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Silver enhancement of polymerised diaminobenzidine: Increased sensitivity for immunoperoxidase staining

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Abstract

Unambiguous identification of lymphocytes is sometimes difficult because of weak immunostaining of the cell membrane immunoglobulins. A simple method of intensifying the diaminobenzidine (DAB) peroxidase reaction was therefore devised. Paraffin wax sections of formalin fixed tonsils and lymphomas were digested with trypsin and immunostained for κ and λ light immunoglobulin chains and CD3 antigen by various peroxidase linked detection systems. After reaction with hydrogen peroxide and DAB the sections were immersed in methenamine silver solution at 60°C for three to seven minutes. The light brown stain on the cell membranes of the mantle zone lymphocytes became dark brown and the stronger stain of the plasma cells became black. Mantle zone B lymphocytes and CD3 positive T lymphocytes were precisely outlined even at low magnification and the lymphomas were easily classified as monoclonal or polyclonal. At high magnification, staining was clearer than with the immunogold-silver stain. Cryostat and paraffin wax sections of other tissues immunostained for various antigens showed similar intensification.

Silver methenamine provides an easy means of increasing the sensitivity and visual impact of an immunoperoxidase/DAB reaction in any preparation.

Since the establishment of the peroxidase anti-peroxidase (PAP) method[1] and the avidin-biotin complex (ABC) method[2] various other ways of increasing the sensitivity of immunostaining methods have been proposed, with the aim of identifying very small quantities of antigen and of increasing the visual impact of the reaction. Among the earlier suggestions were the addition of imidazole[3] or heavy metal salts such as cobalt chloride or nickel sulphate[4] to the diaminobenzidine (DAB) peroxidase development medium to darken the colour of the normal brown end-product. A different tack was taken by Holgate *et al*[5] with the introduction of the immunogold-silver staining (IGSS) method for light microscopy, the scarcely visible red colour of the colloidal gold marker being made intensely black by deposition of silver from a silver lactate solution. This additional intensification allowed the depiction of the surface membrane immunoglobulins of lymphocytes in paraffin wax sections to a much greater extent than ordinary immunoperoxidase techniques.[6] Subsequent work by this group resulted in modification of the fixative to give even better localisation of lymphocyte surface antigens.[7,8] Even without this fixative reasonable IGSS staining can be achieved, certainly better than that provided by a standard three-layer immunoperoxidase method, but an inherent problem of the IGSS method is a fine grained non-specific deposit of silver over the section.

Scopsi and Larsson carried out an in vitro survey of the sensitivity of available immunoperoxidase methods, including the addition of imidazole and heavy metals to the DAB incubating solution and post-silvering of the reaction product.[9] Their results indicated that one of the most sensitive methods was to intensify the oxidised DAB product with silver from a methenamine silver solution.[10] Because this solution is part of a commonly used method of staining basement membranes and fungi,[11,12] it is readily available in most histology laboratories, and we have found it to be a convenient and valuable way of increasing the sensitivity of three-layer immunoperoxidase staining to the level achieved by IGSS.

Methods

The methenamine silver intensification can be used on any immunoperoxidase preparation after the peroxidase/H_2O_2/DAB reaction has been carried out to give a brown deposit. The preparations are washed in running tap water, then thoroughly rinsed in distilled water, and placed in preheated methenamine silver solution at 60°C for three to five minutes or, occasionally, longer. The intensification can be carried out at room temperature but proceeds at a much slower rate. Sections can be stored indefinitely in distilled water before the silver intensification is carried out, and preparations previously stained weakly by immunoperoxidase and DAB can be retrospectively intensified. In this case it is best to remove haematoxylin from counterstained nuclei with acid alcohol before the silver intensification is carried out.

The composition of the stock methenamine silver solution is as follows: 0·125% silver nitrate in 1·5% hexamine; this solution can be stored at 4°C. Just before use, 2 ml of 5% sodium tetraborate is added to 50 ml of the stock silver solution, giving a pH of 8·0. The

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solution is filtered into a Coplin jar and heated in a water bath at 60°C, protected from strong light.

Silver deposition is progressive and can be monitored microscopically. Preparations must be well rinsed in distilled water before being replaced in the silver solution. When a satisfactory level of staining has been reached, the preparations are rinsed in distilled water. If overstained, they may be "toned" in 0·2% gold chloride for 30 seconds. This procedure can give a more pronounced blackening of the reaction product than progressive silvering alone. The preparations are then fixed for 30 seconds in 5% sodium thiosulphate solution, which also removes excess silver, and can then be counterstained as required, dehydrated, and mounted in a permanent mountant.

Occasionally sections may show deposition of silver on connective tissue/reticulin fibres, but this is not a problem with intensification times of up to 10 minutes. Enterochromaffin cells in formalin fixed, paraffin wax sections are blackened after seven minutes so the method is unsuitable for enhancing immunostained gut endocrine cells. If poly-L-lysine is used as an adhesive to coat slides silver may be deposited on the areas of the slide not covered by the tissue after the longer incubation times. This is not aesthetic but does not affect the stained area of the tissue itself.

Test preparations used in this study were mainly tonsil and lymphoma samples fixed in neutral buffered formalin and embedded in paraffin wax, or fresh-frozen for cryostat sections that were post-fixed in acetone.

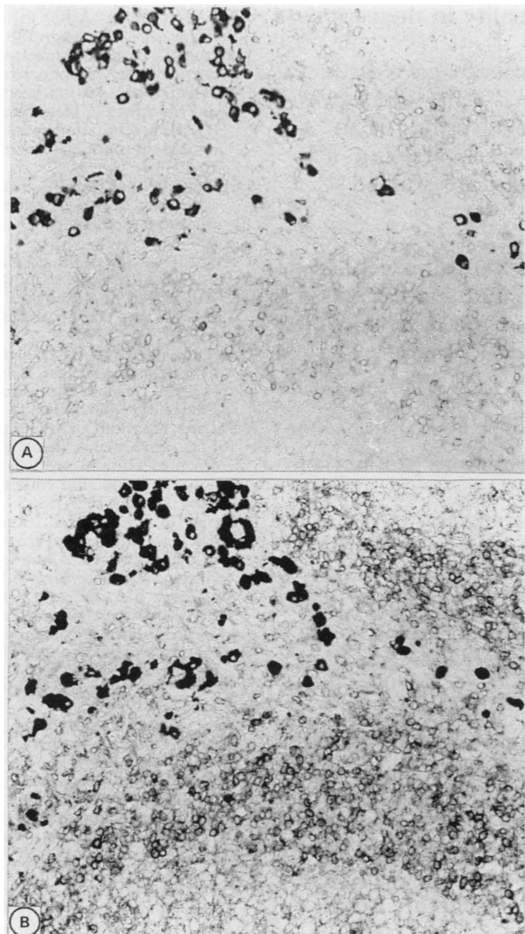

Figure 1A Formalin-fixed paraffin wax section of tonsil, digested with trypsin, and immunostained for κ light immunoglobulin chains by the PAP method with DAB as chromogen. Plasma cells are well stained; in the mantle zones cell surface immunoglobulin is lightly stained.
Figure 1B Serial section to 1A, identically treated but subjected to methenamine silver solution at 60°C for five minutes. Mantle zone cells now show strong staining of surface immunoglobulin. Nuclei are not counterstained.

Paraffin wax sections were immunostained with rabbit polyclonal antibodies for κ and λ immunoglobulin light chains and the CD3 antigen using the peroxidase anti-peroxidase (PAP), avidin-biotin complex (ABC), and biotinylated second antibody-peroxidase-labelled streptavidin methods. The sections were treated with trypsin (Sigma T8128, 0·1% in 0·1% calcium chloride, pH 7·8) at 37°C for five to 10 minutes before blocking of endogenous peroxidase with 0·3% aqueous H_2O_2 for 30 minutes. Non-specific binding of antiserum protein was prevented by covering the sections with 5% normal swine serum in phosphate buffered saline (PBS), pH 7·2 before applying the primary antibody. The antibodies to κ and λ light chains were used at a dilution of 1 in 3000 and the CD3 antibody at 1 in 20 in PBS containing 0·1% bovine serum albumin and 0·1% sodium azide, and incubation was carried out overnight at 4°C. Incubation in subsequent immunoreagents was for 30 minutes at room temperature. IGSS staining was carried out in parallel on many preparations,[5] and in some cases the special dichromate-PLP fixative[7] was compared with the standard neutral buffered formalin.

Cryostat sections were immunostained with monoclonal antibodies for various B and T lymphocyte markers by an indirect method using peroxidase-conjugated rabbit anti-mouse immunoglobulins. Endogenous peroxidase was blocked by incubation for 30 minutes at 37°C in a solution of sodium azide and glucose with glucose oxidase,[13] and background staining was prevented by incubation in 5% normal rabbit serum.

Additional material was stained for other routinely useful diagnostic antigens. The peroxidase on all preparations was developed with 0·025% DAB in phosphate buffered saline, pH 7·2, containing 0·03% H_2O_2.

The 5 nm colloidal gold adsorbed goat anti-rabbit immunoglobulin used in the IGSS method was supplied by Janssen and all other immune reagents by Dako.

Results

PARAFFIN WAX SECTIONS

The conventional staining methods for κ and λ produced a good dark brown reaction in the cytoplasm of the plasma cells, but surface immunoglobulin could not be distinguished satisfactorily, although after the biotinylated second antibody/labelled streptavidin procedure a light brown stain was present, only distinguishable in sections without a counterstain. Staining was more intense after dichromate-PLP fixation, but still suboptimal. After silver intensification the cytoplasmic immunoglobulin in the plasma cells and immunoblasts became intensely black and the cell membranes of immunostained cells became dark brown and were easily seen in contrast with a light haematoxylin counterstain (fig 1). The IGSS method, used in comparison, produced black cell surface staining. At low power the B cell areas were well marked with both methods, but at high power the IGSS method produced a diffuse silver precipitate which made it difficult to distinguish between

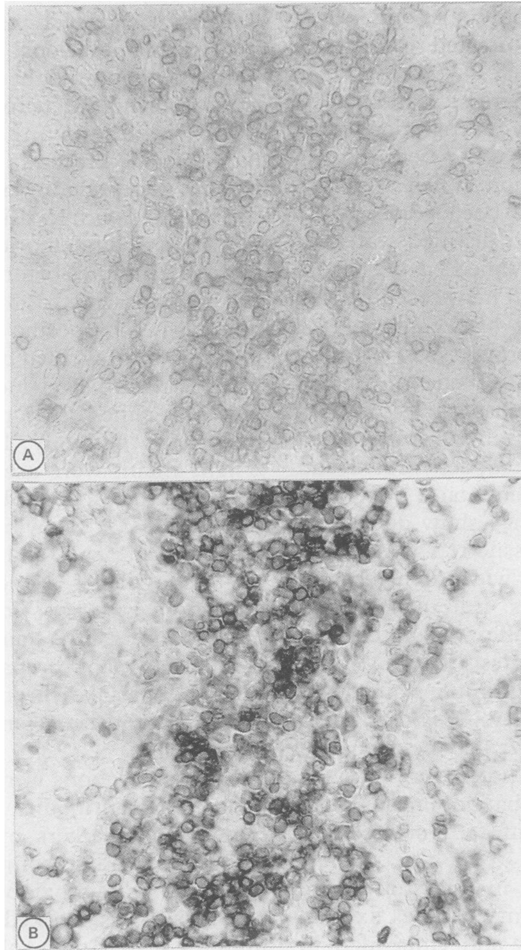

Figure 2A Section from the same block as shown in fig 1, digested with trypsin and immunostained for CD3 by a peroxidase-linked avidin-biotin method. The interfollicular T lymphocytes are weakly positive.
Figure 2B As fig 2A after immersion in methenamine silver solution at 60°C for five minutes. The T lymphocytes are now fully evident. Nuclei are not counterstained.

positive and negative cells. The DAB-methenamine silver intensification method provided a clean background and permitted firm characterisation of κ or λ monoclonality in the various lymphomas studied.

The polyclonal CD3 antibody gave distinct but weak staining of the T cells, which was greatly intensified by post-silvering (fig 2). A similar improvement to weak staining was achieved for all the other antigens studied.

CRYOSTAT SECTIONS

The B and T cell markers were all adequately stained by the indirect immunoperoxidase method, but post-silvering of the DAB reaction product provided much better contrast.

Discussion

Polymerised DAB, the reaction product of peroxidase-mediated oxidation of DAB, catalyses the reduction of alkaline silver salt solutions to metallic silver, which is deposited on the DAB precipitate. As in all silver impregnation methods, the metallic silver itself then acts as an autocatalyst, raising the rate of deposition and allowing an expanding shell of silver to be built up at the site of reaction. This increases both the contrast and the area of the immunostaining reaction and could be carried to excess so that individual areas of staining are indistinguishable. Thus a judicious but subjective compromise must be reached between intense staining and discrete localisation.

Post-silvering of the DAB raises the sensitivity of a three-layer immunoperoxidase technique to the level of the IGSS method, so that lymphocyte surface immunoglobulins can be localised even on paraffin wax sections from tissue conventionally fixed in neutral buffered formalin. The weak immunoperoxidase/DAB staining shown here could certainly have been improved by other means but has been used to illustrate the general point that a poor result from any peroxidase/DAB preparation can be greatly improved by intensification with silver, though it must be said that any background staining will be intensified to the same extent as the specific staining.

Post-silvering of immunoperoxidase/DAB reactions has several advantages over other methods of intensification. The decision as to whether to carry out intensification need not be taken until the reaction is complete, and retrospective intensification can be carried out, whereas cobalt or nickel salts must be added to the medium at the incubation stage and a decision to carry out the IGSS method must be taken from the outset. The methenamine-silver method is considerably simpler than the other methods, even using the commercially available DAB/silver intensification kit (Amersham). No hazardous substances are used, unlike a recent suggestion for osmium intensification,[14] and as the silver deposit is stable, the increased contrast it provides means that, like the IGSS method, it can be combined with many different special stains or counterstains.

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