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Characterization of B-Cell Epitopes on IpaB, an Invasion-Associated Antigen of *Shigella flexneri*: Identification of an Immunodominant Domain Recognized during Natural Infection

SIMONA BARZU,[1] FARIDA NATO,[2] SYLVIE ROUYRE,[2] JEAN-CLAUDE MAZIE,[2] PHILIPPE SANSONETTI,[1] AND ARMELLE PHALIPON[1]*

Unité de Pathogénie Microbienne Moléculaire and Institut National de la Santé et de la Recherche Médicale, U199,[1] and Hybridolab,[2] Institut Pasteur, 75724 Paris Cedex 15, France

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The invasion plasmid antigen B (IpaB), a 62-kDa plasmid-encoded protein associated with the ability of shigellae to invade epithelial cells, is the bacterial antigen most strongly and consistently recognized by the host during infection. The strong systemic and mucosal immune responses observed against this invasin prompted us to map its B-cell epitopes. For this purpose, IpaB was first overexpressed in *Shigella flexneri* and used to raise rabbit polyclonal antiserum and murine monoclonal antibodies, which were subsequently used to screen a λgt11 *ipaB* library. Inserts of recombinant DNA clones that were specifically recognized by the antisera and antibodies were sequenced, and three distinct determinants were identified. Further characterization of these determinants showed that they were recognized by sera from patients convalescent from shigellosis, suggesting that they are relevant to the humoral response during natural infection. Moreover, the IpaB region comprising the three determinants was systematically recognized by all sera from infected patients that we tested, whereas other regions of the protein were not. These data suggest that this region, located between amino acid residues 147 and 258, is the major immunogenic domain of the invasin in the course of natural infection.

Shigella spp. cause dysentery in humans by invading the colonic mucosa. Invasion is a multistep process that encompasses entry of the bacterium into epithelial cells by induced phagocytosis, intracellular growth, intra- and intercellular spread, and eventually, killing of the host cells (27). The virulence properties of *Shigella flexneri* are associated with the presence of a 220-kb plasmid that is found in all invasive isolates (28). A 35-kb fragment of this virulence plasmid that includes the *ipa* (invasion plasmid antigen) genes is necessary and sufficient for expression of the entry functions (15). Subsequent analysis of this region has indicated that the *ipaB*, *ipaC*, *ipaD*, and *ipaA* genes are clustered in an operon and that the IpaB, -C, and -D proteins are essential for the entry process (5, 12, 29). IpaB is an invasin involved in triggering entry after initial adhesion to the target cell membrane has occurred and is also involved in lysis of the membrane-bound phagocytic vacuole (12). It has recently been shown that these proteins are secreted at the surface of the bacteria by a *Shigella* sp.-specific secretory apparatus (1, 2, 30).

Natural infection of humans and intragastric experimental infection in macaque monkeys elicits specific serum immunoglobulin G (IgG) antibodies (20) as well as intestinal secretory IgA antibodies (7, 22) directed both against the lipopolysaccharide, which carries the serotype-specific determinants, and against bacterial proteins, among which IpaB and IpaC are the most strongly and consistently recognized (10). The significance of the systemic and mucosal immune responses for protection of individuals against shigellosis is still unknown. Previous studies have suggested that either natural infection or the administration of experimental oral vaccines protects humans against a subsequent homologous challenge (8, 9, 11, 16). The role that anti-Ipa antibodies play in protection remains elusive. It has been recently suggested, on the basis of epidemiological studies, that anti-Ipa secretory IgA antibodies present in mucosal secretions limit the spread and severity of the illness (22). No data concerning the role of systemic anti-Ipa antibodies are available.

To study the immunogenicity and to analyze the functional domains of these proteins, we identified and characterized B-cell epitopes on IpaB and IpaC, each of whose corresponding genes had already been cloned and sequenced (4). We previously mapped four independent epitopes on IpaC that are recognized by sera from convalescent humans or monkeys, thus suggesting their relevance in natural infection (23). In this study, we identified three B-cell epitopes on IpaB. We show that they are recognized by sera from humans convalescent from shigellosis and that they are clustered as a major immunogenic domain of IpaB which is uniformly recognized during natural infection.

MATERIALS AND METHODS

Plasmids, phages, and bacterial strains. Plasmid pIpaB (12) is a transcriptional fusion coupling the *ipaB* gene to the *tac* promoter in the vector pTTQ18. Plasmid pTZB was constructed as follows: the 2.54-kb *Pst*I fragment carrying the *ipaB* gene was purified from plasmid pHS4108 (15) and cloned into the *Pst*I restriction site of vector pTZ (Pharmacia). Construction of plasmids pMAL-B1, pMAL-B2, and pMAL-B3 will be described in this work. The λgt11 cloning kit and Gigapack II Gold packaging extracts were obtained from Stratagene (La Jolla, Calif.) and used according to the manufacturer's instructions. M90T, an invasive strain of *S. flexneri* belonging to serotype 5, and BS176, its noninvasive, plasmidless derivative, have been previously described (28).

* Corresponding author.

Escherichia coli MC1061 [Δ(*lac* IPOZYA)X74 *galU galK strA*) was used for cloning, *E. coli* JM04 [F^- *lac* ΔU169 *araD139 strA thi purE*::Tn*5* (Ade^-) Δ*fepA-ompT metA*$^+$ Δ*malE444* Rif^r] was used for expression of MalE fusion proteins, and *S. flexneri* M90T, carrying plasmid pIpaB, was used for overexpression of IpaB. λgt11 recombinant phages were propagated in *E. coli* Y1090r− [Δ(*lacU169*) Δ(*lon*) *ara D139 strA supF trpC22*::Tn*10* (pMC9) *hsdR* (r_k^- m_k^-)] (32).

Genetic procedures. Isolation of plasmids, purification and restriction of DNA fragments, DNA ligation, and transformation of *E. coli* and *S. flexneri* were carried out as described by Sambrook et al. (25). Nucleotide sequences were determined by the dideoxy chain termination procedure (26).

Construction and expression of MalE fusion proteins. Three recombinant plasmids were constructed by cloning each of three different fragments from the *ipaB* gene into the expression vector pMAL-p2 (New England Biolabs), in frame with the 3′ end of the *malE* open reading frame. Recombinant plasmid pMAL-B1 was obtained as follows: first, a 700-bp *Hin*fI restriction fragment was purified from the plasmid pTZB, blunt ended, and digested with *Nsi*I. The resultant 442-bp *Nsi*I-*Hin*fI DNA fragment (see Fig. 3A) was ligated into pMAL-p2, which had been previously digested with *Hin*dIII, blunt ended, and further digested with *Pst*I in order to generate a cohesive *Nsi*I-compatible end. Plasmid pMAL-B2 was obtained by ligating the 1,139-bp *Nsi*I fragment (see Fig. 3A), which had been purified from pTZB, into the *Pst*I restriction site of pMAL-p2. Plasmid pMAL-B3 was constructed by ligating the blunt-ended 916-bp *Spe*I-*Dde*I restriction fragment (see Fig. 3A) from pTZB into the *Xmn*I site of pMAL-p2. For expression of the fusion proteins, a 1:100 dilution of overnight cultures of *E. coli* JM04 harboring the recombinant plasmids was used to inoculate Trypto Casein Soya broth supplemented with ampicillin (100 μg ml^{-1}). Bacteria were grown at 37°C to an optical density of 0.5 at 600 nm. Expression of fusion proteins was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the culture was harvested after growth for an additional 1 h and 30 min at 37°C.

Overexpression and partial purification of IpaB. Overexpression of IpaB with *S. flexneri* M90T harboring the plasmid pIpaB (12) was obtained as described above for the expression of MalE fusion proteins, except that bacteria were grown for 3 h after induction with IPTG. After the bacteria were recovered by centrifugation, they were resuspended in 50 mM Tris (pH 7.4) at 4°C, sonicated, and harvested by centrifugation for 30 min at 27,000 × *g*. The pellet was then solubilized in 8 M urea (pH 6.8). After a second 15-min centrifugation at 100,000 × *g*, IpaB, which remained insoluble in this buffer, was recovered from the pellet. After separation on a sodium dodecyl sulfate (SDS)-polyacrylamide gel and staining with Coomassie brilliant blue, the protein was recovered from the gel by excising the corresponding band and electroeluting the protein in Laemmli buffer. The protein was concentrated by ultracentrifugation in Centricon 30 filters (Microconcentrator; Amicon). To be sure that no degradation occurred during the step of purification, IpaB was further tested by SDS-polyacrylamide gel electrophoresis (PAGE) before being used for immunization (data not shown).

SDS-PAGE and immunoblotting. Whole cells were solubilized at 100°C for 5 min in Laemmli buffer and electrophoresed through a 10% polyacrylamide gel with a 4% stacking gel (14). The amounts of lysate proteins used were standardized by loading on each lane 5 μl of bacterial cultures at a concentration of 3 × 10^9 bacteria per ml. Proteins were stained with Coomassie brilliant blue or Western blotted (immunoblotted) onto nitrocellulose by the method of Burnette (6). The transferred proteins were probed with either rabbit or monkey serum at a 1:100 dilution, with human serum at a 1:20 dilution, or with purified monoclonal antibodies (MAbs; 1 μg ml^{-1}). Secondary antibodies were either alkaline phosphatase-labeled goat anti-rabbit or anti-mouse antibodies (diluted 1:5,000) (Stratagene) or horseradish peroxidase-labelled goat anti-human antibodies (diluted 1:5,000) (Nordic Immunological Laboratories).

Immunization of rabbits and mice. IpaB was used to immunize male New Zealand rabbits (Charles River) and 6-week-old female BALB/c mice (Janvier) according to the protocol previously described by Phalipon et al. (23).

Production, screening, and selection of hybridomas. Cell fusions were performed as previously described (23). Hybridoma culture supernatants were initially tested against partially purified IpaB by an enzyme-linked immunosorbent assay (ELISA). Culture supernatants that tested positive by ELISA were subsequently tested by an immunoblot assay against whole-cell SDS lysates of M90T (the wild-type strain) and BS176 (a strain that does not carry the *ipaB* gene). Hybridomas producing antibodies reactive with M90T, but not BS176, were cloned twice by limiting dilution. Ascites fluid was prepared in pristane-primed BALB/c mice by intraperitoneal injection of 10^6 cloned hybrid cells per mouse. The immunoglobulin subclass of each MAb was determined by using a mouse MAb isotyping kit (Holland Biotechnology, Leiden, Holland).

ELISA. ELISAs were performed essentially as previously described (17). Polystyrene plates were coated with 20 ng of IpaB, and horseradish peroxidase anti-mouse immunoglobulin (Nordic) was used as the secondary antibody (dilution, 1:5,000). Affinity of antibodies for IpaB was determined as described by Phalipon et al. (23). The affinities of MAbs H16, H4, and F22 were shown to be 4 × 10^9 M^{-1}, 5 × 10^9 M^{-1}, and 6 × 10^8 M^{-1}, respectively.

Construction and screening of the λgt11 library. Plasmid pTZB was first methylated by *Eco*RI methylase in order to prevent any subsequent *Eco*RI digestion of the *ipaB* gene. The 2.54-kb *Pst*I fragment carrying the *ipaB* gene was isolated by 7% agarose gel preparative electrophoresis, purified by Geneclean (Bio 101), and sonicated to generate random DNA fragments. These DNA fragments were end repaired by treatment with the Klenow enzyme in the presence of deoxynucleoside triphosphates, and then ligated to *Eco*RI linkers. Cohesive ends were generated by *Eco*RI digestion, and 75- to 300-bp DNA fragments were isolated by 6% PAGE followed by Elutip-d purification (Schleicher & Schuell). These fragments were ligated to dephosphorylated λgt11 arms and then packaged into λgt11 phage heads. The recombinant phages were amplified in *E. coli* Y1090r− and screened according to the manufacturer's instructions, by using an anti-IpaB polyclonal rabbit serum (diluted 1:500) which had been preabsorbed on *E. coli* Y1090r−.

PCR for sequencing DNA inserts in antibody-reactive λgt11 clones. Two oligonucleotide primers were synthesized by use of an Applied Biosystems automated oligonucleotide synthesizer. One primer (5′GGCACATGGCTGAATATCGA3′) corresponded to the λgt11 sequence located 55 bp upstream of the *Eco*RI restriction site; the other (5′GGCCTGCCCGG TTATTATTA3′) corresponded to the sequence located 55 bp downstream of the same *Eco*RI site. Primers were used at 20 pmol each, and the target DNA fragment was used at 1 ng. DNA amplification was performed in 60 mM Tris-HCl–17 mM ammonium sulfate–6.7 mM $MgCl_2$–10 mM β-mercap-

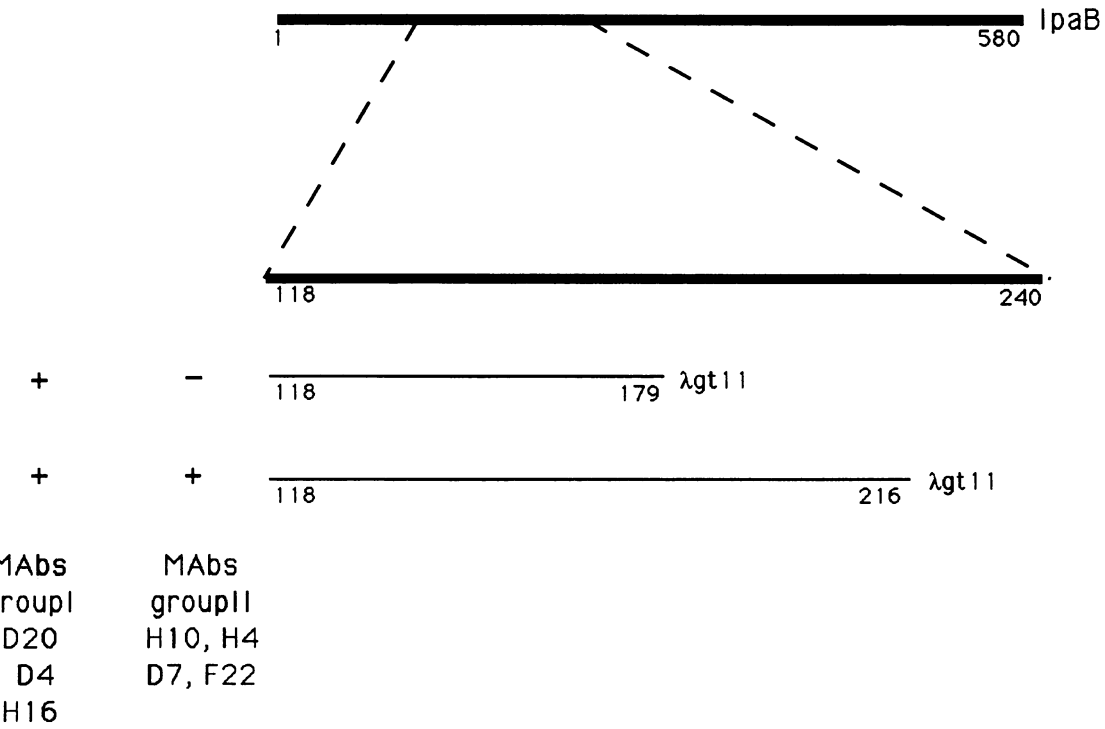

FIG. 1. Screening of the λgt11 library. Schematic of IpaB and the corresponding amino acid residues of inserts of two λgt11 clones selected for their reactivity with the rabbit anti-IpaB serum. The amino-terminal end of inserts of all other reactive λgt11 clones was also at aa 118; the carboxy-terminal ends of the inserts of these other clones all fell between aa 179 and 216. Reactivity of λgt11 clones with MAbs is indicated as positive (+) or negative (−).

toethanol–5 mM EDTA with 30 cycles, each consisting of denaturation at 92°C for 2 min, primer annealing at 55°C for 2 min, and extension by the *Taq* polymerase (Perkin-Elmer Cetus) at 72°C for 5 min. Polymerase chain reaction (PCR) products were purified on a Qiagen column (U.S. Biochemicals) before being used as templates for DNA sequencing.

Competition assay to determine antibody binding sites. For this purpose, each MAb was biotin conjugated according to the manufacturer's (IBF) instructions, and ELISAs were performed as described by Phalipon et al. (23).

Competition between MAbs and human convalescent-phase sera for binding to IpaB. Competition assays were performed by the ELISA procedure as previously described (23) except that purified MAbs were added to a constant final concentration of 10^{-5} μg ml^{-1}.

RESULTS

Production of MAbs against IpaB. Two spleen cell-myeloma cell fusions resulted in 400 positive wells containing proliferating hybridoma cells, among which 49 produced antibodies against the partially purified IpaB protein that was used for immunization, as established by ELISA. To ensure specificity of these antibodies, immunoblot analysis of positive culture supernatants was performed with total protein extracts from each *S. flexneri* M90T and BS176 isolate. Among the 49 ELISA-positive supernatants, 23 were specific for IpaB. After amplification, six IpaB-specific hybridomas stably produced high levels of IpaB antibody. These cells were cloned twice, and ascites fluid was prepared. Isotyping showed that each MAb carried the κ chain and was of the IgG1 isotype. Another MAb, also of the IgG1 isotype and called H10, was previously obtained by the same procedure except that the mice were immunized with a fusion protein that contained only the first 258 amino acid (aa) residues of IpaB fused to β-galactosidase and had been expressed in *E. coli* (data not shown).

Screening of the λgt11-*ipaB* library. Approximately 2,000 recombinant plaques were probed with an anti-IpaB rabbit antiserum preabsorbed on *E. coli*. Sixty-four positive plaques were identified. Following reisolation, 32 λgt11 clones were selected and the DNA sequence of each cloned fragment was determined. All selected λgt11 inserts encoded a fragment of IpaB located between aa 118 and 216 (Fig. 1). All clones started at aa 118, thus sharing the same amino terminus, but each differed in length and consequently in carboxy terminus. The shortest sequence ended at aa 179, and the longest ended at aa 216. No other amino acid sequence was detected with the anti-IpaB rabbit antiserum. We tested whether the λgt11 library was representative of the entire *ipaB* gene. Both a 442-bp *Nsi*I-*Hin*fI and a 916-bp *Spe*I-*Dde*I fragment of the *ipaB* gene (see Fig. 3A), corresponding to regions undetected by the anti-IpaB rabbit antiserum, were used as ^{32}P-radiolabelled probes. After hybridization, the percentage of the clones detected with each probe was similar to that (about 1%) found when the library was screened with the IpaB-specific rabbit antiserum. Thus, we concluded that the λgt11 library was representative of the entire *ipaB* gene and that the anti-IpaB rabbit antiserum recognized a restricted immunogenic domain of IpaB.

All MAbs reacted with the λgt11 clones previously selected. On the basis of their differential reactivities with the shortest λgt11 insert, they were classified into two distinct groups (Fig. 1); group I recognized this sequence, whereas group II did not, thus suggesting that at least two distinct

determinants, which map close to one another in terms of the primary sequence of IpaB, were identified.

Identification of three independent epitopes by competition assays. Competition assays for binding to IpaB protein were carried out with each group of MAbs to determine whether MAbs belonging to group I and group II recognized similar or different antigenic determinants (Table 1). In each assay between members of group I (MAbs D20, D4, and H16), binding of the biotinylated MAb decreased in the presence of increasing concentrations of the nonconjugated MAb, suggesting that competition for binding occurred and that therefore each pair of MAbs recognized the same epitope. The epitope recognized by group I was designated epitope I. MAbs in group II appeared to recognize two different epitopes, which were designated epitope II and epitope III. Epitope II was recognized by MAbs H4 and H10, and epitope III was recognized by MAbs F22 and D7. Competition assays carried out between MAbs specific for each epitope confirmed the presence of three independent antigenic determinants.

Epitopes I, II, and III are recognized by sera of humans convalescent from shigellosis. To investigate the relevance of the characterized epitopes in the context of natural infection, competition assays between MAbs H16, H4, and F22 and sera of three patients convalescent from shigellosis were performed. As shown in Fig. 2, similar profiles were obtained for each MAb with each of the three sera tested (graphs A, B, and C). The amount of MAbs H16, H4, and F22 bound to IpaB was proportional to the dilution of

TABLE 1. Competition assays for binding to IpaB

Nonconjugated MAb	Competition[a] against following biotinylated MAb:						
	D20	H16	D4	H4	F22	H10	D7
D20	+	+	+	−	−	NT	NT
H16	+	+	+	NT	NT	NT	NT
D4	+	+	+	NT	NT	NT	NT
H4	−	NT	NT	+	−	NT	NT
F22	−	NT	NT	−	+	NT	NT
H10	NT	NT	NT	+	−	+	−
D7	NT	NT	NT	−	+	−	+

[a] +, competition occurs; −, no competition occurs; NT, competition not tested.

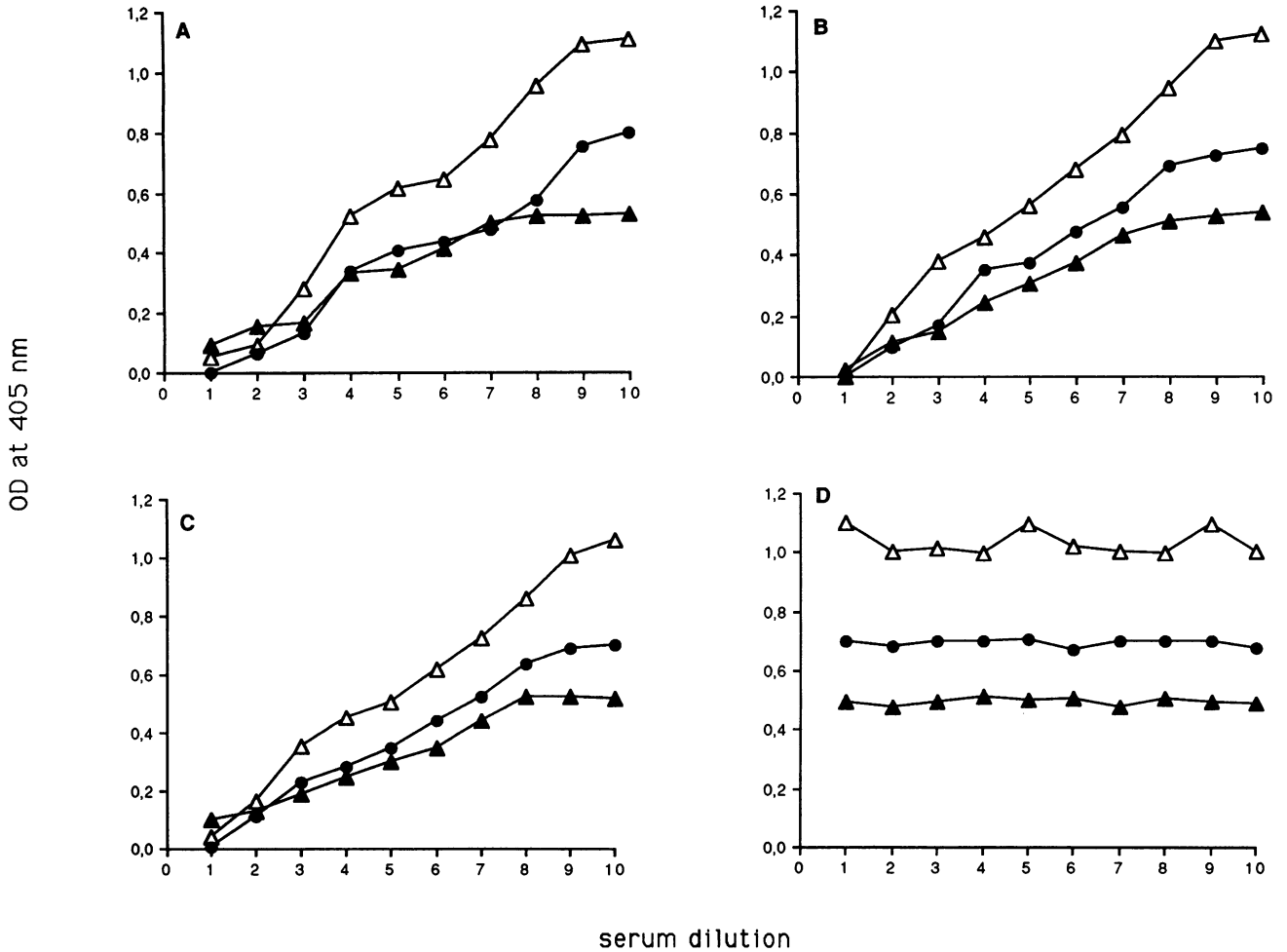

FIG. 2. Competition for binding to IpaB between MAbs and sera from humans convalescent from shigellosis. MAbs and human sera were mixed before being assayed. MAbs were used at a constant concentration, whereas sera were added at decreasing concentrations. A_{405} represents the binding of the MAb. Numbers on the horizontal axis represent twofold dilutions of human serum from 1:50 to 1:25,600. Three serum samples from convalescent humans (graphs A, B, and C) were tested, and a nonimmune serum was used as a control (graph D). Symbols: ●, MAb H16 (epitope I); △, MAb H4 (epitope II); ▲, MAb F22 (epitope III).

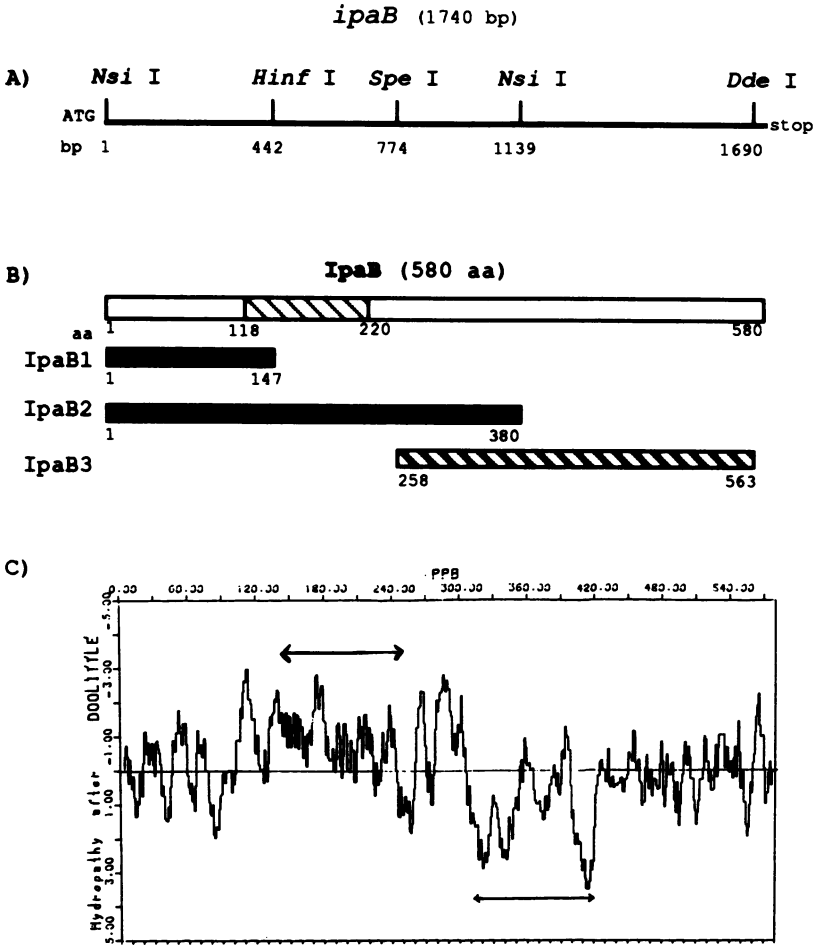

FIG. 3. Construction of the MalE fusion proteins. (A) In the restriction map of *ipaB*, *ipaB* is represented as a black line. Restriction sites used for cloning of *ipaB* DNA fragments into pMAL-p2 are indicated. The 442-bp *Nsi*I-*Hin*fI fragment, the 1,139-bp *Nsi*I-*Nsi*I fragment, and the 916-bp *Spe*I-*Dde*I fragment were subcloned in frame into pMAL-p2 as described in Materials and Methods, thus leading to the fusion proteins MalE-IpaB1, MalE-IpaB2, and MalE-IpaB3, respectively. (B) For the MalE fusion proteins, the amino acid sequence of IpaB is represented as a white bar within which the domain encompassing the three characterized determinants is represented as a lightly cross-hatched box (aa 118 to 220). Below are shown the segments of IpaB (IpaB1, IpaB2, and IpaB3) which were expressed as fusions to MalE. The numbering of amino acid residues is indicated below each protein sequence. (C) The hydropathy profile of IpaB is according to that of Kyte and Doolittle (13). The immunodominant region containing the three antigenic determinants is indicated by an arrow above the profile; the hydrophobic segment of the protein is indicated by an arrow below the profile.

convalescent-phase sera. A plateau similar to that observed in the presence of nonimmune sera (graph D) was reached beyond a dilution of 1:640 of human sera for the binding of MAbs F22 and H16 and 1:1,280 for MAb H4. As the affinities of MAbs were comparable and the anti-IpaB antibody titer was similar in each human serum sample tested (data not shown), we concluded that the sera had similar titers of antibodies directed against epitopes I, II, and III. Thus, these epitopes are relevant to the humoral response against IpaB during natural infection.

Epitopes I, II, and III are clustered as a major immunogenic domain of IpaB recognized during natural infection. Whether the region encompassing the characterized epitopes was a major immunogenic domain of IpaB recognized during natural infection was further analyzed. Fusion proteins between MalE and various IpaB fragments were constructed (Fig. 3A) and tested for their reactivities with sera from patients infected by different *Shigella* species.

The fusion protein MalE-IpaB1 encompassed IpaB aa 1 to 147, the fusion protein MalE-IpaB2 encompassed aa 1 to 380 (i.e., the region containing the characterized epitopes), and the fusion protein MalE-IpaB3 encompassed aa 258 to 563 (Fig. 3B). Expression of MalE-IpaB1 (60 kDa), MalE-IpaB2 (85 kDa), and MalE-IpaB3 (75 kDa) in *E. coli* JM04 was analyzed by Western blot with an anti-MalE rabbit antiserum (Fig. 4A). A band corresponding to each fusion protein at the expected molecular mass was observed, although some degradation occurred, and the levels of expression were lower than those obtained for MalE fusion proteins constructed with other *S. flexneri* antigens (18).

Twenty-eight human sera from adults or children were tested. Twelve were collected from patients infected by *S. flexneri* in Madagascar (four sera), in New Caledonia (five sera), and in the Central Africa Republic (three sera). Two sera originated from patients infected by *Shigella bodyi* in Madagascar, whereas two others came from patients infected by *Shigella sonnei* in New Caledonia. The remaining

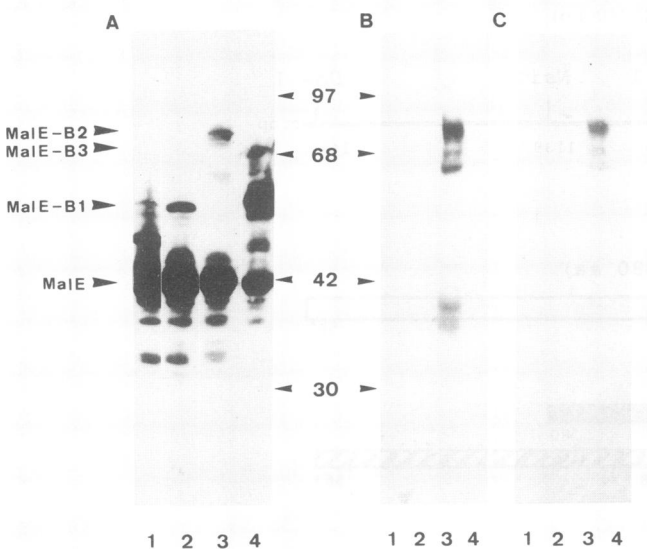

FIG. 4. Expression of the MalE fusion proteins in *E. coli* and recognition by serum from a human convalescent from shigellosis. Whole-cell lysates of *E. coli* JM04 expressing MalE (lanes 1), MalE-IpaB1 (lanes 2), MalE-IpaB2 (lanes 3), and MalE-IpaB3 (lanes 4) were analyzed by Western blot with anti-MalE antibodies (A), a human serum (B), and a MAb directed against epitope II used as a control (C). Bands corresponding to each fusion protein are indicated by large arrowheads. Molecular mass markers (in kilodaltons) are shown (small arrowheads).

11 sera were collected from patients infected by a *Shigella* sp. in New Caledonia; the species has not been determined.

Except for one serum sample collected from a patient infected by an undetermined *Shigella* species, which recognized both MalE-IpaB2 and MalE-IpaB3 (data not shown), all sera recognized only one fusion protein, MalE-IpaB2, which includes epitopes I, II, and III, as shown for one representative serum sample in Fig. 4B. When reactivities of human sera were tested with MalE fusion proteins in nondenaturating conditions (dot blotting without SDS), similar results were obtained (data not shown). These data suggest that we have identified the major immunogenic domain of IpaB, not only during experimental immunization of rabbits and mice but also in the course of natural infection in humans.

On the basis of the overlap in sequence among the three MalE fusion proteins (Fig. 3B), we concluded that the major immunogenic domain of IpaB was located between aa 147 and 258. As shown in Fig. 3C, this corresponds to the most hydrophilic region of IpaB.

DISCUSSION

To evaluate the basis for the strong immunogenicity of IpaB and to further define the functional domains of this invasin, its B-cell epitopes were characterized. For this purpose, an *ipaB* λgt11 expression library was constructed and immunoreactive clones were selected with specific rabbit antiserum and murine MAbs. By both sequence analysis of the selected clones and competition assays of binding of MAbs to IpaB, we identified three distinct epitopes which lie within the amino-terminal half of IpaB. This domain is a part of a larger region previously described by Mills et al. (19) as a region encoding several antigenic determinants. In our study, IpaB was partially purified in denaturating conditions (SDS-PAGE), whereas in the former study, the protein was recovered after a preparative isoelectric focusing procedure (nondenaturating conditions). It is interesting to note that both approaches led to the obtention of MAbs which exclusively recognize epitopes localized within the amino-terminal domain.

We have shown that IpaB epitopes I, II, and III are relevant during natural infection in humans. Using three fusion proteins expressing each a different part of IpaB, we demonstrated that the serum immune response is predominantly directed against an immunodominant sequence consisting of aa 147 to 258, in which epitopes I, II, and III are all clustered. The absence of reactivity with the other parts of IpaB is really due to the absence in the human sera of antibodies recognizing epitopes located within these regions, since the reactivities of the human sera tested with the fusion proteins in denaturating versus nondenaturating conditions were found to be similar.

Only 1 serum among the 28 sera that we tested recognized both the immunodominant domain and the carboxy-terminal part of IpaB. Oaks and Turbyfill (21) have described a conformational epitope of IpaB which is not located within this immunodominant domain and consists of three distinct noncontiguous regions comprising aa 99 to 110, 136 to 148, and 170 to 181. Unfortunately, no data about the recognition of such an epitope by human sera are available; however, it is recognized by sera from infected monkeys. Taken together, these results indicate that B-cell clones producing antibodies against epitopes localized elsewhere on IpaB are elicited during natural infection. Nevertheless, our results provided by the study of human sera from patients infected with different *Shigella* species (*S. flexneri*, *S. sonnei*, *S. bodyi* and *S. dysenteriae*) in different parts of the world suggest that such B-cell clones are scarcely produced.

IpaB is highly conserved, not only among serotypes, but also among *Shigella* species. For example, only 9 aa residues differ between IpaB in *S. flexneri* 5 and IpaB in *S. dysenteriae* 1 (31). Two of these changes are located within the major immunogenic domain that we have characterized. These findings may explain that immune sera from patients infected with one *Shigella* species also recognize the major immunogenic domain of other species.

The immunogenicity of IpaB may be related to its localization and its conformational structure within the bacteria. The invasin is both present at the bacterial surface (2, 30) and secreted in culture supernatants (1, 2, 30). MAbs directed against epitopes I, II, and III reacted with bacteria in a whole-cell ELISA (3), suggesting that these epitopes and therefore the immunodominant domain actually are accessible on the surface of the bacteria. No data about the structure of IpaB are yet available. Some predictions of secondary structure indicate that IpaB may interact with cell membranes and may exist as a multimer (24). The hydropathy profile reveals that the major immunogenic domain is located within the most hydrophilic region of IpaB, which is flanked by a hydrophobic and an amphipathic segment. This hydrophilic domain might be the only part of the protein that is accessible, the remainder of the protein being anchored in the cell membrane or buried in a complex. Whether this immunodominant domain is involved in the entry of bacteria into the cells and lysis of the phagocytic membrane vacuole remains to be determined.

It will be important to determine whether epitopes I, II, and III elicit antibodies protective against *Shigella* invasion. Inhibition or reduction of *S. flexneri* invasion in the presence of selected MAbs is currently being tested, in vitro in HeLa

cell invasion assays and in vivo in the guinea pig keratoconjunctivitis assay (Sereny test). To complement our understanding of the immune response to IpaB, it will be interesting to explore in more detail the mucosal immune response, as it has been suggested that anti-Ipa secretory IgA antibodies limit the severity of the illness (22). Answers to such questions are essential to the development of orally administered anti-invasive subunit vaccines.

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