Letters to the Editor

Sensitive system for visualising biotinylated DNA probes hybridised in situ

A recent article by Burns *et al*[1] referred to a silver amplification system that was crucial to the success of the demonstration, by in situ hybridisation, of specific sequences of DNA in the sex chromosomes. The silver amplification system was used to visualise complexes of diaminobenzidine (DAB), and uses the principles of a method established by ourselves—namely, that complexes of DAB bind gold salts,[2] and that the avidity of DAB for gold salts (gold chloride in particular) is so great that it can be used to generate an insoluble sulphide on which the silver amplification procedure depends.[3 4]

We were surprised that as we were the first to have used these sequential steps to enhance DAB visualisation no mention was made of our work and that the reference given for the use of gold salts was to a brief letter,[5] which, in fact, makes no mention of the use of gold. In our publications we have acknowledged the earlier work of Siegesmund *et al* who, in an *x* ray microanalysis study, showed the affinity of DAB for gold salts.[6]

We would not like your readers to believe that the application of the silver amplification procedure described by Burns *et al* is confined to in situ hybridisation technology. The method by which the DAB is generated is irrelevant: silver amplification based on the principles that we have described can be used for various applications, ranging from work on artificial materials such as nitrocellulose to tissue sections for the light and electron microscope.

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Dr Burns and colleagues reply as follows:

The objectives of our article were to describe a system for the fast, sensitive detection of biotinylated DNA sequences by in situ hybridisation, and show one of its applications—namely, cell sexing.[1] This system has two components: methodology for hybridisation of cloned biotinylated DNA to complementary nucleic acids in fixed cells and detection of the site of hybridisation of biotinylated DNA by immunoperoxidase histochemistry in which the diaminobenzidine (DAB)/hydrogen peroxide reaction product is amplified by gold and silver precipitation.

It was not our purpose to review methods for enhancing DAB/hydrogen peroxide products. Dr Newman and others claim that the "principles" used in the detection system[1] were first described by them and are surprised that their work was not cited.[2 3] This claim is wrong. For this and other reasons (see below) their work was not referenced. Heavy metals such as osmium have been used to enhance DAB/hydrogen peroxide reaction products since the late 1960s. Gold chloride and other metals were introduced for the same purpose in the 1970s and 1980s.[5 6] There is a typographical error in the paper[4] referred to by Dr Newman and others: the formula given for gold chloride is $AuCl_2$; gold has a valency of 1 or 3, and $AuCl_2$ does not exist. Timm[7] showed that "the sulphides of insoluble heavy metals catalyse the deposition of silver in the presence of a reducing reagent".[2] In fact gold sulphide was visualised by silver in tissue in 1981 and apparently as early as 1935.[8] Gallyas *et al* described a light insensitive silver reagent to amplify DAB/hydrogen peroxide complexes,[9] and a year later Holgate *et al* used a light sensitive developer to amplify colloidal gold immunohistochemically.[10] Historically, the statement made in the first paragraph of Dr Newman and others' letter is therefore erroneous and misleading.

The protocol described by the Welsh group for gold and silver enhancement of DAB/hydrogen peroxide products described in "principle" in a letter[2] and abstract.[3] The letter states that "after immunoperoxidase staining, the sections were treated with 0·1% gold chloride . . . immersed in neutralised sodium sulphide . . . and exposed to the silver developing solution of Gallyas *et al*".[2] Gold chloride is a generic name for sodium chloroaurate ($NaAuCl_4$), chloroauric acid ($HAuCl_4$), gold monochloride (AuCl), and auric chloride ($AuCl_3$): the molecular weights and pH of aqueous solutions of these gold chlorides differ. The chemical formula and pH of gold chloride used by us is detailed.[1] "Neutralised sodium sulphide" is also chemically meaningless: was it 0·1 mol/l, pH 7·5, or 10 mol/l, pH 7·0? Additionally, the silver reagent described by Gallyas *et al* is unstable, forms precipitates in a test tube, and is unsuitable for tissue localisation of biotinylated DNA.[1] The modified silver reagent[1] is stable, does not precipitate in a test tube, and is used routinely in this and other laboratories (D Ward, unpublished data).

Dr Newman and others imply that the readers of the *Journal of Clinical Pathology* might not deduce that the amplification procedure could be used in other applications. We assumed this to be self evident. Finally they refer to the use of silver for amplifying DAB/hydrogen peroxide signals on nitrocellulose filters, which assumes they have unpublished data to support this claim. We advise that gold and silver should not be used for the detection of biotinylated nucleic acids on nitrocellulose filters. Much greater sensitivity (fg quantities of DNA) can be detected on filters by other more sensitive non-radioisotopic methods.[11]

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Distinguishing lymphoma and small cell anaplastic carcinoma of the thyroid by immunocytochemistry

I read with interest the report by Burt *et al*[1] regarding the problem of differential diagnosis between lymphoma and small cell anaplastic carcinoma of the thyroid. A similar study has been performed in our department in Leicester on a smaller number of cases (19). In addition to thyroglobulin and epithelial membrane antigen antisera, our study also included a cytokeratin antibody (CAM 5·2). It was found that this antibody was more sensitive for detecting epithelial malignancies than epithelial membrane antigen. A combination of common leucocyte antigen and CAM 5·2 resolved the differential diagnosis in all but two of the cases. The use of this or a related cytokeratin antibody, together with common leucocyte antigen is thus suggested for this diagnostic problem. Epithelial membrane antigen, apart from reduced sensitivity compared with cytokeratin, suffers from the additional disadvantage of being reactive in a proportion of lymphomas.[2]

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T lymphocyte numbers and serum E rosette inhibitory substance

It is well established that T lymphocyte numbers are diminished in certain disease conditions. These include protein calorie malnutrition[1 2] plasmodium falciparum infection,[3 4] measles infection,[5 6] and systemic lupus erythematosus.[7] Others are HBs Ag positive chronic hepatitis,[8] rheumatoid arthritis,[9] and cancer.[10] Sera from most of these patients inhibit both in vitro phytohaemagglutinin transformation of lymphocytes,[5 11] and E rosette formation by normal human lymphocytes.[12–17]

There is circumstantial evidence to suggest that the presence of E rosette inhibitory substance (probably an immune complex) could be partly responsible for the diminished number of E rosettes that are recorded in these patients. Treatment with levamisole considerably increases the number of E rosette forming T lymphocytes in vivo and in vitro in several disease conditions, in which patients commonly possess circulating immune complexes and low E rosetting lymphocytes.[10 18–20] We also observed recently that, in common with children who had protein calorie malnutrition, children with malaria or measles infections had increased titres of circulating immune complexes, serum E rosette inhibitory substance(s), and diminished numbers of circulating E rosettes.[16]

It is therefore necessary for workers carrying out studies of lymphocyte subpopulations to be cautious in interpreting findings of diminished E rosettes. The percentage of E rosettes observed in such cases may not represent the total circulating E rosette numbers present. From our experience a test for the presence of E rosette inhibitory substance(s) in such patients is useful, as there may be some circulating T lymphocytes that are not capable of forming E rosettes in vitro, probably as a result of the previous binding of their surface receptors to inhibitory substances in vivo.[14]

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