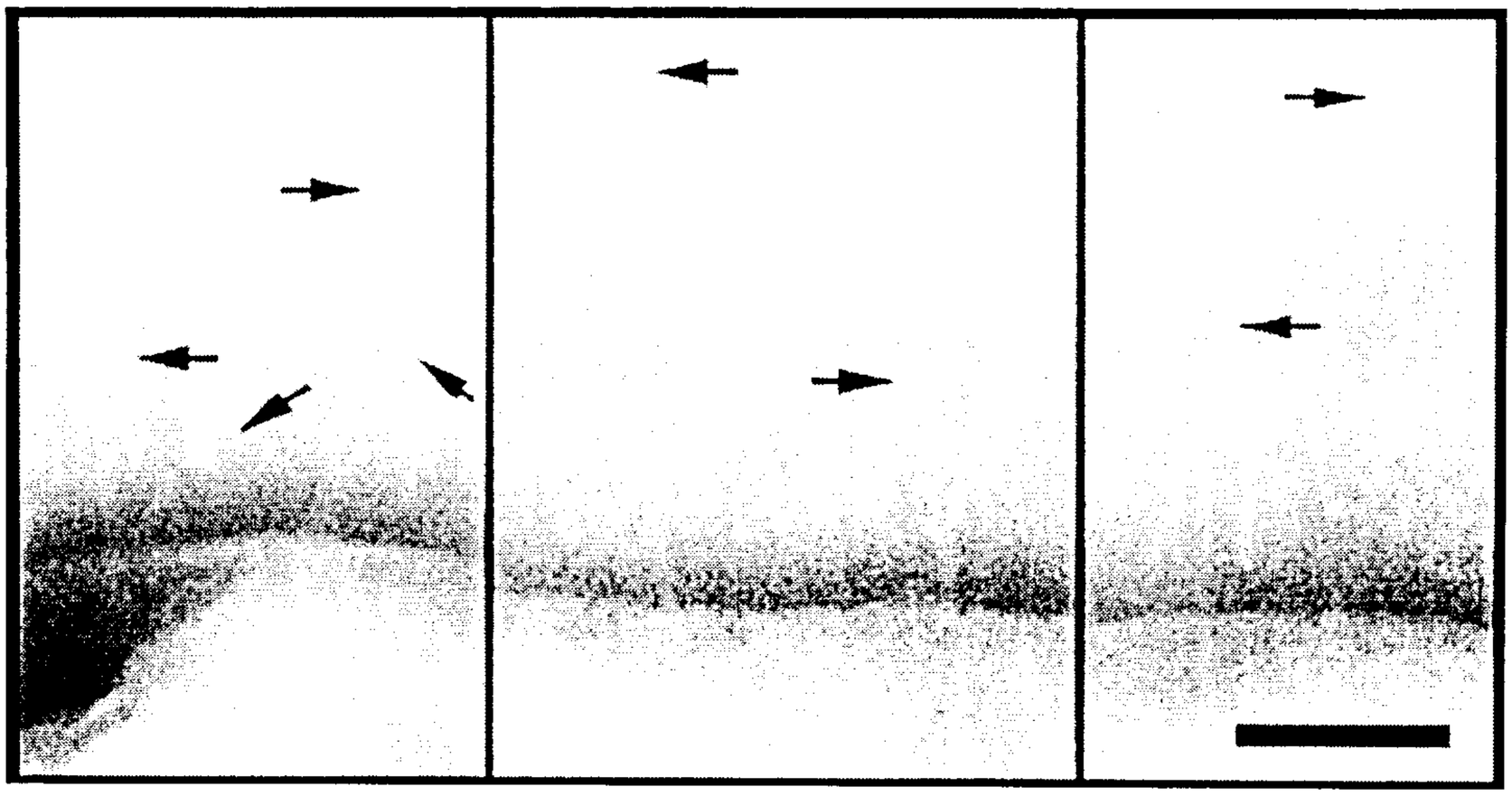

FIGURE 1

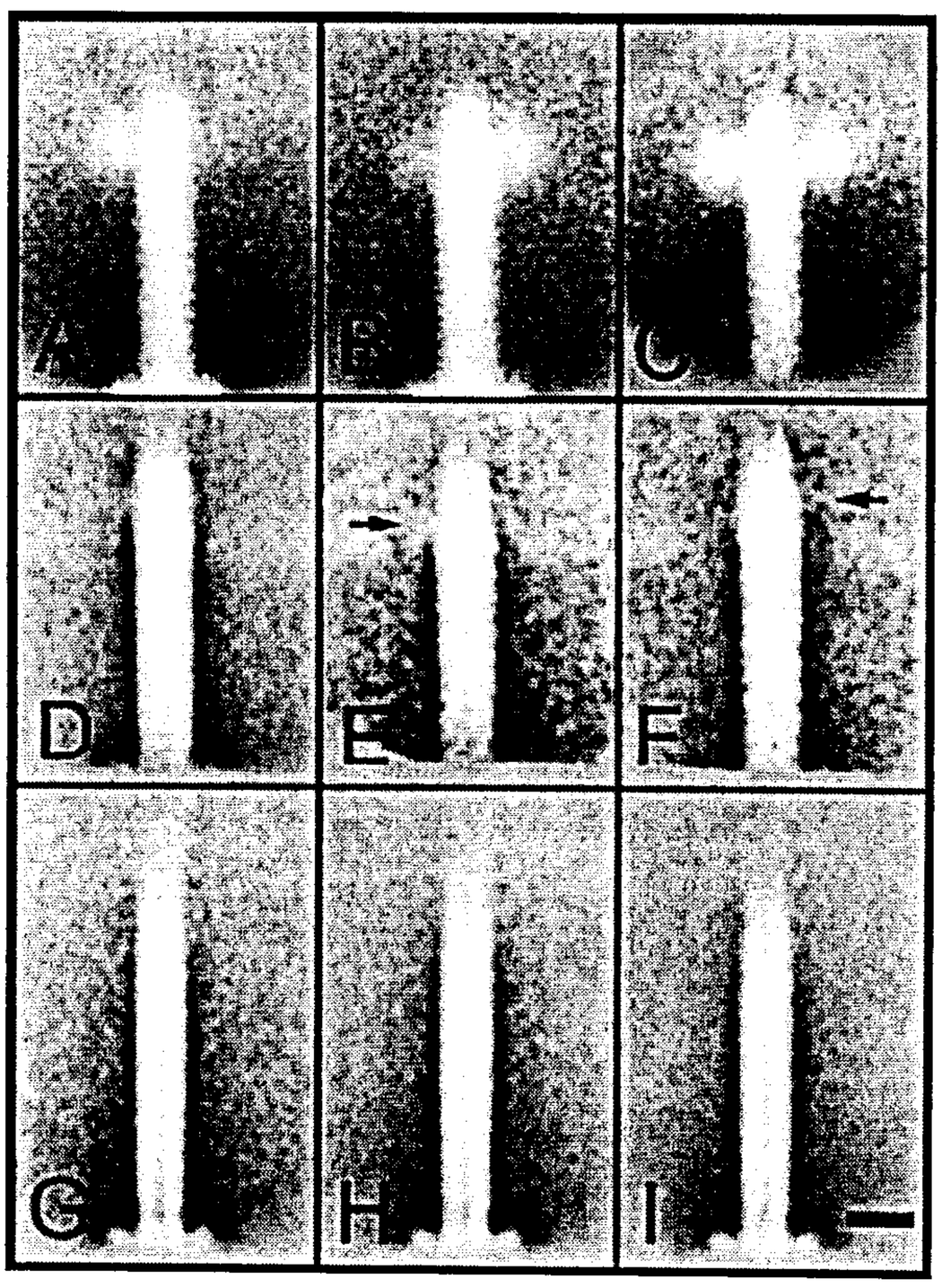

FIGURE 2

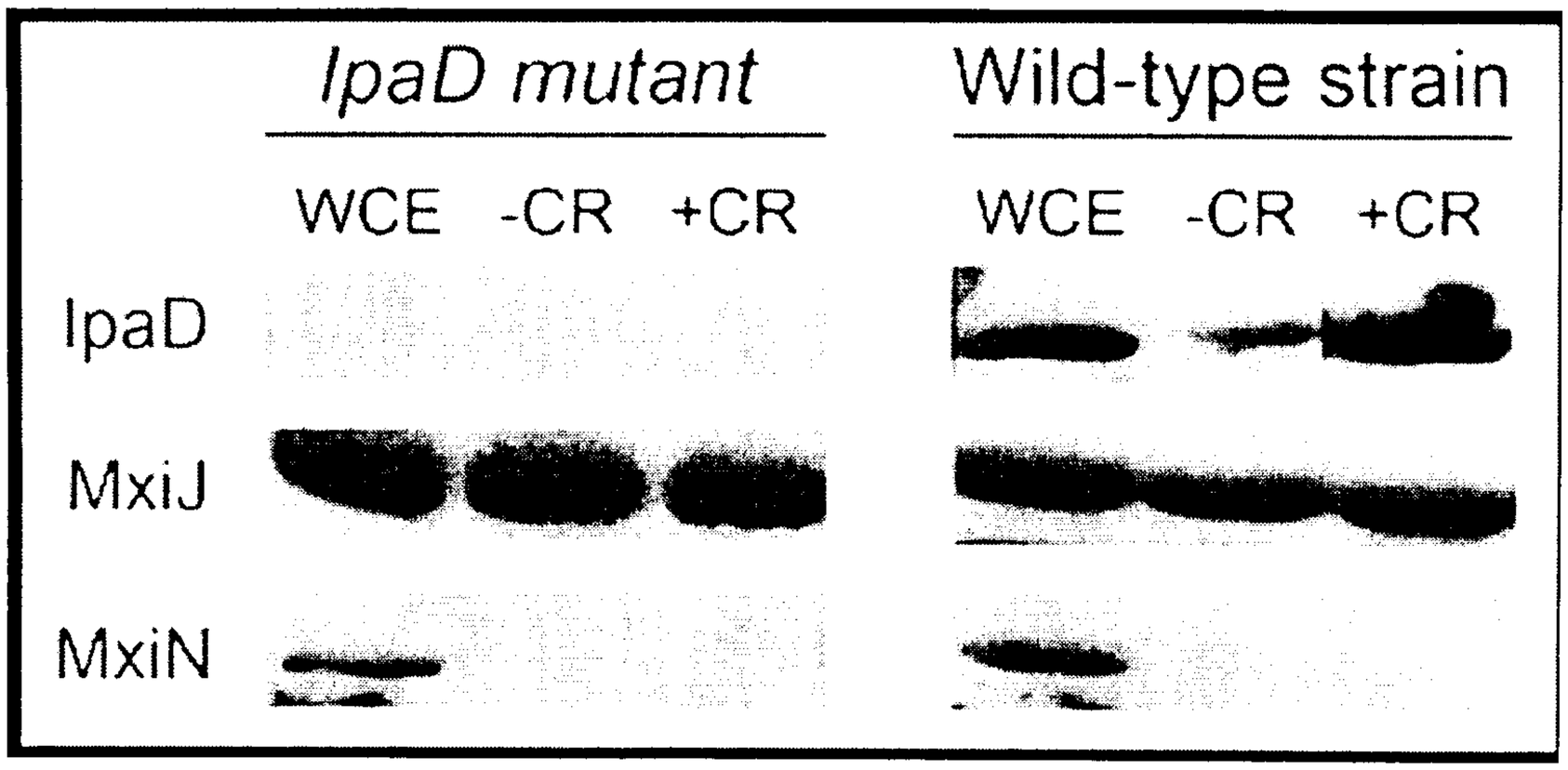

FIGURE 3

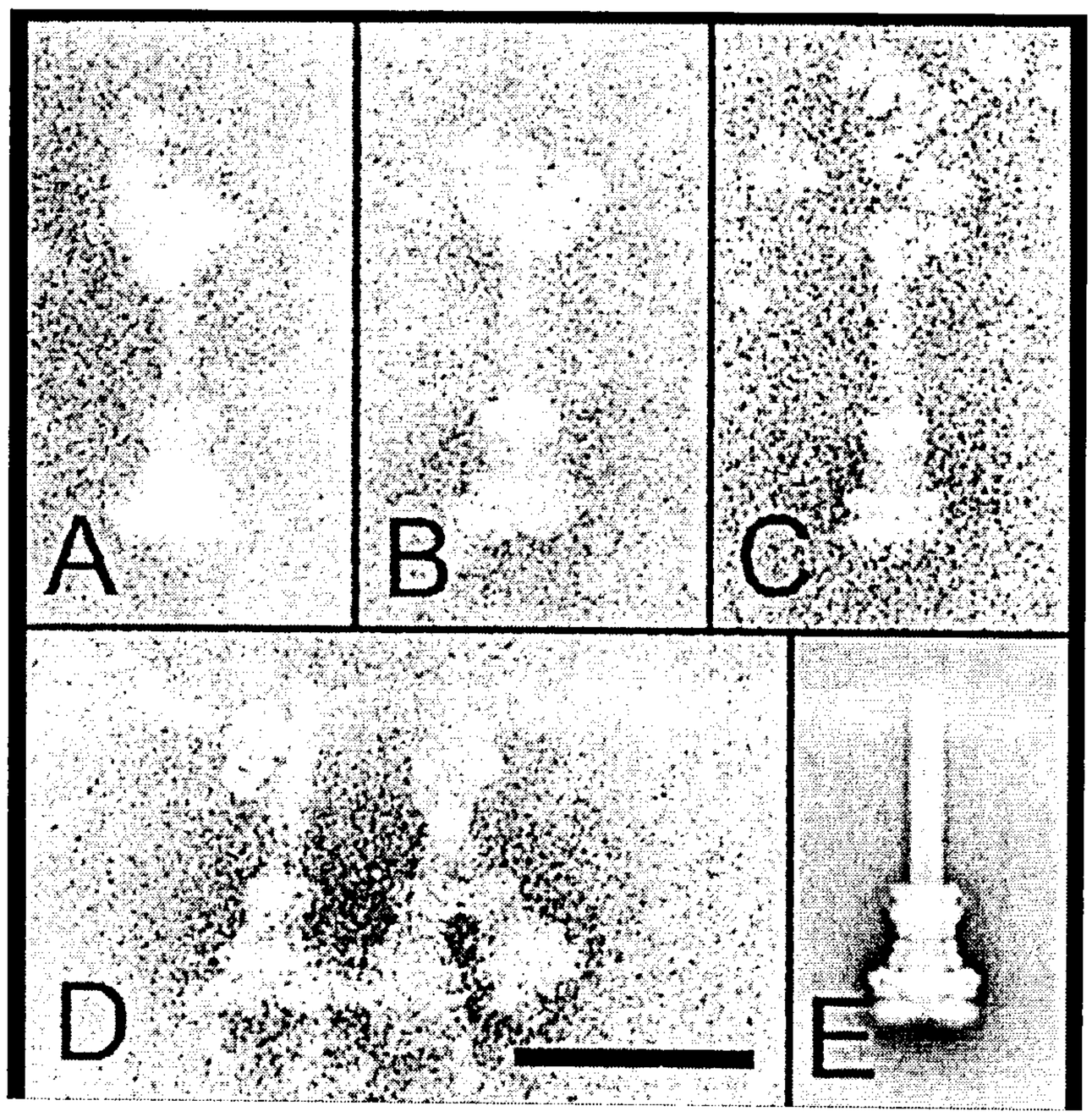

FIGURE 4

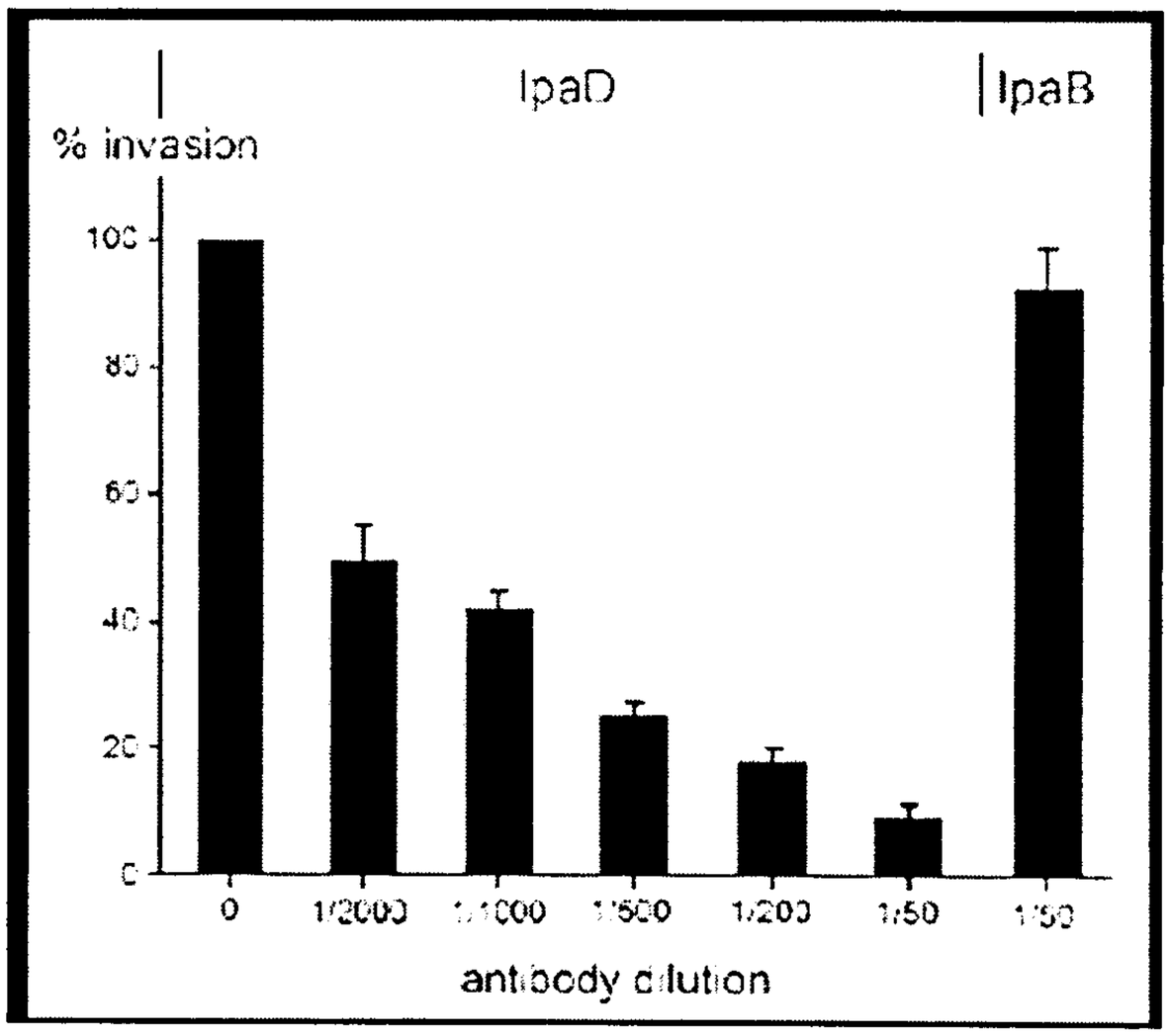
IpaD
IpaB
% invasion
100
80
60
40
20
0
0
1/2000
1/1000
1/500
1/200
1/50
1/10
antibody dilution

FIGURE 5