Fluorescent Staining of Intracellular and Extracellular Bacteria in Blood

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The fluorescent dye ethidium bromide stains *Escherichia coli* and *Staphylococcus aureus* in whole blood. The staining is rapid, relatively specific, and does not require fixation of the sample. Furthermore, stained bacteria can be seen microscopically without the need for a final wash to remove unbound dye. By using lysozyme, a *S. aureus*-specific lytic enzyme, we have demonstrated that *S. aureus* can be stained with ethidium bromide even after phagocytosis. After short periods of incubation with the dye (less than 5 min), bacteria, both intracellular and extracellular, were the predominant fluorescent particles. With increasing time of incubation, blood cell components, notably leukocyte nuclei, began to fluoresce.

Fluorescence microscopy is being used increasingly in both clinical and research laboratories. Immunofluorescence microscopy in particular is now a well-established technique in a variety of diagnostic tests, primarily because of the high sensitivity of fluorescence, coupled with the high specificity of antibodies. Although they do not provide the exquisite specificity of fluorescently tagged antibodies, fluorescent dyes themselves can be relatively specific for particular cells or cell structures and can be used in rapid and inexpensive staining protocols. Examples of these applications include the use of acridine orange to selectively stain bacteria in dried smears of clinical blood culture samples (8, 10) and the use of 4'-6-diamidino-2-phenylindole (16, 15) and Hoechst 33258 (2, 14) dyes to indicate viral, chlamydial, or mycoplasmal contamination of tissue culture cells.

Ethidium bromide, a phenanthrididine dye which binds to nucleic acids, is a fluorescent dye which has been used extensively to stain both procaryotic (12, 13, 16) and eucaryotic (1, 3, 4, 11, 19) cells. Although ethidium bromide is known to bind tightly to double-stranded nucleic acids in vitro (6, 9), the binding of ethidium bromide to cells may be influenced by factors other than nucleic acid content alone. In mutant rho° yeast cells, staining by ethidium bromide appears not to be solely due to DNA binding (3), and in unfixed cultured L cells, the staining appears to be dependent on factors other than total DNA content of the cell (11). To avoid variables involved with stain penetration of cells, most ethidium bromide staining protocols involve fixation of cells, followed by incubation with dye for 15 min or more. In this paper, we report an ethidium bromide staining protocol which involves no fixation of bacterial cells and in which we incubated bacteria with dye for less than 5 min before microscopic observation. By using this protocol, we demonstrated rapid and preferential labeling of both intracellular and extracellular *Escherichia coli* and *Staphylococcus aureus* in the presence of whole blood.

**MATERIALS AND METHODS**

Ethidium bromide was purchased from Sigma Chemical Co., St. Louis, Mo. It was found to migrate on silica gel thin-layer chromatography with an ethanol–H₂O (50:1) solvent system as essentially a single species. Lysozyme was also purchased from Sigma and prepared as a stock solution at a concentration of 240 U/ml in water. All other chemicals were reagent grade.

*S. aureus* ATCC 25923 and *E. coli* ATCC E11775 were purchased from the American Type Culture Collection, Rockville, Md., and routinely grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C to log phase, washed once with normal saline (0.85% NaCl), and resuspended in normal saline for use. Quantitation of viable *S. aureus* and *E. coli* was accomplished by pour plate analysis with Trypticase soy broth agar.

Human blood was drawn into sodium polyanetholsulfonate (SPS)- or heparin-containing Vacutainer blood collection tubes (Becton Dickinson and Co., Paramus, N.J.) and used for experiments within 2 h. Cell-free plasma was produced by centrifuging heparinized human blood at 3,500 × g for 15 min and filtering the supernatant through a 0.2-μm (pore size) filter.

**Fluorescent staining of *S. aureus* and *E. coli* in whole blood.** *S. aureus* or *E. coli* (10⁶ CFU) was added in a 0.01-ml volume to 1 ml of heparinized or SPS-anticoagulated blood. The samples were then either stained immediately or preincubated for 30 min at 37°C to allow phagocytosis to occur. Staining was accomplished by incubating 0.7 ml of sample with 0.2 ml of staining buffer (100 mM sodium borate, 60 mM EDTA, 0.05% formaldehyde, 0.05% Triton X-100, pH 9.2) and 0.1 ml of ethidium bromide (100 μg/ml in water). This mixture was then incubated for 1 min at room temperature, and 0.01 ml was withdrawn for microscopic observation. Light microscopy and ethidium bromide fluorescence were viewed with an Olympus BHA microscope equipped for epilumination by using a mercury arc lamp with a green dichroic, 590 barrier filter and IF545 and BG36 excitation filters. For photomicrographic purposes, the Olympus photomicrographic PM-10 system (equipped with an FK 3.3 eyepiece) and a PM-CP Polaroid camera back (type 667 film, Polaroid Corp., Cambridge, Mass.) were employed. The microscope objective used for all photographs was a ×100-magnification oil immersion lens. Total magnifications are supplied for each photomicrograph.

**Treatment of *S. aureus* with lysozyme.** *S. aureus* (10⁶ CFU) was added in a 0.01-ml volume to 1 ml of normal saline, cell-free plasma, or heparinized, or SPS-anticoagulated human blood. The mixtures were incubated at 37°C for 30 min and then further incubated with ±2.4 U of lysozyme for an additional 10 min at 37°C. Quantitation of viable *S. aureus* was accomplished by pour plate analysis of the
sample with Trypticase soy broth agar. Control experiments demonstrated that lyso- 
that concentration during plating.

RESULTS

To characterize the fluorescent labeling of bacteria with ethidium bromide, washed cultures of either E. coli or S. aureus at concentrations of 10^8 CFU/ml were added to freshly drawn human blood anticoagulated with SPS. The mixture was then stained with ethidium bromide as described above and diluted with normal saline to allow easy microscopic observation. Figures 1A and 2A are fluorescence photomicrographs showing the ethidium bromide staining of E. coli and S. aureus, respectively. These are wet preparations involving no fixation and incubation with dye for only 1 min. The transmitted light photomicrographs showing the same respective fields are presented in Fig. 1B 

Staining of bacteria by ethidium bromide is a very rapid process, occurring within a few minutes. Leukocyte nuclei are also stained by ethidium bromide but require longer incubation periods with the dye, and only a relatively small percentage stain readily. Five minutes after ethidium bro-
than 99% of the *S. aureus* organisms incubated in cell-free plasma or normal saline and then treated with lysostaphin were killed by the enzyme. As mentioned above, *S. aureus* incubated in heparinized blood was susceptible to phagocytosis. After a 30-min incubation period at 37°C in heparinized blood, 25% of the *S. aureus* organisms appeared by fluorescence microscopy to be associated with, and presumably phagocytized by, leukocytes. Lysostaphin treatment of that preparation (see Table 1) resulted in ca. 78% reduction in bacterial viability, indicating that 22% of the *S. aureus* population was protected from the effects of the enzyme. Furthermore, *S. aureus* incubated in SPS-anticoagulated blood was less susceptible to phagocytosis as determined by microscopy, and there was a corresponding decrease in the number of bacteria protected from the effects of lysostaphin (Table 1). Thus, lysostaphin, under the conditions described here, appeared to lyse virtually all extracellular *S. aureus*

but did not appreciably affect intracellular (phagocytized) *S. aureus*.

Figures 4A and B are fluorescence and transmitted-light photomicrographs of *S. aureus* in heparinized human blood treated with lysostaphin and stained with ethidium bromide. There are no free or extracellular *S. aureus* organisms visible in this preparation because of the lytic effects of the enzyme. The stained *S. aureus* organisms seen in the fluorescence photomicrograph are intracellular and yet clearly stained by ethidium bromide.

**DISCUSSION**

The addition of ethidium bromide and staining buffer to human blood containing *E. coli* or *S. aureus* has been shown to result in the rapid and preferential staining of the bacteria,
with minimal staining of the blood cells. The method is rapid and simple and does not require a fixative or final wash step before microscopic analysis. The basis for this preferential staining of the bacteria is not yet understood, although the fact that intracellularly located bacteria (those ingested by leukocytes) are stained very quickly, often before the nuclei of the surrounding leukocyte, would suggest that the dye affinity for bacterial binding sites, rather than permeability properties of cell membranes, may be the primary determinant. With increased time of sample incubation with dye, leukocyte nuclei begin to fluoresce. However, within the first 5 min after dye addition, bacteria, both extracellular and intracellular, are the predominant fluorescent entities. Erythrocytes and platelets did not stain with ethidium bromide under our conditions.

It is difficult to identify phagocytized bacteria by microscopy alone. Extracellular bacteria bound to a leukocyte can often appear to be intracellular. To minimize this possibility, we have used both microscopy and resistance to the lytic enzyme lysostaphin as a measure of the phagocytosis of S. aureus. Lysostaphin is a staphylococcal-specific lytic enzyme widely used in neutrophil functional assays and has been found not to significantly penetrate polymorphonuclear leukocytes (18) or human alveolar macrophages (5). Although a recent paper suggests that lysostaphin penetrates neutrophils (20), we feel that lysostaphin did not appreciably act upon the intracellular, viable S. aureus population in our system because of the close correlation between lysostaphin results and results obtained by microscopy. As indicated above, we found that ethidium bromide fluorescently stains even phagocytized S. aureus. We observed the ethidium bromide staining of phagocytized E. coli as well, although in this case phagocytosis was judged by microscopy alone.

For the purpose of this study, E. coli and S. aureus were used as representative bacteria. We have looked at Pseudomonas aeruginosa, Klebsiella pneumoniae, and Streptococcus faecalis in blood with this staining method, and they too showed similarly specific staining by ethidium bromide. We believe this method will be generally applicable to other bacteria. The whole-blood preparations in this paper were seeded with ~10^5 CFU/ml. Bacterial concentrations below this level may make it difficult and time consuming to evaluate results with a microscope. The concentrations of bacteria encountered in clinical cases of bacteremia are, of course, generally well below these levels. Potentially, however, the specificity of ethidium bromide for bacteria could be utilized in clinical microbiology in several ways. Ethidium bromide staining, rather than Gram or Wright staining, could be used for detection of intracellular or extracellular bacteria in buffy coat preparations (7). The relative lack of background staining would be a great advantage in this area. Another application might be the use of ethidium bromide staining for the detection of bacteria in blood cultures. Acridine orange, a fluorescent dye which shows a color-specific fluorescent staining of bacteria (10), has been shown to be of value in this area. Ethidium bromide may have additional advantages because it can be used directly on a wet preparation and shows minimal background staining.

Ethidium bromide in the proper staining buffer preferentially stains bacteria in complex samples. This simple, rapid, and inexpensive staining protocol may be applicable not only to the detection of bacteria in blood but also in other systems or body fluids in which it is necessary to detect bacteria against a background of eucaryotic cells.

LITERATURE CITED


