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Plasmid-Mediated Early Killing of Eucaryotic Cells by *Shigella flexneri* as Studied by Infection of J774 Macrophages

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In *Shigella flexneri* a 220-kilobase plasmid encodes the ability to invade nonprofessional phagocytes by a mechanism similar to phagocytosis. In this report, the continuous macrophage cell line J774 was used to study the intracellular fate of both invasive and noninvasive strains. pWR100, the virulence plasmid of *S. flexneri* serotype 5, mediated very efficient and rapid killing of J774 macrophages, as measured by cellular detachment and uptake of trypan blue. For this to occur, the bacteria had to be within the cells, since the macrophages were protected by cytochalasin D. A battery of strains differing in their levels of Shiga toxin production showed that inhibition of protein synthesis by Shiga toxin, as measured by [^{35}S]methionine incorporation into infected macrophages, was not required for early killing of cells. Damage to J774 macrophages rather correlated with the ability of invasive bacteria to rapidly and efficiently lyse the membrane of the phagocytic vacuole. The role of the release of bacteria within the cytosol for subsequent expression of cytotoxic activity is discussed, and mitochondria are proposed as a potential target for this activity.

The enteric pathogen *Shigella flexneri* can invade the human colonic mucosa. The main factors in the pathogenesis of this invasive process are the ability of virulent organisms to induce their own phagocytosis by epithelial cells, the rapid intracellular growth and finally the killing of host cells (6, 9, 18). A 220-kilobase plasmid is required for the induction of phagocytosis (15) and efficient multiplication within HeLa cells (16). A plasmid-encoded contact hemolytic activity has been correlated both with the capacity of the invasive bacterium to induce its own phagocytosis (1) and to escape the phagosomal vacuole through membrane disruption (16). In the same series of experiments, no correlation could be observed between the rate of intracellular multiplication and the level of Shiga toxin production, which had been proposed as one of the ways by which invasive bacteria preferentially incorporate amino acids through the shutoff of host cell protein synthesis (5). Shiga toxin produced during intracellular growth may also contribute to the killing of host cells (5). However, Hale and Formal demonstrated a delayed (i.e., 8 to 24 h) cytolytic effect of extracellular cytotoxin on HeLa cells, whereas invasive *S. dysenteriae* 1 induced early cell damage even in the toxin-resistant cell line Henle 407. These experiments suggested that toxic products independent of Shiga toxin may account for early cytolytic activity (4).

The obvious limitation of these models is that nonprofessional phagocytes such as HeLa cells are not susceptible to noninvasive mutants of *S. flexneri*. Thus, it is impossible to compare the intracellular behavior of invasive bacteria with their noninvasive plasmidless derivatives and to study the intracellular cytotoxic effect of enteric pathogens which are normally noninvasive but produce high levels of Shiga or Shiga-like toxin. We circumvented this problem by using the continuous macrophage cell line J774 (17). These professional phagocytes become infected by both invasive and noninvasive microorganisms. In these experiments it was demonstrated that pWR100, the virulence plasmid of *S. flexneri* serotype 5 strain M90T, played a critical role in inducing rapid killing of host cells within 6 h of infection regardless of the level of Shiga toxin production. A noninvasive plasmidless derivative of M90T and a strain of enterohemorrhagic *Escherichia coli* which produced high levels of Shiga-like toxin produced no cellular damage. The killing ability of invasive bacteria was correlated with lysis of the phagocytic vacuole, which reflected a membrane-damaging capacity of these strains, and it may allow direct expression of other virulence attributes within the cytosol.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains and their relevant characteristics are listed in Table 1.

Medium and growth conditions. Bacteria were routinely grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 37°C. Exponential-phase cultures were used to infect macrophages.

Routine procedure for infection of macrophages. J774 macrophages were maintained in RPMI 1640 (Flow Laboratories, Inc., McLean, Va.) supplemented with 15% complement-inactivated fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 2 mM glutamine (Gibco). No antibiotics were added. At 18 h before infection, cells were plated at a density of 7×10^5 per plate in 35-mm tissue culture dishes (Becton Dickinson Labware, Oxnard, Calif.). After the semiconfluent monolayers were washed with Earle balanced salt solution (EBSS; Gibco), the cells were infected by adding 1 ml of bacterial suspension in RPMI without serum and incubating for 20 min at 37°C in 5% CO_2. The multiplicity of infection (MOI) was standardized to obtain an average number of five intracellular bacteria per macrophage after this incubation. The MOI was ca. 150 bacteria per cell for the invasive strains M90T and 395-1(pWR110) and 1,500 bacteria per cell for the noninvasive strains BS176, E30846, and 395-1. The monolayers were then washed three times with EBSS and covered with 2 ml of RPMI supplemented with 2 mM glutamine. Gentamicin at a final concentration equivalent to the MBC for the strain under study was added to kill extracellular bacteria and prevent reinfection of the cells. Plates were reincubated at 37°C and removed every

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TABLE 1. Bacterial strains and designations

Species	Strain	Relevant characteristics (source)	HeLa cell invasion	Titration (CD_{50}/mg of protein)[a]
S. flexneri serotype 5	M90T	Contains virulence plasmid pWR100 (15)	+	55
	BS176	Derivative of M90T cured of pWR100 (16)	−	55
E. coli K12	395-1	Rough plasmidless laboratory strain (14)	−	14
	395-1(pWR110)	Transconjugant carrying pWR110, a Tn*5*-labeled derivative of pWR100 (14)	+	14
E. coli O157:H7	E30846	Noninvasive enterohemorrhagic strain (8)	−	485

[a] Shiga toxin on J774 macrophages calculated by (cytotoxic dose for 50% of cells)/(protein in milligrams).

hour for 6 h, washed extensively with EBSS, Giemsa stained, and observed under a light microscope at ×1,000.

Assay for cellular detachment and killing. The cells were infected as described above, except that the macrophages were labeled for 18 h before infection in a culture medium containing 0.5 μCi of [^{3}H]uridine per ml (Amersham Corp., Buckinghamshire, England). Cells were then washed three times with EBSS before addition of the bacterial suspension. After the incubation and washing procedure described above, the plates were removed every hour for 6 h and processed as follows. After three washings with EBSS, the percentage of nonviable macrophages among cells that still adhered to the dish was determined by trypan blue staining at a concentration of 0.2% in saline (12). The percentage of residual macrophages was then determined by measuring the amount of radioactivity remaining in the dish. Adherent cells were lysed with 1 ml of 0.5% sodium deoxycholate in distilled water, and 100 μl of the lysate was precipitated in 900 μl of 5% trichloroacetic acid. This was passed through a GFC filter (Whatman Ltd., Maidston, England), washed three times with 5% trichloroacetic acid and backed at 80°C for 30 min before being soaked for counting in an NCS-OCS preparation in a 1/9 ratio (Amersham).

In some experiments, phagocytosis was inhibited by incubating the cell monolayers for 30 min in 1 μg of cytochalasin D (Sigma Chemical Co., St. Louis, Mo.) per ml in RPMI before infection with a bacterial suspension containing a final concentration of 1 μg of cytochalasin D per ml. Stock solutions of cytochalasin D were prepared in dimethyl sulfoxide (DMSO, Sigma). Negative controls with equivalent concentrations of DMSO were always included in these experiments. Infection of the monolayers at an MOI of 150 bacteria per cell was carried out by incubating J774 monolayers with bacterial suspensions at 37°C continuously for 90 min without antibiotics.

Assay for protein synthesis. The cells were infected as already described until the end of the 20-min incubation period. The monolayers were then washed three times, and 2 ml of RPMI 1640 without methionine (Biochrom K.G., Berlin) supplemented with 2 mM glutamine, gentamicin, and 1 μCi/ml of [^{35}S]methionine ([^{35}S]methionine > 800 Ci/mmol; Amersham) was added. Plates were removed every hour and washed three times in EBSS. Residual cells were lysed, and trichloroacetic acid-precipitated material was processed as already described, except that NCS was omitted.

Preparation of samples for electron microscopy. Samples were processed for electron microscopy as described in a recent paper (16).

Titration and neutralization of Shiga toxin. Bacterial extracts for Shiga and Shiga-like toxin titration were prepared according to O'Brien et al. (11), except that bacteria were grown in M9 medium ($NaH_2PO_4 \cdot 12H_2O$, 15.12 g/liter; KH_2PO_4, 3 g/liter; NaCl, 0.5 g/liter; NH_4Cl, 1 g/liter; $MgSO_4 \cdot 7H_2O$, 0.051 g/liter; Casamino Acids (Difco), 5 g/liter; glucose, 2 g/liter; thiamine, 50 mg/liter; nicotinic acid, 10 mg/liter; tryptophan, 20 mg/liter) supplemented with 200 μg of human transferrin (Sigma) per ml. Toxin titration was performed on J774 macrophages by determining the 50% cytotoxic dose in a microtiter assay (3). Results were standardized according to the protein concentration in the bacterial lysates. Neutralization was performed with an anti-Shiga toxin rabbit serum (11).

RESULTS

Light microscopic observation of infected macrophages. J774 macrophages permitted the study of the intracellular activity of both invasive and noninvasive bacteria. Preliminary experiments demonstrated striking differences between monolayers infected with invasive and noninvasive microorganisms. A characteristic pattern of infection by M90T and its plasmidless derivative BS176 is shown in Fig. 1. Monolayers infected by M90T showed a decrease in the number of adherent cells with time and a rapid killing of remaining cells. By 6 h most of the infected macrophages appeared as ghosts, whereas noninfected cells were intact. The amount of intracellular bacteria was monitored by counting 200 infected cells every hour. A fourfold increase in the number of bacteria within infected macrophages was observed after 6 h. This is certainly an underestimation due to early detachment or lysis of heavily infected cells. In contrast, monolayers infected with BS176 showed no significant alteration in the number or aspect of macrophages. Light microscopic evaluation of intracellular growth indicated a doubling of the number of intracellular bacteria within 6 h. These observations suggested a correlation between the invasive phenotype and rapid and efficient killing of infected macrophages. The roles of plasmid pWR100 and Shiga toxin production in the rapid killing process were studied in further experiments.

Kinetics of killing of J774 macrophages. Strains were selected for their characteristic phenotypes of invasiveness and production of Shiga or Shiga-like toxin. The origin and relevant properties of these strains are summarized in Table 1. They all demonstrated similar growth rates in tryptic soy broth at 37°C.

The effect of these strains on J774 macrophages is shown in Fig. 2. Plasmidless *S. flexneri* and *E. coli* strains had no significant effect on macrophage viability when compared with uninfected controls. On the other hand, M90T was very efficient at destroying J774 monolayers. According to our criteria for cell mortality (detachment and lysis as measured by a decrease in residual radioactivity, and trypan blue positivity), 65% of the cells were dead at 1 h, and 95% were dead at 6 h. 395-1(pWR110) showed an equivalent capacity to kill macrophages. The striking differences in the ability of

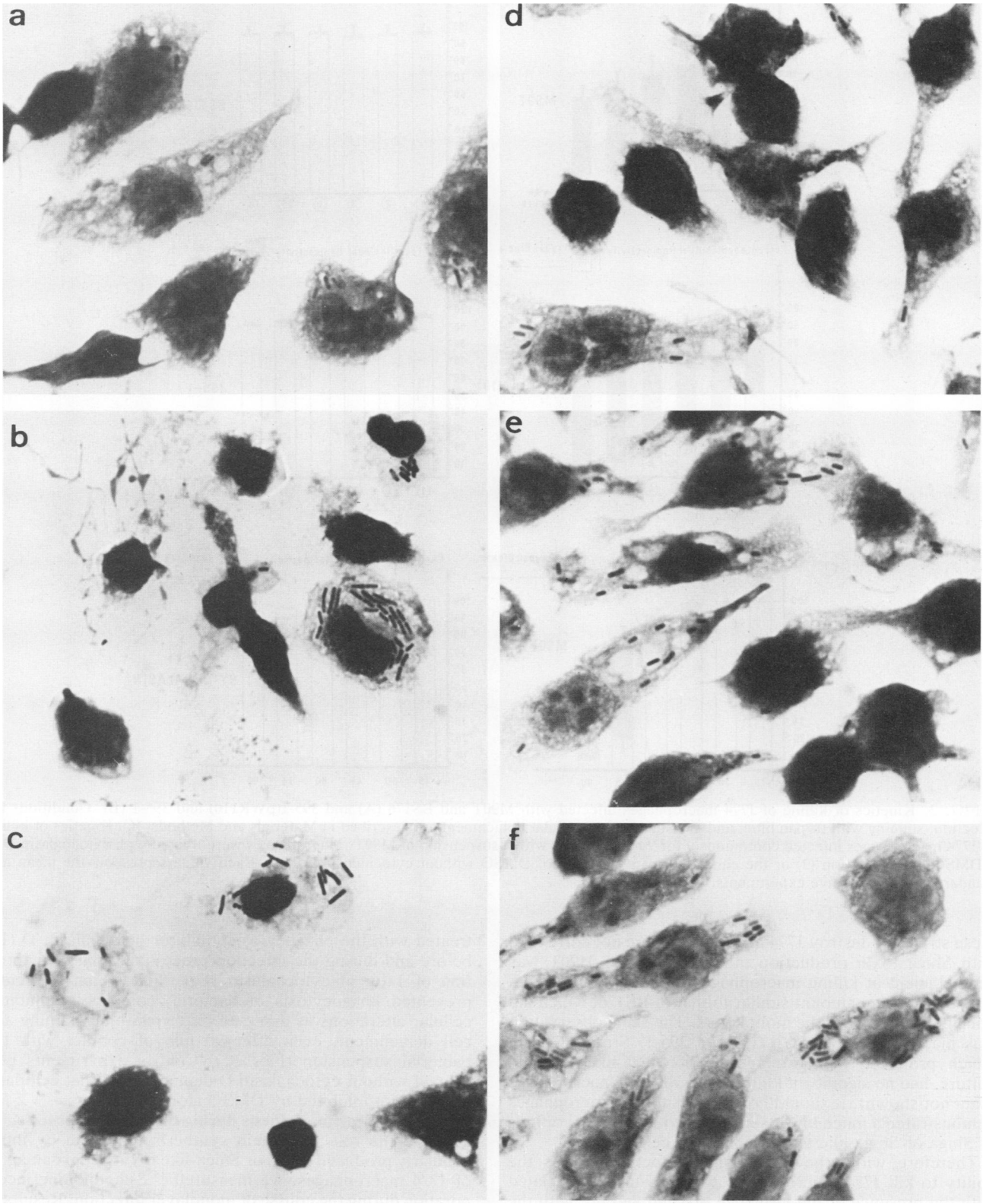

FIG. 1. Light micrographs of J774 macrophages infected with M90T (a, b, and c) and BS176 (d, e, and f) prepared as described in Materials and Methods. Incubation times at 37°C were 1 h (a and d), 4 h (b and e), and 6 h (c and f).

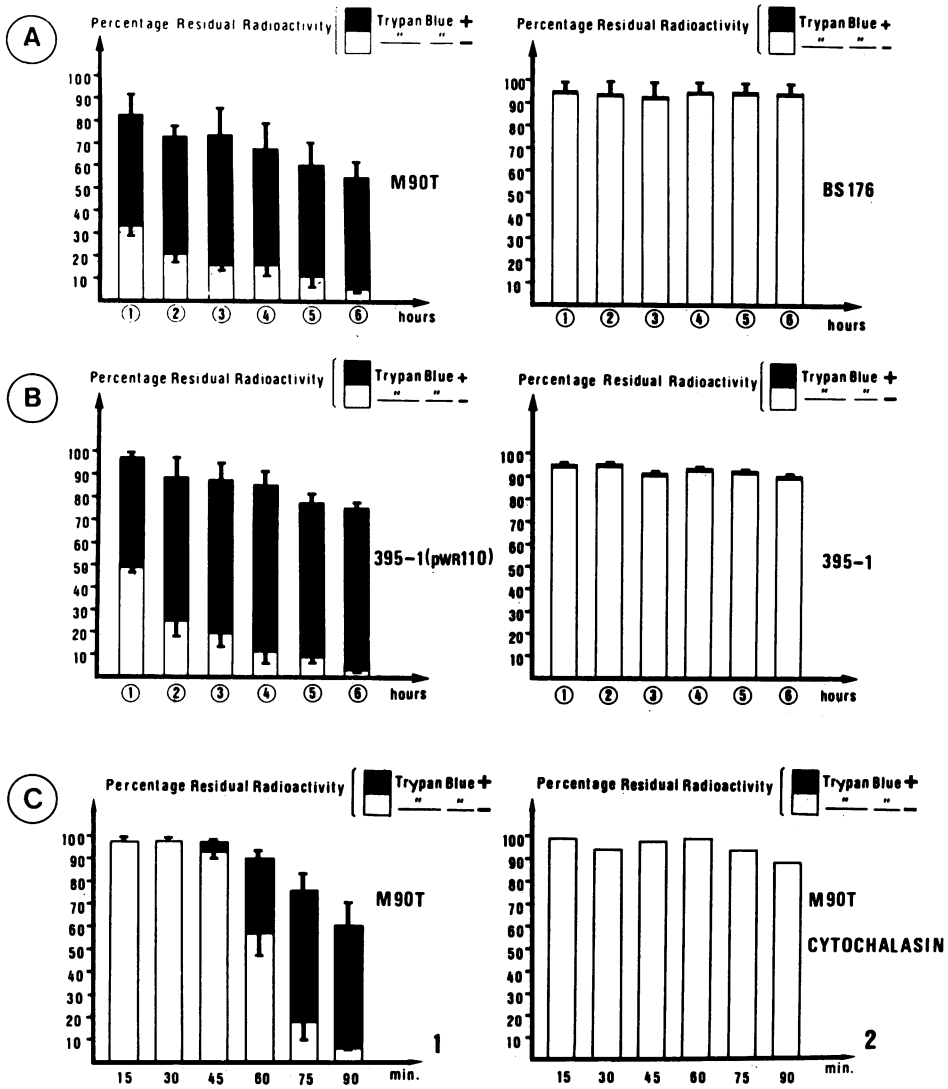

FIG. 2. Kinetics of killing of J774 macrophages infected with M90T and BS176 (A) and 395-1(pWR110) and 395-1 (B). Conditions for infection, staining with trypan blue, and measurement of cellular detachment are described in Materials and Methods. (C) Kinetics of killing of J774 macrophages infected continuously for 90 min at 37°C with a suspension of M90T. Macrophages were treated with cytochalasin D in a DMSO stock solution (2) or the equivalent concentration of DMSO without cytochalasin (D) (1). Each value represents the mean and standard deviation of five experiments.

these strains to destroy J774 monolayers were not correlated with Shiga toxin production in culture media. M90T was very efficient at killing macrophages, although it produced Shiga toxin in an amount similar to that of BS176, which did not significantly alter the monolayers. The same observation was made with 395-1(pWR110) and 395-1. Strain E30846, which produced higher quantities of Shiga-like toxin in culture, had no significant killing effect on J774 macrophages (data not shown). It should be noted that J774 macrophages demonstrated a much lower sensitivity to crude preparations of Shiga or Shiga-like toxin than HeLa cells did.

Therefore, within the first 6 h of the infection process, the ability to kill J774 macrophages appeared to be correlated with the presence of the virulence plasmid and not with the level of cytotoxin production.

To ascertain that macrophage alterations were caused by intracellular bacteria and not by simple contact between invasive bacteria and the macrophage cell surface, cells were treated with the phagocytosis inhibitor cytochalasin D (19) before and during the infection process. A final concentration of 1 μg of cytochalasin D per ml, which efficiently prevented phagocytosis of bacteria, completely inhibited cellular alterations as assessed by trypan blue staining and cell detachment, even after 90 min of contact with the bacterial suspension (Fig. 2C). Control experiments performed without cytochalasin D demonstrated that cell damage was not inhibited by DMSO alone.

Assay for protein synthesis during the infection process. To evaluate the role of protein synthesis inhibition by intracellularly produced Shiga or Shiga-like toxin in the damaging of J774 macrophages, we measured [^{35}S]methionine incorporation during the course of monolayer infection. Results of these experiments are presented in Fig. 3. Three categories of strains were defined according to the kinetics of protein synthesis during a 6-h infection of the monolayer. Category 1 consisted of 395-1, which did not significantly alter protein

synthesis. In category 2, E30846 and BS176 significantly altered the rate of incorporation which started at 2 h and reached a plateau by 5 h. In category 3, M90T and 395-1(pWR110) altered the rate of protein synthesis after 1 h of infection as seen by a highly reduced rate of incorporatin within the next 4 h. Therefore, purely cytotoxic bacteria displayed late inhibition of protein synthesis, whereas invasive microorganisms displayed early inhibition, which probably reflected rapid damage to the cells with subsequent reduction of their metabolism.

Morphological study by electron microscopy. To examine the mechanisms by which invasive pathogens kill macrophages, electron microscopic analysis was performed on infected cells. The results exhibited several features that are relevant to the intracellular behavior of both invasive and noninvasive strains. Invasive strains such as M90T and 395-1(pWR110) exhibited early lysis of the membrane-bound vacuole (ca. 100% after 15 min of infection) (Fig. 4a and b). The noninvasive plasmidless derivative, BS176, exhibited no membrane lysis after 2 h (Fig. 4c). After 4 h of infection, phagolysosomal fusion could be observed (Fig. 4d); after 6 h, large coalescent vacuoles containing lysed bacteria could be seen (Fig. 4e). Macrophages were able to sustain heavy infection by the high Shiga-like toxin producer *E. coli* 30846 without evidence of cell alteration even after 6 h (Fig. 4f). These results obtained with noninvasive bacteria correlate perfectly with the data presented in Fig. 2.

In macrophages infected by M90T, 395-1(pWR110), or BS176, intracellular bacteria were always near at least one mitochondria. Frequently, several mitochondria surrounded the bacterium, especially within the first hour of infection. In the cases of M90T and 395-1(pWR110), they exhibited direct contact with the microorganisms in the absence of phagosomal membrane (Fig. 4a and b). In addition, the mitochondria appeared smaller with a disruption of their internal morphology. For BS176, mitochondria were physically separated from the bacterium by the phagosomal membrane. They exhibited a perfectly normal aspect.

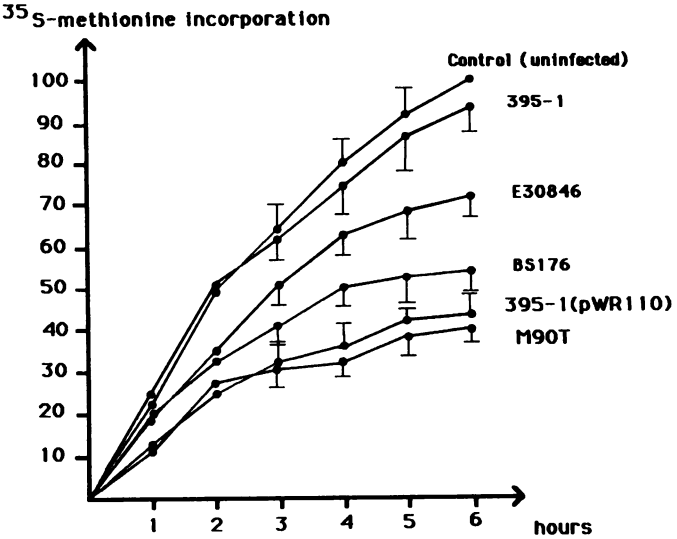

FIG. 3. Kinetics of protein synthesis in infected J774 macrophages. The experimental procedure was described in Materials and Methods. For each time point, [^{35}S]methionine incorporation by residual macrophages was standardized according to cellular detachment and expressed as a percentage of incorporation in uninfected monolayers at 6 h. Data were calculated by using the following equation: (radioactivity incorporated within residual macrophages)/(percentage of residual macrophages × radioactivity incorporated within uninfected control at 6 h). Each value represents the mean and standard deviation of five experiments.

DISCUSSION

The mechanisms by which intracellular bacteria may kill host cells are unclear (10). This is particularly true for *S. flexneri*. Among potential mechanisms, inhibition of protein synthesis by Shiga toxin is a likely candidate for promoting both intracellular multiplication and killing (5), although mutants that produce low levels of Shiga toxin show no alteration of their pathogenicity in animal models (2). In addition, bacterial products other than Shiga toxin might mediate early cytolytic effects (4). A recent study of the intracellular multiplication of different invasive microorganisms such as *S. flexneri* and *E. coli* K-12 carrying the virulence plasmid pWR110 (16) demonstrated a lack of correlation between the rate of intracellular multiplication and the level of Shiga toxin production by these strains. Alternatively, results suggested a mechanism involving early lysis of the membrane-bound phagosome by a plasmid-encoded contact hemolytic activity. This experimental model, in which HeLa cells are sensitive to infection only by invasive strains, has the obvious limitation of preventing comparisons between the behavior of invasive and noninvasive, plasmid-cured bacteria for both intracellular multiplication and killing of host cells.

This limitation could be overcome by using the continuous macrophage line J774, which is susceptible to infection by both invasive and noninvasive microorganisms. This allowed comparison of the intracellular behavior of invasive and noninvasive bacteria, as well as high and low producers of Shiga and Shiga-like toxin. However, a 10-fold-higher MOI of noninvasive bacteria was required to obtain the equivalent average number of intracellular bacteria as with invasive bacteria. This indicated that the plasmid-encoded function which induces phagocytosis in HeLa cells strikingly enhanced the level of phagocytosis by professional phagocytes. This observation suggests that during the course of the natural infection, *S. flexneri* may be efficiently phagocytosed by professional phagocytes. Moreover, during natural infection macrophages may show a much higher bactericidal activity than do J774 macrophages and lead to *Shigella* destruction as previously suggested (13). Experiments are under way to confirm this hypothesis and evaluate its implication in *Shigella* killing by human macrophages and polymorphonuclear leukocytes.

We demonstrated here that the *S. flexneri* virulence plasmid pWR100 was necessary for rapid and efficient killing of infected J774 macrophages regardless of the host strain carrying the plasmid. At 6 h, both M90T and *E. coli* K-12(pWR110) produced significant cell detachment (45 and 25%, respectively) and created sufficient cellular alterations to render more than 90% of the remaining macrophages trypan blue positive. In contrast, noninvasive strains such as 395-1, BS176, and E30846 produced no cellular damage during the same period (data not shown). During the 6 h of the experiment, no correlation could be observed between cell killing and the level of Shiga or Shiga-like toxin production by the strains. The relative resistance of J774 macrophages to extracellular Shiga or Shiga-like toxin further strengthened the hypothesis of a non-toxin-dependent killing mechanism. In addition, the kinetics of inhibition of protein synthesis in infected macrophages could not account for early cell killing, since noninvasive E30846 and BS176,

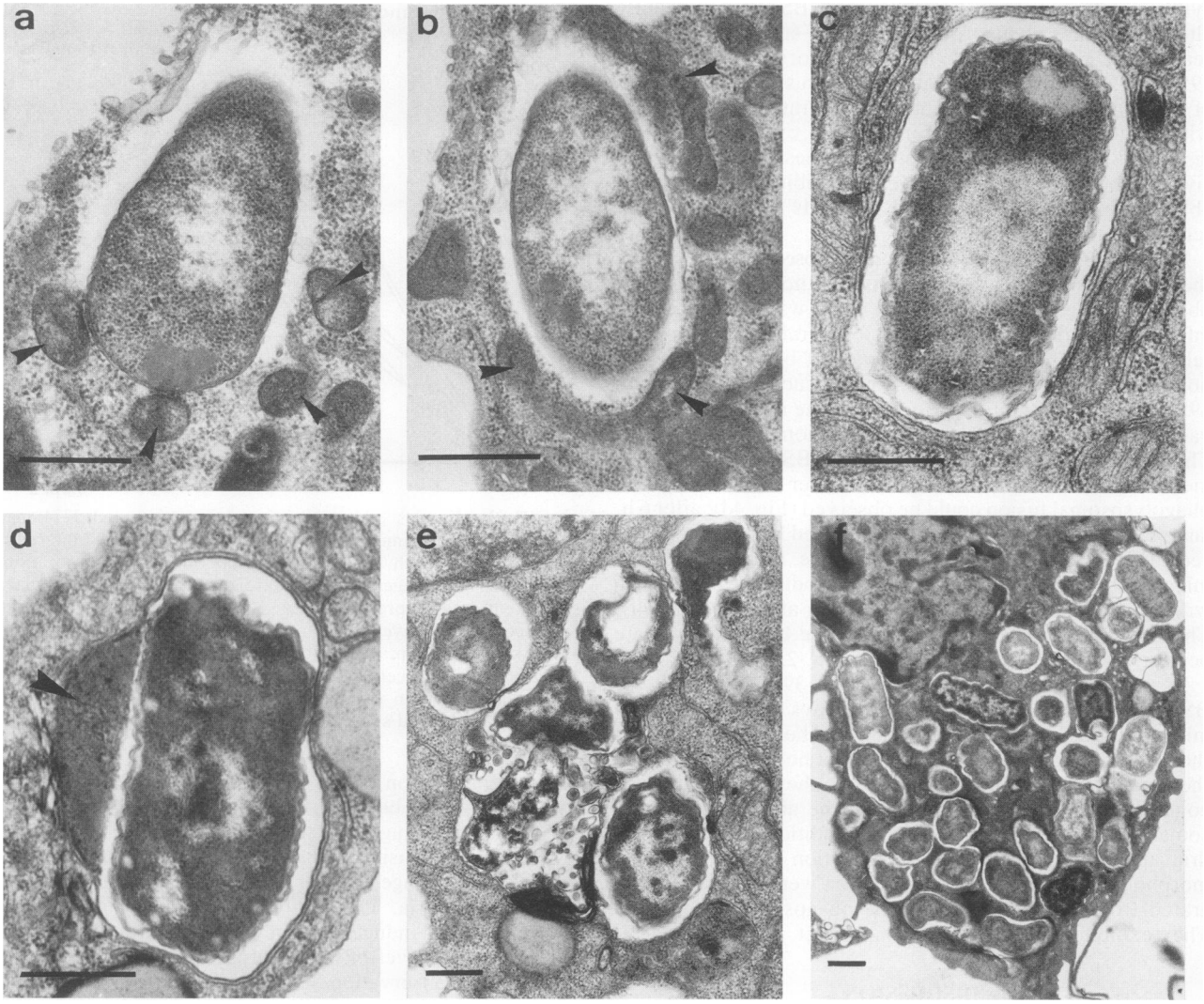

FIG. 4. Electron micrographs of infected J774 macrophages. (a) Infection by M90T after 15 min. Arrowheads point to mitochondria surrounding the bacterium. (b) Infection by 395-1(pWR110) after 15 min. Arrowheads point to mitochondria surrounding the bacterium. (c) Infection by BS176 after 2 h. (d) Infection by BS176 after 4 h. Arrowhead points to a region of phagolysosomal fusion. (e) Infection by BS176 after 6 h. (f) Infection by E30846 after 6 h. Bars, 0.5

which significantly inhibited protein synthesis 2 h after infection, did not kill cells. On the other hand, strain M90T damaged 66% of the cells within 1 h, although protein synthesis in residual cells reached a plateau only after 5 h of infection. This indication that cell damages largely occurred before protein synthesis was shut off by the action of Shiga toxin on the 60S ribosomal subunit was strengthened by the observation that 395-1(pWR110), which did not produce a significant amount of Shiga toxin, was still able to produce substantial cellular damage. However, it would be noted that the kinetics of protein synthesis in macrophages infected by M90T or 395-1(pWR110) are very difficult to assess. In these cases, early killing of the macrophages resulted in a lack of [^{35}S]methionine incorporation that could not be related to Shiga toxin. On the other hand, detachment of the most heavily infected macrophages enriched for slightly infected macrophages that should still synthesize proteins in reasonable conditions. In any event, another mechanism of killing mediated by the *Shigella* virulence plasmid has to be found. Expression of this damaging function was observed only with intracellular bacteria, since cytochalasin D, which inhibits phagocytosis, protected macrophages from killing by the invasive strain M90T. To characterize this plasmid-mediated function, infected macrophages were observed by electron microscopy. This study demonstrated that efficient cell killing correlated with the ability of invasive strains to lyse the membrane-bound vacuole very rapidly after phagocytosis (i.e., 15 min). This was consistent with our recent observation that early lysis of the phagocytic vacuole and efficient multiplication within HeLa cells were correlated with a contact hemolytic activity encoded by the plasmid (1, 16). In the present system, rapid killing and detachment of macrophages prevented quantification of intracellular multiplication. Light microscopy indicated a significant degree of intracellular multiplication of invasive strains (Fig. 1). On the other hand, noninvasive bacteria which did not damage macrophages even at 6 h remained trapped within intact phagocytic vacuoles, thus allowing further phagolysosomal fusion and bacterial destruction (Fig. 4d and e). This would suggest a mechanism in which

invasive bacteria avoid intracellular killing by lysing the phagocytic vacuole, thus escaping phagolysosomal fusion.

Contact hemolytic activity may directly or indirectly account for cell killing. In the former hypothesis, this membrane-damaging function may have a deleterious effect on the intracellular membrane system of the cell such as endoplasmic reticulum, Golgi apparatus, lysosomes, and mitochondria and would subsequently alter the cytoplasmic membrane from the inside of the cell. This is consistent with the rapid loss of trypan blue exclusion observed with cells infected by invasive strains, since the uptake of this dye indicates both a loss of surface membrane function and cell death by membrane criteria (12). In the latter hypothesis, rapid lysis of the phagocytic vacuole could lead to direct expression of other cytotoxic activities and facilitate contact of the invading microorganism with intracytoplasmic organelles. A striking accumulation of mitochondria was observed around the invasive M90T and 395-1(pWR110) strains (Fig. 4a and b). Some of these mitochondria even established close contact with the bacterium (Fig. 4a). In addition, important morphological alterations could be observed within these organelles such as disruption of the inner membrane folding. Mitochondria could also be observed surrounding the phagocytic vacuoles containing noninvasive pathogens (Fig. 4c). In these cases, the mitochondria appeared intact.

The accumulation of mitochondria around phagocytic vacuoles has already been reported with *Legionella pneumophila*-infected macrophages (7). It probably reflects a normal cellular response during phagocytosis. In this case of invasive *S. flexneri* the bacterium may take advantage of lysing the phagosome to interact with mitochondria and kill cells. Experiments are under way to study the perturbations of cellular respiration that could occur early after invasion.

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