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Double Immunofluorescence Microscopic Technique for Accurate Differentiation of Extracellularly and Intracellularly Located Bacteria in Cell Culture

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A double immunofluorescence staining technique is described for differentiation between cell-attached (extracellular) and ingested (intracellular) bacteria by HEp-2 cells in cell culture monolayers. This method is based upon the observation that membranes of viable mammalian cells are impermeable for antibodies but are rendered permeable by treatment with fixatives. Consequently, extracellular bacteria can be stained by specific rhodamine-labeled antibodies before fixation, and intracellular bacteria can be visualized by treatment with specific fluorescein-labeled antibodies after fixation. The accuracy and simplicity of this method is demonstrated with HEp-2 cell culture monolayers as target cells and an isogenic pair of *Yersinia enterocolitica*, one of which is phagocytosis resistant and the other of which is phagocytosis sensitive. Furthermore, it is shown that this staining technique is also applicable for studying the interaction of bacteria with macrophages and fibroblasts.

In vitro tissue culture systems are frequently used for investigating the interaction between microbial pathogens and mammalian cells (7, 12). The process of infection can be divided into two main phases, i.e., adherence (extracellular location) and internalization (intracellular location of the bacteria). With conventional bright field light microscopy it is impossible to distinguish accurately intracellularly located from extracellularly attached bacteria. To overcome this obstacle, several indirect methods have been developed, e.g., antibiotic treatment to eliminate extracellular bacteria (13, 17), optical sectioning through the cells by light microscopy (13), differential interference contrast microscopy (1, 2), identification of cell-adherent bacteria by immunofluorescence microscopy (4, 11), and fluorescence quenching technique (9). However, these methods are not exact, and sometimes they lead to artifacts. For example, extracellularly attached bacteria are not completely eliminated by antibiotics (this paper), optical sectioning requires high numbers of cell-associated bacteria, differential interference contrast microscopy cannot distinguish bacteria located closely underneath the cell membrane from those attached to the outer cell membrane, and monolabeling immunofluorescence microscopy is troublesome because of the need to count a large number of cell-associated bacteria of fixed and unfixed specimens to get a statistical estimation of intra- and extracellular bacteria. Finally, the fluorescence quenching method uses bacteria whose surfaces are chemically modified by fluorescein isothiocyanate coupling. Of course, the localization of cell-associated bacteria can be exactly determined by electron microscopy. However, this method does not give a three-dimensional image of the total distribution of the bacteria associated with cells, and it is not convenient for routine use with a large number of samples.

In this paper, we describe a simple double immunofluorescence staining technique suitable for discriminating directly between intracellularly and extracellularly located bacteria. This method is based upon the observation that antibodies are not able to diffuse freely into viable mammalian cells but can do so after the mammalian cell membrane has been treated with weak fixatives (3, 4, 11). Consequently, it should be possible to stain extracellular bacteria by, e.g., tetramethylrhodamine isothiocyanate (TRITC)-labeled specific antibodies before fixation and then to stain intracellular bacteria by, e.g., fluorescein isothiocyanate (FITC)-labeled specific antibodies after fixation of the cell monolayer. As a test bacterium we used an enteropathogenic *Yersinia enterocolitica* strain of serotype O:3 which harbors a 46-megadalton virulence plasmid and a plasmid-cured avirulent derivative (10). This isogenic pair was chosen because the plasmid-bearing strain was suggested to be resistant to phagocytosis by HEp-2 human epithelial cells, whereas the plasmidless derivative can apparently be internalized by HEp-2 cells (16). Furthermore, it is shown that this staining technique is not restricted to HEp-2 cells but also applies to the interaction of bacteria with other cell types, e.g., fibroblasts or macrophages.

MATERIALS AND METHODS

Bacterial strains and culture conditions. An isogenic pair of *Y. enterocolitica* of serotype O:3 (strain y-108-P, harboring a 46-megadalton virulence plasmid, and its plasmidless derivative, y-108-C; see reference 10), *Shigella sonnei*, and *Salmonella enteritidis* (both clinical isolates) were used as test strains. The bacteria were cultivated in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) overnight at 26°C. The next day the cultures (stationary phase) were diluted 1:20 with brain heart infusion broth and were shaken with aeration for 2 to 3 h at 37°C. After the cultures had reached an optical density (at 600 nm) of ca. 0.3 (logarithmic phase), the bacteria were harvested by centrifugation, washed in phosphate-buffered saline (PBS), and then suspended in cell culture medium without fetal calf serum and antibiotics (for details, see below) to an optical density of ca. 0.1 to 0.15. This bacterial concentration which was used for infection of all cultures corresponds to 5×10^7 to 1×10^8 CFU/ml as determined by plating appropriate dilutions of the suspension

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onto solid medium and counting the number of colonies grown the next day.

Antisera. The preparation procedures of antisera for immunofluorescence staining were not critical in our experiments. We found that antisera against *Y. enterocolitica* serotype O:3 obtained from rabbits after intravenous immunization with heat-killed (10), Formalin-killed, or live bacteria (5) and from guinea pigs after intracutaneous immunization with heat-killed bacteria emulsified in Freund incomplete adjuvant (5×10^8 bacteria per ml emulsified with an equal volume of adjuvant [8]) could be used equally. For example, rabbit anti-*Yersinia* serum (Formalin-killed antigen) exhibiting a tube agglutination titer of 1:1,600 could be used as 1:100 dilution for the immunofluorescence stain. *Shigella sonnei* and *Salmonella enteritidis* were stained with commercial O-serotyping sera diluted 1:40 (rabbit anti-*Shigella sonnei*, smooth form, and rabbit anti-*Salmonella* serotype O:9 were purchased from Behring, Marburg, Federal Republic of Germany). The second anti-rabbit immunoglobulin G (IgG) antibodies as TRITC conjugate and as FITC conjugate were from Sigma, Heidelberg, Federal Republic of Germany (IgG fraction of antisera and affinity-isolated antigen-specific antibodies gave similar results).

Cell cultures. HEp-2 cells (human epithelial cells), ATCC strain CCL-23 (Flow Laboratories, Meckenheim, Federal Republic of Germany), were grown in a basal medium (minimal essential medium with Earle salts, nonessential amino acids, and 5 mM glutamine) supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer and 5% fetal calf serum (Flow). Human fibroblast cells (Flow 5000, Flow) were maintained in the same medium as described for HEp-2 cells but supplemented with 10% calf serum. HEp-2 cells and fibroblasts were grown to confluent monolayers before exposure to bacteria. Unstimulated resident macrophages were obtained by washing out directly the peritoneal cavity of white mice (strain NMRI, Hannover) with Dulbecco modified Eagle medium-glutamine (Flow) supplemented with 10 U of heparin per ml (washing medium) (15). The washed macrophages were suspended in Dulbecco modified Eagle medium supplemented with 5 mM glutamine, 44 mM sodium bicarbonate, and 10% fetal calf serum (macrophage culture medium). Cell suspensions (ca. 300 μl of 10^6 cells per ml) were layered over glass cover slips in a moist chamber and cultured at 37°C. After 2 h of incubation, the cover slips were washed free of nonadherent cells with warm (37°C) washing medium. The cover slips with the attached macrophages were then placed into glass tubes filled with 3 ml of macrophage culture medium and incubated for 10 to 14 h in a slantwise position before being infected with bacteria.

Infection procedure. The cover slips with the cell monolayers were placed into slanted glass tubes filled with bacteria suspended in 2 ml of culture medium lacking fetal calf serum (5×10^7 to 10×10^7 CFU/ml). After 30 min of incubation at 37°C, the monolayers were washed by dipping the cover slips two times into sterile PBS. Incubation was then continued in sterile culture medium without fetal calf serum at 37°C for 60 or 90 min as indicated. In some experiments gentamicin (E. Merck AG, Darmstadt, Federal Republic of Germany) was added (50 μg/ml) to eliminate cell-attached bacteria. In the phagocytosis inhibition experiments, the culture medium was supplemented with 8 μg of cytochalasin B (Sigma) per ml dissolved in 1 mg of dimethyl sulfoxide per ml. The infection process was stopped by dipping the cover slips three times in cold PBS (15°C), followed by incubation with the corresponding rabbit antiserum at 20°C (see below).

Double fluorescent-antibody test. After washing the infected cell monolayers, the cover slips were overlaid with 0.2 to 0.3 ml of the corresponding rabbit antiserum diluted in PBS and incubated for 15 to 20 min at 20°C (this temperature was found to be low enough to inhibit phagocytic activity). Thereafter, excess antiserum was washed off by dipping the cover slips four times into PBS (this short washing time was sufficient as indicated by low background fluorescence). After air-drying at 20°C, the monolayers were fixed in pure methanol (Merck, Darmstadt) at −15°C for 1 to 2 min. The cover slips were again air-dried and subsequently overlaid with TRITC-labeled goat anti-rabbit IgG serum (Sigma) diluted 1:50 in PBS and incubated for 15 to 20 min at 37°C to stain extracellularly located bacteria. After being washed by dipping three times into PBS, the monolayers were treated a second time with the corresponding rabbit antiserum at 37°C for 30 min. At this step, antibodies were able to react with intracellular bacteria. After washing off excess antiserum, the second stain was performed with FITC-labeled goat anti-rabbit IgG serum (Sigma) diluted 1:200 in PBS (15 to 20 min at 37°C). The cells were again rinsed with PBS and then mounted in glycerol-PBS (9:1 [vol/vol]) under a glass cover slip. The specimens were examined with the Leitz fluorescence microscope Orthoplan with epifluorescent illumination and a ×53 oil immersion lens (final magnification, ×636). The number of extracellular bacteria was counted by the TRITC filter system, and the total number of cell-associated bacteria (intracellular plus extracellular bacteria) was determined by the FITC filter system.

After the fluorescent antibody stain, a methylene blue poststain (Löffler methylene blue for 30 s) enables the same specimen to be examined by both bright field light microscopy and fluorescence microscopy. Photomicrographs of representative cells were taken with an ultra-high-speed reversed film (640-T; 3M) for tungsten light (ASA 640).

Determination of the number of viable bacteria in cell culture medium during the infection process. Fifty-microliter samples were removed from the cell culture at time zero (cover slips were dipped into the bacterial suspension), 30 min (end of the attachment period), 35 min (washed cover slips were replaced into sterile fresh culture medium without supplements and with added gentamicin), 60 min, and 90 min (end of infection period). The 50-μl samples were centrifuged (microcentrifuge, Eppendorf) for 5 min. The supernatant was discarded, and the pellet was suspended in PBS. Appropriate dilutions were plated onto Müller-Hinton agar. Colonies were counted after incubation of the plates at 30°C for 20 h.

RESULTS

Interaction of *Y. enterocolitica* with HEp-2 cells. In Fig. 1, three micrographs of the same area of a HEp-2 cell monolayer infected with the avirulent plasmidless strain y-108-C are recorded by immunofluorescence and bright field microscopy. The extracellular bacteria (Fig. 1A) are visualized as red rods by the TRITC filter system. After FITC filtering, additional bacteria appear (Fig. 1B). Obviously, these are intracellular, but those which emit green and red fluorescence are extracellular. The reason for double staining of extracellular bacteria is probably due to the fact that the binding sites of the rabbit anti-*Yersinia* antibodies attached to the extracellular bacteria are not sufficiently saturated by reaction with goat anti-rabbit TRITC conjugate in the initial staining step. Therefore, sites are available for binding goat anti-rabbit FITC conjugate. This interpretation is supported by using rabbit anti-*Yersinia* serum and goat

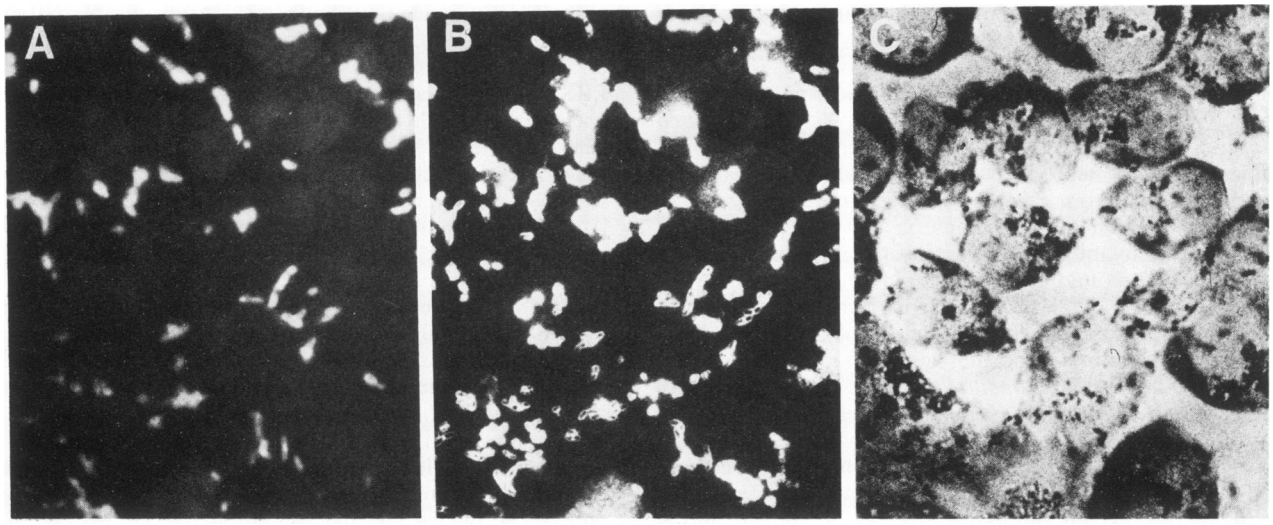

FIG. 1. Avirulent *Y. enterocolitica* strain y-108-C (plasmid negative) after incubation with HEp-2 cell culture monolayers. The same area of the cell monolayer examined by fluorescence microscopy and bright field light microscopy. (A) TRITC fluorescence, showing (red) rods which are extracellularly located. (B) FITC fluorescence, showing (green and green-yellow) rods which represent extracellular and intracellular bacteria. (C) Methylene blue stain, demonstrating that intracellular bacteria cannot be distinguished from extracellular bacteria (30 min of preincubation, three washings, and 60 min of postincubation).

anti-rabbit TRITC conjugate for staining extracellular bacteria and guinea pig anti-*Yersinia* and goat anti-guinea pig FITC conjugate for staining intracellular bacteria (data not shown). In this case, after FITC filtering extracellular bacteria appeared as weak orange rods (residual fluorescence of the rhodamine dye), and the intracellular bacteria appeared green.

In Fig. 2, an accurate quantitation of the number of cell-associated bacteria per HEp-2 cell and the percentage of intracellular and extracellular microorganisms is shown. About 80% of the cell-associated plasmidless bacteria are internalized by HEp-2 cells after 120 min.

When HEp-2 cells were treated with 8 μg of cytochalasin B per ml during infection with the plasmidless strain y-108-C, nearly 100% of the cell-associated bacteria fluoresced red (Fig. 3A) and green (Fig. 3B), indicating the extracellular location of the majority of cell-associated bacteria. This result is also expected, because cytochalasin B inhibits the phagocytic ability of mammalian cells (18).

HEp-2 cells infected with the virulent plasmid-bearing strain y-108-P are shown in Fig. 4. These micrographs resemble those in Fig. 3 which were treated with cytochalasin B. Intracellular bacteria (indicated by arrows in Fig. 4B) are rarely found. The quantitative evaluation of this experiment (Fig. 5) indicates that the virulent strain was essentially not phagocytized by HEp-2 cells. A striking result, however, was obtained when the virulent *Yersinia* strain was treated with 50 μg of gentamicin per ml for 90 min after the cell attachment period (t = 35 min, see above). As shown by the histogram in Fig. 6, a large portion of extracellular bacteria remained cell attached despite the bactericidal concentration of gentamicin and subsequent PBS washings.

Furthermore, ca. 30 to 40% of cell-associated bacteria appeared to be internalized by HEp-2 cells. Obviously gentamicin treatment had impaired the anti-phagocytic properties of the virulent *Yersinia* strain. Parallel to these experiments, the number of viable bacteria of the culture medium was determined. Within the attachment period (t = 0 min to t = 30 min; see above) the bacterial concentration remained approximately constant (about 10^8/ml). After washing and removing the monolayers into fresh medium, the bacterial concentration decreased to ca. 3×10^4/ml (t = 35 min). Within the following hour (t = 90 min), the bacterial concentration of the antibiotic-free medium increased to 5 ×

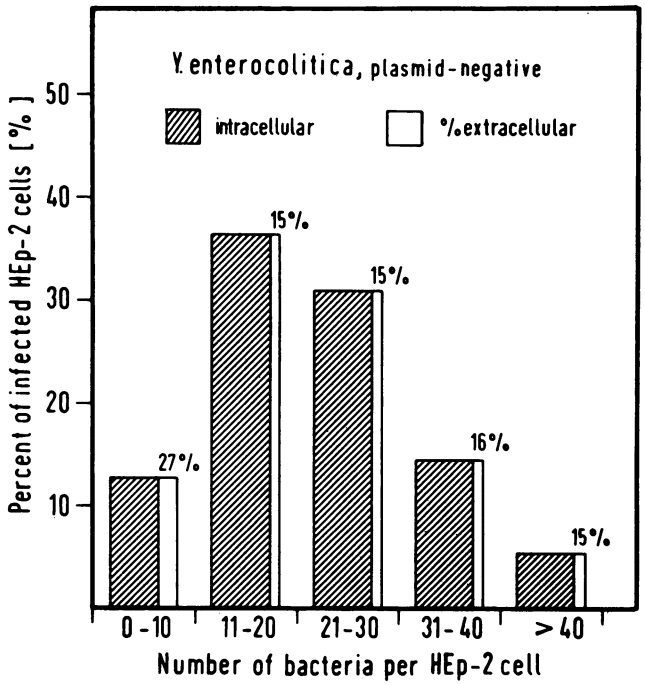

FIG. 2. Avirulent *Y. enterocolitica* strain y-108-C after incubation with HEp-2 cells (30 min of preincubation, followed by washings and 90 min of postincubation). Distribution of cell-associated bacteria after enumerating 100 HEp-2 cells by fluorescence microscopy. Data are grouped according to numbers of cell-associated bacteria and are represented as the percentage of cells in each group. Furthermore, the percentage of extracellularly located bacteria of each group is indicated.

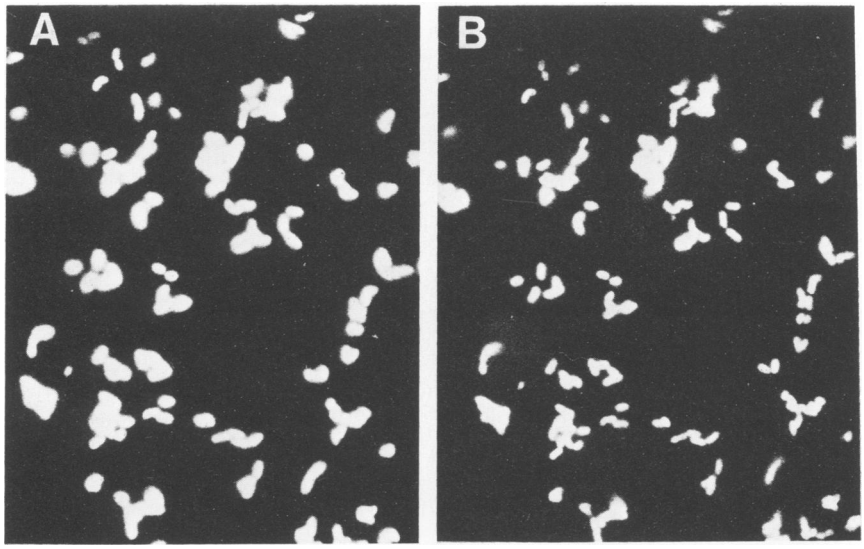

FIG. 3. Avirulent *Y. enterocolitica* strain y-108-C after incubation with cytochalasin B-treated HEp-2 cells. (A) TRITC fluorescence. (B) FITC fluorescence. The two micrographs show the same bacteria, indicating that all cell-associated bacteria are extracellularly located (30 min of preincubation, three washings, and 60 min of postincubation).

10^4/ml, whereas the number of viable bacteria in the gentamicin-containing medium decreased to 1.5×10^4/ml.

Interaction of *Y. enterocolitica* with macrophage and fibroblast monolayers. To investigate whether the double immunofluorescence discrimination method is generally applicable, we studied the interaction of *Y. enterocolitica* with resident mouse peritoneal macrophages and human fibroblast cell monolayers. The infection procedure was identical with that described for HEp-2 cells.

Figure 7 shows the results of macrophage monolayers infected with the plasmid-bearing strain y-108-P. Only a few bacteria are recognized after TRITC filtering (Fig. 7A), whereas the overwhelming mass of cell-associated bacteria appears after FITC filtering (Fig. 7B). These results indicate that resident macrophages attached to glass slides are able to phagocytize the virulent *Yersinia* strain. For comparison, a Giemsa stain of infected macrophages of another area of the same specimen is shown in Fig. 7C.

In Fig. 8, human fibroblasts monolayers are shown, which were infected with the plasmidless *Y. enterocolitica* strain y-108-C. Like HEp-2 cells, fibroblasts are obviously able to phagocytize the avirulent *Yersinia* strain: about 50% of cell-associated bacteria are internalized (emission only of green fluorescence).

Interaction of *Shigella sonnei* and *Salmonella enteritidis* with HEp-2 cells. In these experiments, we used commercial serotyping sera for immunostains. Figure 9 shows TRITC- and FITC-fluorescing shigellae (Fig. 9A and B) and salmonellae (Fig. 9C and D), respectively, which had infected HEp-2 cells according to the general infection protocol. In both cases, a few bacteria are identified as intracellular (indicated by arrows). Probably the kinetics of

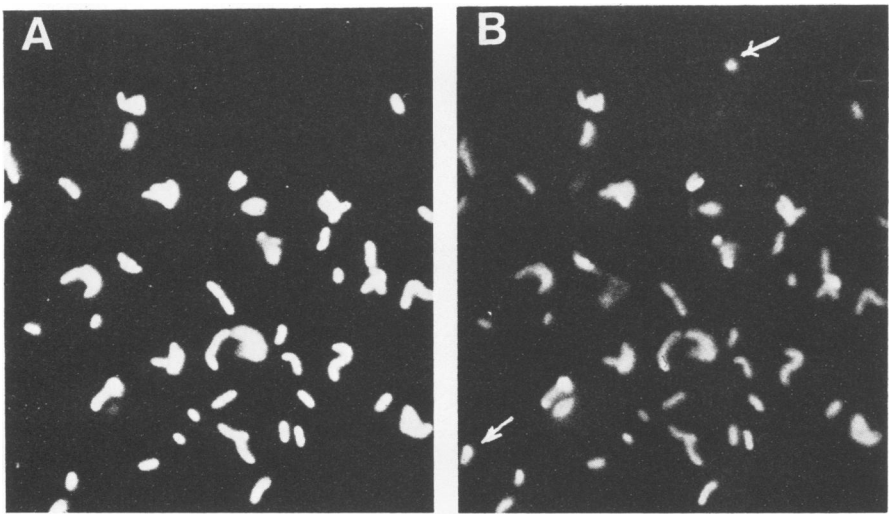

FIG. 4. Virulent *Y. enterocolitica* strain y-108-P (plasmid positive) after incubation with HEp-2 cells. (A) TRITC fluorescence. (B) FITC fluorescence, showing two rods (indicated by arrows) which are not shown by the TRITC micrograph. These bacteria are suggested to be internalized by the HEp-2 cell (30 min of preincubation, three washings, 60 min of postincubation).

internalization of these strains is slower than that of avirulent *Y. enterocolitica* (see Fig. 1).

DISCUSSION

Cell cultures are frequently used as model systems to elucidate the interaction mechanism of bacteria with mammalian cells. For discrimination of intracellular from extracellular bacteria, a single immunofluorescence stain with two samples (one for the determination of extracellular bacteria and one for counting the total number of cell-associated bacteria) was described recently (11). We have modified this technique with a double immunofluorescence stain which enables us to determine the accurate localization of cell-associated bacteria from one sample. The practicability and reliability of this technique is tested with various *Enterobacteriaceae* strains and various cell lines. In the first experiments, HEp-2 cell monolayers infected with a virulent (plasmid-positive) *Y. enterocolitica* strain and with an avirulent (plasmid-negative) derivative were investigated. Using the double immunofluorescence technique, we found that the virulent strain was not internalized by HEp-2 cells, whereas more than 80% of the plasmidless derivative was phagocytized after ca. 90 min. These results agree well with those described by Vesikari et al. (16) by scanning electron microscopy. Furthermore, these results show that cell invasiveness is not associated with virulence of *Y. enterocolitica*.

The next experiment was performed to demonstrate that cytochalasin B-treated HEp-2 cells are not able to phagocytize the avirulent *Yersinia* strain. In fact, by the double immunofluorescence stain, the majority of cell-associated bacteria were visible as orange rods, indicating their extracellular location. Furthermore, this result shows that *Y. enterocolitica* is not able to actively penetrate HEp-2 cells.

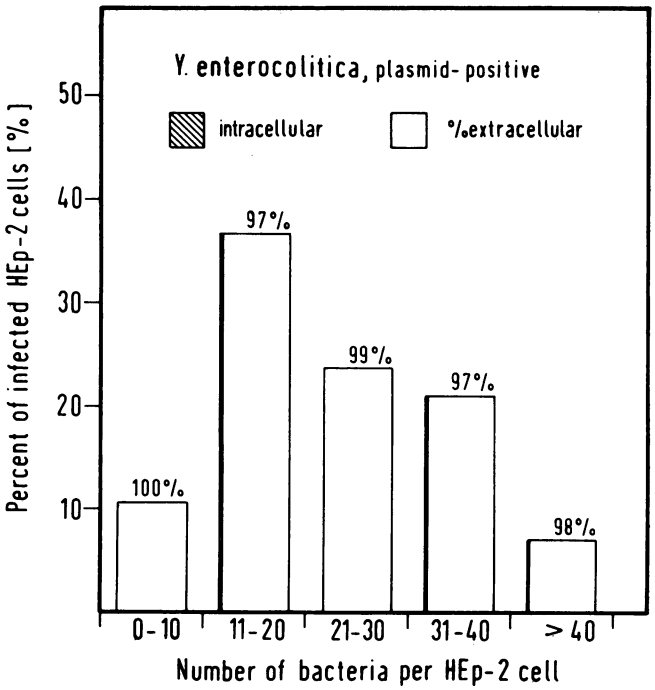

FIG. 5. Distribution of cell-associated virulent *Y. enterocolitica* bacteria (strain y-108-P) after incubation with HEp-2 cells (30 min of preincubation, followed by washings and 90 min of postincubation). For further details see the legend to Fig. 2.

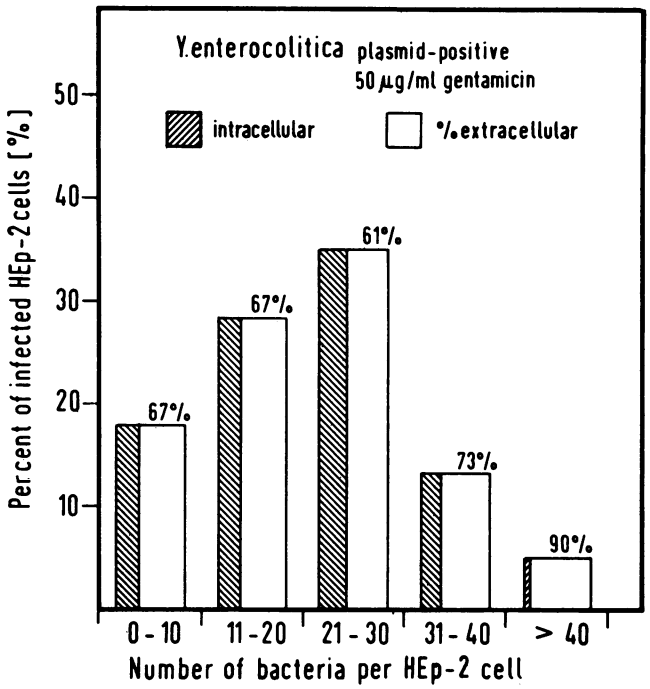

FIG. 6. Distribution of cell-associated virulent *Y. enterocolitica* bacteria (strain y-108-P) after incubation with HEp-2 cells for 30 min, followed by washings and incubation with 50 µg of gentamicin per ml for 90 min (for further details see the legend to Fig. 2).

A common procedure for the determination of the number of intracellular bacteria by using gentamicin for the elimination of cell-attached bacteria was checked (13, 17). As a test strain we used the plasmid-positive *Yersinia* strain, because this strain is not internalized by HEp-2 cells under standard conditions. Gentamicin treatment for 90 min followed by three washes of the monolayers only partly eliminated cell-attached bacteria. However, more suprisingly, we found that a large portion of cell-associated bacteria had been phagocytized. Apparently, the high bactericidal concentration of gentamicin (14) renders the cell-attached bacteria susceptible to phagocytosis by HEp-2 cells. In summary, these results demonstrate that antibiotic treatment is not a suitable method to detach cell-associated bacteria.

To show that the double immunofluorescence stain is also applicable to various *Enterobacteriaceae* strains with commercial antisera, HEp-2 cells were infected with clinical isolates of *Shigella sonnei* and *Salmonella enteritidis*. As we have demonstrated, typing sera are as useful as self-made sera. About 10 to 15% of cell-associated *Shigella* and *Salmonella* bacteria were identified as intracellularly located. These results are comparable with those obtained by interference contrast microscopy (2).

For the sake of completeness, mouse macrophages and human fibroblasts were infected with *Y. enterocolitica*. Fibroblast cell monolayers showed phagocytic properties similar to those of HEp-2 cells: a large portion of the avirulent *Yersinia* strain was found to be internalized. In contrast to these results, mouse macrophages attached to a glass support were even able to phagocytize the virulent *Y. enterocolitica* strain. This result is not surprising, because unlike the fibroblasts and HEp-2 cells, macrophages are professional phagocytes.

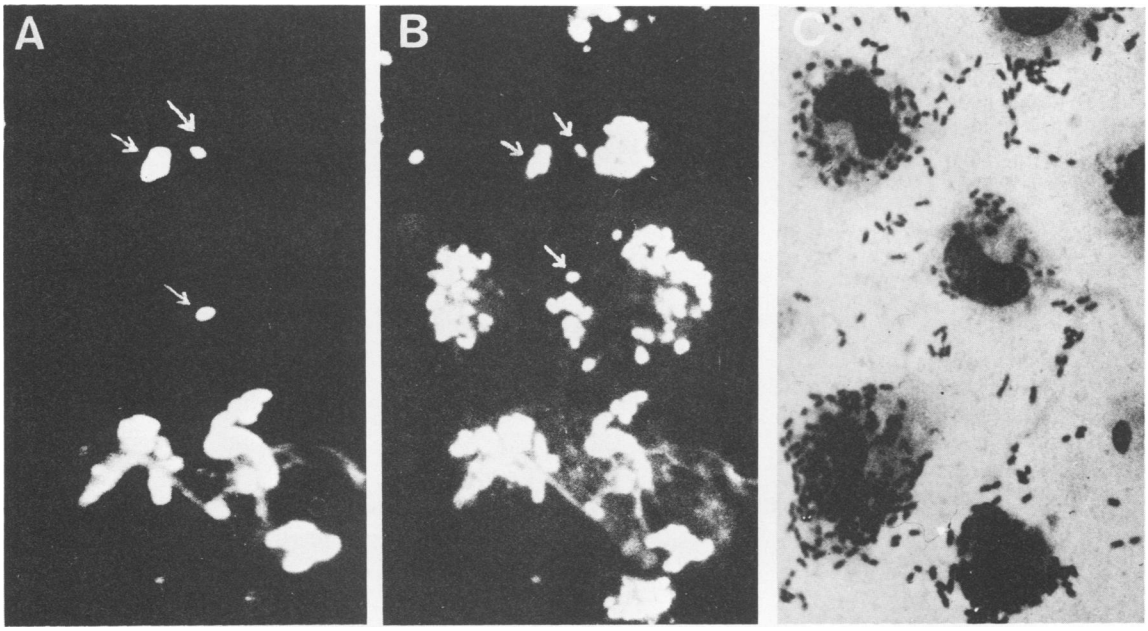

FIG. 7. Virulent *Y. enterocolitica* strain y-108-P after incubation with mouse macrophage monolayers (30 min of preincubation, three washings, and 60 min of postincubation). (A) TRITC fluorescence, showing several cell-associated rods (indicated by arrows) which are extracellularly located. (B) FITC fluorescence, showing that the majority of cell-associated bacteria is intracellularly located. (C) Light microscopic photograph of another area of the same sample, but stained according to Giemsa.

The double immunofluorescence method presented here enables us to make a simple and accurate differentiation between intra- and extracellular location of cell-associated bacteria. This staining method can be applied to nonprofessional as well as to professional phagocytic cell lines. The immunization procedure for producing antisera for immunostaining does not seem to be critical. As antisera against many different bacteria are commercially available, the double immunofluorescence method is suitable for the routine examination of the interaction of bacteria with mammalian cells. This method seems to be superior to time-consuming electron microscopy and to differential interfer-

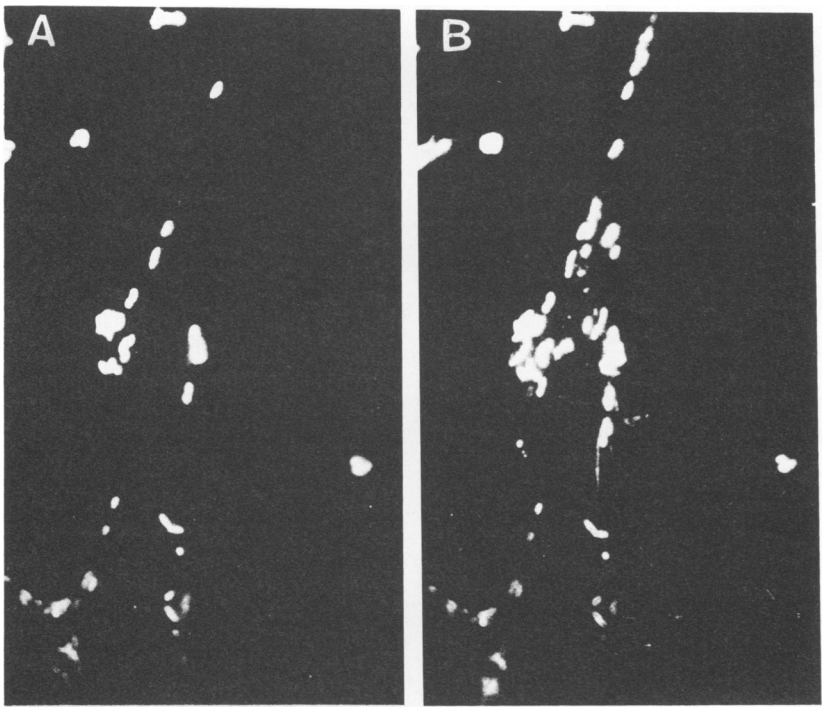

FIG. 8. Avirulent *Y. enterocolitica* strain y-108-C after incubation with human fibroblast monolayers. (A) TRITC fluorescence and (B) FITC fluorescence (30 min of preincubation, three washings, and 60 min of postincubation).

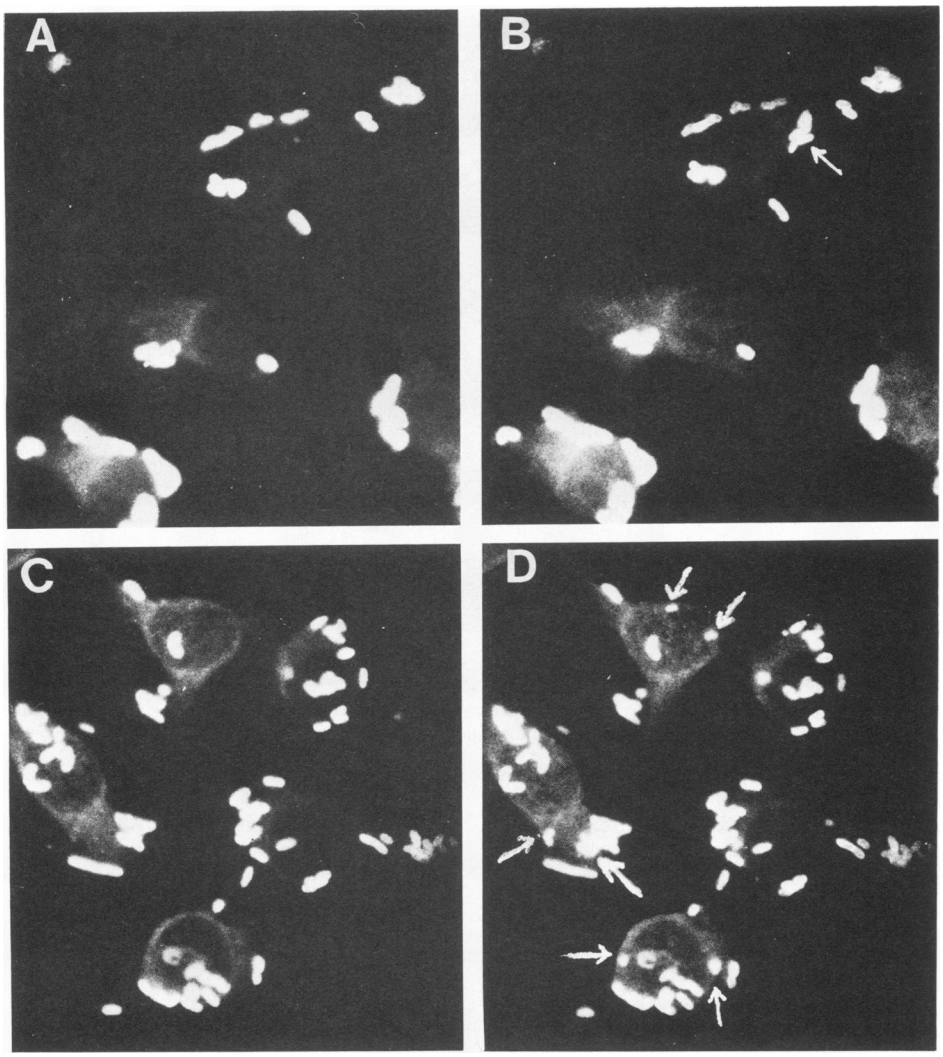

FIG. 9. Top, Infection of HEp-2 cells by *Shigella sonnei* (30 min of preincubation, three washings, and 60 min of postincubation). (A) TRITC fluorescence and (B) FITC fluorescence showing several intracellular bacteria (indicated by an arrow). Bottom, Infection of HEp-2 cells by *Salmonella enteritidis* (30 min of preincubation, three washings, and 60 min of postincubation). (C) TRITC fluorescence and (D) FITC fluorescence showing several intracellular bacteria (indicated by arrows).

ence contrast microscopy which only roughly recognized extracellular bacteria. Furthermore, the double immunofluorescence stain makes visible bacterial degradation within the phagocytic cell.

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