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IpaB, a *Shigella flexneri* Invasin, Colocalizes with Interleukin-1β-Converting Enzyme in the Cytoplasm of Macrophages

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Shigellae are the most prevalent etiological agents of dysentery. A crucial step in shigella pathogenesis is the induction of macrophage apoptosis. The invasion plasmid antigen B (IpaB) is necessary and sufficient to induce macrophage programmed cell death. IpaB activates apoptosis by binding to interleukin-1β (IL-1β)-converting enzyme (ICE) or a highly homologous protease. Here, we show that IpaB is disseminated throughout the cytoplasm of shigella-infected macrophages as detected by both immunofluorescence and immunoelectron microscopy. The cytoplasmic distribution of IpaB requires phagosome escape, and it is specific to IpaB, since lipopolysaccharide, used here as a bacterial marker, remains closely associated with the bacteria. In double-labeling experiments, we show that IpaB and ICE colocalize in the cytoplasm of the macrophage, suggesting that soon after secretion, IpaB binds to ICE to initiate apoptosis and to promote the cleavage of IL-1β.

Bacillary dysentery is a severe bloody form of diarrhea which is prevalent in underdeveloped countries and, if left untreated, can be fatal in children. Shigellae, the major etiological agents of dysentery, are gram-negative rods of the family *Enterobacteriaceae* that invade the colonic submucosa. In its endemic form, the disease is primarily caused by *Shigella flexneri* (5, 22).

A current model for the onset of shigellosis can be summarized as follows: (i) bacterial entry into the colonic mucosa through M cells; (ii) colonization of the lymphoid follicles, which are thickly populated with macrophages; (iii) phagocytosis of the bacteria by macrophages, followed by the escape from the phagolysosome into the cytoplasm of these cells; (iv) induction of apoptosis in macrophages; (v) concurrent release of interleukin-1 (IL-1); and ultimately (vi) initiation of inflammation leading to tissue damage and further bacterial invasion. Thus, it appears that macrophage apoptosis is a crucial point during the onset of shigellosis (22).

Shigella invasion and cytotoxicity genes are located on a plasmid encoding the invasion plasmid antigen (Ipa) operon and a type III secretion apparatus. The secreted invasins IpaB, IpaC, and IpaD are encoded on this operon (7). IpaB and IpaC are sufficient to promote bacterial invasion (9). IpaB, a 62-kDa protein, is necessary and sufficient to induce programmed cell death in macrophages (1, 21).

Recent findings have shown that IpaB binds to IL-1β-converting enzyme (ICE) or a close homolog. This binding is crucial in the induction of apoptosis in macrophages infected with virulent *S. flexneri* (1). ICE activation is involved in both maturation of IL-1β and apoptosis and was originally identified as a cysteine protease capable of cleaving IL-1β to its mature form. This protease is highly homologous to the product of *ced3*, a *Caenorhabditis elegans* gene crucial for developmental cell death. There are now more than 10 ICE/*ced3* product homologs that have been identified in mammals and that can be divided into two large groups based on sequence homology and substrate specificity. The prototypes for these two families are ICE itself and CPP32 (also called apopain and yama). All of these homologs induce apoptosis when overexpressed in mammalian cells, but only ICE cleaves IL-1β (3, 6, 19). In shigella infections the dual function of ICE in cell death and as a proinflammatory molecule is evident (1).

Subcellular localization of IpaB in infected macrophages. To elucidate the mechanism by which IpaB induces apoptosis, we determined the cellular localization of secreted IpaB in infected macrophages. We stained the cell for IpaB by indirect immunofluorescence with a precleaned anti-IpaB rabbit polyclonal antiserum (kindly provided by Armelle Phallipon, Institut Pasteur) and for the bacteria and the nucleus with the DNA-binding dye propidium iodide (PI) (4). Cells were infected for 20, 40, and 60 min with either the wild-type strain of *S. flexneri*, M90T (15), or the negative control BS176, an isogenic strain that lacks the pathogenicity plasmid (14), and processed for immunofluorescence as described previously (1).

The slides were analyzed with a Molecular Dynamics confocal microscope. The optical sections were filtered with an alpha filter and reconstructed in three-dimensional projections (Fig. 1). Both the macrophage nuclei and the bacteria are identified (Fig. 1B, D, F, and H). IpaB was detected by indirect immunofluorescence with a secondary antibody labeled with fluorescein (Fig. 1A, C, E, and G). IpaB was detected in the cytoplasm of macrophages infected with the wild-type strain of *S. flexneri* (M90T) 20 min after infection (Fig. 1C and D). This bacterial protein was highly abundant in proximity to the bacteria and is also seen distributed throughout the cytoplasm of the macrophage, appearing as discrete aggregates. The amount of IpaB present in the cytoplasm of cells is in direct relation to the number of bacteria in the cell: heavily infected cells present more IpaB than cells infected with few bacteria. Cells infected with the plasmid-cured nonpathogenic strain BS176 were not stained by the anti-IpaB antibody (Fig. 1A and B).

Cells infected for 40 min (Fig. 1E and F) showed a pattern similar to that of cells infected for 20 min. Interestingly, 60 min after infection there is a smaller amount of antigen in cells whenever nuclear apoptotic morphology is apparent (Fig. 1G and H). The absence of IpaB in apoptotic cells might be due to the leakiness of the dying cell's plasma membrane.

Localization of IpaB in macrophages with immunoelectron microscopy. In order to determine whether IpaB was localized to specific organelles in the macrophage's cytoplasm, we in-

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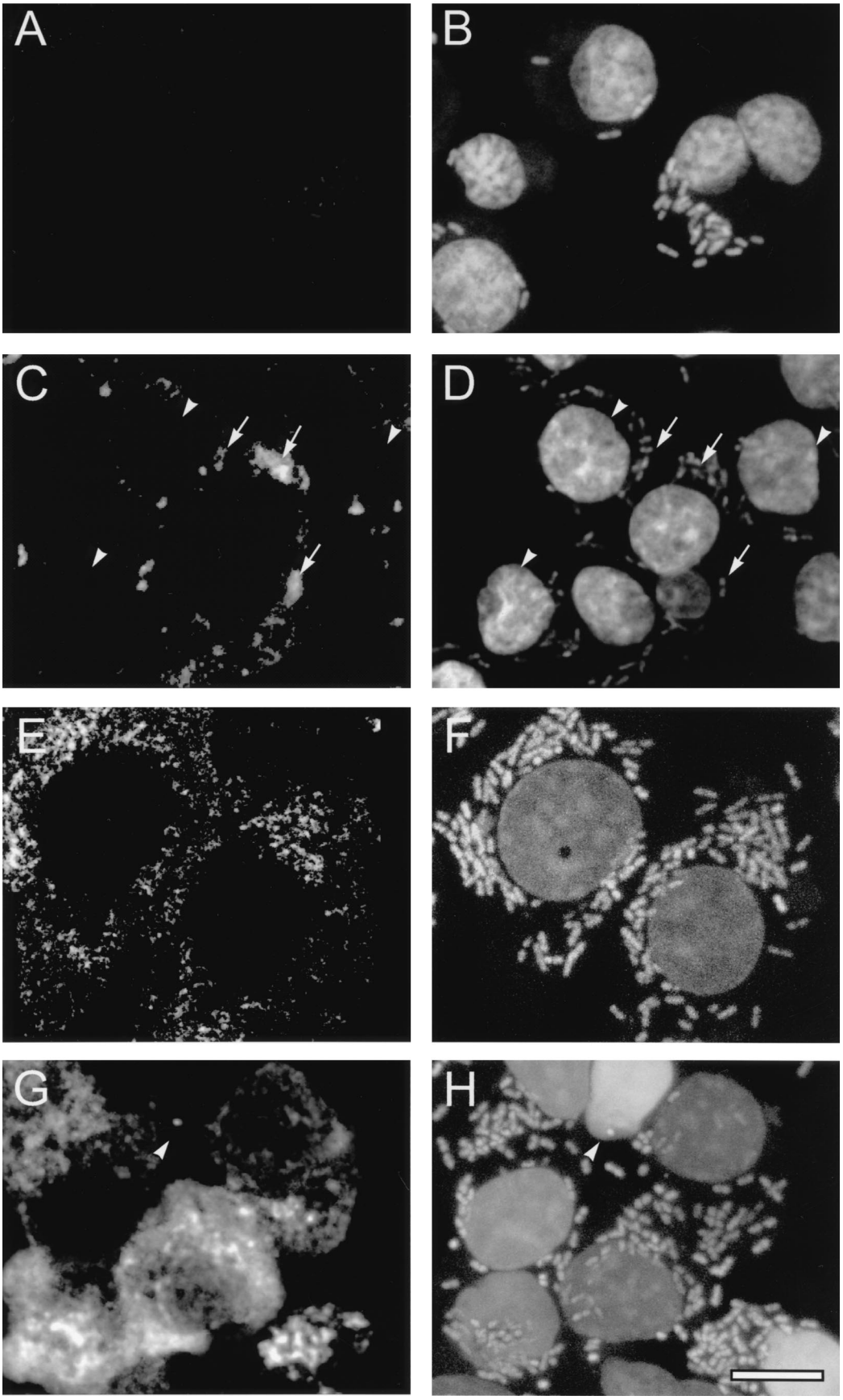
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FIG. 1. Subcellular localization of IpaB in *S. flexneri*-infected J774 macrophages. IpaB was detected by indirect immunofluorescence with fluorescein (A, C, E, and G), and both macrophage nuclei (arrowheads) and bacteria (arrows) were stained with the DNA-binding dye (B, D, F, and H). Exactly the same field is shown for each pair, with arrows and arrowheads localized to the same positions. (A and B) Cells infected with the nonpathogenic, plasmid-cured strain BS176 for 20 min show no immunoreactivity against IpaB. (C and D) Macrophages infected with wild-type strain M90T for 20 min. IpaB is abundant on the bacteria and is also distributed distally from the bacteria, appearing as aggregates. (E and F) J774 cells infected with M90T for 40 min. The distribution of IpaB is similar to that in panel C, although there is less immunoreactive material close to the bacteria. (G and H) Macrophages infected with M90T for 60 min. IpaB appears to be less abundant in cells undergoing apoptosis, identified by the chromatin condensation in the nuclei (arrowheads). Bar = 10 μm.

fected cells with shigella strains for 20 min and processed them for immunoelectron microscopy. Infected cells were fixed in a solution of glutaraldehyde (0.4%) and paraformaldehyde (4.0%), dehydrated, and embedded in LR White. Sections were blocked in ovalbumin (0.5%) incubated with rabbit polyclonal antibody against IpaB containing 0.2% sodium azide, 0.1% Triton X-100, 0.1% Tween 20, and 0.5% bovine serum albumin for 90 min. The samples were rinsed and incubated

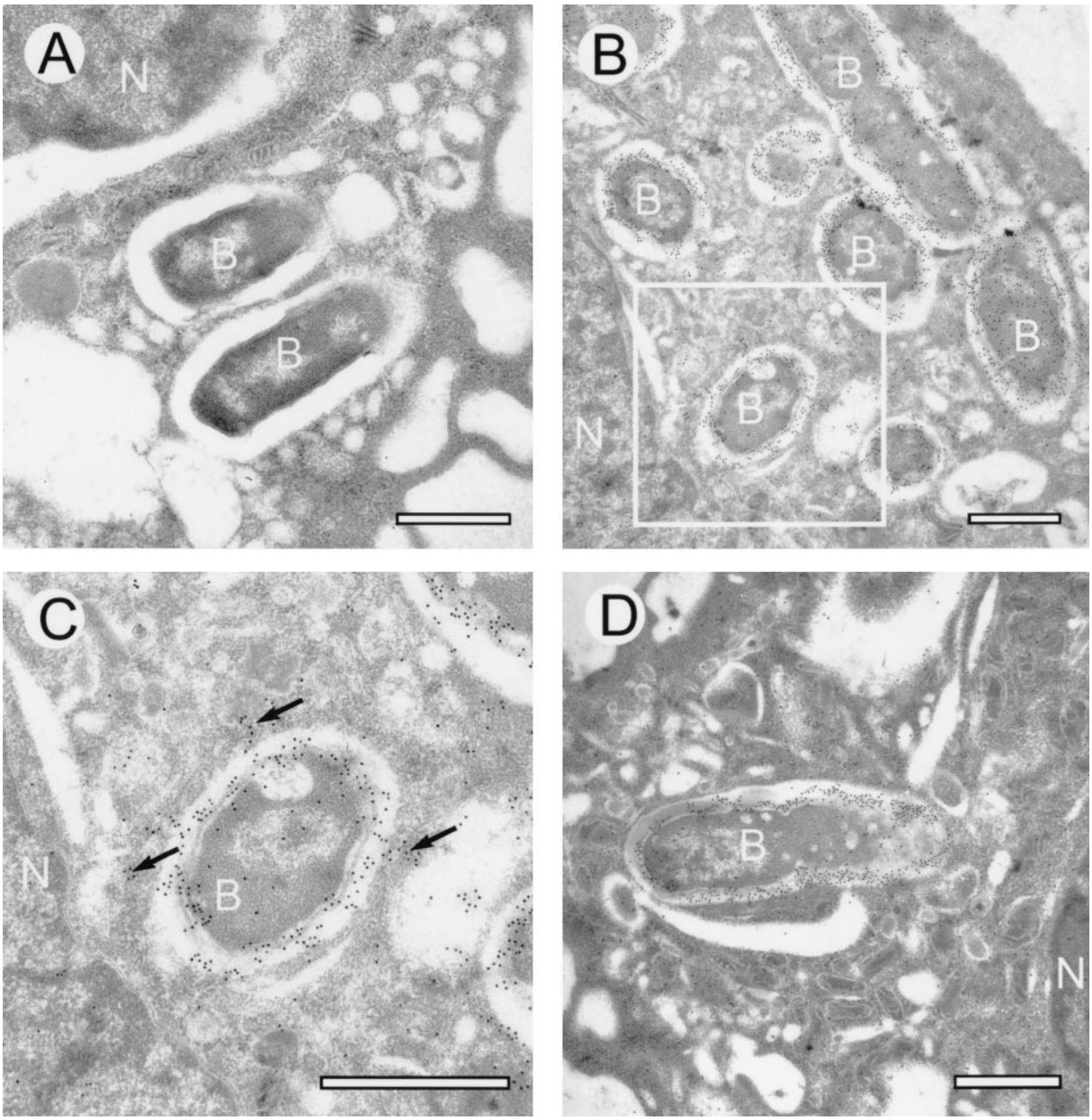

FIG. 2. Immunoelectron localization of IpaB in the cytoplasm of *S. flexneri*-infected macrophages. J774 cells were infected for 20 min with shigella strains and processed for immunoelectron microscopy as described in the text. (A) Macrophages infected with plasmid-cured strain BS176; (B) J774 cells infected with wild-type strain M90T; (C) higher magnification of area outlined in panel B; (D) a different macrophage infected with M90T. Gold particles show that IpaB is abundant in the area surrounding the bacteria and it is also localized in the cytoplasm of the macrophage (arrows). There is no specific association of IpaB with a particular organelle. IpaB was not detectable in cells infected with control strain BS176. N, nucleus; B, bacterium. Bars = 1 μm.

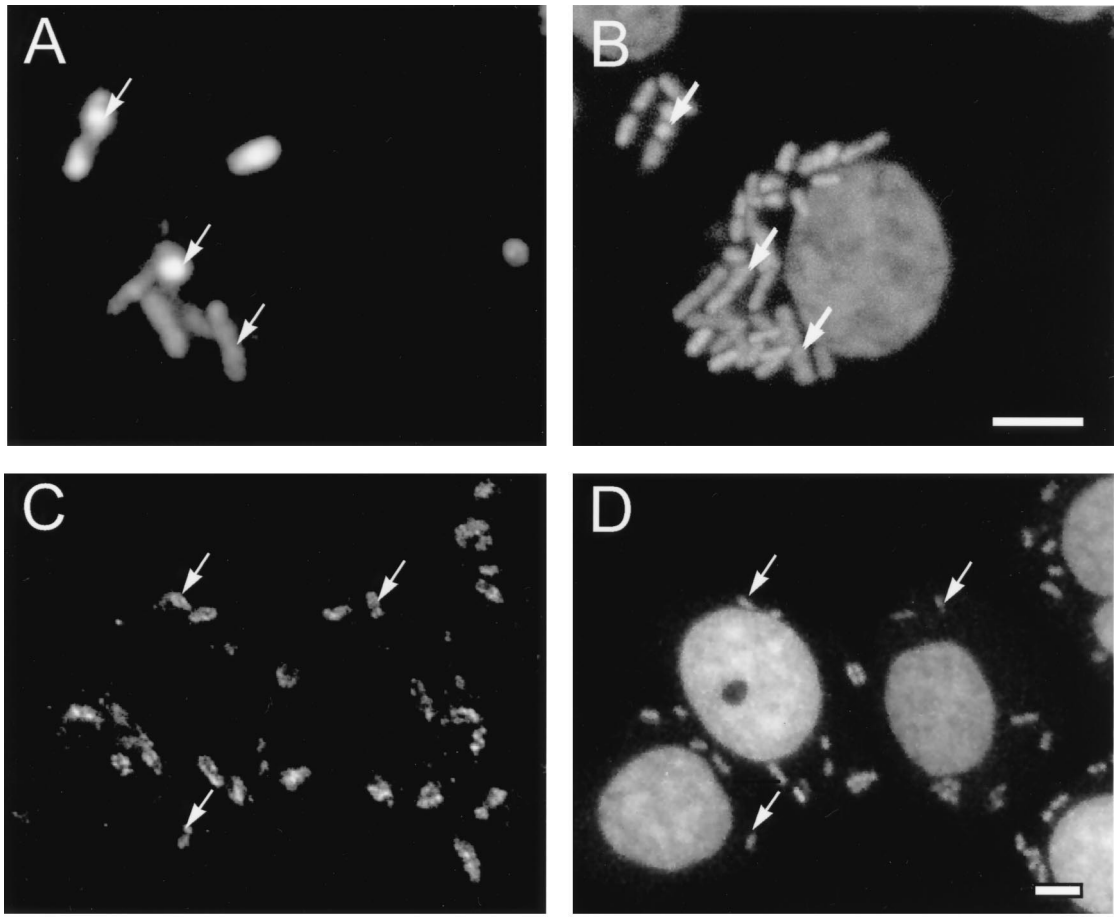

FIG. 3. The cytoplasmic localization of IpaB in the macrophage is specific to this invasin. J774 cells were infected with shigella, and IpaB or LPS was detected by indirect immunofluorescence with fluorescein (A and C). Both macrophage nuclei and bacteria (arrows) were stained with the DNA-binding dye PI (B and D). Exactly the same field is shown for each pair, with arrows localized to the same positions. (A and B) J774 cells were infected with a Δ*ipaD* strain for 60 min. The Δ*ipaD* mutant is not invasive and cannot escape the phagolysosome of macrophages but produces normal amounts of IpaB, which was localized exclusively to the bacteria in these cells. Interestingly, not all the bacteria stained positively for IpaB. (C and D) J774 cells infected with the wild-type strain M90T for 60 min. LPS immunoreactivity colocalized completely with bacteria, demonstrating that the cytoplasmic localization of IpaB is specific, since other bacterial components are not disseminated. Bars = 5 μm.

with protein A conjugated with gold colloidal particles (15 nm) for 1 h. After a final wash the sections were counterstained with uranyl acetate and lead citrate and observed under a Siemens Elmiskop 1A electron microscope.

Very few gold particles were detected in macrophages which were infected with BS176 (Fig. 2A). In contrast, abundant gold particles were present in cells infected with M90T. The distribution of IpaB was similar to that observed with immunofluorescence: IpaB was abundant on the bacterial surface and was also present free in the cytoplasm (Fig. 2B through D). Figure 2C shows what appear to be small clusters of IpaB in the cytoplasm of the macrophage. The gold particles do not colocalize with any organelle or membrane, indicating that IpaB is free in the cytoplasm.

We quantified the number of gold particles in different intracellular compartments in cells infected with either BS176 or M90T. Eight independent micrographs of cells infected with M90T and six of macrophages infected with BS176 were mapped using the National Institutes of Health image program. We counted the number of gold particles and calculated the areas of three different sections of each micrograph: bacteria, cytoplasm, and background. In M90T-infected cells the numbers (means ± standard deviations) of gold particles were 13.3 ± 4, 1.0 ± 0.4, and 0.4 ± 0.3 particles/μm^2 and in BS176-infected cells there were 0.05 ± 0.04, 0.06 ± 0.06, and 0.03 ± 0.07 particles/μm^2, which corresponded to bacteria, cytoplasm, and background, respectively. The number of gold particles directly on the bacteria in M90T-infected cells was significantly different from the labeling either of bacteria in BS176-infected cells ($P = 0.0001$) or of the background in M90T-infected cells ($P = 0.0001$) as determined by the nonparametric Mann-Whitney statistical test. The cytoplasm of macrophages infected with M90T was also different both from the cytoplasm of BS176-infected cells ($P = 0.0061$) and from the background of micrographs of M90T-infected cells ($P = 0.0281$). Taken together, these data indicate that the cytoplasmic label is specific in M90T-infected cells. There was no specific association of IpaB with a particular organelle. Interestingly, most IpaB localized to the interface between the bacterium and the cytoplasm. It is possible that the interaction between IpaB and ICE or an ICE homolog actually happens at this interface.

IpaB localizes specifically to the cytoplasm of macrophages. To determine the specificity of the cytoplasmic labeling with anti-IpaB antibodies in wild-type-shigella-infected cells, we investigated the distribution of IpaB in macrophages infected with the Δ*ipaD* strain (10). This strain, which is not invasive or

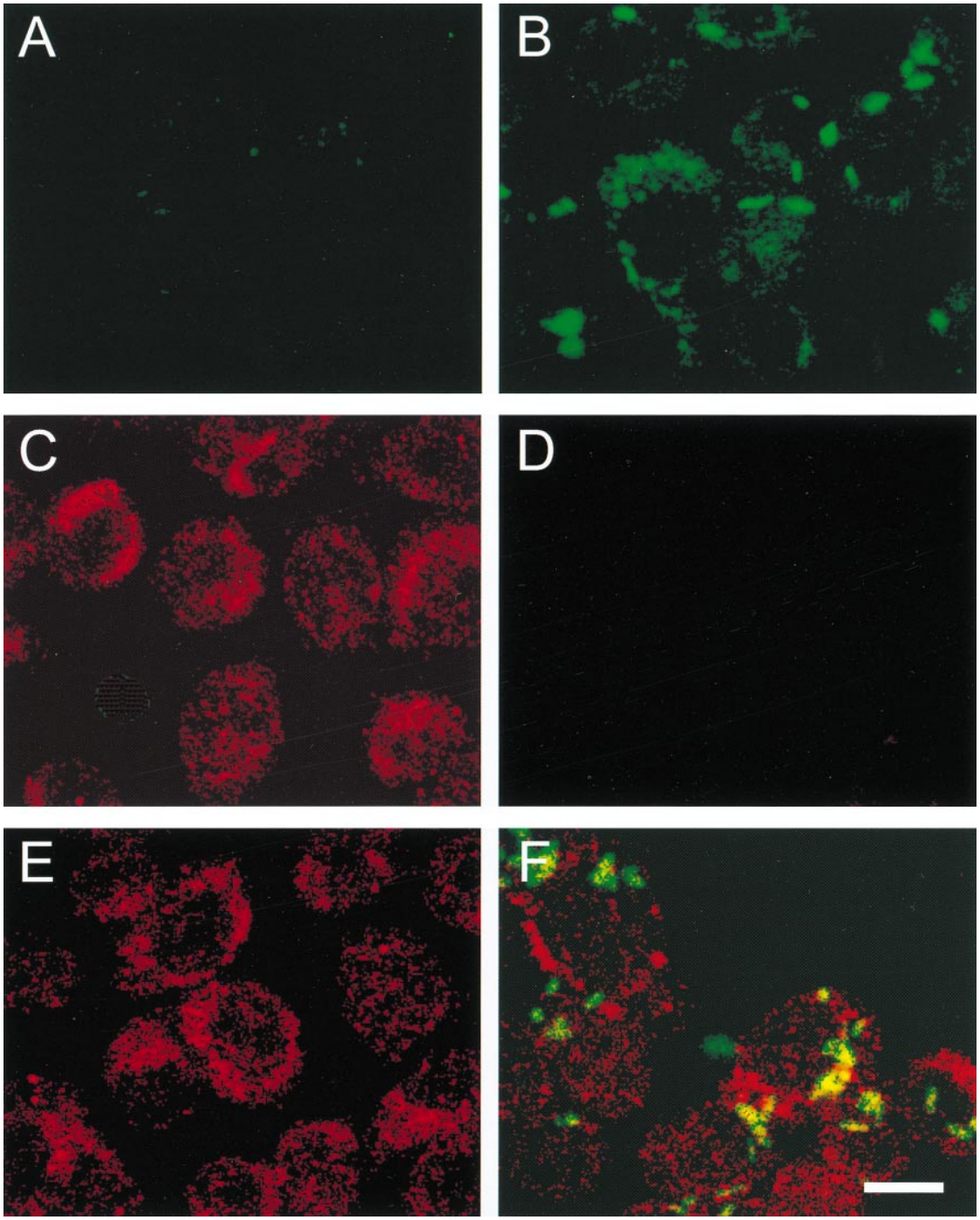

FIG. 4. IpaB colocalizes with ICE. J774 cells were infected with shigella, and IpaB or ICE was detected by indirect immunofluorescence with fluorescein or Texas red, respectively. (A) As a control, macrophages were infected with a Δ*ipaB* strain complemented with *ipaB* and stained with an anti-Flag antibody and a secondary fluoresceinated antibody. As expected, there was no immunoreactivity in these infected cells. (B) J774 cells infected with a Δ*ipaB* strain complemented with *flag-ipaB* and stained with an anti-Flag antibody. The subcellular localization of the Flag-IpaB chimeric protein is similar to that of wild-type IpaB (Fig. 1), which was localized to the bacteria and to the macrophage cytoplasm. (C) J774 cells were stained with an anti-ICE antibody. ICE appears to be distributed through the cytoplasm of the macrophages, and some aggregates are evident. (D) J774 cells were stained with an anti-ICE antibody, which was competed with the immunogenic peptide. The specificity of the ICE antibody was demonstrated by blocking the immunostaining with a peptide specific for the recognition site of the anti-ICE antibody. (E) J774 cells infected with a Δ*ipaB* strain complemented with *ipaB* were double stained with anti-ICE and anti-Flag antibodies. (F) J774 cells infected with a Δ*ipaB* strain complemented with *flag-ipaB* were double stained with both anti-ICE and anti-Flag antibodies. Arrows indicate areas where IpaB and ICE colocalize. Bar = 10 μm.

cytotoxic, produces and secretes normal amounts of IpaB but cannot escape from the phagocytic vacuole into the cytoplasm of the macrophage. Sixty minutes after infection, IpaB localized exclusively to the bacteria (Fig. 3A and B). Surprisingly, only a few intracellular bacteria were labeled with the anti-IpaB serum. These results might indicate either that IpaB is down-regulated or that IpaB is rapidly degraded inside the macrophages' phagolysosome.

To determine the subcellular localization of a different bacterial macromolecule within infected macrophages, we tested the localization of lipopolysaccharide (LPS) with a polyclonal anti-*S. flexneri* serotype V serum. LPS remained closely associated with the invasive bacteria even 60 min after infection (Fig. 3C and D). Similar distributions were observed with the wild-type, plasmid-cured Δ*ipaB* (10) and Δ*ipaD* strains (data not shown). These results suggest that the widespread distribution of IpaB in the cell is specific and dependent on the bacteria escaping the phagosome and reaching the host cell cytoplasm. Dacosta et al. (2) showed that LPS was present in vacuoles of macrophages infected with a noninvasive derivative of *Shigella dysenteriae* 4 h after infection, but these investigators did not determine the fate of invasins under these conditions. Further studies on the expression and stability of shigella virulence factors within macrophage phagolysosomes are necessary to determine why some bacteria contained in phagosomes are not labeled with the anti-IpaB antibody.

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are capable of secreting and translocating two virulence factors, YopE and YopH, into the cytoplasm of HeLa cells without bacterial penetration (11, 12, 17). In contrast, the results presented here show that shigella does not "inject" IpaB into the macrophage's cytoplasm; rather, it has to deliver this protein after the bacterium escapes from the phagosome. Furthermore, we have previously shown that IpaB only partially colocalized with the endosome-phagolysosome marker lysosome-associated membrane protein 1 (LAMP-1) (1), indicating that shigella escapes from the phagosome very soon after infection and secretes IpaB directly into the host cell cytoplasm. Interestingly, YopE and YopH as well as IpaB are secreted through highly homologous type III secretion apparatuses (13, 18). The difference in invasin delivery among these bacteria might reflect important differences in virulence factor secretion.

IpaB colocalizes with ICE in the cytoplasm of macrophages. We have recently shown that IpaB induces apoptosis by binding to ICE (1). We investigated whether IpaB and ICE colocalize in infected cells. Both the anti-IpaB and the commercially available anti-ICE antisera were made in rabbits, making double-labeling experiments difficult to control. Thus, the experiment was done with an anti-ICE rabbit polyclonal and an anti-Flag murine monoclonal antibody. We tagged IpaB with the Flag epitope by PCR from the plasmid p179 (8). The PCR product was cloned into pUC19 (New England Biolabs, Beverly, Mass.) and transferred to a Δ*ipaB* strain (10). Flag-IpaB was expressed in shigella, as determined by Western blot with the M2ab monoclonal antibody (International Biotechnology, Inc., New Haven, Conn.), and complemented the macrophages' cytotoxicity (data not shown), indicating that the Flag-tagged IpaB was a functional protein.

To determine the specificity of the anti-Flag antibody, we infected J774 cells with a Δ*ipaB* strain complemented with *flag-ipaB* and labeled the cells with anti-Flag antibody. As shown in Fig. 4B, these cells were clearly labeled with the anti-Flag antibody. Furthermore, the distribution of Flag-IpaB was very similar to the distribution of wild-type IpaB detected with the anti-IpaB antiserum shown in Fig. 1. As expected, the anti-Flag antibody did not recognize any epitope in cells infected with a Δ*ipaB* strain complemented with *ipaB* (Fig. 4A).

ICE and its homologs are made as proenzymes of around 45 kDa that are subsequently cleaved to generate two subunits of 20 and 10 kDa. A current model proposes that two 20-kDa subunits and two 10 kDa subunits oligomerize to form the active enzyme (3, 6, 19). Noninfected macrophages were stained with an anti-ICE (10-kDa subunit) antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.). ICE was localized to the cytoplasm of J774 cells (Fig. 4C). This distribution corresponds to the localization of ICE in activated monocytes reported by Singer et al. (16). As a control for the specificity of the anti-ICE antibody, we incubated the cells in the presence of the anti-ICE antibody and a peptide (20 μg/ml; Santa Cruz Biotechnology) with the sequence that corresponds to the recognition site of the antiserum. This competing peptide completely inhibited the binding of the ICE antibody to the cells as shown in Fig. 4D, thereby demonstrating the specificity of the staining.

To localize IpaB and ICE, we infected cells with a Δ*ipaB* strain complemented with *flag-ipaB* and then incubated with anti-ICE and anti-Flag antibodies (Fig. 4F). The staining patterns for ICE and Flag-IpaB are distinct, demonstrating the specificity of the antibodies. Nevertheless, these two molecules colocalize in certain areas of the cytoplasm, especially in close proximity to bacteria. Some of the colocalization regions are indicated in Fig. 4F. As a control, we double stained J774 cells infected with a Δ*ipaB* strain complemented with *ipaB* (Fig. 4E). The distribution of ICE is similar to the one shown in Fig. 4C, in which the cells were stained only with anti-ICE antibody, indicating that *Shigella* invasion does not lead to ICE redistribution. These cells were not labeled with the anti-Flag antibody.

Interestingly, IpaB and ICE colocalize in various areas of the infected cells. Most of the IpaB-ICE colocalization appears to be in association with bacteria, where IpaB is abundant. These data indicate that IpaB and ICE interact soon after the shigella escapes from the phagolysosome. Moreover, the immunoelectron micrographs shown in Fig. 2 indicate that IpaB is released into the cytosolic compartment where ICE resides and is most abundant on the bacterium-cytoplasm interface. Thus, productive interactions between IpaB and ICE-like molecules could take place both in solution in the cytoplasm and in association with bacteria.

Taken together, the data presented in this report correlate with our previous observations that shigella induces apoptosis very soon after macrophage infection (23) and that shigella-induced apoptosis proceeds as a consequence of interaction with ICE (1). The sequence of events in the interaction of shigella and its host macrophage are likely to be as follows: (i) phagocytosis of shigella, (ii) bacterial vacuolar escape, (iii) secretion of IpaB, (iv) distribution of IpaB through the macrophage's cytoplasm, (v) binding and activation of ICE, and (vi) induction of apoptosis and cleavage of IL-1β (20). Thus, the dissemination of IpaB in the cytoplasm of macrophages is a crucial step in the interaction of this pathogen with the host.

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