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Immunoperoxidase techniques: the deleterious effect of sodium azide on the activity of peroxidase conjugates

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SUMMARY The effect of including sodium azide as a bacteriostatic agent in solutions used to dilute antibodies conjugated with the enzyme horseradish peroxidase was examined. An enzyme-linked immunosorbent assay (ELISA) and an immunohistochemical method were used and both techniques demonstrated an inhibitory effect of sodium azide on the activity of the peroxidase conjugates. It is concluded that the use of sodium azide in solutions used to dilute peroxidase conjugates is to be avoided.

In immunohistochemical studies antibodies conjugated with the enzyme horseradish peroxidase are widely used. The peroxidase enzyme is used to produce localised areas of tissue staining by its ability to precipitate a coloured product when incubated with a suitable substrate, often diaminobenzidine, DAB (3,4,3',4'-tetra-aminobiphenyl hydrochloride)[1]. It is common practice to have peroxidase conjugates in solution in a buffer such as phosphate-buffered saline (PBS), to which sodium azide may be added for its bacteriostatic properties. Commercially available preparations of peroxidase conjugates may also include sodium azide as a preservative—for example, DAKO-Immunoglobulins a/s, Copenhagen.

However, there is good evidence that sodium azide is an inhibitor of the peroxidase enzyme. Klapper and Hackett[2] demonstrated a 57% inhibition of the oxidation of menadiol by peroxidase by a sodium azide concentration of 4·2 mmol/l and a 95% inhibition by a sodium azide concentration of 42·0 mmol/l. Similar evidence was provided by Herzog and Fahimi,[3] who measured the activity of peroxidase by its ability to oxidise 3,3'diaminobenzidine tetrahydrochloride (DAB) and found that in their system 50% inhibition of activity could be achieved by a sodium azide concentration of 2·0 mmol/l and 95% inhibition by an azide concentration of 10 mmol/l.

Since sodium azide may be included as a preservative in buffers at concentrations ranging between 3·0 mmol/l (0·02%) and 15·0 mmol/l (0·1%), it is possible that peroxidase activity may be significantly inhibited in conjugate preparations. Prior to making bulk dilutions of conjugates and freezing in aliquots that could be subsequently used directly for immunohistochemical staining we examined the effect of azide using both an enzyme linked immunosorbent assay (ELISA) and an immunohistochemical method.

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Material and methods

ELISA STUDY

The methodology used for the ELISA was based on that described by Voller *et al.*[4] Rabbit immunoglobulin (IgG) at a concentration of 5 μg/ml in coating buffer was applied to the wells of microtitre plates (Dynatech Laboratories, Billingshurst, Sussex) and incubated at 4°C overnight. After washing the wells three times with azide-free PBS containing 0·05% Tween 20, 0·1 ml of a sheep antirabbit peroxidase conjugate diluted 1/64 000 in the same PBS was applied to some of the wells in each plate and incubated at room temperature for two hours. After washing unbound conjugate from the wells using three changes of PBS Tween, 0·1 ml of the peroxidase substrate *o*-phenylenediamine, (0·4 g/l) in the presence of hydrogen peroxide (0·012%) in citrate buffer pH 5·0 was applied to the wells. A coloured reaction product was generated during the ensuing 30-minute incubation period. The reaction was stopped by the addition of 0·1 ml sulphuric acid (2·5 mol/l). The intensity of the colour was measured spectrophotometrically at 492 nm using a

Titertek Multiskan spectrophotometer (Flow Laboratories, Irvine, Ayrshire).

This assay was repeated seven days later using further wells on the same microtitre plates. In addition to incubating with freshly diluted conjugate at a similar dilution, replicate conjugates which had been stored frozen for the intervening seven days diluted 1/200 in either 1% ovalbumin in PBS containing 0·02% sodium azide, or 1% ovalbumin in azide-free PBS were also tested. These stored conjugates were further diluted for the purpose of the ELISA to 1/64 000 using azide-free PBS containing 0·05% Tween 20. Results for the stored conjugates were calculated relative to the freshly diluted conjugate and could be compared with the readings obtained using freshly diluted conjugate on the same microtitre plates from seven days previously.

IMMUNOPEROXIDASE STUDY

A parallel study was made using an indirect immunohistochemical staining procedure.[5] First antibodies raised against a variety of different antigens (see Table 2) were incubated on tissue sections for one hour, and after washing the slides appropriate antispecies conjugates were applied and incubated for a further hour. The sections used were approximately 4 μm thick, cut from formalin-fixed, paraffin-embedded blocks of tissue. The conjugates had been stored frozen, and diluted ready for use, either in 1% ovalbumin in azide-free PBS or in 1% ovalbumin in PBS containing 0·02% sodium azide. The visible intensity of staining was assessed and is recorded in Table 2. No negative controls were used in this study as the object was to compare intensity of staining with and without azide, rather than to establish the specificity of the antibodies.

The antibody to carcinoembryonic antigen (CEA) was raised in a rabbit, and affinity-purified using a column containing CEA bound to agarose gel beads which included N-hydroxy succinamide esters (AffiGel 10, BioRad Laboratories, Watford, Herts). The affinity purified antibody was subsequently absorbed with a human spleen extract. This was prepared by homogenising normal spleen in distilled water, adding an equivalent volume of perchloric acid (1·2 mol/l) stirring for 30 min. at room temperature and centrifuging (1000 g, 4°C, 20 min). The supernatant was extensively dialysed against distilled water and then lyophilised.

Results and discussion

The differences in spectrophotometer readings obtained by the ELISA technique are presented in Table 1 and the immunoperoxidase results in Table 2.

Clearly when a conjugate has been stored frozen in the presence of azide the intensity of colour produced in an ELISA is measurably reduced compared with the same conjugate stored frozen in the absence of azide. This effect is equally well demonstrated when similarly stored conjugate is used in immunohistochemical staining (Table 2) and this is illustrated in the photographs of a squamous carcinoma of the lung stained for carcinoembryonic antigen using conjugate diluted 1/200 in PBS with and without azide (Figure). Non-specific cross-reactivity in the affinity purified CEA antiserum had previously been noted in polymorphonuclear cells in sections stained with this antibody. This was due to the presence of non-specific cross-reacting antigen (NCA) in these cells.[6] This cross-reactivity was removed by the addition of 100 mg perchloric acid extract of human spleen/ml affinity-purified antiserum.

In some cases there were larger differences between the slides treated with conjugates with and without azide. We suspect that where the dilution was optimal a slight difference in conjugate activity would have more effect than when an unnecessarily high concentration of first antibody was used. For example, in this study the antibodies to somatostatin and glucagon were used at concentrations which were too strong to enable a precise assessment of differences in staining intensity to be made. These first antibodies are now used at a greater dilution.

The results obtained using peroxidase conjugated with antibodies are in agreement with the earlier

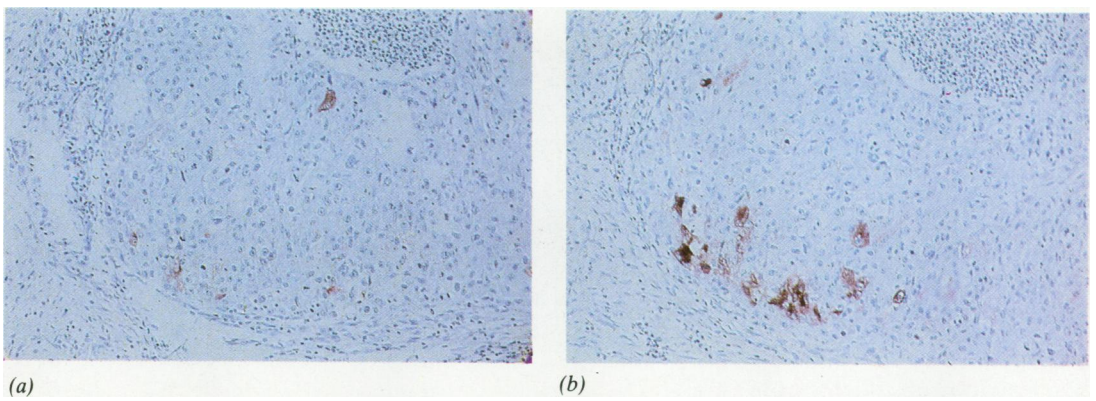
Table 1 *Effect of sodium azide on the storage of diluted conjugate as measured using an enzyme-linked immunosorbent assay*

Initial absorbance on first day (azide-free)	*Absorbance after 7 days stored at −20°C (azide-free)*	*Absorbance after 7 days stored at −20°C (with 0·02% azide)*
1·04	1·48	0·82
1·01	1·15	0·79
1·04	1·18	0·90
*Means ± SEM		
1·03 ± 0·01	1·27 ± 0·11	0·84 ± 0·03

*Values are blank corrected spectrophotometer readings from three separate assays.

Table 2 *Effect of sodium azide on the storage of diluted conjugate as assessed using immunoperoxidase staining*

Tissue	*First antibody*	*Conjugate with azide*	*Conjugate without azide*
		Sheep antirabbit conjugate (1/200)	
Carcinoma of the lung	CEA absorbed with NCA	+	++
Carcinoma of the colon	CEA absorbed with NCA	Feeble	++
Placenta (1st trimester)	α-HCG	+	+++
Carcinoma of the thyroid	Calcitonin	+	++++
Carcinoma of the breast	Epithelial membrane antigen	+	+++
Prostate	Prostatic acid phosphatase	Feeble	++
Normal pancreas	Somatostatin	++	++++
	Glucagon	+++	++++
		Donkey antisheep conjugate (1/100)	
Tonsil	IgA	Feeble	+
	IgG	++	++++
	IgM	−	+
Lactating breast	Casein	+	++
		Goat antimouse monoclonal (1/100)	
Carcinoma of the breast	Blood group substance A	Feeble	++
Carcinoma of the cervix	Blood group substance B	Feeble	++
Tonsil	Ig κ chain	−	+
	Ig λ chain	Feeble	++



(a) (b)

(a) Section of a squamous carcinoma of the lung stained for CEA by the indirect method with a rabbit anti-CEA first antibody, followed by sheep antirabbit second antibody conjugated with horseradish peroxidase. This conjugate had previously been frozen for seven days in 1% ovalbumin in PBS containing 0·02% sodium azide. (b) Serial section of the same tissue, stained using the same procedure, the only difference being that the conjugate had been stored frozen in 1% ovalbumin in PBS which was azide-free. The reaction product is clearly much stronger and with more positive cells than in (a). The polymorphs in a microabscess (top right) are unstained by this absorbed antiserum.

observations that azide is an inhibitor of the peroxidase enzyme. We now determine the optimum dilution of conjugate on several tissues using a variety of first antibodies and then store suitable aliquots of conjugate at −20°C diluted in 1% ovalbumin in azide-free PBS. This eliminates one source of experimental variation.

Conclusion

On the basis of the results from this study it appears that the use of sodium azide as a preservative in diluted preparations of conjugate is to be avoided. However, peroxidase conjugates have been prepared in this laboratory and stored undiluted at 4°C in the presence of 0·1% azide[7] for up to 18 months without apparent detriment. Similarly we have not noted any deleterious effect of including azide in preparations of unconjugated antibodies such as the first antibodies used in our staining procedures.

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