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Sensitive in situ hybridisation technique using biotin-streptavidin-polyalkaline phosphatase complex

F A LEWIS, SHARON GRIFFITHS, ROBINA DUNNICLIFF, M WELLS, N DUDDING, C C BIRD*

*From the Departments of Pathology, University of Leeds, Leeds, and the *University Medical School, Edinburgh, Scotland*

SUMMARY A sensitive in situ hybridisation technique, using a biotin-streptavidin-polyalkaline phosphatase complex detection system, was successfully applied to smears of fresh cultured cells, frozen sections, and formalin fixed paraffin processed tissue: the procedure was successful for DNA-DNA hybridisations using a variety of DNA probes. The detection method is rapid, reliable, and economical producing a purplish-blue precipitate at the site of hybridisation and clearly visible by low power light microscopy.

In situ hybridisation with labelled gene probes is visualised by methods specific for the labels used. Autoradiography has been used to detect radiolabelled probes,[1] but despite the sensitivity of this technique the use of such highly radioactive material is unsuitable for most routine applications because of the special containment facilities required. Biotinylated probes have been used as an alternative and are detected immunologically using antibiotin antibodies, together with fluorescence or enzyme labelled second antibodies, or by the extremely high affinity reaction occurring between biotin and the protein tetramer streptavidin. Streptavidin can be subsequently visualised by direct conjugation to an enzyme label, or a biotinylated enzyme can be used in a secondary reaction. Peroxidase has generally been used as the enzyme label in these reactions,[2] but to achieve high sensitivities it has been found necessary to perform a silver enhancement reaction. In our experience the introduction of such non-specific enhancement reactions to increase sensitivity renders the method unreliable. To overcome this difficulty we developed a simple and reliable method for the detection of in situ hybridisations with biotinylated probes, using a biotin-streptavidin-polyalkaline phosphatase complex, which matches the sensitivities claimed for silver enhanced peroxidase methods. This finding has recently been confirmed by Unger *et al*[3] using a colorimetric detection method similar to the one described in this paper.

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Material and methods

PRETREATMENT

Whole cells Smears of a cultured lymphoma cell line were prepared on poly-l-lysine coated single or multiple well slides (CA Hendly, Essex) and fixed for 30 minutes in 4% paraformaldehyde in a buffer containing 0·1M phosphate (pH 7·4), 0·1M sucrose, and 20mM vanadyl sulphate. After fixation the slides were washed briefly in the same buffer without the paraformaldehyde and immediately extracted for two × five minutes in 0·1M Tris-hydrochloric acid (pH 7·4), containing 0·25% Triton X-100 and 0·25% Nonidet P40 to permeabilise partially and extract cell membranes. The slides were then washed with several changes of 0·1M Tris-hydrochloric acid (pH 7·2) and immersed in 20% acetic acid in water at 4°C for 15 seconds. The slides were again washed with several changes of 0·1M Tris-hydrochloric acid (pH 7·2) and incubated in 20% glycerol in water for 30 minutes. The slides were finally rinsed in two × SSC (one × SSC = 0·15M sodium chloride, 15mM sodium citrate; pH 7) before prehybridisation processing.

Fresh tissue Cryostat sections (10μ) of fresh specimens, previously frozen in liquid nitrogen were placed on poly-l-lysine coated single well slides and pretreated as for whole cells as described above.

Fixed tissue Specimens were fixed in buffered formalin and embedded in paraffin wax in the usual way. Sections (3–5 μm) were placed on poly-l-lysine coated single well slides and hot plated for several days to ensure maximum tissue adhesion. The sections were dewaxed in xylene at 37°C for 30 minutes, xylene at

room temperature for 10 minutes, and absolute alcohol for two × 10 minutes. The sections were hydrated through graded alcohols to distilled water, immersed in phosphate buffered saline for five minutes, and processed according to the method described by Brigati *et al*,[4] with some modification. Briefly, the slides were immersed in 0·02M hydrochloric acid for 10 minutes, washed in phosphate buffered saline for 10 minutes, immersed in phosphate buffered saline containing 0·01% Triton X-100 for one and a half minutes, and washed in phosphate buffered saline for 10 minutes. The slides were then immersed in 20% acetic acid in water for 15 seconds at 4°C, washed through several changes of phosphate buffered saline, and treated with a solution of proteinase K (Gibco-BRL, Paisley, Scotland) in 50 mM Tris-hydrochloric acid (pH 7·6) and 5 mM edetic acid at 37°C for 10 minutes. The concentration of proteinase K was 0·1–0·5 mg/ml. The slides were washed twice in phosphate buffered saline, containing 2 mg/ml glycine, for five minutes each and post-fixed in 4% paraformaldehyde in phosphate buffered saline for five minutes, washed for a further two × five minutes in phosphate buffered saline and glycine, dehydrated through graded ethanols, and stored in absolute ethanol before hybridisation mixture was applied.

DNA probes The DNA probes studied were plasmid pBR 322 (Gibco-BRL) for use as a negative control probe, pHY 2·1, a Y chromosome specific probe,[5] and human papilloma virus probes HPV6b,[6] 11,[7] 16,[8] and 18.[9] The probes were biotinylated with biotin-11-dUTP (Gibco-BRL), using a nick translation kit (Gibco-BRL) and following the recommended protocol. Unincorporated dNTPs were separated from the biotinylated DNA by the spun column technique[10] on Sephadex G50 (Pharmacia Ltd, Milton Keynes). In addition, DNA was prepared and purified from a male tonsil by the method of Bentley and Rabbitts[11] and biotinylated as described above for the DNA probes. This biotinylated tonsil DNA was used as a "total DNA" probe. The biotinylated probes were prepared at a concentration of 200 ng/ml in a hybridisation buffer containing two × SSC, 10% dextran sulphate, 50 mM Tris-hydrochloric acid (pH 7·2), two × Denhardt's solution (one × Denhardt's = 0·01% Ficoll, 0·01% bovine serum albumin, 0·01% polyvinyl pyrrolidone), 0·4 mg/ml single stranded salmon sperm DNA, 50% formamide. Before use the probes were made single stranded by heating for 10 minutes in a boiling water bath followed by rapid cooling on ice to prevent reannealing.

PREHYBRIDISATION

After pretreatment the cell smears and cryostat sections were immersed in two × SSC briefly, then incubated with two × SSC 50% formamide for 10 minutes, and prehybridised at room temperature with hybridisation buffer not containing probes for one hour. Before the hybridisation mixture was applied the slides were dipped briefly into two × SSC and excess liquid was wiped away. Sections from paraffin wax embedded specimens did not undergo any prehybridisation after pretreatment.

IN SITU HYBRIDISATION

Hybridisation mixture (50 μl) containing the probes were pipetted on to the prepared sections or cell smears. The wells were covered with a piece of gel bond (Miles Laboratories Ltd, Slough), hydrophobic side down, and sealed with rubber solution or clear nail varnish. The slides were heated at 90°C for 10 minutes to denature cellular DNA and transferred to an incubator at 42°C in a humidified box. The incubation was allowed to proceed for 18–42 hours at 42°C. After this time the gel bond covering the wells was carefully removed and the slides were immersed in two × SSC. Sequentially the slides were washed in two × SSC at 60°C with agitation for 20 minutes, 0·2 × SSC for five minutes, 0·2 × SSC at 42°C with agitation for 20 minutes, 0·1 × SSC for 10 minutes, and finally two × SSC for two minutes prior to detection of the hybridisation signal.

DETECTION OF HYBRIDISATION SIGNAL

The slides were processed using a DNA detection kit (Gibco-BRL) with some modifications to the recommended protocol. Briefly, the slides were immersed in 2% bovine serum albumin in buffer 1 (0·1M Trihydrochloric acid (pH 7·5), 0·1M sodium chloride, 2 mM magnesium chloride, 0·05% Triton X-100) for five minutes to block non-specific streptavidin binding sites. The slides were drained of excess buffer and placed in a humidity chamber. A solution of streptavidin in buffer 1 (2 μg/ml) was pipetted on to the slides and incubated at room temperature for 20 minutes. The slides were washed for three × five minutes in buffer 1 with agitation and excess buffer was removed; the slides were returned to the humidified box. A solution of biotinylated polyalkaline phosphatase (1 μg/ml) in buffer 1 was applied to the slides and incubated for a further 20 minutes at room temperature. The slides were washed for three × five minutes in buffer 1 with agitation and immersed in buffer 2 (0·1M Tris-hydrochloric acid (pH 9·5), 0·1M sodium chloride, 50 mM $MgCl_2$) for one hour. The slides were returned to the humidified box and development reagent was applied to the slides. Development reagent contained 33 μl of nitroblue tetrazolium (75 mg/ml in 70% dimethylformamide) and 25 μl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml in dimethylformamide) in 7·5 ml of buffer 2. The slides were

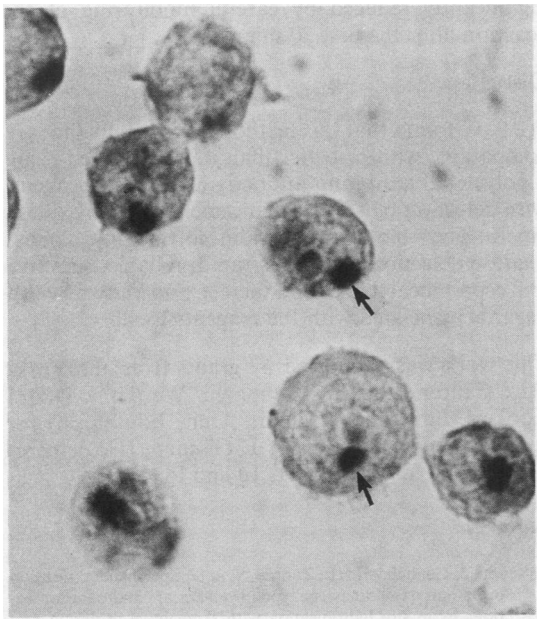

Fig 1 *Fresh cultured male lymphoma cell line was probed with pHY 2·1. Y body is localised (arrowed) in nucleus as round purple-blue area.*

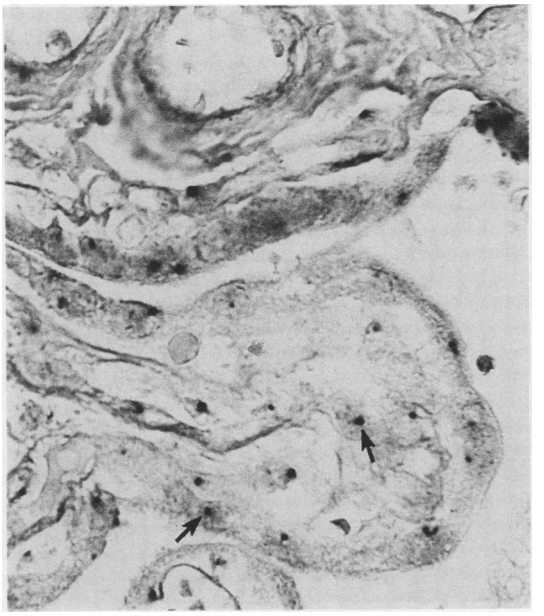

Fig 3 *Formalin fixed paraffin embedded section of placenta was probed with biotinylated pHY 2·1 showing presence of cells (arrowed) containing Y body.*

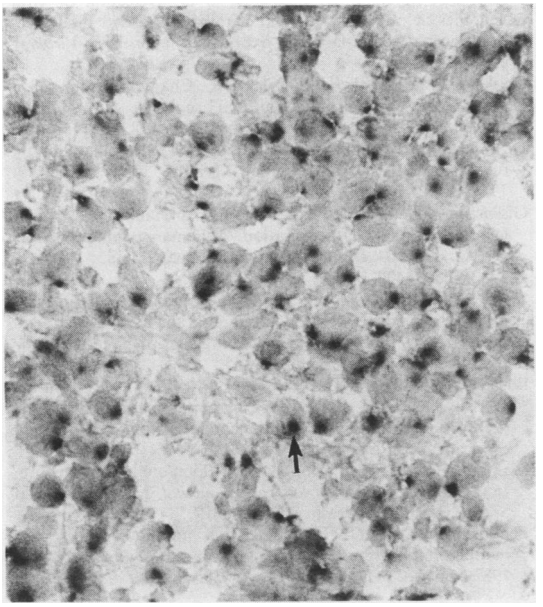

Fig 2 *Fresh frozen cryostat section of male lymphoma was probed with biotinylated pHY 2·1 showing presence of Y bodies (arrowed) in nuclei of most cells.*

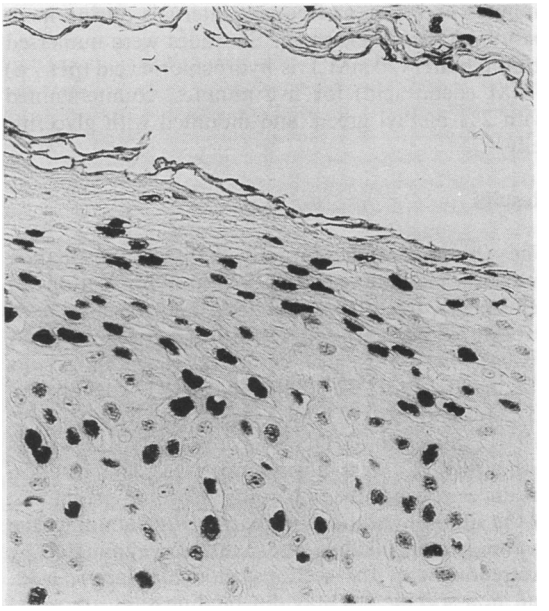

Fig 4 *Formalin fixed paraffin embedded section of genital wart was probed with biotinylated HPV 6b showing presence of virus in koilocytes.*

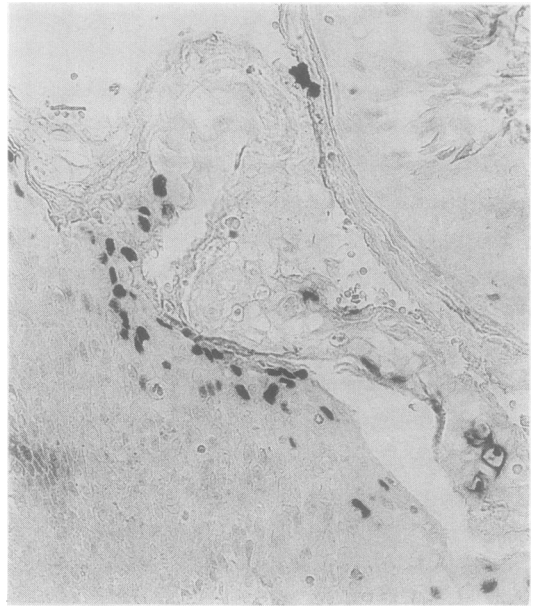

Fig 5 *Formalin fixed paraffin embedded section of vulval intraepithelial neoplasia grade probed with biotinylated HPV 16 showing presence of virus in koilocytes.*

incubated in subdued light from 30 minutes to four hours. Colour development was monitored after 30 minutes and then at frequent intervals during incubation. After development the slides were immersed in stop buffer (20 mM Tris-hydrochloric acid (pH 7·5) 5 mM edetic acid) for five minutes, counterstained with 2% methyl green, and mounted with glycerine jelly.

Results

The biotin-streptavidin-polyalkaline phosphatase detection method visualised in situ hybridisations of cell smears, fresh frozen sections, and formalin fixed paraffin embedded sections with a variety of DNA probes. Figs 1–5 give some examples of results obtained with pHY2·1, HPV6b, and HPV16 probes. In addition, we obtained successful results with HPV11 and "total DNA" probes. Hybridisations using pBR322 were always negative. The reaction produces a water insoluble purplish-blue precipitate at the site of hybridisation. Background staining due to endogenous alkaline phosphatase is eliminated by pretreatment of the sections with 20% acetic acid, and non-specific streptavidin binding sites are satisfactorily blocked with bovine serum albumin. Overdevelopment of the colour reaction can produce a high level of non-specific staining, but this was significantly reduced by careful monitoring of the reaction after the first 30 minutes.

Discussion

We have found that this method gives results that are comparable with or better than those obtained using a polyclonal antibiotin antibody as primary reagent, with detection by silver enhancement of a peroxidase label. The biotin-streptavidin-polyalkaline phosphatase detection method is rapid, reliable, sensitive and economical to use, and there are no known health hazards associated with the reagents used.

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Requests for reprints to: Professor CC Bird, Department of Pathology, University Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland.