JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1991, p. 830–831
0095-1137/91/040830-02$02.00/0

Vol. 29, No. 4

Acridine Orange Stain for Determining Intracellular Enteropathogens in HeLa Cells

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Received 15 October 1990/Accepted 23 January 1991

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

[a] Invasion was determined by acridine orange-crystal violet staining.
[b] Association was determined by Giemsa staining.

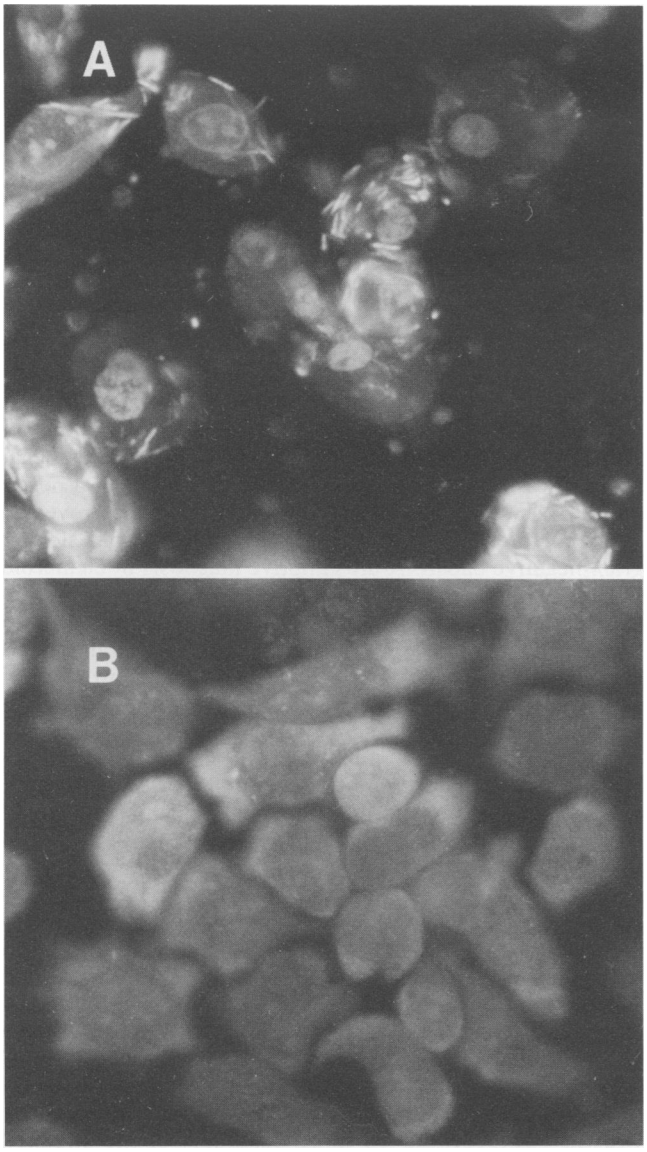

FIG. 1. Acridine orange-crystal violet-stained monolayers showing fluorescent intracellular *S. flexneri* 20001 (A) and nonfluorescing enterotoxigenic *E. coli* H-10407 (B).

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

REFERENCES

1. **Bhigale, S. R., K. D. Sharma, and R. S. Kamat.** 1983. Role of heat labile antigens of *Shigella flexneri* in HeLa cell invasion. J. Med. Microbiol. **16**:37–43.
2. **Clerc, P., and P. J. Sansonetti.** 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. Infect. Immun. **55**:2681–2688.
3. **Dupont, H. L., S. B. Formal, R. B. Hornick, M. J. Snyder, J. P. Libonati, D. J. Sheahan, E. H. LaBrec, and J. P. Kalas.** 1971. Pathogenesis of *Escherichia coli* diarrhea. N. Engl. J. Med. **285**:1–9.
4. **Evans, D. J., Jr., and D. G. Evans.** 1973. The characteristics associated with enteropathogenic *Escherichia coli* isolated from man. Infect. Immun. **8**:322–323.
5. **Giannella, R. A., S. B. Formal, G. J. Dammin, and H. Collins.** 1973. Pathogenesis of salmonellosis. Studies on fluid secretion, mucosal invasion, and morphologic reaction in the rabbit ileum. J. Clin. Invest. **52**:441–453.
6. **Giannella, R. A., O. Washington, P. Gemski, and S. B. Formal.** 1973. Invasion of HeLa cells by *Salmonella typhimurium*: a model for study of invasiveness of *Salmonella*. J. Infect. Dis. **128**:69–75.
7. **Goldner, M., H. Farkas-Himsley, A. Kormendy, and M. Skinner.** 1983. Bacterial phagocytosis monitored by fluorescence and extracellular quenching: ingestion and intracellular killing. Lab. Med. **14**:291–294.
8. **Hale, T. L., and P. F. Bonventre.** 1979. *Shigella* infection of Henle intestinal epithelial cells: role of the bacterium. Infect. Immun. **24**:879–886.
9. **Hale, T. L., P. J. Sansonetti, P. A. Schad, S. Amatin, and S. B. Formal.** 1983. Characterization of virulence plasmids and plasmid-associated outer membrane proteins on *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. Infect. Immun. **40**:340–350.
10. **Kihlstrom, E.** 1977. Infection of HeLa cells with *Salmonella typhimurium* 395 MS and MR10 bacteria. Infect. Immun. **17**: 290–295.
11. **Kihlstrom, E., and L. Edebo.** 1976. Association of viable and inactivated *Salmonella typhimurium* 395 MS and MR10 with HeLa cells. Infect. Immun. **17**:851–857.
12. **Kopecko, D. J., P. J. Sansonetti, L. S. Baron, and S. B. Formal.** 1981. Invasive bacterial pathogens of the intestine: *Shigella* virulence plasmids and potential vaccine approaches, p. 111–122. *In* S. B. Levy, R. C. Clowes, and E. L. Koenig (ed.), Molecular biology, pathogenicity, and ecology of bacterial plasmids. Plenum Publishing Corp., New York.
13. **LaBrec, E. H., H. Schneider, T. H. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.
14. **Lee, W. H., P. P. McGrath, P. H. Carter, and E. L. Eide.** 1977. The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Can. J. Microbiol. **23**:1714–1722.
15. **Lindberg, A. A.** 1980. Bacterial virulence factors with particular reference to *Salmonella* bacteria. Scand. J. Infect. Dis. Suppl. **24**:86–92.
16. **Miliotis, M. D., H. J. Koornhof, and J. I. Phillips.** 1989. Invasive potential of noncytotoxic enteropathogenic *Escherichia coli* in an in vitro Henle 407 cell model. Infect. Immun. **57**:1928–1935.
17. **Niesel, D. W., C. E. Chambers, and S. L. Stockman.** 1985. Quantitation of HeLa cell monolayer invasion by *Shigella* and *Salmonella* species. J. Clin. Microbiol. **22**:897–902.
18. **Ogawa, H., A. Nakamura, and R. Nakaya.** 1968. Virulence and epithelial cell invasiveness of dysentery bacilli. Jpn. J. Med. Sci. Biol. **20**:315–328.
19. **Ogawa, H., A. Nakamura, and R. Sakazaki.** 1968. Pathogenic properties of enteropathogenic *Escherichia coli* from diarrheal children and adults. Jpn. J. Med. Sci. Biol. **21**:333–349.
20. **Okamura, N., T. Nagel, R. Nakaya, S. Honda, M. Murakami, and K. Hisatume.** 1983. HeLa cell invasiveness and O antigen *Shigella flexneri* as separate and prerequisite attributes of virulence to evoke keratoconjunctivitis in guinea pigs. Infect. Immun. **39**:505–513.
21. **Pruzanski, W., S. Saito, and D. W. Nitzan.** 1983. The influence of lysostaphin on phagocytosis, intracellular bactericidal activity, and chemotaxis of human polymorphonuclear cells. J. Lab. Clin. Med. **102**:298–305.
22. **Robins-Browne, R. M., M. R. Jacobs, and H. J. Koornhof.** 1978. *Escherichia coli* gastroenteritis in adults. S. Afr. Med. J. **53**:93–96.
23. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
24. **West, S. S.** 1969. Quantitative microscopy in bacteriology. Ann. N.Y. Acad. Sci. **157**:111–122.