TECHNICAL NOTE

ABC-Immunoperoxidase Staining of Cytologic Preparations:
Improvement of Specificity

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Immunoperoxidase (IP) methods perfected on formalin-fixed, paraffin-embedded tissues (FFPE) and then applied to aspirate smears may result in high background staining and a significant number of false-positive results. This is especially true if polyclonal primary antibodies are used or if aspirates and fluids contain a high interstitial or serum protein content. Because of a recurring problem with antiserum to alpha-fetoprotein (AFP), AFP was selected as the primary test antibody with which to evaluate our avidin-biotin complex (ABC)-IP method. The same method, with diaminobenzidine (DAB) or aminoethylcarbazole (AEC) chromogens, was performed on six types of cytologic preparations of a fresh liver specimen. The liver did not stain for AFP in FFPE and frozen tissue; therefore, it could be used to evaluate potential false-positive staining of direct touch imprints, washed aspirate smears, and cytospins that were both air-dried and alcohol-carbowax–fixed. Initial chromogen incubation times were standardized to give identical results on AFP-positive fixed hepatoma and fetal liver controls. Cytologic preparations immunostained with ABC-IP with AEC chromogen resulted in varying background and hepatocyte staining. In comparison, the ABC-IP method using DAB chromogen resulted in no false-positive results and a clean background. The ABC-IP method with AEC standardized for sensitivity on a fixed tissue control required a markedly shortened chromogen incubation time to preclude significant false-positive staining of cytology specimens. It appears that use of AEC chromogen for this antibody with incubation time standardized on a FFPE tissue control and then applied to cytologic preparations also amplifies nonspecific and background staining, contributing difficulty in assessing a true-positive result. In contrast, the ABC-IP method with DAB chromogen standardized on fixed tissues can be directly translated to various types of cytologic preparations. Knowledge of this pitfall should be especially useful in trouble-shooting other immunoreactions since it is often difficult to obtain and store cytologic quality-control materials for immunohistochemistry. Diagn Cytopathol 1990;6:134–138.

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Immunohistochemical staining of cytologic preparations is usually performed with methods, reagent titers, and controls tested in fixed tissue samples. However, immunoperoxidase (IP) methods perfected on formalin-fixed, paraffin-embedded tissues (FFPE) and then applied to aspirate smears may result in high background staining, which serves to reduce sensitivity of antigen detection and produce a significant number of false-positive results due to nonspecific binding of antibodies. To avoid these potential pitfalls in diagnostic interpretation, it is often preferable to immunostain FFPE cell blocks prepared from aspirates and fluids.

Specificity problems most commonly arise when cocktails of primary monoclonal antibodies of high protein concentration or polyclonal primary antibodies are used and when aspirates and body fluids contain a high interstitial or serum protein content. These factors can be addressed by modifications in immunohistochemical method and/or specimen washing or Ficoll separation of aspirates. The latter are not routine methods of aspirate preparation and may result in loss of diagnostic cells.

Over the past 3 years, we have used an immunohistochemical method standardized on fixed tissues to immunostain 250 cytology aspirates with numerous antibodies. Review of our experience has revealed one case of false-positive staining of a thyroid follicular adenoma with polyclonal antiserum to calcitonin and two cases of false-positive staining of liver aspirate smears, but not the corresponding cell blocks, with a polyclonal antibody to alpha-fetoprotein (AFP). The reason for these false immunoreactions has not been readily resolved from daily quality-control trouble-shooting. Therefore, we have tested aspirate cytospins and touch imprints of an AFP-negative liver specimen to recreate conditions of high protein content in order to examine modifications of cytologic specimen processing and the avidin-biotin complex (ABC)-IP staining method to identify the cause of false-positive AFP staining in cytologic preparations.

Materials and Methods

Reagents

Reagents included Isolyte multielectrolyte salt wash solution (Kendall McGaw, Irvine, CA), alcohol/carbowax spray fixative (SurgiPath, Grayslake, IL), anti-AFP antiserum (1:400) (Dako, Carpinteria, CA), normal goat serum (Vector, Burlingame, CA), normal rabbit serum (Vector), biotinylated goat antirabbit IgG (1:200) (Vector), avidin-biotin complex (1:80) (Vector), DAB (Sigma D-5637, St. Louis, MO), AEC (Sigma A-5754), *N-N* dimethyl formamide (Fisher D-4254, Fair Lawn, NJ), trizma hydrochloride (Sigma T-3253), trizma base (Sigma T-1503).

Test Tissue

A fresh liver resection of focal nodular hyperplasia that was negative by the ABC-IP method outlined below for AFP in both FFPE and frozen tissue was used to make the cytologic test slides.

Test Cytologic Preparations

The six types of test cytologic slides examined in duplicate were air-dried and alcohol-carbowax spray fixed cytologic preparations of (1) direct touch imprints; (2) Isolyte-washed aspirate smears; and (3) cytospins from Isolyte-washed aspirates. Immunostaining was performed using our modification of the ABC-IP method of Hsu et al.,[1] which had been perfected on fixed and frozen tissue sections (see below).

Primary Antiserum Titration and Negative Controls

A FFPE hepatoma and fetal liver were used to optimize the dilution of rabbit anti-AFP antiserum (1:400) and standardize initial chromogen incubation times to give identical sensitivity for AEC (20 min) and DAB (5 min) chromogens used in the same ABC-IP method. No protease predigestion was employed. Substitution (buffer and nonimmune rabbit serum) negative controls demonstrated no endogenous peroxidase or avidin binding activity in the test material.

AEC Incubation Time Study

To assess the contribution of chromogen incubation time to the lack of immunostaining specificity, Isolyte-washed air-dried aspirate smears of the fresh liver were stained with the basic ABC-IP method (outlined below) with AEC chromogen incubation times of 20, 15, 10, 7, and 3 min.

ABC-Immunoperoxidase Method

1. Block endogenous peroxidase activity with 3% H2O2 (5 min). Rinse briefly in distilled water.
2. Rinse in phosphate-buffered saline (PBS) three times, 5 min each.
3. Incubate with 5% goat suppressor serum for 30 min at room temp (RT) to reduce nonspecific protein binding.
4. Decant suppressor serum, and apply diluted primary antibody 1 hr at RT. Dilution should be optimized on formalin-fixed control block.
5. Wash with PBS three times for 5 min each.
6. Incubate with biotinylated secondary antibody (1:200) for 30 min at RT.
7. Wash with PBS three times for 5 min each.
8. Incubate with ABC reagent (1:80) for 1 hr.
9. Wash in PBS three times for 5 min each.
10. Perform chromogen staining reaction as follows.

DAB (3.3-Diaminobenzidine-Tetrahydrochloride)

1. Add 30 mg DAB to 50 ml tris buffer, pH 7.6 (see below).
2. When dissolved, add 0.5 ml 3% H_2O_2.
3. Keep solution shielded from light by covering flask with aluminum foil.
4. Do not use beyond 45 min or if solution develops anything but a trace of color. Filter before use.
5. Stain for 5 min (optimized on formalin-fixed control block).
6. Wash in PBS twice.
7. Counterstain in Mayer's hematoxylin for 1–3 min.
8. Wash in running tap water 10–15 min.
9. Dehydrate with increasing concentrations of ethanol:70%, 95%, and absolute for 3 min each.
10. Clear with xylene three times 3 min each.
11. Coverslip with Permount.

Tris-buffered saline (0.05 M), pH 7.6, includes the following: 6.06 g Tris HCl; 1.39 g Tris base; 15 g NaCl. Dissolve into 1,000 ml of distilled water.

AEC (3-Amino-9-Ethylcarbazole)

1. Prepare working solution from stock solution (see below).
2. Add the 3% hydrogen peroxide immediately before use and mix using a disposable glass pipette.
3. Stain for 20 min at RT (optimized on formalin-fixed control block).
4. Wash in PBS three times.
5. Wash in running tap water for 5 min.
6. Counterstain in Mayer's hematoxylin.
7. Wash in running tap water.
8. Coverslip with liquified glycerol-gelatin.

AEC stock solution includes 240 mg of 3-amino-9-ethylcarbazole and 100 ml of *N-N* dimethylformamide.

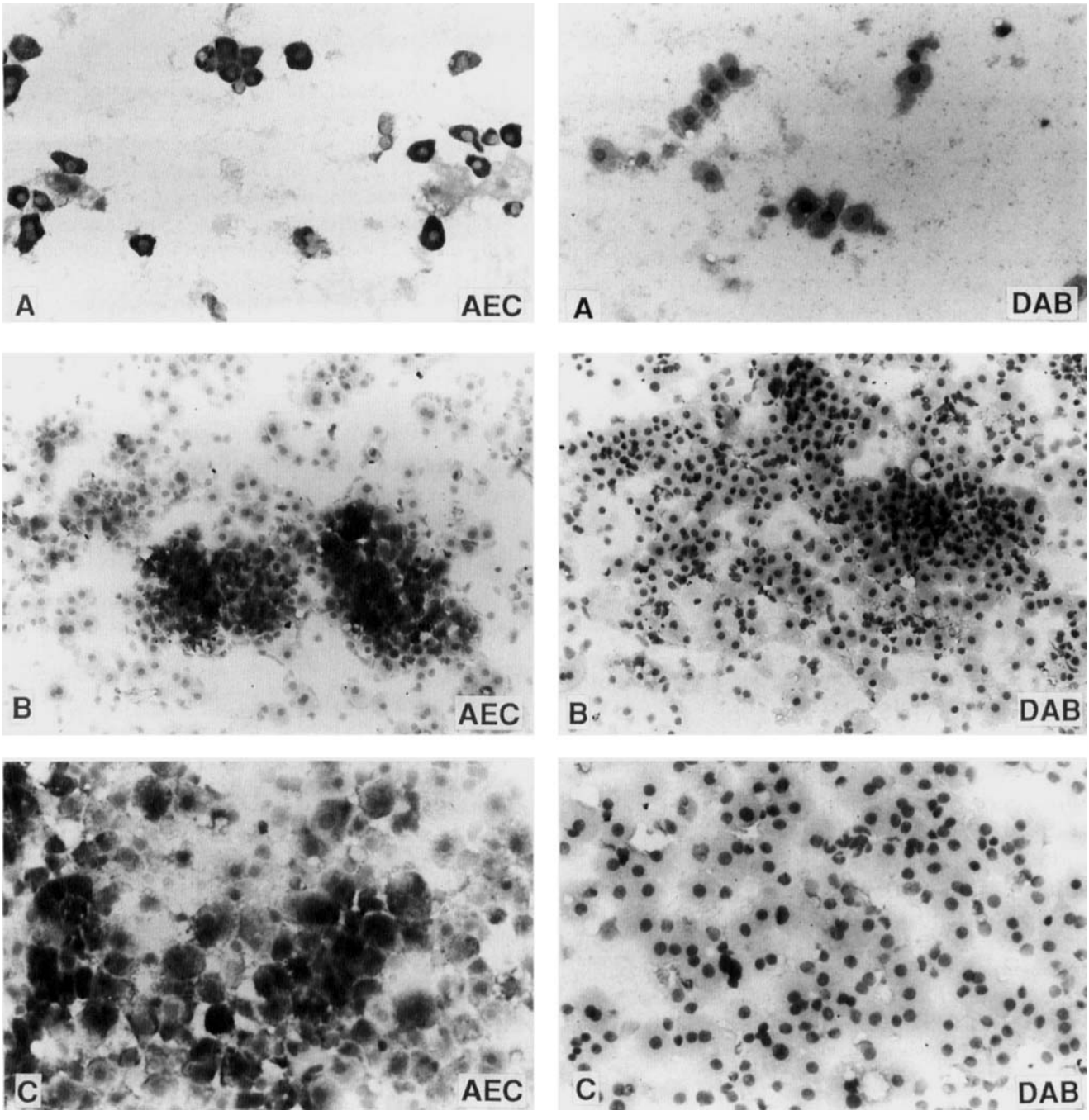

Fig. 1. This panel compares immunostaining with AEC (vertical left) and DAB (vertical right) chromogens and hematoxylin counterstain for the following cytologic preparations of fresh liver: **(A)** direct touch imprints, spray-fixed (original magnification ×80); **(B)** washed aspirate smears, spray-fixed (original magnification ×80); **(C)** cytospins from washed aspirates, air-dried (original magnification ×80). All types of cytologic preparations obtained false-positive staining with AEC but not DAB.

Dissolve 240 mg AEC in 100 ml dimethylformamide. Store at 4°C.

Acetate buffer (0.2 M), pH 5.2, includes 790 ml of 0.2 M sodium acetate (27.22 g/L; 27.22 g sodium acetate · 3 H_2O to 1,000 ml DH_2O) and 210 ml of 0.2 M acetic acid (11.4 ml/L; 11.4 ml glacial acetic acid to 1,000 ml DH_2O). Combine 790 ml 0.2 M sodium acetate and 210 ml 0.2 M acetic acid (pH 5.2). Adjust pH with 1 N HCl or 1 N NaOH. Store in refrigerator at 4°C.

AEC working solution includes 100 ml of 0.2 M acetate buffer, pH 5.2, 0.04 ml of 30% hydrogen peroxide, and 10 ml of AEC stock solution.

Results

All six types of cytologic preparations stained with DAB chromogen (optimized on fixed tissue) had no false-positive hepatocyte staining and a clean background. All preparations stained with AEC (optimized on fixed tissue) exhibited false-positive hepatocyte staining of variable intensity and patchy distribution of both single dispersed cells and small cell clumps (Fig. 1).

The AEC time study performed on isolyte-washed air-dried aspirate smears revealed a progressive decrease in the intensity of false-positive hepatocyte staining with decreasing incubation time (Fig. 2). No staining was obtained with AEC chromogen incubation times of 7 min or less. However, this abbreviated incubation time resulted in a markedly reduced sensitivity in the detection of AFP in the FFPE-positive control tissues.

Discussion

It is often difficult to obtain and store both antigen-positive and -negative cytologic materials appropriate for quality-controlling immunohistochemical reactions performed on cytologic aspirate and smear preparations. For many immunohistochemistry laboratories, this situation is often not addressed, fixed tissue controls are utilized, and unexpected false-positive and background staining in cytologic preparations is observed.[2] Others have recently addressed the influence of fixation and storage of cytologic cell preparations on immunoreactivity with selected monoclonal and polyclonal antibodies.[3] Immunohistochemical study of cytology cell blocks prepared from aspirates and fluids is appropriately controlled like other FFPE materials but not always available. In this study, we have found that differences in the chromogen reaction of a standard ABC-IP method standardized on fixed tissue controls accounted for the false-positive staining with anti-AFP that we also observed in clinical specimens. With the use of DAB chromogen optimized on FFPE control blocks, all cytologic specimen processing techniques with and without a saline wash-step or spray-fixation resulted in a clean background and no false-positive reactions with polyclonal anti-AFP. In contrast, the same basic ABC-IP method with AEC chromogen standardized on the same fixed tissue controls could not be performed on direct imprints, aspirate smears, or

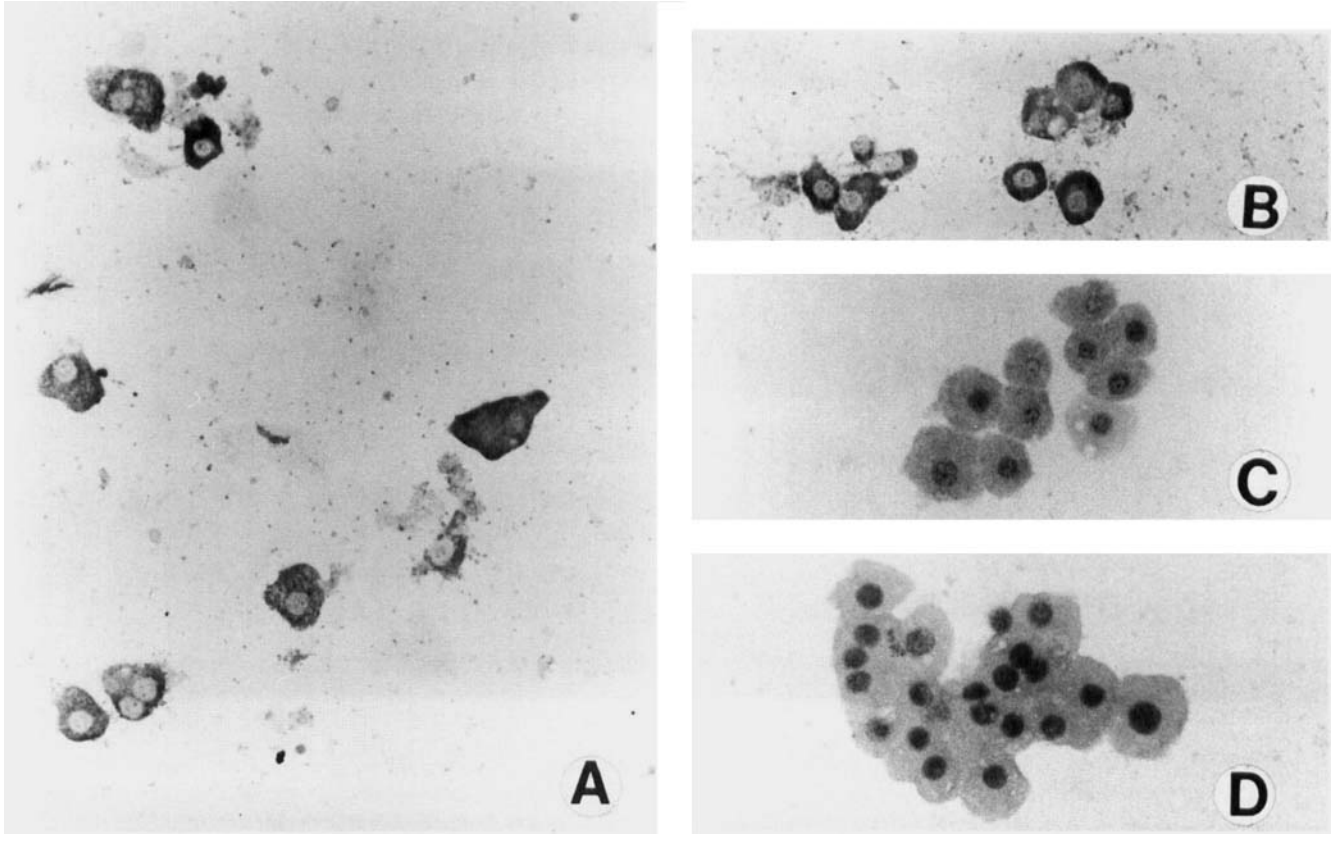

Fig. 2. Immunostains of washed, air-dried aspirate smears performed with AEC chromogen and hematoxylin counterstain are illustrated for incubation times of (**A**) 20, (**B**) 10, (**C**) 7, and (**D**) 3 min. A markedly shortened chromogen incubation time compared with that determined on the fixed-tissue positive control was required to achieve the same immunostaining specificity as with the DAB method.

cytospin preparations without considerable background and false-positive staining reactions. Nonspecific staining with AEC occurred despite the use of a cell wash procedure to reduce the potential for antibody binding to highly concentrated serum and interstitial proteins. In order to achieve the same immunostaining specificity in cytologic preparations, the AEC chromogen reaction in this ABC-immunoperoxidase detection system required more rigid control with a markedly shortened chromogen incubation time compared with the optimal time determined on the fixed-tissue positive control. In our experience, the AEC chromogen reaction in FFPE tissues has appeared more intense (with negative background) than that of DAB chromogen and is therefore preferable to detect low-density antigens. However, it appears that use of AEC chromogen with incubation time standardized on a FFPE tissue control and then applied to cytologic preparations also amplifies nonspecific and background staining, contributing difficulty to assessing a true-positive result. Since the numerous antibodies used in diagnostic immunohistochemistry are optimized for sensitivity, knowledge of this potential pitfall in the immunohistochemical staining of cytologic preparations should be of benefit to others who experience similar unexpected false-positive immunoreactions.

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