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NOTES

Use of Avidin-Biotinylated Horseradish Peroxidase Complex for Visualization of Spirochetes

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The use of avidin-biotinylated peroxidase as a simple technique for light microscopic visualization of spirochetes is described. The three major genera of spirochetes—*Treponema*, *Borrelia*, and *Leptospira*—were stained with the avidin complex.

The morphological examination of spirochetes has generally been accomplished by dark-field examination. This method is tedious; it poses visualization difficulties which are in part due to lability of the organisms and artifacts and in part due to physical limitations. Staining of most spirochetes with various aniline dyes has also proven to be generally unsatisfactory (3).

We have found that a modification of the avidin-biotinylated peroxidase complex (ABC) staining method of Hsu et al. (4) provides a suitable, technically reproducible means for visualization of spirochetes by light microscopy. Avidin has a strong affinity for the vitamin biotin. The binding of avidin to spirochetes can be demonstrated by the use of commercially prepared biotin-labeled horseradish peroxidase.

The samples included in this preliminary study were air-dried smears of organism-containing specimens on glass slides. Fixation was achieved by a 15-min immersion in either acetone or 95% ethyl alcohol; our comparison of five smears fixed in acetone with five fixed in 95% ethyl alcohol revealed no appreciable difference in the staining quality of the fixatives. Slides were washed and flooded for 5 min with Tris buffer (pH 7.6), drained, and blotted dry. They were then flooded with Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, Calif.), incubated for 15 min, drained, and blotted dry. (Please note that Vectastain ABC reagents used in our procedure were from a kit designed to identify human immunoglobulin G (IgG) in tissue sections. Since these reagents had been titrated to give optimal reactions for human IgG, they were not necessarily optimal for spirochete staining; therefore, to obtain optimal staining of spirochetes, we titrated the ABC reagents provided in each kit by varying the ratio of the ABC reactants. In some cases, the concentration of ABC had to be increased 16-fold.) Tris buffer was again used to flood the slides, which were incubated for 15 min, drained, and blotted dry.

Either 3,3-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) can be used as a chromagen with hydrogen peroxide as the substrate; we used them as follows. For AEC, 20 mg of AEC was dissolved in 4.9 ml of *N*,*N*-dimethylformamide and 95 ml of 0.1 M acetate buffer (pH 5.2). Shortly before application, 0.93 ml of 3% hydrogen peroxide was added to the AEC solution and filtered. Slides were flooded with AEC plus H_2O_2, incubated for 20 min, and then washed for 5 min in tap water. Cover slips were mounted with glycerin, and the edges were sealed with clear fingernail polish. For DAB, 0.1% DAB in 0.1 M Tris buffer was mixed with an equal volume of 0.02% hydrogen peroxide. Slides were flooded with DAB plus H_2O_2, incubated for 5 min, and then washed for 5 min in tap water. The stained smears were dehydrated by immersing slides in 95% ethanol followed by absolute alcohol and finally xylene (6). Cover slips were then immediately mounted with synthetic resin mounting medium (Preservaslide; Matheson Coleman Bell, Norwood, Ohio); this gave a more permanent preparation than did the AEC-processed slides.

Use of these staining methods enables us to demonstrate the various morphological forms of spirochetes in direct smears of dental plaque (Fig. 1). Acetone-fixed smears of *Treponema pallidum* (Beckman Instruments, Inc., Fuller-

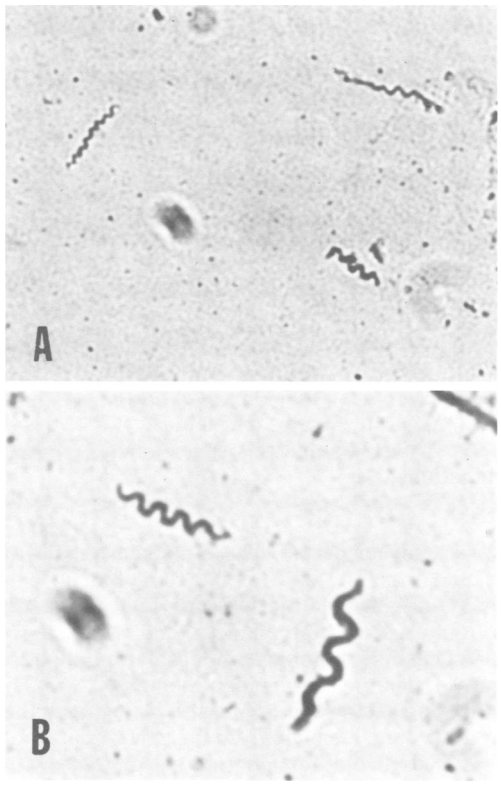

FIG. 1. Spirochetes in smears of dental plaque stained by the ABC method. (A) Approximately ×500. (B) Approximately ×1,000.

ton, Calif.) were also successfully stained. Organisms were demonstrated on control slides by the indirect immunofluorescent method (1).

Borrelia hermsi, originally isolated from a patient with relapsing fever, was maintained in vivo by serial passage of infected blood in CF-1 mice (2). This isolate was also cultured in vitro (5). *B. hermsi* in blood films from infected animals as well as smears from cultures were readily visualized by staining with our modified ABC procedure.

Leptospira interrogans serotype *icterohaemmorrhagiae* ATCC 23581 was stained in smears after growth in Fletcher medium at 30°C. The staining of these organisms was paler than that of *B. hermsi* or *T. pallidum*. The organisms were easily visualized, and morphological detail was clearly distinct.

Our method seems to depend on the binding of avidin to the spirochetes. Elimination of the various major components of the reaction demonstrated that avidin is necessary for the binding of biotin to the organisms and the subsequent staining. Control slides of *T. pallidum* treated with only the biotinylated peroxidase reagent and the AEC or DAB substrate gave negative results, thus indicating the necessity of the presence of avidin for positive staining.

The demonstration of the binding of avidin to spirochetes also has implications in areas other than morphological study. Interpretations of immunological reactions with the ABC-immunoperoxidase method of visualization would have to take into account any nonspecific staining of spirochetes. Also, the binding of avidin to spirochetes suggests that this substance might interfere with biotin uptake by the organism.

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