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Shigella flexneri Transformants Expressing Type 1 (Mannose-Specific) Fimbriae Bind to, Activate, and Are Killed by Phagocytic Cells

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***Shigella flexneri* M90T (invasive) and BS176 (noninvasive) are typical nonfimbriated organisms that do not bind to or activate phagocytic cells. We demonstrate that *S. flexneri* M90Tp and BS176p, obtained by transformation of the strains named above with the cluster of genes encoding type 1 (mannose-specific) fimbriae of *Escherichia coli*, express the functional fimbriae, as shown by electron microscopy, by binding of antifimbria antibodies and by yeast cell aggregation. The transformants, but not the parental strains, bound to human granulocytes and mouse peritoneal macrophages. This binding was inhibited by methyl α-D-mannoside but not by methyl α-D-galactoside. The bound bacteria induced oxidative burst activation and degranulation of the granulocytes in vitro. With mouse peritoneal macrophages, the binding of the fimbriated bacteria induced degranulation in vitro. Injection of the bacteria into mouse peritoneum also induced degranulation of the macrophages in vivo; no such effect was observed with the nonfimbriated strains. The bound fimbriated transformants were effectively killed by the human granulocytes in vitro in the absence of opsonins or after opsonization with human anti-*S. flexneri* antiserum. The nonfimbriated strains were killed only after opsonization. These results provide further evidence for the role of type 1 fimbriae in lectin-mediated nonopsonic phagocytosis.**

Many members of the family *Enterobacteriaceae* express on their surface mannose-specific lectins in the form of type 1 fimbriae which mediate the adhesion of the bacteria to mammalian cells, including phagocytes, in the absence of opsonins (26). The sugar-specific adhesion to the phagocytes is frequently followed by activation of the oxidative burst, degranulation of the phagocytes, and the ingestion and killing of the bacteria, a phenomenon designated lectinophagocytosis (22). Type 1 fimbriae are threadlike appendages consisting of about 1,000 protein subunits. Eight genes, from A to H, are required for the expression of the fimbriae, only one of which, the H gene, codes for the mannose-binding subunit (15). *Shigella flexneri* strains are generally nonfimbriated (20) and, unless opsonized, are unable to bind to phagocytic cells or to induce an oxidative burst in these cells. Verdon et al. (27) have recently introduced a plasmid (obtained from P. E. Orndorff) which contains the cluster of *Escherichia coli* genes controlling the production of type 1 fimbriae into two isogenic strains of *S. flexneri*, the invasive M90T and the noninvasive BS176, to obtain the derivative recombinant strains M90Tp and BS176p, respectively.

In this study, we describe experiments aimed at assessing the ability of these transformants to bind to yeast cells, human granulocytes, and mouse peritoneal macrophages in the absence of opsonins in a mannose-specific manner and to cause activation of, and to be killed by, the phagocytic cells. As a control, we used type 1-fimbriated *E. coli* 346, which was employed previously in studies of lectinophagocytosis (2, 3, 5, 11).

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MATERIALS AND METHODS

Chemicals. Methyl α-D-mannoside (MeαMan) and methyl α-D-galactoside (MeαGal) were purchased from Pfanstiehl Laboratories (Waukegan, Ill.); 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (MeUmb-β-GlcNAc) and 4-methylumbelliferone (MeUmb) were from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals were from commercial sources and were of the highest purity available.

Mice. Male C3HeB/FeJ mice (Jackson Laboratory Animal Resources, Bar Harbor, Maine) were 5 to 7 weeks of age. They were fed regular animal food and water.

Media and growth conditions of bacterial strains. Transformation of the nonfimbriated strains M90T and BS176 was done with plasmid pSH2 obtained from *E. coli* ORN103 provided by P. E. Orndorff. Plasmid DNA isolation was performed by the technique of Birnboim and Doly (4). This plasmid, which contained the total type 1-fimbria operon, was transferred to the nonfimbriated strains by the method of Lederberg and Cohen (16). *S. flexneri* transformants BS176p and M90Tp (27) were grown in Trypticase soy broth containing chloramphenicol at a final concentration of 20 μg/ml to ensure that they retained the plasmid encoding the gene for type 1 fimbriae; parental strains BS176 and M90T (9, 18, 24) were grown in the absence of chloramphenicol. A uropathogenic isolate of *E. coli* (strain 346) (19) was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). To obtain heavily fimbriated bacteria, the *E. coli* or *Shigella* strains were cultured at 37°C for 24 h under static conditions. The bacteria were collected by centrifugation (3,000 × *g*, 4°C, 10 min) and washed and resuspended in phosphate-buffered saline (PBS; 0.15 M NaCl in 0.01 M KH_2PO_4–0.009 M Na_2HPO_4 phosphate buffer [pH 7.4]) containing 2 mM Ca^+ and 1 mM Mg^+ (PBS-CaMg). The

mannose-specific activity of the type 1-fimbriated bacteria was measured by yeast cell aggregation (21). The rate of yeast cell aggregation was determined from the tangent of the steepest slope in the curve produced by increasing light transmittance and is expressed as the rate change of optical density in millimeters per minute.

Antisera. Antifimbrial antibodies were raised in rabbits by the intracutaneous injection of 200 μg of purified *E. coli* 346 fimbriae (8) suspended in 0.5 ml of 0.15 M NaCl in a mixture with 0.5 ml of complete Freund's adjuvant. After 6 weeks, a booster of 400 μg of fimbriae in 0.5 ml of 0.15 M NaCl was injected intracutaneously, and the animals were bled 8 days later. Control and human antisera to *S. flexneri* obtained from a soldier before and after infection with *S. flexneri* 2a was a gift of Dani Cohen, Medical Corps, Israel Defense Forces. The antiserum strongly agglutinated the *S. flexneri* strains used at a titer of 1:20 in an agglutination assay and bound to the bacteria at a dilution of 1:200, as measured with an enzyme-linked immunosorbent assay (ELISA) (6).

Treatment with antisera. To opsonize the *Shigella* strains, the bacteria (2×10^8/ml in PBS) were incubated at 37°C for 30 min with 10% human antiserum diluted 1:10 in PBS and then washed three times with PBS. The type 1-fimbriated *E. coli* was opsonized (2×10^8 bacteria per ml in PBS) for 30 min at 37°C with 10% human serum, isolated from the blood of normal donors obtained from the Sheba Hospital Blood Bank (Tel Hashomer, Israel). The bacteria were then washed three times with PBS and finally resuspended (2×10^8/ml) in the same buffer.

Electron microscopy. Bacteria were treated with 2% formaldehyde, mounted on polycarbonated grids, and negatively stained with 0.5% uranyl acetate as described previously (8).

Binding assay. Binding of rabbit antifimbrial antibody to the different strains of *S. flexneri* and to type 1-fimbriated *E. coli* or binding of the bacteria to phagocytic cells was examined by the ELISA at 37°C for 1 h, by using the corresponding antibacterial antiserum and goat antirabbit immunoglobulin G conjugated with horseradish peroxidase (5).

Chemiluminescence assay. The oxidative burst activation of granulocytes was determined by a chemiluminescence assay and measured in a Lumacounter M2080, which digitally displays the amount of photons obtained every 10 s when phagocytic cells are allowed to react with a stimulant in the presence of 10^{-5} M Luminol (11). Phorbol-12-myristate-13-acetate, was used as a control for the effect of sugar concentration.

Degranulation in vitro. Granulocytes were isolated from the buffy coat of the blood of normal donors, obtained from Sheba Hospital Blood Bank, by centrifugation on a Ficoll gradient and dextran sedimentation (7). Mouse peritoneal macrophages were isolated after intraperitoneal injection of 10 ml of PBS containing 3% bovine serum albumin and 0.45% glucose (PBS-BSA). Eight milliliters of the peritoneal fluid was aspirated, and the cells were collected by centrifugation (500 × *g*, 4°C, 10 min) and washed four times with PBS-BSA. The phagocytic cells (10^6 in 0.4 ml of PBS-BSA) were incubated with different numbers of fimbriated or nonfimbriated *S. flexneri* in the presence or absence of 50 mM MeαMan or MeαGal for 1 h at 37°C. Controls consisted of phagocytic cells alone or phagocytic cells with sugar only. At the end of the incubation period, 4 ml of cold PBS was added to each cell suspension, which was immediately centrifuged (500 × *g*, 4°C, 15 min). The cell pellets were discarded, and the supernatants were recentrifuged (3,000 × *g*, 4°C, 15 min) to remove the bacteria. The clear supernatants were kept frozen at −70°C and assayed on the following day. Degranulation was estimated by measuring *N*-acetyl-β-D-glucosaminidase (βGlcNAc-ase) activity (3, 14).

Degranulation in vivo. Degranulation in vivo was done essentially as described previously (3). Briefly, the bacteria (10^6 to 10^{10} cells per ml in PBS-CaMg) were injected intraperitoneally into male C3HeB/FeJ mice. Control animals received PBS-CaMg alone. After 30 min, the animals were sacrificed and the peritoneal macrophages were recovered by lavage at 4°C with 5 ml of PBS containing 50 mM MeαMan; samples with blood were discarded. The lavage fluid was immediately centrifuged (500 × *g*, 4°C, 10 min) to remove peritoneal cells and then centrifuged (3,000 × *g*, 4°C, 15 min) to remove bacteria; the clear supernatant was stored overnight at −70°C and then assayed for βGlcNAc-ase activity.

Assay of βGlcNAc-ase activity. The enzyme was assayed by a modification (3) of the method of Harrison and Bowers (14), in which the fluorescence of the MeUmb released by hydrolysis of MeUmb-β-GlcNAc is measured in a spectrofluorimeter (model MPF-3L, Perkin-Elmer) at an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Enzyme activity is expressed as the amount of MeUmb released per minute of incubation time per total volume of peritoneal lavage fluid. Each determination was performed in duplicate.

Killing of bacteria by granulocytes. To each well of a microtiter plate (96-well, flat-bottom microtiter plates; Linbro/Titertek; Flow Laboratories, Inc., McLean, Va.), granulocytes (5×10^5 in 50 μl of PBS-CaMg) were added. The plates were incubated at 37°C for 30 min in 5% CO_2 in air, the supernatant containing nonadherent cells was removed by aspiration, and the residual cells were washed three times with PBS-CaMg. The number of cells bound to each well was $(2 \pm 0.45) \times 10^5$, as determined by methylene blue staining by using a standard curve to convert absorbance into number of bound cells (10). The adherent cells were incubated with 100 μl of PBS-CaMg containing 3% BSA for 30 min at 37°C, and the buffer was removed by aspiration. Bacteria (2×10^7 in 50 μl of PBS-CaMg), in the absence or presence of 100 mM MeαMan, were added to each well, and the plate was incubated at 4°C for 1 h. In parallel, the bacteria were added after opsonization with anti-*Shigella* antiserum. The type 1-fimbriated *E. coli* was added after opsonization with 10% normal human serum. The supernatant containing unbound bacteria was aspirated, and the attached cells were washed three times with ice-cold PBS-CaMg. The plate was further incubated for different periods at 37°C before the attached cells were lysed with 1% sodium deoxycholate. The lysates were diluted 1:5, 1:50, and 1:500 in PBS-CaMg, and aliquots of 20 μl were plated on nutrient agar in petri dishes. Incubation was for 24 h at 37°C, at which time the number of colonies was counted. Killing of bacteria was calculated by comparing the number of colonies formed after incubation of the human granulocytes with the bacteria at 37°C to that obtained without incubation at this temperature. Incubation for 30 min at 37°C of the opsonized *S. flexneri* or *E. coli* in the absence of granulocytes revealed that no killing of the bacteria occurred.

RESULTS

Fimbriation of bacteria. When the bacteria were grown under conditions that promote heavy fimbriation, the transformants M90Tp and BS176p expressed fimbriae on their

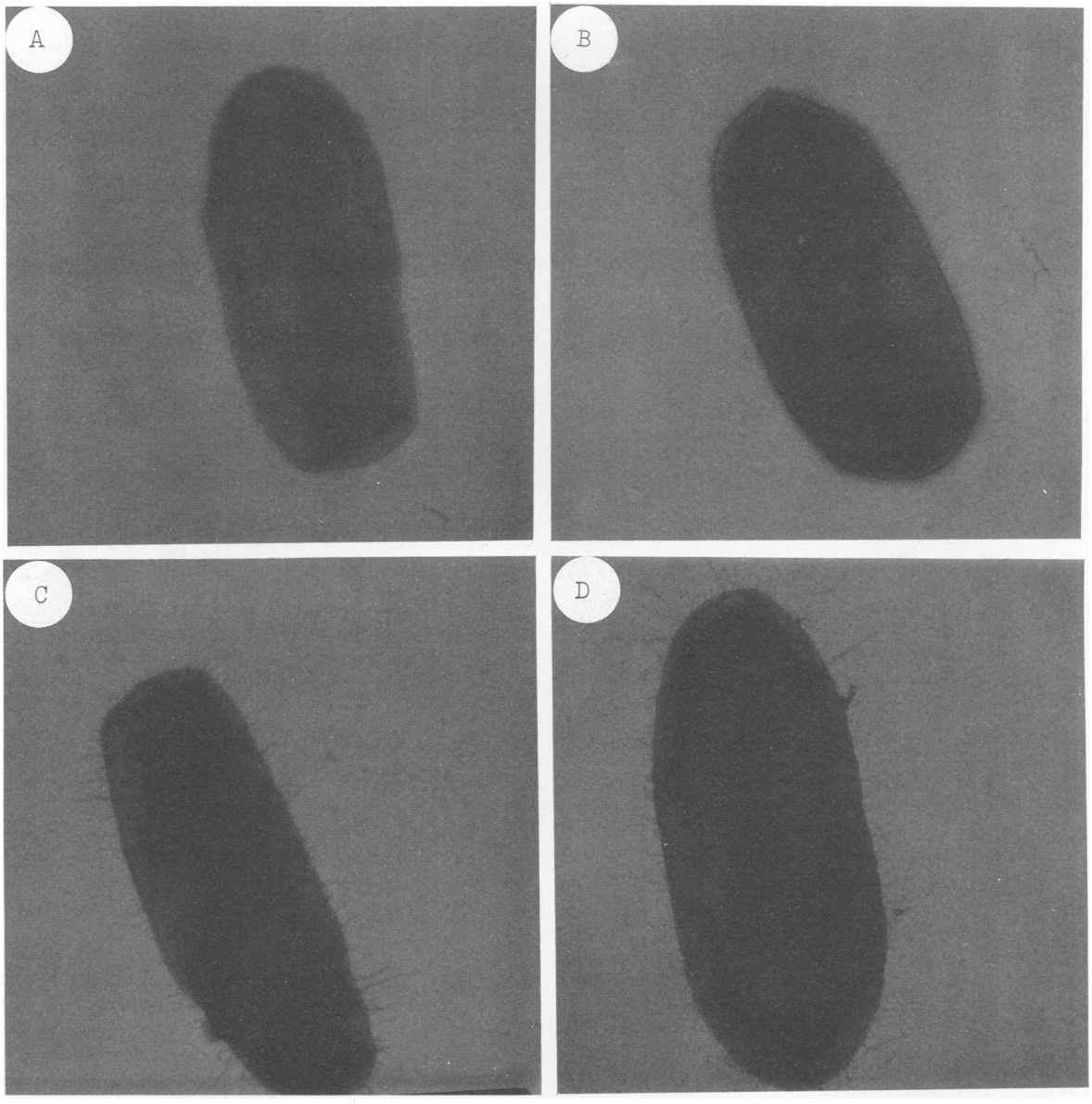

FIG. 1. Electron micrographs negatively stained with 0.5% uranyl acetate of and *S. flexneri* M90T (A), BS176 (B), M90Tp (C), and BS176p (D). Magnification, ×24,000.

surface as shown by electron microscopy, whereas the parental strains did not (Fig. 1). Antifimbria antibodies failed to bind the latter strains but bound readily to their transformants (Fig. 2). The transformants aggregated yeast cells in a concentration-dependent and MeαMan-inhibitable manner (Fig. 3). No detectable yeast cell aggregation was observed with the parental strains up to a concentration of 4×10^7 bacteria per ml.

Activation of the phagocytic cells and killing of the bacteria. Similarly to type 1-fimbriated *E. coli*, the fimbriated *S. flexneri* strains also bound to human granulocytes and mouse peritoneal macrophages in a mannose-inhibitable manner (Fig. 4). This binding resulted in the activation of an oxidative burst of the granulocytes, as monitored by the generation of chemiluminescence. The activation was dose dependent and inhibited by MeαMan (Fig. 5). The nonfimbriated *Shigella* strains M90T and BS176 failed to activate an oxidative burst of the granulocytes but did so after opsonization with human anti-*Shigella* antiserum. At a bacterium/phagocyte ratio of 144:1, the maximal activation of the oxidative burst with type 1-fimbriated *E. coli* was considerably higher (two to three times) than that with fimbriated *S. flexneri*.

The fimbriated strain BS176p induced the in vitro degranulation of human granulocytes and mouse peritoneal macrophages. Degranulation increased with an increase in the ratio of bacterium/phagocyte and was inhibited by MeαMan but not by MeαGal. The nonfimbriated strain BS176 failed to induce degranulation (Fig. 6). Similarly, whereas strain M90Tp induced degranulation with both types of phagocytic cells, the nonfimbriated strain M90T did not (data not shown).

Injection of BS176p into the peritoneal cavity of mice caused degranulation, as evidenced by the significant release

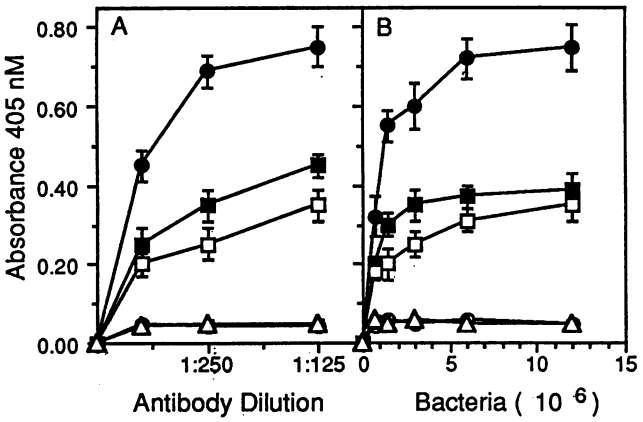

FIG. 2. (A) Binding of rabbit antifimbria antiserum at different dilutions to different strains of bacteria (1.2×10^7 bacteria per tube). (B) Binding of the antiserum (diluted 1/100) to different numbers of bacteria. Binding was measured by ELISA (5). Symbols: ○, M90T; △, BS176; ■, M90Tp; □, BS176p; ●, type 1-fimbriated *E. coli*.

of βGlcNAc-ase into the peritoneal cavity, similarly to results obtained with type 1-fimbriated *E. coli* (3). With the nonfimbriated strain BS176, βGlcNAc-ase release was significantly lower (Fig. 7). Both of the fimbriated strains, M90Tp and BS176p, were extremely sensitive to killing by human granulocytes (Fig. 8), whereas the nonfimbriated strains, M90T and BS176, were resistant to killing under nonopsonic conditions but were sensitive to killing when opsonized with human anti-*Shigella* antiserum.

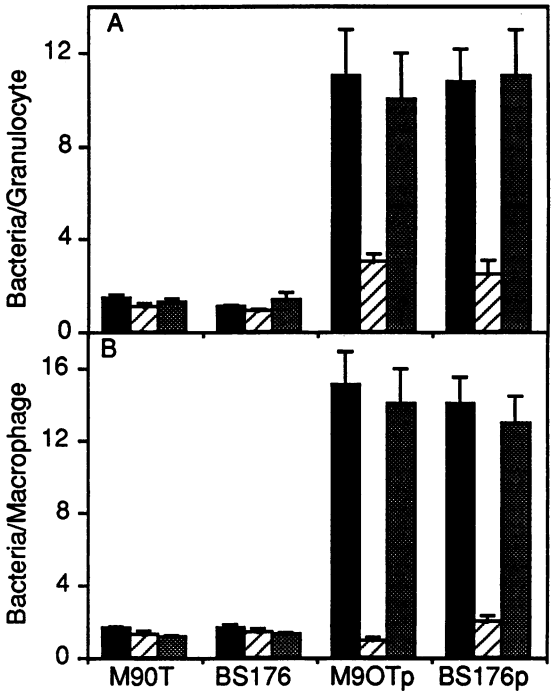

FIG. 4. Binding of different strains of *S. flexneri* to human granulocytes (A) and to mouse peritoneal macrophages (B). The phagocytes were incubated with the bacteria at a ratio of 100:1 in the absence (■) or presence of 100 mM MeαMan (▨) or MeαGal (▩) for 30 min at 4°C; unbound bacteria were removed by washing and bound bacteria determined by ELISA (5).

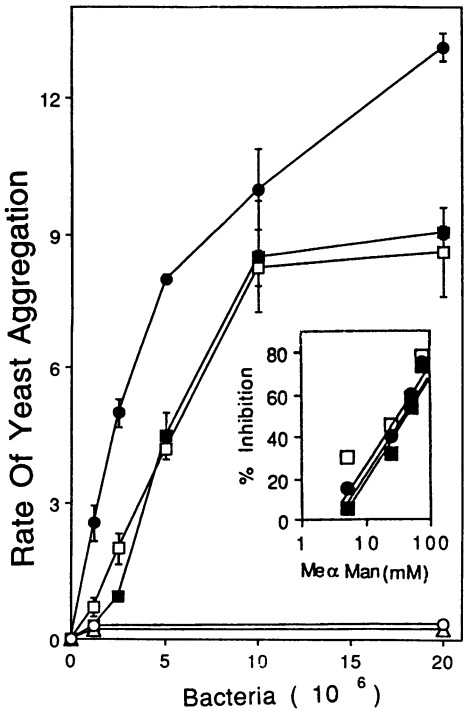

FIG. 3. Rate of yeast cell aggregation (measured as change of transmission per minute) induced by fimbriated bacteria. The effect of MeαMan on aggregation is shown in the inset. Symbols: ○, M90T; △, BS176; ■, M90Tp; □, BS176p; ●, type 1-fimbriated *E. coli*.

DISCUSSION

The purpose of the present study was to investigate the role of type 1 fimbriae in phagocytosis of nonfimbriated and fimbriated *S. flexneri* as measured by binding, activation of the oxidative burst, degranulation in vitro and in vivo, and killing of the bacteria by the granulocytes. The fimbriated bacteria were obtained by transformation of the nonfimbriated strains with the cluster of genes encoding type 1 fimbriae of *E. coli*. The results indicate that the transformants expressed the fimbriae, as shown by electron microscopy, binding of antifimbria antibodies, and yeast cell aggregation which is inhibited by MeαMan. Moreover, the fimbriated, but not the nonfimbriated strains, bound to human granulocytes and to mouse peritoneal macrophages. This binding resulted in oxidative burst activation and degranulation of the granulocytes in vitro. With mouse peritoneal macrophages, the binding of the fimbriated bacteria induced degranulation both in vitro and in vivo. Under nonopsonic conditions, the fimbriated bacteria were effectively killed by the granulocytes, whereas the nonfimbriated bacteria were resistant even in the presence of normal human serum. Opsonization of the nonfimbriated bacteria was required to induce activation of the granulocytes and their killing of the bacteria. Our findings show that type 1-fimbriated *S. flexneri*, in the absence of opsonins, had similar effects on human and mouse phagocytic cells as those found with type 1-fimbriated *E. coli*, although the level of activation with *S. flexneri* was two to three times lower than that with *E. coli*. This difference is most likely related to the smaller number of fimbriae per bacterial cell, as indicated by the lower level of yeast cell aggregation and the lower

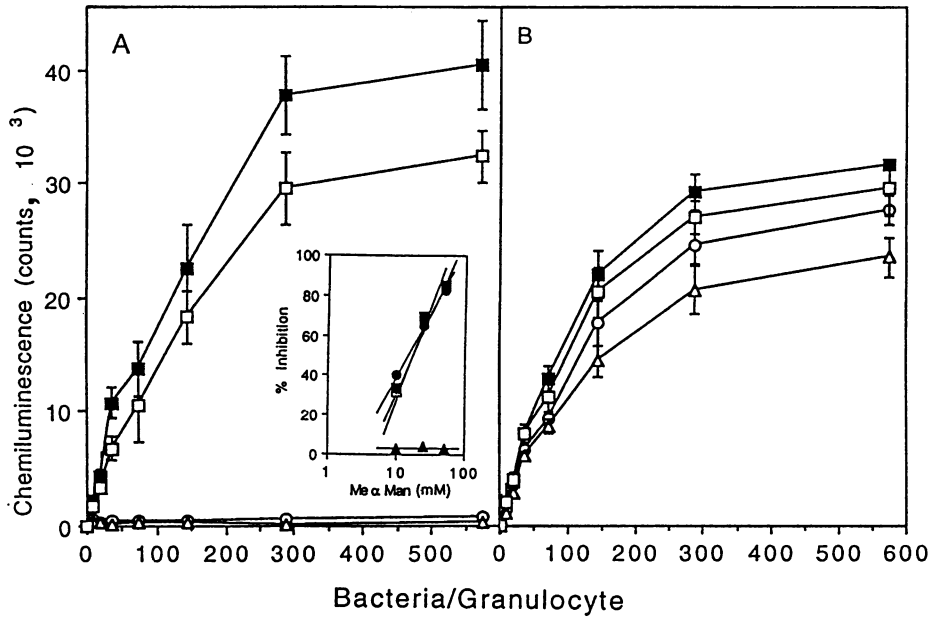

FIG. 5. (A) Dose response curve for bacterial effect on chemiluminescence generation by human granulocytes in the absence of serum. The effect of different concentrations of MeαMan is shown in inset. The bacteria or PMA were incubated with MeαMan for 45 min at 37°C. Aliquots of bacteria (100 μl) or PMA were added to give a final ratio of 144 bacteria per cell or a final concentration of 66 ng of PMA per ml. Chemiluminescence generation was determined after 30 min. The maximal activation of the oxidative burst by type 1-fimbriated *E. coli* and PMA was 44,000 and 42,000 photons, respectively. (B) Dose-response curve for bacterial effect on chemiluminescence generation by human granulocytes in the presence of anti-*S. flexneri* antiserum. Symbols: ○, M90T; △, BS176; ■, M90Tp; □, BS176p; ●, *E. coli*; ▲, PMA.

binding of the antifimbria antibodies to *S. flexneri* strains as compared with *E. coli*. Our results add further support to other reports (1, 5, 12, 13, 17, 23, 25), which indicate that type 1 fimbriae may have dual roles in host-pathogen interactions. In some locations, such as the gut, fimbriation appears to be an advantage for adherence and colonization. In circulating blood, or areas rich in phagocytic cells, fimbriae may be a disadvantage for the bacteria. The expression of fimbriae has been shown to be controlled by environmental signals. That such signals differ in different habitats may explain how, in some cases, a fimbriated pathogen can evade host defenses.

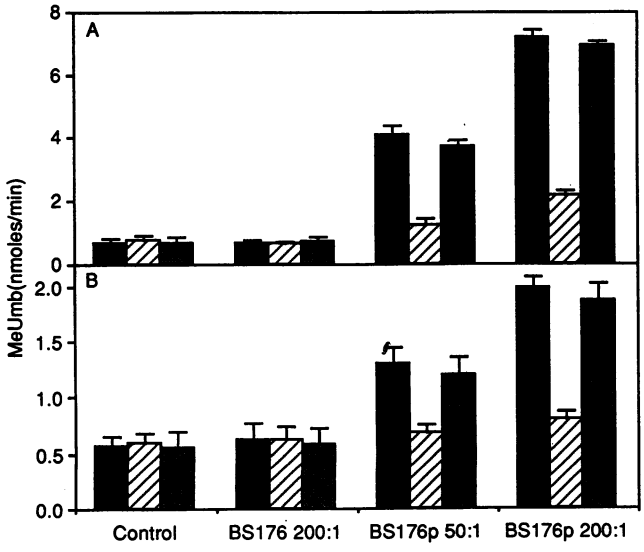

FIG. 6. In vitro release of βGlcNAc-ase from 10^6 human granulocytes (A) or mouse peritoneal macrophages (B) by different bacterium/phagocyte ratios after 1 h of incubation at 37°C. Symbols: ■, phagocytes alone; ▨, phagocytes with bacteria in the presence of 50 mM MeαMan; ▩, phagocytes with bacteria in the presence of 50 mM MeαGal (▩).

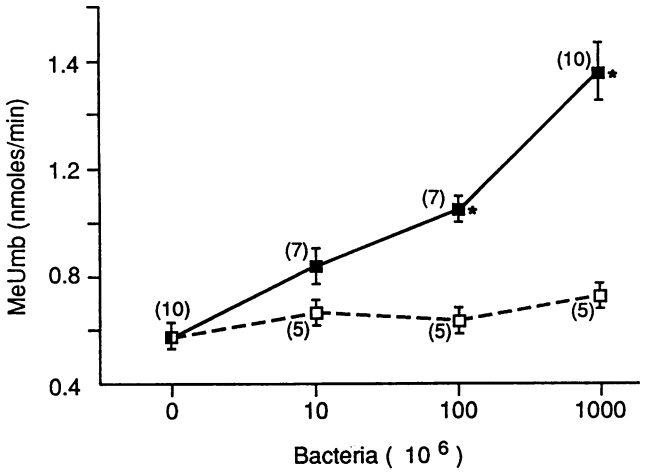

FIG. 7. βGlcNAc-ase release into the peritoneal cavity of male C3HeB/FeJ mice, after injection of different concentrations of *S. flexneri* BS176p (■) or BS176 (□) for 30 min. Symbols and bars represent means and standard errors of the means, respectively; *, $P < 0.01$ compared with controls. Statistical calculations were done by means of computer-assisted one-factor analysis of variance by using Statview +518. Single groups were compared with the 99% confidence interval method.

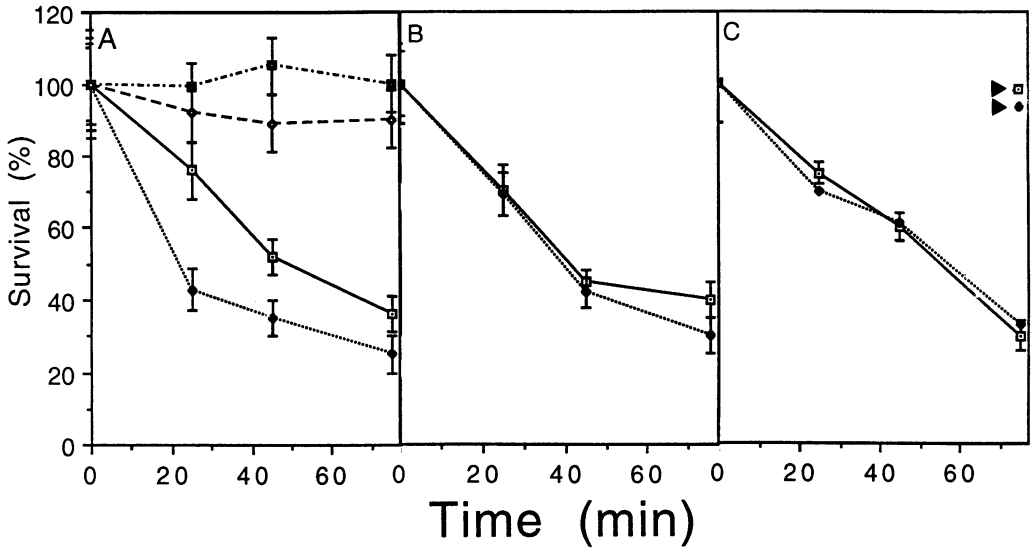

FIG. 8. Killing of *S. flexneri* strains by human granulocytes under different conditions. Nonfimbriated (A) and fimbriated (B and C) bacteria were incubated with the granulocytes at a ratio of 100:1 for 1 h at 4°C and for different periods at 37°C. The granulocytes were lysed, and the numbers of colony-forming bacteria were counted. (A) Killing was assayed in the absence of serum (strains M90T [□] and BS176 [◇]) and in the presence of 10% human anti-*S. flexneri* antiserum (strains M90T [⊡] and BS176 [◆]). The survival (at 75 min of incubation) of opsonized or nonopsonized bacteria was not affected by the presence of MeαMan (100 mM). (B) Killing of strains M90Tp (⊡) and BS176p (◆) in the presence of 10% human anti-*S. flexneri* antiserum by human granulocytes. (C) Killing of M90Tp (⊡) and BS176p (◆) in the absence of opsonins; incubation (for 75 min) of M90Tp (▶⊡) or BS176p (▶◆) in the presence of 100 mM MeαMan prevented the killing of the bacteria.

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