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An alkaline-phosphatase staining method in avidin-biotin immunohistochemistry *

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Summary. An avidin-biotin alkaline-phosphatase (ABAP) staining method has been developed for the labeling of tissue sections and cell smears. The introduction of alkaline phosphatase as a marker enzyme through an avidin bridge results in excellent immunocytochemical labeling of different antigens using poly- and monoclonal antibodies. This technique avoids problems with endogenous peroxidase activity that sometimes occur using peroxidase staining procedures. The introduction of a preformed avidin-biotin alkaline-phosphatase complex (ABAPC) makes the presented technique as simple to handle as the widely used avidin biotin-peroxidase complex method (ABC). The ABAPC technique could be combined with other enzymatic labelings for double immunoenzymatic staining.

Introduction

Avidin, a glycoprotein with a molecular weight of 68,000 daltons found in egg white, and biotin (i.e., vitamin H) have a very high affinity for each other (Green 1963). The interaction of biotin and avidin is remarkably rapid and stable. Since biotin can be covalently linked to immunoglobulins as well as to enzymes, and avidin can be labeled with fluorescent compounds, colloidal gold, or enzymes, researchers have used these reagents in various methodological modifications.

One of the most innovative immunohistochemical applications of avidin-biotin interaction has been the avidin-biotin peroxidase-complex method of Hsu et al. (1981). However, endogenous peroxidase activity in many cells and tissues obscures the specific peroxidase labeling reaction. Procedures to block endogenous enzyme activity may sometimes destroy labile antigens and can affect cell morphology (Stein 1983). Furthermore, the double labeling of two different antigens on one section by the application of different peroxidase substrates is capricious and not easy to perform in routine immunohistochemistry.

We have therefore developed an avidin-biotin alkaline-phosphatase (ABAP) staining method to localize antigens in paraffin- embedded material as well as in blood smears, which avoids the disadvantages of peroxidase labeling and takes advantage of avidin-biotin interactions.

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Materials and methods

The following tissue samples were used: human goiter (surgically removed) fixed in buffered formaldehyde; a specimen of human gastric cancer fixed in Carnoys solution; acetone fixed blood smears from healthy volunteers.

The following antisera and substances were used: anti-human cytokeratin 35βH11 (Enzo Biochemicals, NY); anti-human cathepsin-G polyclonal sheep (Serotec, UK); anti-human thyroglobulin polyclonal rabbit (kindly provided by Henning, Berlin); ABC peroxidase kit; avidin DH; avidin/alkaline phosphatase; biotin-labeled alkaline phosphatase; biotin antimouse immunoglobulin; biotin antirabbit immunoglobulin; biotin antisheep immunoglobulin (Vector, USA); naphtol AS-MX salt; BB-blue salt; fast-red TR salt; levamisole (Sigma, FRG); DAB (Fluka, FRG).

Immunostaining procedures. In accordance with the ABC-peroxidase technique, tissue sections were deparaffinized in xylene, brought down through graded alcohols, and washed in phosphate-buffered solution (PBS). Blood smears were only rinsed in PBS. Further steps were performed in an incubation chamber (Ormanns and Pfeifer 1981) at room temperature (RT). The slides were incubated with optimally diluted antibodies (i.e., cathepsin, 1:200; cytokeratin, 1:2,000; thyroglobulin, 1:800) for 2 h and washed in PBS; biotin-labeled second antibodies (dilution, 1:200) were then applied for 30 min. Alkaline-phosphatase labeling was performed as shown in Table 1. Various concentrations of avidin DH, avidin alkaline phosphatase, and biotin-labeled alkaline phosphatase were tested. Preformed complexes of enzyme-labeled avidin and biotin were also tested. The complexes were made by incubating various concentrations of avidin DH, or avidin/alkaline phosphatase and biotin/alkaline phosphatase for 30 min at RT. Either PBS (pH 7.3; 0.0125 M) or hydrogen carbonate buffer (pH 9.4; 0.1 M; Bussolati and Gugliotta 1983) were used as the diluting buffer.

The final reaction was achieved by incubating the sections with alkaline-phosphatase substrate (Mason et al. 1983). This was prepared by dissolving 2 mg Naphtol AS-MX in 0.2 ml dimethylsulfoxide (DMSO) in a tube. The solution was added to 9.8 ml 0.1 M Trisbuffer containing 1 mmol levamisol in order to block endogenous alkaline-phosphatase activity. Immediately before use, fast-red TR salt or BB-blue salt was added at a concentration of 1 mg/ml; the solution was then directly filtered onto the slide. The incubation time was between 10 min and overnight. After an intensive rinse in tap water, the slides were counterstained with hematoxylin, air dried, and mounted in Eukitt.

Controls. In parallel tissue sections and using the same primary and secondary antibodies, the ABC-peroxidase method was applied in order to compare it with the alkaline-phosphatase method. Additional controls were performed as follows: omission of the primary antibody and replacement of the primary antibodies by normal rabbit, sheep, or mouse serum.

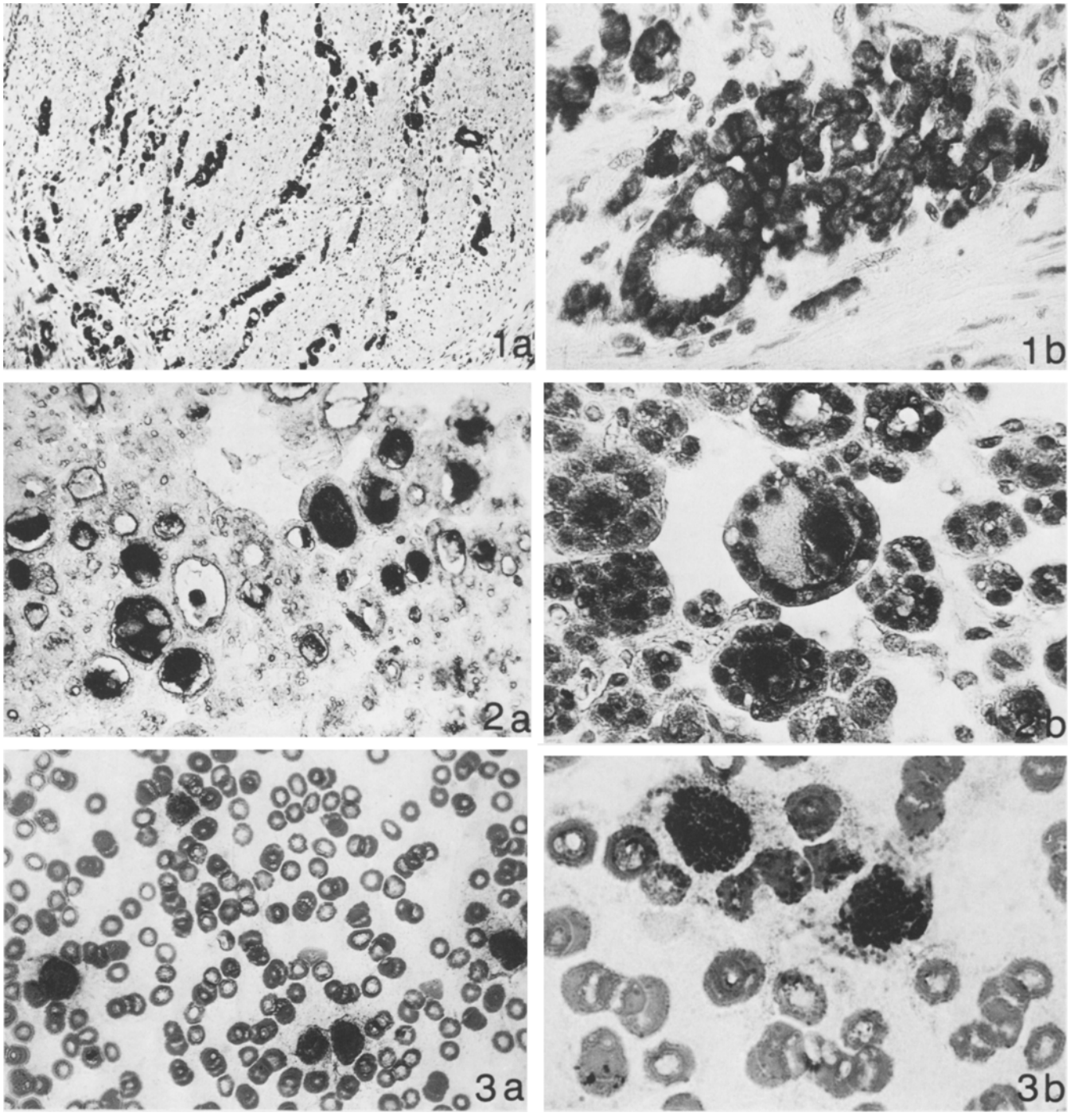

Fig. 1a, b. Results of avidin-biotin alkaline-phosphatase complex (ABAPC) staining in paraffin sections. Naphtol AS-MX/fast red was used as substrate for the demonstration of enzyme activity; counterstaining with hematoxylin. Gastric carcinoma stained with a monoclonal antibody specific for epithelial cytokeratin (35βH11). **a** Heavily stained tumor cells infiltrating the layers of the muscularis propria ($\times$170). **b** Same tumor with distinctly positive tumor glands ($\times$360)

Fig. 2a, b. ABAPC staining in paraffin sections as in Fig. 1. Thyroid tissue stained with polyclonal antithyroglobulin. **a** Endemic goitre with strong reaction in the colloid ($\times$220). **b** Follicular adenoma with positive staining of colloid as well as thyroid cells ($\times$440)

Fig. 3a, b. Blood smears stained with polyclonal anticathepsin G. **a** Positive reaction only in polymorphonuclear leukocytes ($\times$440). **b** Strong staining of azurophile granules in polymorphonuclear leukocytes ($\times$1,100)

Results

Each of the antibodies under investigation gave sharply localized immunocytochemical staining reactions both with the ABAP and avidin-biotin alkaline-phosphatase complex (ABAPC) techniques (Figs. 1–3). The labeling intensity and localization were almost identical to those obtained using ABC-peroxidase staining, although the immunoalkaline-phosphatase reaction was sometimes slightly granular compared to the DAB reaction in peroxidase staining. Increased

Table 1. Synopsis of immunoalkaline labeling procedures

Step				
1	Primary antibody	Primary antibody	Primary antibody	Primary antibody
2	Biotin-labeled second antibody (1:200)	Same procedure	Same procedure	Same procedure
3	Avidin DH (10 µg/ml)	Avidin-ALP (5,000–500 IU/ml)	Step 3 omitted	Step 3 omitted
4	Biotin-ALP (5,000–500 IU/ml)	Biotin-ALP (5,000–500 IU/ml)	Preformed complexes: Avidin-ALP (5,000–500 IU/ml); Biotin-ALP (5,000–500 IU/ml)	Preformed complexes: Avidin DH (10 µg/ml); Biotin-ALP (5,000–500 IU/ml)
5	Alkaline-phosphatase substrate	Same procedure	Same procedure	Same procedure

Avidin DH = modified avidin supplied by Vector. Avidin-ALP = avidin alkaline phosphatase conjugate; Biotin-ALP = biotin alkaline-phosphatase conjugate.

Enhancement of staining could be achieved by repeating steps 2–4 before the final application of the alkaline-phosphatase substrate (step 5). The optimal combination for immunohistochemistry was preformed complexes of Avidin DH (10 µg) incubated with 1,000–2,000 IU/ml Biotin-ALP

concentrations of avidin/alkaline phosphatase or biotin/alkaline phosphatase shortened the reaction time by up to 10 min and resulted in intensive staining which sometimes masked good cytological morphology. Good reactions were obtained by using 4,000 IU/ml avidin/alkaline phosphatase and 4,000 IU/ml biotin/alkaline phosphatase in a bridge technique. An almost identical result was achieved using the same concentrations of labeled enzymes in preformed complexes. The replacement of labeled avidin by avidin DH did not diminish the intensity of the staining reaction. It was found that 10 µg avidin DH and 2,000 IU/ml biotin/alkaline phosphatase in preformed complexes gave an optimal "signal-to-noise" ratio (Table 1). Enhancement of the staining reaction could be achieved by repeating steps 2 and 4 before the final application of the alkaline-phosphatase substrate.

Unwanted staining, which was sometimes derived from collagen in tissue, was demonstrable in the immunoalkaline as well as in the immunoperoxidase technique. Although alkaline-phosphatase activity is inhibited by inorganic phosphate (Ishikawa et al. 1983), the use of PBS as diluting buffer did not interfere with staining intensity when compared to results obtained using hydrogen carbonate buffer.

Discussion

Our results indicate that alkaline phosphatase can be used as a marker enzyme in avidin-biotin immunohistochemistry. Reagents giving an optimal staining result can be easily prepared from commercially available components. Instead of using the biotin-peroxidase solution supplied with the ABC kit (Vector), biotin/alkaline phosphatase can be introduced to achieve a very sensitive staining. This method seems to be superior to the currently employed alkaline-antialkaline phosphatase (APAAP) technique. Unlike the latter method (Cordell et al. 1984) which may require APAAP made in different animal species, the ABAPC method relies on a general biotinylated secondary antibody and a universal avidin-biotinylated alkaline-phosphatase complex. This feature is most important because anti alkaline-phosphatase antibodies made in many species are not commercially available. The additional steps which are often needed to adopt the APAAP system to one's own requirements are not only time consuming but may also produce increased background staining.

Compared to biotin-peroxidase staining, relatively large amounts of alkaline phosphatase as marker enzyme must be introduced to achieve optimal staining. This is due to the fact that the enzyme is not very stable and loses some of its activity in very dilute solutions. The coupling process of alkaline phosphatase to biotin may also alter the enzymatic activity (Guesdon et al. 1979). These unwanted interactions can be balanced by increasing the incubation time with the enzyme substrates and by repeating incubation steps.

The excellent results obtained with preformed ABAPCs must have been due to the formation of large complexes containing multiple alkaline-phosphatase molecules. It is likely that the formation of this complex is similar to that of avidin-biotin peroxidase aggregates, although their structure has not yet been determined.

The technique presented is a universal immunostaining procedure that can also be combined with peroxidase or other marker enzymes to localize two different antigens in the same section. Therefore the ABAP method would seem to be a good alternative technique for immunohistochemical studies.

References

Bussolati G, Gugliotta P (1983) Nonspecific staining of mast cells by avidin-biotin-peroxidase complexes (ABC) J Histochem Cytochem 31:1419–1421

Cordell LJ, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, Macdonald S, Pulford KA, Stein H, Mason DY (1984) Immunoenzymatic labeling of monoclonal antibodies using complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem 32:219–229

Green NM (1963) The use of ^{14}C biotin for kinetic studies and for assay. Biochem J 89:585–591

Guesdon JL, Ternynck T, Avrameas S (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. J Histochem Cytochem 27:1131–1139

Hsu SM, Raine L, Fanger H (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580

Ishikawa E, Imagawa M, Hashida S, Yoshitake S, Hamaguchi Y, Ueno T (1983) Enzyme labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. J Immunoassay 4:209–327

Mason DY, Abdulaziz U, Falini B, Stein H (1983) Double immunoenzymatic labeling. In: Polak JM, Van Noorden S (eds) Immunocytochemistry. John Wright and Sons, Bristol, pp 113–128

Ormanns W, Pfeifer U (1981) A simple method for incubation of tissue sections in immunohistochemistry. Histochemistry 72:315–319

Stein H (1983) Immunhistochemie am Knochenmarksgefrierschnitt und -ausstrich. Verh Dtsch Ges Pathol 67:2526