

## Acridine Orange Stain for Determining Intracellular Enteropathogens in HeLa Cells

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**Green-fluorescent intracellular enteropathogenic bacteria were observed after infected HeLa cell monolayers were stained with acridine orange and counterstained with crystal violet at least 3 h after infection.**

The invasive abilities of enteroinvasive bacteria, such as shigellae, salmonellae, enteroinvasive *Escherichia coli*, *Yersinia enterocolitica*, and *Y. pseudotuberculosis*, in cell culture monolayers correlate well with their potential to produce disease in humans (3, 5, 6, 13-15, 18, 19, 23).

Various techniques have been used to differentiate internalized and surface-attached bacteria in cultured cells: direct microscopy with specific and nonspecific staining techniques, including electron microscopy and immunofluorescent bacterium-specific antibodies (2, 8-13, 18-20, 23); enumeration of intracellular bacteria by plate counts after monolayer cell lysis (1, 8, 10); and agarose overlay of infected tissue culture (17). All of these techniques are cumbersome and time consuming.

In this study, a fluorescent acridine orange stain technique, previously described for determining the presence of gram-positive bacteria in professional phagocytes (21), was used to differentiate intracellular from extracellular gram-negative bacteria in cultured HeLa cells previously used to detect in vitro enteroinvasiveness (2, 6, 10, 13, 14, 17).

The six enteropathogenic strains used in this study included one strain each of enteroinvasive *E. coli* EI314 (O124:K?) (22); enterotoxigenic *E. coli* H-10407 (O78:K89:H11) (4); enteropathogenic *E. coli* E10783 (O126:H2), known to be highly adherent and minimally invasive (16); *Salmonella enteritidis* SE-3; *Shigella flexneri* 20001; and *Y. enterocolitica* FIN obtained from our culture collection. Cell monolayers on Lab-Tek eight-chambered slides (Baxter Scientific Products, Columbia, Md.) were infected with bacteria in duplicate for 3 and 7 h as described previously (15). After these periods, one set of slides was stained with Giemsa reagent (Fisher Scientific, Pittsburgh, Pa.) and examined at  $\times 40$  magnification under light microscopy to determine total cell-bacterium association. The percentage of cultured cells infected with bacteria and the number of bacteria per cell were calculated.

At the same time intervals, duplicate sets were washed in Hanks balanced salt solution (Gibco Laboratories Inc., Grand Island, N.Y.), stained with 0.01% acridine orange in Gey's solution (Gibco) for 45 s, rinsed with Hanks balanced salt solution, and counterstained with 0.05% crystal violet in 0.15 N NaCl for 45 s. After the coverslips were rinsed with Hanks balanced salt solution, they were mounted on the slides and sealed with colorless nail polish (20). Slides were then viewed under a fluorescence microscope by using incident light at  $\times 20$  magnification for screening and  $\times 40$  magnification for quantitation. The number of green (viable) fluorescent bacteria in 30 cells was counted, and the mean number of bacteria per cell was determined, as appropriate.

All infecting bacteria took up the acridine orange and

fluoresced; crystal violet quenched the fluorescence of extracellular adhering bacteria so that only fluorescent intracellular bacteria would be visible under fluorescent light microscopy (7). The acridine orange-crystal violet staining technique revealed viable green-fluorescing intracellular organisms (Table 1; Fig. 1). Nonviable intracellular bacteria would have stained red as described previously (7, 21). The change in color is attributed to the increase in the amount of acridine orange interchelating with the phosphate-sugar backbone of DNA as the DNA becomes denatured in nonviable cells (24). Invasion by all of the strains tested, except enterotoxigenic *E. coli* H-10407, which is known to be noninvasive (4), was evident 3 h after infection.

Table 1 also demonstrates the difference between (i) associated (i.e., adherent and internalized) bacteria, as determined by Giemsa stain, and (ii) organisms only internalized, as determined by acridine orange-crystal violet treatment. As shown in Table 1, numbers of truly invasive bacteria, such as yersiniae, salmonellae, shigellae, and enteroinvasive *E. coli*, did not differ substantially with the two staining techniques 7 h after infection. However, significantly fewer minimally invasive enteropathogenic *E. coli* E10783 bacteria were evident in the acridine orange-treated monolayers than in those stained with Giemsa reagent ( $P < 0.05$ , Student's *t* test). Also, no fluorescing bacteria were seen in acridine orange-stained monolayers infected with the noninvasive enterotoxigenic *E. coli* strain (Fig. 1). We had previously found similar results by infecting cultured Henle 407 cells with enteropathogenic, enteroinvasive, and enterotoxigenic *E. coli* strains (16).

The above data demonstrate that a simple, quick staining technique using acridine orange and crystal violet can be used for rapid determination of HeLa cell penetration by gram-negative enteropathogens in vitro.

TABLE 1. Comparison of infected HeLa cells stained with acridine orange-crystal violet and Giemsa 7 h after infection

| Strain  | Mean ( $\pm$ SEM) % of cells |                         | Mean ( $\pm$ SEM) no. of bacteria/cell |                 |
|---------|------------------------------|-------------------------|--|-----------------|
|         | Invaded <sup>a</sup>         | Associated <sup>b</sup> | Invading                               | Associating     |
| EI314   | 60.5 $\pm$ 3.75              | 70.5 $\pm$ 1.74         | 7.9 $\pm$ 0.73                         | 7.7 $\pm$ 0.84  |
| H-10407 | 0                            | 52.1 $\pm$ 10.6         | 0                                      | 4.4 $\pm$ 0.78  |
| E10783  | 48.0 $\pm$ 3.01              | 86.7 $\pm$ 2.71         | 4.0 $\pm$ 0.04                         | 13.5 $\pm$ 0.61 |
| FIN     | 95.7 $\pm$ 2.96              | 97.0 $\pm$ 1.53         | >20                                    | >20             |
| SH20001 | 10.7 $\pm$ 0.91              | 11.1 $\pm$ 1.1          | >20                                    | >20             |
| SE-3    | 8.0 $\pm$ 0.83               | 11.3 $\pm$ 1.84         | 3.8 $\pm$ 0.85                         | 4.0 $\pm$ 0.65  |

<sup>a</sup> Invasion was determined by acridine orange-crystal violet staining.

<sup>b</sup> Association was determined by Giemsa staining.

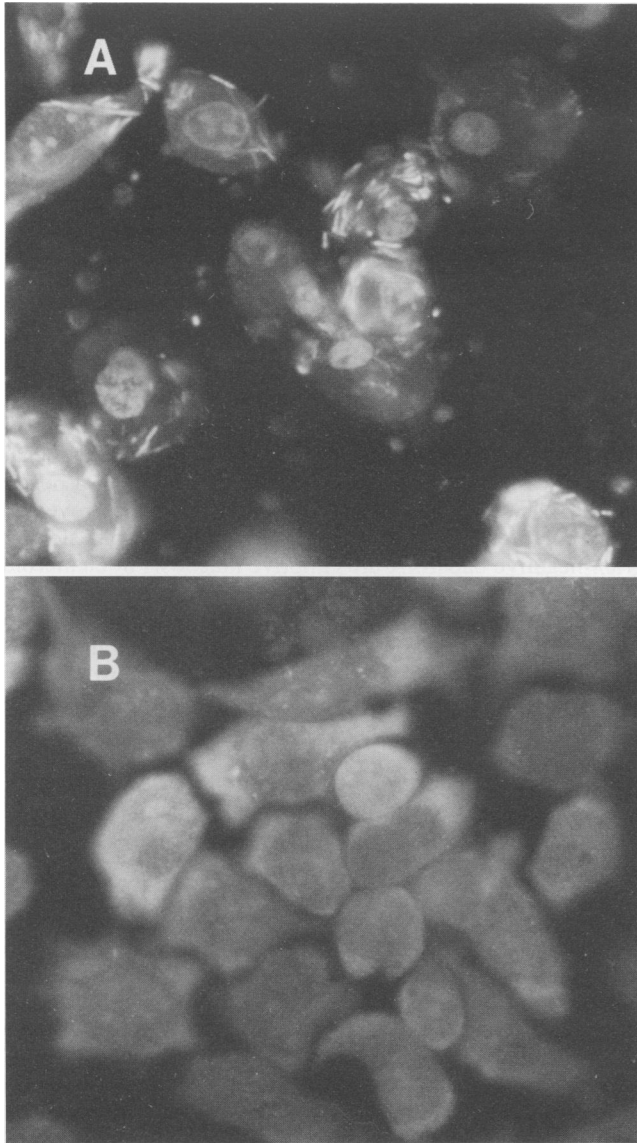


FIG. 1. Acridine orange-crystal violet-stained monolayers showing fluorescent intracellular *S. flexneri* 20001 (A) and nonfluorescing enterotoxigenic *E. coli* H-10407 (B).

Statistical analyses were performed by using an IBM personal computer Epistat program, version A2.00, of Tracy L. Gustafson, Round Rock, Tex.

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