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Association of Viable and Inactivated *Salmonella typhimurium* 395 MS and MR 10 with HeLa Cells

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The mouse-virulent *Salmonella typhimurium* 395 MS, containing a complete lipopolysaccharide (LPS) structure with S-specific repeating units, and the nonvirulent, LPS-defective mutant 395 MR 10 (chemotype Rd), derived from it, were studied for their tendency to interact with HeLa cells. In the definition of interaction no distinction has been made between intracellular and cell membrane-attached bacteria. R10 bacteria were found to have a greater tendency to interact than MS bacteria. This difference was seen as early as 1 h after the start of incubation, but it became more pronounced beyond 3 h. Heat-killed and ultraviolet-killed R10 bacteria interacted with HeLa cells less than living ones. Killed MS bacteria interacted to an extent similar to that of living ones. These results are discussed in relation to the susceptibility of the bacteria to phagocytosis by professional phagocytic cells and to the physicochemical properties of the bacteria as measured by their distribution in a two-polymer, aqueous-phase system.

Most microbial pathogens gain access to the host tissue at mucosal surfaces such as those of the respiratory, gastrointestinal, and urinary tracts. For several viruses, well-defined, specific receptors have been determined. For most bacteria the animal cell surface structures responsible for attachment are less well known. Furthermore, it has not been satisfactorily elucidated how much the bacterial activity and the mammalian cell, respectively, contribute to bacterial attachment and ingestion.

Professional phagocytes, e.g., polymorphonuclear leukocytes, which combat the invasive process, engulf bacteria in an energy-requiring process largely dependent on the hexose-monophosphate pathway of the leukocyte (10). Engulfment is initiated after an initial attachment step. Attachment and engulfment of *Salmonella typhimurium* is facilitated after S → R mutation (2, 18) and after opsonization with immunoglobulin G (21). Both processes increase the hydrophobicity, as shown by partition in a two-polymer aqueous-phase system (19-21) and measurements of the contact angle of a drop of saline on a layer of cells (2). From theoretical calculations the increased surface tension (hydrophobicity) is of great importance for attachment (13). We hypothesized that similar mechanisms might operate in the internalization of bacteria into other mammalian cells that are not professional phagocytes, notwithstanding the paradoxical consequence of this hypothesis that nonvirulent R-mutants might be more invasive to the cells than the virulent parent S-bacteria. The attachment necessary for internalization has been investigated with HeLa cells in vitro, the cell line originating from an adenocarcinoma of the cervix uteri.

MATERIALS AND METHODS

Bacterial strains. The smooth, mouse-virulent strain *S. typhimurium* 395 MS and its R-mutant 395 MR10 (chemotype Rd), derived from it, have been characterized earlier (4, 9, 12).

Cultivation and killing of bacteria. All strains were kept at 4°C on agar slants before use. The bacteria were inoculated into 15 ml of nutrient broth (Difco) and incubated at 37°C for 18 h. The bacteria were harvested by centrifugation (1,100 × *g*, 15 min). One batch was suspended in phosphate-buffered saline solution, pH 7.3 (PBS), exposed to 56°C for 60 min, and tested for sterility. Another batch was washed twice in PBS, suspended in PBS to 2 × 10^8 bacteria/ml (estimated with a Turner spectrophotometer at 650 nm), exposed to ultraviolet (UV) radiation for 15 min, and tested for sterility. A third batch was washed twice in PBS and kept viable.

Mammalian cell culture. The HeLa cell line (ATCC strain CCL 2, human serum, research grade) was obtained from Flow Laboratories, Irvine, Scotland. Specimens of cells were kept in liquid nitrogen. Such cells were initiated at intervals not longer than 3 months. The cells were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum and 100 IU of penicillin and 100 IU of streptomycin per ml. Cells were maintained as monolayers in plastic tissue culture bottles in a humidi-

fied incubator with an atmosphere of 5% CO_2 and 95% air at 37°C.

The monolayer was treated with 0.25% trypsin to detach the cells, and the detached cells were used to prepare monolayers on cover slips. The cover slips, 18 by 18 mm, were attached with vaseline to the bottom of a tissue culture petri dish, 5 cm in diameter, and each dish was seeded with 2 ml of HeLa cell suspension and 1 ml of fresh medium. The dishes were incubated for approximately 72 h in the humidified incubator, with changes of medium every 24 h, the last change without penicillin and streptomycin. This gave 80 to 90% confluent monolayers. The cells were regularly tested for mycoplasma contamination by staining with orcein (5). All cell culture material was purchased from Flow Laboratories, Irvine, Scotland.

Interaction procedure. After 72 h of incubation, the medium was poured off from the petri dishes containing cover slips, the bottom of the petri dish was rinsed three times in 37°C PBS, 3 ml of bacterial suspension was added, and the petri dishes were incubated again in the humidified incubator. The concentrations of bacteria added were: for inactivated bacteria, 4.0×10^7/ml; for viable bacteria, 2.5×10^6, 10^7, 4.0×10^7, 1.6×10^8, and 6.4×10^8/ml. All bacteria were suspended in Earle balanced salt solution, pH 7.3 (EBSS; Flow Laboratories) before exposure to the HeLa cells. At indicated intervals, cover slips were taken, rinsed three times in 37°C PBS, fixed in methanol-acetic acid (3:1) for 5 min, rinsed three times in deionized water 37°C, stained with Giemsa stain at pH 7.0 for 15 min, rinsed three times in deionized water 37°C, and observed with an immersion objective after putting immersion oil onto the cell layer (11).

Two hundred HeLa cells were randomly counted on each cover slip and ordered into six groups, containing 0, 1, 2, 3 to 5, 6 to 10, and more than 10 bacteria per HeLa cell, respectively.

Bacterial growth. About 4×10^7 MS or R10 bacteria/ml in 3 ml of EBSS were incubated in test tubes at 37°C for 1.5, 3, or 5 h, when samples for viable count were removed. Viable counts were performed by spreading 0.1 ml onto nutrient agar plates and incubating overnight at 37°C. The net growth of bacteria in the presence of HeLa cells was estimated by suspending 4×10^7 bacteria (MS or R10) per ml in 3 ml of EBSS and incubating with monolayers of HeLa cells in petri dishes without cover slips for 1.5, 3, or 5 h. At each time, samples were removed from the cell culture medium for viable counts. The corresponding monolayers were rinsed three times in 37°C PBS and detached from the petri dishes with 0.25% trypsin and a rubber policeman. The HeLa cells were suspended in 3 ml of EBSS plus 24 ml of PBS and disintegrated with a modified LoX-press at an extrusion pressure of 5.3×10^6 to 6.1×10^6 Pa (1 kp/cm^2 = 0.980665×10^5; Pa = pascal) (23). Assessment of the disintegration achieved was performed by microscopic examination of wet mounts. After pressing, no intact HeLa cells remained. Samples were removed from the homogenate for viable count. These bacteria were regarded as HeLa cell associated. The sum of the total number of bacteria in the culture medium, including the three rinsings, and the HeLa cell-associated bacteria was expressed as percentage of initially added bacteria.

RESULTS

Effect of concentration of *S. typhimurium* MS and MR10 bacteria on their association with HeLa cells. Within the concentration range tested, 0.25×10^7 to 64×10^7 bacteria/ml, the association of *S. typhimurium* S-bacteria to HeLa cells in 3 h was small (Fig. 1). A greater effect of the bacterial concentration was noted for R-bacteria such that the lowest concentration tested, 0.25×10^7/ml, gave a lower association than the others (Fig. 2). At the highest concentration tested, several HeLa cells had obtained a low number of bacteria (1 to 5 bacteria/HeLa cell), whereas the number of HeLa cells showing more than five bacteria associated was remarkably low. These results indicate an adverse effect on association by high concentrations of bacteria. Therefore, 4.0×10^7 bacteria/ml was chosen for inoculum unless stated otherwise.

Association of *S. typhimurium* 395 MS with HeLa cells. The association of *S. typhimurium* 395 MS with HeLa cells after incubation for 1 h was small, rising very little within the next 4

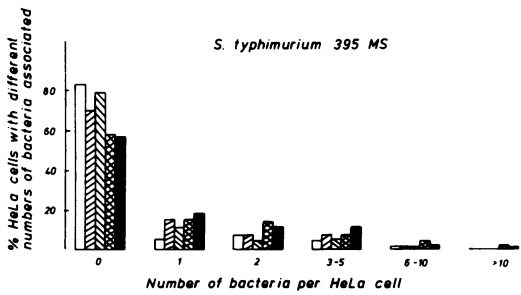

FIG. 1. *Association of different concentrations of viable S. typhimurium 395 MS with HeLa cells after 3 h of incubation. Symbols: □, 2.5×10^6 bacteria/ml; ▨, 10^7 bacteria/ml; ▧, 4.0×10^7 bacteria/ml; ■, 1.6×10^8 bacteria/ml; ■, 6.4×10^8 bacteria/ml.*

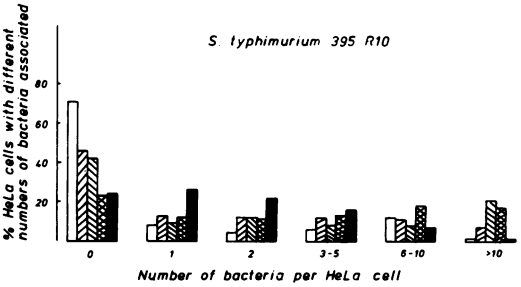

FIG. 2. *Association of different concentrations of viable S. typhimurium 395 MR 10 with HeLa cells after 3 h of incubation. Symbols: see Fig. 1.*

h. Most of the HeLa cells, 68 to 86%, did not show a single attached bacterium (Fig. 3). UV irradiation or heating at 56°C for 60 min (Fig. 3) reduced the number of associating bacteria slightly ($P < 0.10$, Student's t test).

Association of *S. typhimurium* R-bacteria with HeLa cells. After incubation for 1 h with viable *S. typhimurium* 395 MR 10, more than 50% of the HeLa cells already showed association with at least one bacterium (Fig. 3). With increasing incubation time, a larger proportion of HeLa cells showed bacteria, such that after 5 h only 16% were free from bacteria. With increasing incubation time, the number of bacteria per HeLa cell increased steadily, so that after 5 h 44% of the HeLa cells showed association with more than 10 bacteria. In one experiment a high percentage of HeLa cells showed association with more than 10 bacteria after incubation for both 3 and 5 h (Fig. 3).

Killing the bacteria with UV irradiation or heating at 56°C for 60 min removed the tendency to association nearly completely ($P < 0.02$, Student's t test; Fig. 3). No statistically significant difference in association was observed between incubation for 1, 3, and 5 h for UV-killed bacteria and heat-killed 395 MS. For heat-killed 395 MR 10 there was a slight increase in association after incubation for 5 h ($P < 0.10$, Student's t test; Fig. 4).

To reduce the possible effect on association by multiplication of viable bacteria to a greater number, 4×10^7 viable bacteria/ml and 10^8 UV- or heat-killed bacteria were inoculated into HeLa cell cultures in parallel experiments and incubated for 5 h (Table 1). More HeLa cells showed association of viable bacteria in spite of their smaller inoculum.

Change of the viable population of *S. typhimurium* in incubation medium with and without HeLa cells. The number of viable bacteria in the medium was investigated by viable counts after incubation of 4×10^7 bacteria/ml in EBSS at 37°C (Fig. 5). After 5 h of incubation only 30% of the inoculum was recovered, the same for MS and R10.

Population changes under the conditions of the association tests were investigated by performing the above experiments in the presence of HeLa cells. The viable count was determined by adding that of the medium to that of the disintegrated HeLa cells (Fig. 5). Both the MS and the R10 population expanded, the increase being approximately 85 and 130%, respectively, after 5 h.

DISCUSSION

Viable R10 bacteria inoculated into a culture of HeLa cells became associated to the HeLa cells (Fig. 1-3). This tendency proceeds with incubation such that after 5 h only 16% of the HeLa cells do not show any associated bacteria. In contrast, the association of MS bacteria is much smaller and does not increase conspicuously with time. This difference between R10 and MS bacteria cannot be due to different multiplication rates, since viable counts for MS and R10 on samples from bacteria inoculated into the medium alone and from bacteria added to HeLa cells were rather close to each other. Neither is there reason to believe that MS should be killed intracellularly to a greater extent than R10, since S-bacteria usually survive better than R-bacteria in granulocytes and macrophages (6) and in homogenates from granulocytes (22).

Ogawa et al. (16) were unable to demonstrate invasion of HeLa cells by various strains of salmonella, including a strain of *S. typhimurium*. We presume that those salmonella strains were virulent and smooth, since they had been submitted to the laboratory for identification. In contrast are the results of Giannella et al. (7), in which a number of *S. typhimurium* S and R strains were shown to invade HeLa cells. In the latter experiments the only strains not showing invasion were three genetically marked standard laboratory strains which were not compared as S-R pairs. When they compared the smooth, virulent strain TML with strain TML-47B2, a one-step rough mutant of TML unable to synthesize O-repeating units (mean lethal dose for mice, $\log_{10} = 5.4$), both strains were able to invade. In fact the mutant strain "appeared comparably invasive to its smooth parent." Parallel findings were observed when smooth strain SL 1027 and its rough derivatives SL 1034 and SL 1036 were compared. No quantitative data were given, though. To reconcile their data with those of Ogawa et al. (16), Giannella et al. suggested that the 5-h incubation time for salmonella plus HeLa cells used by Ogawa et al. was too short; according to their findings, invasion was just beginning at this time. As Ogawa et al. (16) commented on the results of Ito (1960, see reference 16), who stated that freshly isolated strains of *S. typhi* and *S. paratyphi* A could multiply in HeLa cells, they considered the discrepancy from their results to be largely due to the difference in infection period. In an infection period of 2 to 5 h, the cell infection rate was very low (1% or less) and was 5 to 10% in subsequent incubation for 3 days (Ito, see 16). We support the conclusion by Ogawa et al. (16) showing the comparatively low infection rate of *Salmonella* S bacteria within a 5-h incubation period.

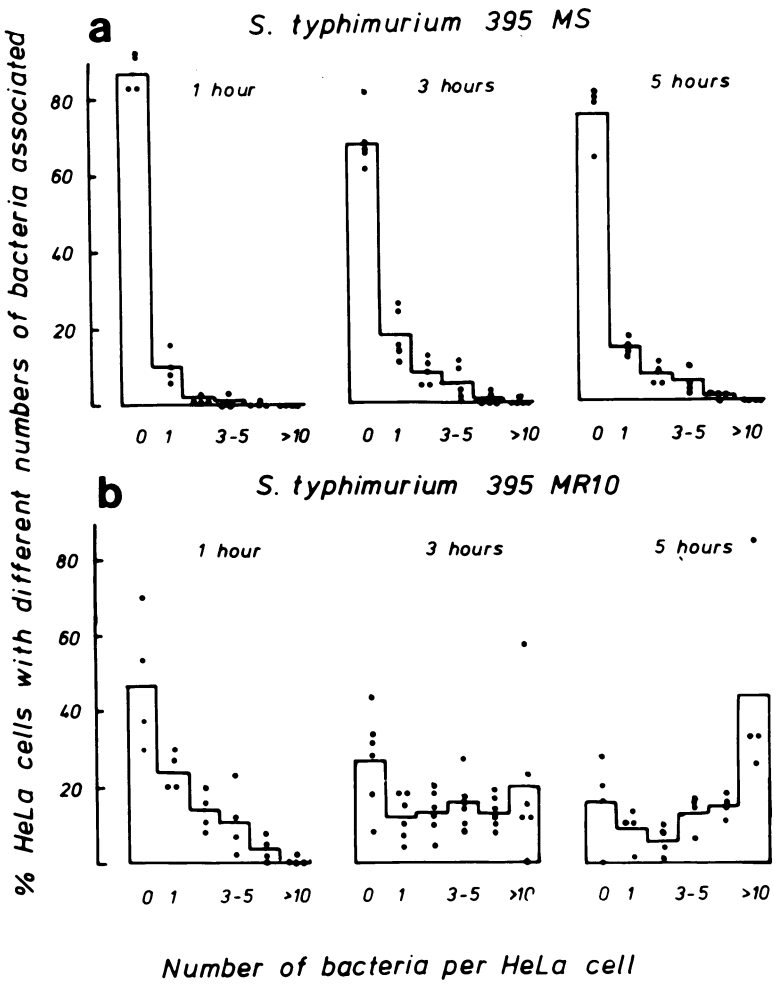

FIG. 3. *Association of viable S. typhimurium 395 MS and MR 10 with HeLa cells after 1, 3, and 5 h of incubation (a, b) and viable and inactivated S. typhimurium 395 MS and MR 10 after 5 h of incubation (c, d).*

The higher association rate of *S. typhimurium* 395 MR10 already after 1 to 3 h in comparison with its parent S-strain is in apparent contradiction to the results of Giannella et al. (7), who noted infectivity for HeLa cells of both S and R strains of *S. typhimurium*. Both Ogawa et al. (15, 16) and Giannella et al. (7) used 10 to 20% calf serum in the culture as well as in the infection medium. Ogawa et al. (15) stated that serum enhanced the uptake of bacilli. In cinematographic observations on the penetration by *Shigella flexneri* into, inter alia, HeLa cells, the events leading to incorporation were compared to phagocytosis (15). Serum and precolostral calf serum are known to opsonize S and R strains of *S. typhimurium* for phagocytosis by polymorphonuclear leukocytes (18), and precolostral calf serum has a marked bactericidal effect on *S. typhimurium* 395 MRO (an Ra mutant) at a dilution of 1:32 (3). Thus, serum presumably influences the association between salmonella and HeLa cells, via both the surface properties and the physiological activity of the bacterium.

The smaller tendency of MS rather than R10 bacteria to associate to HeLa cells is analogous to their difference in liability to phagocytosis by rabbit polymorphonuclear leukocytes (18). This difference, which is related to the surface properties of the bacteria, has been analyzed by partition in an aqueous two-polymer phase system made from dextran and polyethylene glycol (19, 20) and by measurement of the contact angle of a drop of saline on a layer of bacteria (2). The conclusion is that MS bacteria are hydrophilic and nearly uncharged, whereas R10

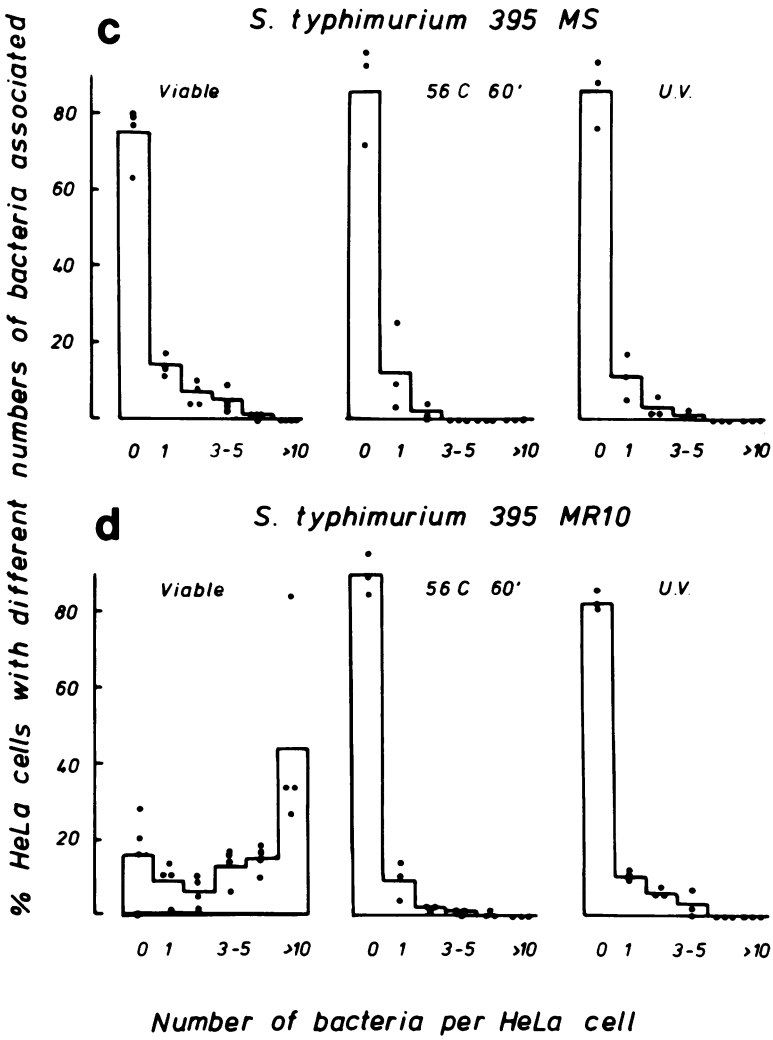

FIG. 3—*Continued*

are less hydrophilic and have a negative charge. Reduced hydrophilicity should, for theoretical reasons, favor attachment to structures that themselves are not too hydrophilic (13). However, although smaller than the association of R bacteria, the association of S bacteria with epithelial mammalian cells cannot be negligible since natural infection usually takes place that way. The higher infectivity of S bacteria is, however, presumably due to their higher resistance to host defense mechanisms, which allows multiplication of a few invading cells (24).

The differences shown between S and R bacteria for *S. typhimurium* 395 M are probably not restricted to this strain. Preliminary data have shown similar differences between *Salmonella minnesota* S 66 (an S-strain) and *S. minnesota* R 595 (chemotype Re). Whereas *S. minnesota* R 595 showed a strong tendency to associate with HeLa cells, that of S 66 was negligible. Parallel differences existed with respect to partition in the two-phase system (E. Kihlström, to be published). These data support the view that association of salmonella bacteria with HeLa cells is related to lipopolysaccharide structure and surface properties of the bacteria.

Viable but not heat-killed (60°C, 1 h) *Shigella flexneri* caused keratoconjunctivitis in guinea pigs, and the same was true for infection of cultured cells (e.g., HeLa cells) in vitro (15). Similarly, in our experiments heat or UV inactivation of R10 abolished the association with HeLa cells. In contrast, living as well as heat-killed *Mycobacterium tuberculosis* were

phagocytosed by HeLa cells (17), and HeLa cells engulfed thorium dioxide particles (1).

The physicochemical properties of R10, determined by partition in the two-phase system, is the same for viable bacteria and bacteria heated at 56°C for 60 min (19), and heat-killed *S. typhimurium* are efficiently engulfed by polymorphonuclear leukocytes (18). Heating at 56°C for 60 min inactivates several enzymes and produces cracks in the cell wall of gram-negative bacteria (8). Few or no such effects are known for UV killing, which is supposed to be more specific for the nucleic acids and nucleotides. However, UV irradiation of myxovirus inactivates neuraminidase (14), which is a possible candidate for enhancement of the association between enterobacteria and HeLa cells, as are other surface-bound catalysts such as the cyclic nucleotide system and the lysophospholipid systems. Such microbial mechanisms acting on the cell surface of nonprofessional phagocytes may function as adjuvants for association and phagocytosis. The present experiments do not distinguish between bacteria attached to HeLa cell surface and bacteria that have become internalized. However, endocytosis in HeLa and other cultured cells is an established phenomenon, and several different bacterial species have been shown to occur inside epithelial cells as R10 do in HeLa cells (Kihlström, to be published). The association as studied above is presumably an indispensable step in the internalization process.

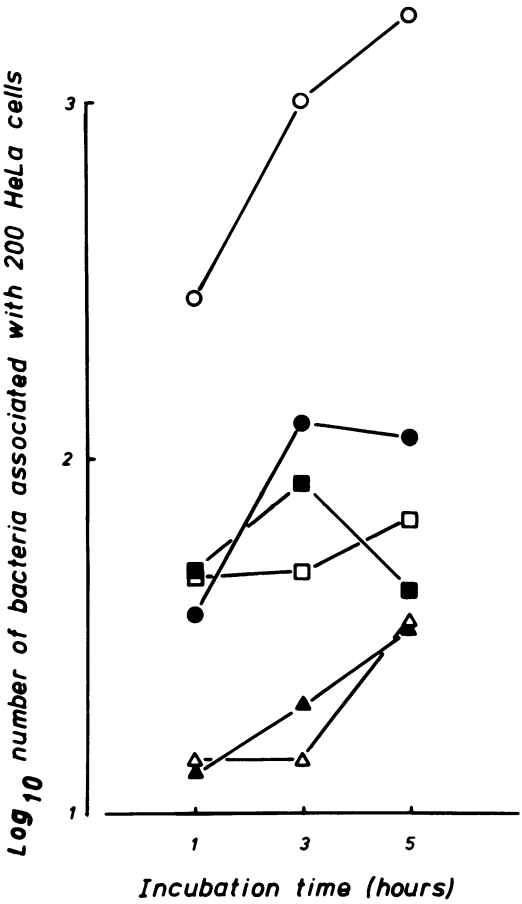

FIG. 4. *Total number of viable or inactivated S. typhimurium 395 MS and MR 10 associated with 200 HeLa cells. Symbols: ●, MS, viable; ■, MS, UV; ▲, MS, 56°C; ○, R10, viable; □, R10, UV; △, R10, 56°C.*

TABLE 1. *Percentage of viable and inactivated S. typhimurium MR10 associated with HeLa cells after 5 h of incubation*

Type of bacteria	No. of bacteria/HeLa cell					
	0 h	1 h	2 h	3-5 h	6-10 h	>10 h
Viable[a]	19	10	11	15	13	32
UV[b]	84	11	5	0	0	0
56°C, 60 min[b]	83	14	2	1	0	0

[a] Initial concentration, 4×10^7/ml.
[b] Initial concentration, 10×10^7/ml.

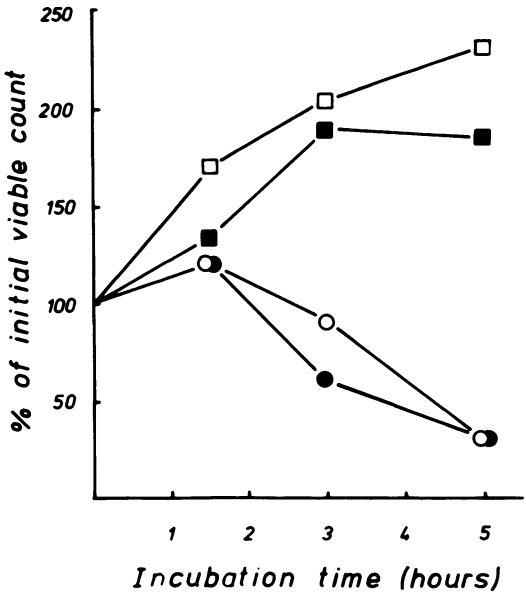

FIG. 5. *Change in viable counts of S. typhimurium 395 MS and MR 10 after incubation in EBSS alone or with a monolayer of HeLa cells. Symbols: ●, MS EBSS; ■, MS EBSS + HeLa; ○, R10 EBSS; □, R10 EBSS + HeLa.*

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