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Induction of Adherence and Degranulation of Polymorphonuclear Leukocytes: a New Expression of the Invasive Phenotype of *Shigella flexneri*

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In the present study, the ability of *Shigella flexneri* to activate polymorphonuclear neutrophils (PMN) was examined. The invasive serotype 5 strain M90T induced strong PMN adherence, which was dependent on both the multiplicity of infection and the duration of incubation. When tested under the same experimental conditions, the noninvasive strain BS176 (cured of the 220-kb virulence plasmid) was less efficient. Indeed, incubation of PMN for 2 h with either M90T or BS176 (multiplicity of infection, 100) induced 51.8% ± 10.5% and 15.2% ± 4.2% adherence, respectively ($n = 3$; $P < 0.05$). Stronger PMN activation by M90T was confirmed by evaluating PMN degranulation induced by the two strains. Whereas M90T triggered significant PMN secretion, BS176 did not. M90T strains with mutations in *ipa* genes were then analyzed. When PMN were incubated with these mutants, their activation was of the same intensity as that obtained with BS176. These data provide the first evidence for PMN activation induced by *S. flexneri*, a process which appears to be mediated by Ipa invasins.

Shigellosis is an invasive infection of the colonic epithelium characterized by the formation of abscesses and ulcerations of the mucosa (27). Understanding the process by which the causative gram-negative bacillus *Shigella* invades the mucosa and causes inflammation-mediated destruction of the tissues is of primary importance. It is now recognized that Ipa invasins are involved in such a phenomenon. Indeed, these proteins, which are secreted upon contact of bacteria with eukaryotic cells (14), seem to be essential in triggering the massive cellular cytoskeletal rearrangements that cause bacterial entry via the formation of membrane protections achieving a macropinocytic event (1). Following entry, Ipa invasins also appear to play an essential role in achieving lysis of the vacuolar membrane, a crucial step allowing shigellae to escape into the cytoplasm of infected cells (9, 23).

Inflammation in dysentery is initiated by basolateral invasion of epithelial cells by *Shigella flexneri* (11, 16) and is characterized by strong mucosal infiltration by polymorphonuclear neutrophils (PMN) preceding infection and destruction of epithelial cells (20). PMN may therefore destabilize epithelial cohesion and facilitate bacterial access to their invasion zone. Such a process differs from the classical scheme of PMN involvement during infections (26). Indeed, if these cells are recognized to play a crucial role in the pathogenesis of inflammation, their primary function concerns host defenses. This is illustrated by the observation that neutropenic patients often present gram-negative bacilli and staphylococcal infections (29). During phagocytosis of microorganisms, the content of cytosolic granules is normally released in phagolysosomes. However, under particular conditions such as "regurgitation during feeding" or "frustrated phagocytosis," microbicidal products could be released in the extracellular environment, leading to tissue destruction (31). In the case of *S. flexneri*, it was demonstrated that PMN facilitate epithelial invasion by opening the paracellular pathway, thus allowing basolateral entry of bacteria (20). In these conditions, recruitment of PMN is advantageous for bacteria in the host-parasite relationship, as already suggested (10).

The transmigration of PMN, which was shown in cell culture studies to be an early step of the cascade of events leading to *S. flexneri* invasion, may be elicited by bacteria themselves (20). However, it has not been determined whether the target of bacterial products was PMN or epithelial cells. Indeed, PMN transmigration might be initiated by the enhancement of adherence molecule expression on the epithelial cell surface. Alternatively, bacteria could induce cytokine and/or chemokine secretion from epithelial cells, thus causing PMN chemotaxis. Nevertheless, recent data (15) have shown that the synthesis of mRNAs of cytokines, such as interleukin 1 (IL-1), IL-6, IL-8, and tumor necrosis alpha, and adherence molecules, such as ICAM-1, are not modified by *S. flexneri* exposure of the apical surface of T84 human colonic epithelial cells grown on filters. It is known, however, that during this period, apical bacteria are able to induce strong transmigration of human PMN through the epithelial layer (20), thus indicating that a direct effect of bacteria or soluble bacterial products may account for increased PMN activity. Therefore, the purpose of this work was to investigate whether PMN are directly activated by *S. flexneri.*

MATERIALS AND METHODS

Bacteria and growth conditions. Bacteria were routinely grown in Trypticase soy broth or on Trypticase soy agar plates (Diagnostics Pasteur, Marnes la Coquette, France).

The following bacteria were used in this study. M90T is an invasive isolate of *S. flexneri* belonging to serotype 5. BS176 is a noninvasive derivative of M90T which has been cured of its 220-kb virulence plasmid (22). *ipa*B and *ipa*C mutants have been obtained by in-frame insertion of a gene cassette in either of these two genes (13). The Δ*ipa* mutant consists of a large deletion of almost the entire *ipa* operon (18).

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Before infection of PMN was performed, bacteria were washed and resuspended to the desired density in Dulbecco modified Eagle's medium (Gibco, Paisley, Scotland) supplemented with thiamine (1 mg/ml), and HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Sigma Chemical Company, St. Louis, Mo.) (15 mM, pH 7.4).

Isolation of human PMN. PMN were isolated from human blood from healthy volunteers (Centre National de Transfusion Sanguine, Paris, France) collected over ACD solution [citrate monohydrated (3.27 g/liter); citrate $(Na)_2$ dihydrated (26.3 g/liter); phosphate (Na) dihydrated (2.51 g/liter); dextrose (23.2 g/liter); pH 5.63]. Briefly, blood was mixed with dextran (Sigma) at a final concentration of 1% and allowed to sediment for 30 min at room temperature. The resulting supernatant was layered over Ficoll-Paque (Pharmacia, Uppsala, Sweden) at 1 volume for 2 volumes of cell suspension and then centrifuged (350 × *g*; 45 min). Erythrocytes remaining in the pellet were lysed by the addition of a lysis buffer [155 mM NH_4Cl, 2.96 mM $KHCO_3$, 3.72 mM $EDTA(Na)_2$]. After gentle agitation for 5 min, the cell suspension was centrifuged (350 × *g*; 10 min) and washed twice with Hanks' balanced salt solution (HBSS) without $CaCl_2$ and $MgCl_2$ (Gibco). Finally, the leukocyte pellet was resuspended in HBSS to reach a cell concentration of 6×10^5 PMN per ml. The viability of recovered PMN was 98.5% ± 1.2%, as determined by the Trypan blue dye exclusion method, and their purity, evaluated with Türk's stain, was 96.4% ± 1.9%.

Adherence assays. Culture plates (96-well; Plastic Products AG, Trasadingen, Switzerland), coated for 1 h with heat-inactivated fetal calf serum (50 μl per well; Boehringer GmbH, Mannheim, Germany) and washed twice with phosphate-buffered saline (PBS), were used as support for PMN adherence. To evaluate the effect of bacteria on PMN adherence, PMN (6×10^4 per well) and bacteria (6×10^5 to 6×10^8 per well) were mixed together and adherence was allowed to proceed for various incubation times at 37°C in a 5% CO_2 atmosphere. The multiplicity of infection (MOI), which corresponds to the number of bacteria added per individual PMN, thus ranged between 10 and 1,000. In some experiments, PMN were incubated without bacteria and stimulated with *N*-formyl-Met-Leu-Phe (FMLP; Sigma) as a control. Nonadherent cells were then removed by two washings with PBS supplemented with 0.1% bovine serum albumin (BSA; Euromedex, Strasbourg, France), and adherent PMN were quantitated according to the method of Bath et al. (4). Briefly, cells remaining in wells were lysed by the addition of 100 μl of hexadecyltrimethylammonium bromide (HTAB) (0.5%; Sigma) diluted in HBSS-BSA, and their myeloperoxidase (MPO) content was determined by adding 100 μl of dianisidine dihydrochloride (0.2 mg/ml; Sigma) and hydrogen peroxide (0.4 mM; Sigma) in PBS-BSA, pH 6. Following a 2- to 5-min incubation, the optical density at 450 nm was measured (Dynatech MR 5000; Dynatech Laboratories, St. Cloud, France). The values obtained were plotted on a standard curve (range of concentrations, 0 to 5×10^4 PMN per well) to determine the number of adherent cells.

PMN degranulation. Release of MPO was selected as a marker of PMN degranulation. PMN (6×10^4 per well) were incubated at 37°C in a 5% CO_2 atmosphere with bacteria (6×10^5 to 6×10^8 per well) or FMLP as a control. In some experiments, cytochalasin B was added (5 μg/ml; Sigma). At the end of the incubation period, the reaction was stopped by centrifugation (80 × *g*; 6 min). Resulting supernatants containing the released enzyme were collected and transferred to another 96-well culture plate to perform the assay.

MPO activity was measured spectrophotometrically by using the same substrate as that used for the adherence assay. Release of enzyme into the supernatant was expressed as the percentage of the total enzyme content obtained by lysing resting PMN with HTAB (0.5%). This protocol was also performed with bacteria alone in order to ensure that they did not interfere with the MPO assay.

LDH measurement. PMN and bacteria were incubated under the same experimental conditions as in the degranulation assay. Supernatants containing released lactate dehydrogenase (LDH) were collected, and pellets were resuspended in 0.5% HTAB in order to extract the residual enzyme. All samples were stored at 4°C overnight. Pellets were then centrifuged again (2,500 × *g*; 10 min; 4°C), and the corresponding supernatants were collected. Enzymatic activity was determined in both supernatants and pellets by using NADH as a substrate (LDH kit; Boehringer). Because this enzyme is highly susceptible to inactivation when released at 37°C, the current procedure was performed to ensure that the amount of LDH secreted was not underestimated. Release of LDH was therefore expressed as the percentage of the total LDH content.

Binding of bacteria to PMN. PMN and bacteria (MOI = 1,000) were incubated together under the same experimental conditions as in the degranulation assay. At the end of the incubation period, the 96-well culture plates were centrifuged (80 × *g*; 6 min) and the PMN pellets were then washed twice in order to remove unbound bacteria, as verified by optical microscopy observation. The final pellets were lysed with sodium deoxycholate (0.5%) and diluted in PBS before being plated. The bacterial colonies were counted after overnight incubation at 37°C.

Statistical analysis of data. Each figure was established by comparing data obtained with various strains processed on the same days and expressed as the mean ± standard error of the mean (SEM) of at least three distinct experiments. This was of importance considering the interassay variability frequently observed in adhesion assays which were highly sensitive to various parameters such as temperature, CO_2 regulation, blood donor, etc. The Student *t* test for unpaired samples was used to assess the statistical significance. In all instances, a *P* value of <0.05 was recognized as significant, and the considered mean values are indicated on the figures.

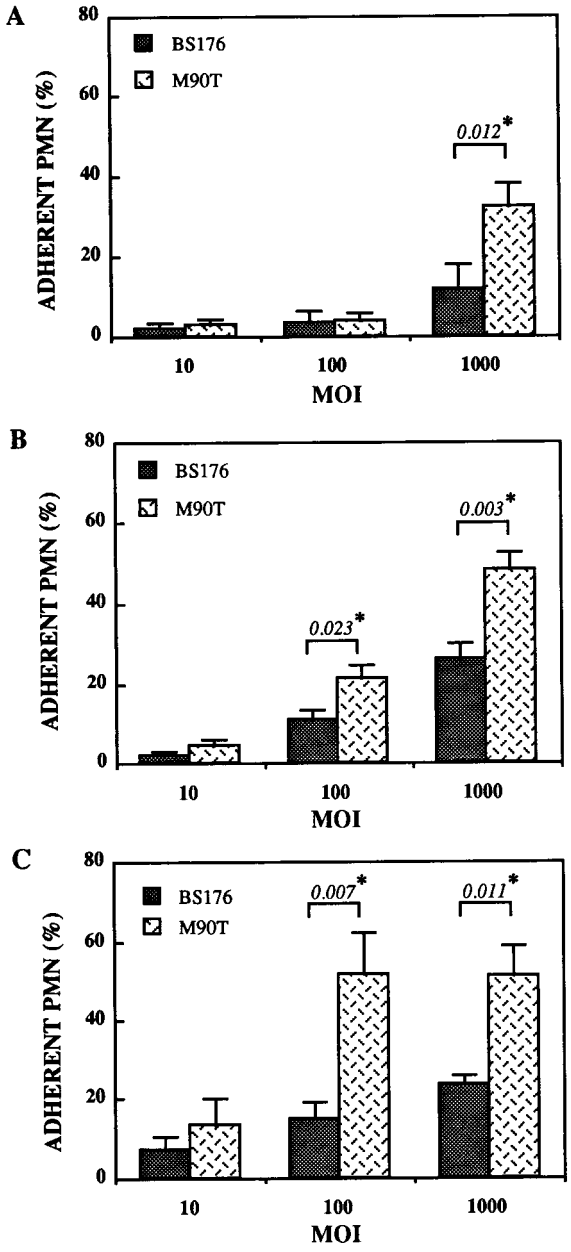

FIG. 1. Kinetics and concentration dependence of *S. flexneri*-induced PMN adherence. PMN and bacteria were mixed together and allowed to adhere for 30 (A), 60 (B), or 120 min (C) on serum-coated wells. At the end of the incubation period, nonadherent cells were removed by washing. The MPO activity remaining associated with the wells and representing adherent PMN was measured and expressed as the percentage of the total amount of enzymatic activity associated with the PMN added. Basal adherence, which was 8.3, 10.8, and 10% at 30 min, 1 h, and 2 h, respectively, was deduced. Each histogram represents the mean ± SEM of three to seven distinct experiments performed at least in quadruplicate. *, $P < 0.05$.

RESULTS

Induction of PMN adherence by *S. flexneri*. As illustrated in Fig. 1, incubation of human PMN with *S. flexneri* resulted in a marked increase of their adherence capacity, this effect depending both on the MOI and on the incubation time. More-

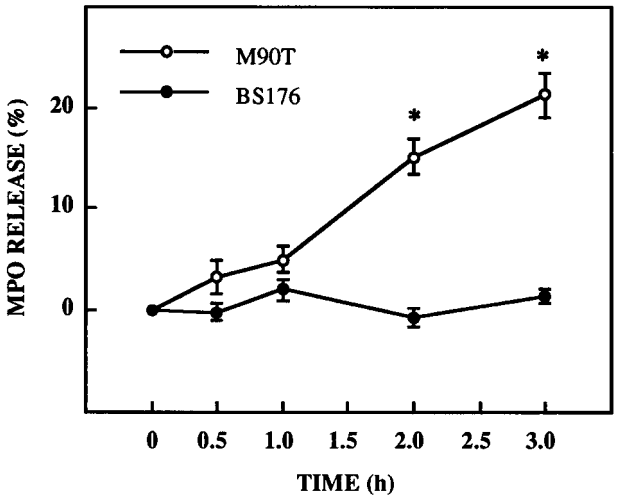

FIG. 2. Effect of *S. flexneri* on PMN degranulation. MPO release was measured in cell-free supernatants of PMN-bacteria mixtures (MOI = 1,000) incubated at 37°C for increasing amounts of time. Release of enzyme is expressed as the percentage of the total MPO content of PMN. Spontaneous secretion, which was 1.4, 2.4, 1.3, and 2.1% at 30 min, 1 h, 2 h, and 3 h, respectively, was deduced. Each point is the mean ± SEM of three to six distinct experiments performed at least in quadruplicate. *, $P < 0.05$.

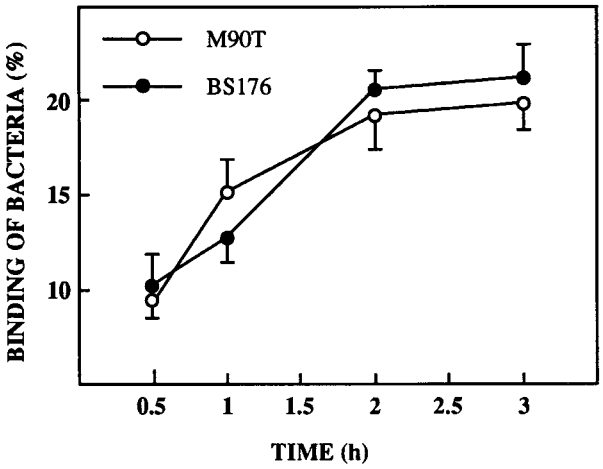

FIG. 3. Binding of *S. flexneri* to PMN. PMN and bacteria (MOI = 1,000) were mixed together and incubated for various amounts of time. Samples were then centrifuged to sediment PMN, which were washed twice. The final pellets containing bound bacteria were lysed and plated. The resulting bacterial colonies were counted, and the number of adherent bacteria is expressed as the percentage of the total amount left in contact with PMN. Each point is the mean ± SEM of three distinct experiments performed in duplicate.

over, these results showed that the number of adherent PMN was significantly higher when cells were allowed to adhere in the presence of the invasive strain M90T than when this occurred in the presence of the noninvasive BS176. Thus, following a 2-h incubation, the percentages of adhering PMN reached 51.8% ± 10.5% and 15.2% ± 4.2% with M90T and BS176, respectively (MOI = 100; $n = 3$; $P < 0.05$). In comparison, PMN adherence in the presence of 1 μM FMLP was 22.9% ± 3% ($n = 3$). In these experiments, background adherence of PMN was approximately 10% (see the legend to Fig. 1) and was deducted.

Degranulation of PMN induced by *S. flexneri*. The ability of *S. flexneri* to trigger PMN degranulation was also investigated. Amounts of MPO released in cell-free supernatants of PMN incubated for various periods with either M90T or BS176 were measured and compared with the total enzyme content measured on lysates of resting cells. These experiments, whose results are depicted in Fig. 2, demonstrated the ability of the invasive strain M90T to induce secretion of MPO from PMN. As observed for adherence, this effect was dependent on the number of added bacteria and on the duration of the incubation. However, induction of PMN degranulation required a higher MOI than adherence. Indeed, upon a 2-h incubation with M90T (MOI = 100), no release was observed, this value being 15.1% ± 1.7% ($n = 6$; $P < 0.05$), with an MOI of 1,000. In contrast, under the same experimental conditions, BS176 failed to trigger a significant release of MPO, even after 3 h of incubation. In the absence of cytochalasin B, FMLP was also devoid of effect (data not shown).

In order to rule out the possibility that the MPO release resulted from PMN lysis induced by M90T, cells and bacteria were mixed and incubated under experimental conditions similar to those used for the MPO assay. Following 1, 2, or 3 h of incubation, LDH activity from both cell-free supernatants and lysed pellets was measured. This procedure was applied to ensure that the percentage of LDH secretion was not underestimated. Indeed, stability of this enzyme is poor, and it is rapidly inactivated at 37°C after secretion has occurred. Results of this series of control experiments ruled out the possibility that a cytotoxic effect of M90T against PMN accounted for their degranulation. When PMN were incubated for 2 h in the presence of either M90T or BS176 (MOI = 1,000), percentages of released LDH were 10.9% ± 3.5% and 8% ± 3.2% ($n = 6$; $P > 0.05$), respectively, while the values for secretion of MPO from the same cells were 15.1% ± 1.7% and 0% ± 0.9% ($n = 6$; $P < 0.05$), respectively. After 3 h of incubation of PMN with M90T, LDH release was slightly higher than after incubation with BS176, but the difference was not statistically significant (data not shown).

Interaction of *S. flexneri* with PMN. When the degranulation assay was performed in the presence of cytochalasin B (5 μg/ml), the kinetics and amplitude of PMN degranulation induced by *S. flexneri* were intensified, but the difference between both strains remained significant. Thus, following a 1-h incubation, MPO release was 18.9% ± 1.6% in the presence of BS176 compared with 29.4% ± 3% with M90T (MOI = 1,000; $n = 6$; $P < 0.05$). In this set of experiments, degranulation induced by 1 μM FMLP was 27.1% ± 4.6% ($n = 6$). Since cytochalasin B prevents the entry of bacteria into cells (7), this result indicated that bacteria exerted their effects outside PMN. We thus looked for the binding of M90T and BS176 strains to PMN. As illustrated in Fig. 3, both bacterial strains bound PMN equally well. While the binding of bacteria to PMN did not represent the mechanism responsible for PMN activation per se, this event seems to be of importance. This was deduced from the fact that supernatants of bacteria (M90T and BS176 strains) were unable, in our experiments, to trigger PMN activation (data not shown).

Role of Ipa invasins in PMN activation by *S. flexneri*. To assess whether Ipa invasins were involved in PMN activation, which was evaluated here in terms of the increase in adherence and induced degranulation, experiments using various *S. flexneri* strains were performed. As shown in Fig. 4, the increase in PMN adherence reached the same level when PMN were incubated with *ipa*B, *ipa*C, or Δ*ipa* mutants. Moreover, the cell response was not significantly different from that observed with the noninvasive strain BS176. In contrast, these values were shown to be statistically lower than that obtained with the M90T strain. Similarly, when these strains were tested for their

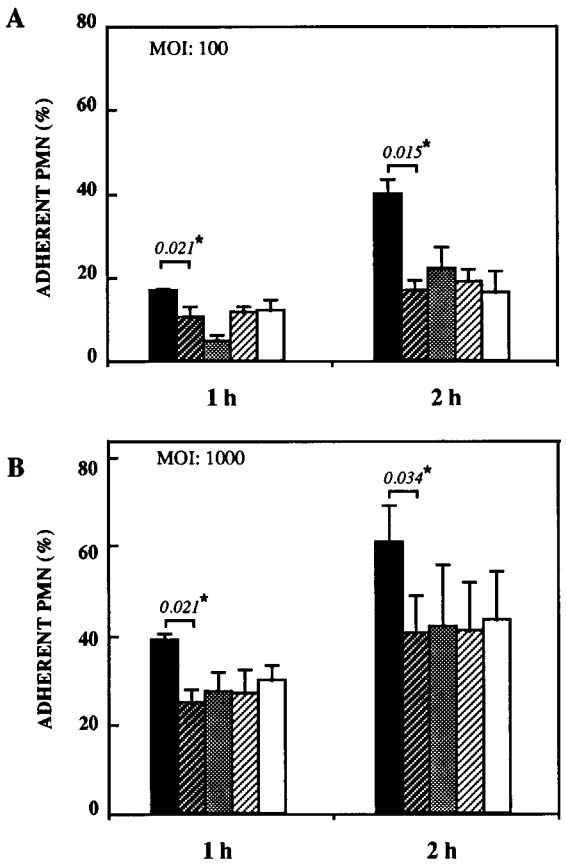

FIG. 4. PMN adherence induced by various *S. flexneri* mutants. Details are as in the legend to Fig. 1; various bacterial strains were used. Each histogram represents the mean ± SEM of three distinct experiments performed in quadruplicate. ■, M90T; ▨, BS176; ▩, *ipa*B; ▨, *ipa*C; □, Δ*ipa*.

ability to induce PMN degranulation, similar negative responses were observed (Fig. 5). Thus, the wild-type M90T strain, which produces a complete set of Ipa invasins, was the only one able to provoke MPO secretion by PMN.

DISCUSSION

The Ipa-related invasive phenotype of *S. flexneri* depends on the nature of the target cell. Comparison of macrophage and epithelial cell infections by *S. flexneri* offers a clear example of a pleiotropic effect of the invasive phenotype which is characteristic of this bacterial species. Recent data suggest that a striking consequence of the Ipa-related invasive phenotype of *S. flexneri* is the elicitation of early inflammation that increases tissue invasion and eventually causes tissue destruction. Provocation of macrophage-programmed cell death seems to be the crucial step for this effect (33, 34). In addition, it has been demonstrated in vitro (20) and in vivo (19) that PMN are the major effectors of the previously described process causing epithelial disruption. The aim of this work was therefore to explore whether another possible expression of the Ipa-mediated *Shigella* invasive phenotype affected the function of PMN, particularly their capacity to adhere and to release their toxic substances.

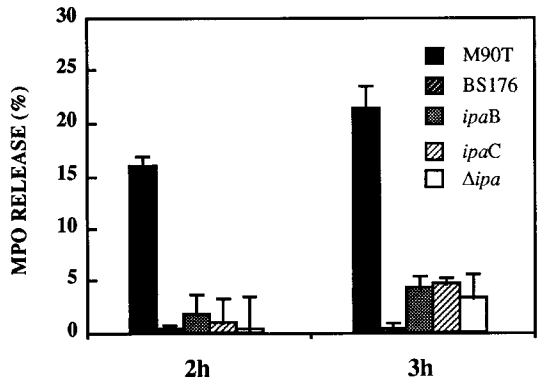

FIG. 5. PMN degranulation induced by various *S. flexneri* mutants. Details are as in the legend to Fig. 2; various bacterial strains were used. Each histogram represents the mean ± SEM of three distinct experiments performed in quadruplicate.

Adherence of PMN, which is regulated by various families of adhesion molecules, is a crucial step in the cascade leading to tissue inflammation. In fact, adherence and cytotoxic effects of these cells are two intimately linked events. PMN contain an arsenal of cytotoxic polypeptides and proteolytic enzymes (5) which are highly effective in inducing tissue destruction (30). Several lines of evidence suggest that the major injurious enzyme released by PMN is elastase (30), a serine proteinase able to induce epithelial cell lysis (17). In fact, it is now recognized that PMN-mediated lethal cell injury results from a synergistic effect between proteinases and reactive oxygen products (28). Interestingly, adherence of PMN contributes to the enhancement of both the exocytosis of PMN granules (21, 32) and the production of oxygen metabolites (8, 24). These data are consistent with the fact that PMN usually perform their cytotoxic effect while adhering to their target surface.

The results described in this paper clearly demonstrate that the Ipa-related invasive phenotype of *S. flexneri* affects PMN behavior, in that it strongly enhances their capacity to adhere to a serum-coated surface upon contact with bacteria. Such a phenomenon, which is mediated by β_2-integrins (3), suggests that *S. flexneri* also favors the interaction of PMN with the cells with which they are led to interact in the intestinal mucosa, particularly epithelial cells which express corresponding counterreceptors (2). As a consequence, it can be speculated that the epithelium could then be damaged. This is well in line with a previous observation that the use of an anti-CD18 monoclonal antibody blocks PMN transmigration, epithelial destruction, and cell invasion by *S. flexneri* both in in vitro systems and in an animal model of tissue invasion. Several cytokines belonging to the inflammatory cascade (25), as well as other products such as LTB_4 (12) and PAF (6), which have been shown to be produced during shigellosis, may account for this effect on PMN as well. However, direct interaction between bacteria and PMN is likely to be an efficient means of activation and, at least, to strongly increase adherence of these cells. Subsequent release of the PMN content and production of oxygen radicals may account for bacterial killing as well as strong tissue destruction (26). Under our experimental conditions, we observed that *S. flexneri* initiated significant PMN degranulation, as evaluated by MPO measurement (Fig. 2 and 5). Of note is that depicted experiments were performed by using a large proportion of bacteria per PMN in order to reduce the delay of PMN reactivity. Indeed, while PMN degranulation was shown to be effective when working with lower MOIs (10 and 100), it required longer incubation periods which were inconsistent with the subsequent measurement of the MPO. Moreover, we failed to demonstrate a significant increase in PMN death related to the presence of invasive shigellae, as estimated by LDH measurement. This result confirmed that an active process of degranulation was initiated by

M90T. However, because of the absorbance of bacteria themselves, we failed to determine whether the leukocyte respiratory burst was also elicited.

The mechanism of *S. flexneri*-induced PMN activation was shown to be unrelated to the entry of bacteria in PMN. In addition, a preferential binding of strain M90T was not evidenced. In fact, the involvement of invasins was highlighted by comparing various mutant strains, and most particularly *ipa*B, *ipa*C, and Δ*ipa*, which activated PMN to the same low extent as the noninvasive strain BS176. In conclusion, we have described here a new facet of the pleiotropic expression of the Ipa-related invasive phenotype of *S. flexneri*. Recent evidence indicates that interaction of invasive bacteria with phagocytic cells is a major step in the development of shigellosis and that actual epithelial invasion may not necessarily lead to major tissue lesions in the absence of the intervention of both macrophages and PMN.

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