Number of white cells, lymphocytes and lymphocyte subpopulations (median and range, $\times 10^9/l$) in venous and skin puncture blood

Sample type	Total white cells	Lymphocytes	CD3+ T cells	CD3+ CD4+ T cells	CD3+ CD8+ T cells	CD19+ B cells	CD3+ CD16+ CD56+ natural killer cells
Venous blood	4.6 (1.3–13.0)	1.46 (0.47–4.45)	1.26 (0.38–3.69)	0.71 (0.23–2.31)	0.49 (0.12–1.32)	0.03 (0–0.36)	0.10 (0.01–0.42)
Skin puncture blood	4.6 (1.6–14.2)	1.51 (0.43–4.62)	1.27 (0.41–3.93)	0.74 (0.25–2.50)	0.47 (0.09–1.66)	0.03 (0–0.38)	0.14 (0.02–0.48)
p value	0.009	0.03	0.036	0.033	0.12	0.05	0.049

Results

Samples were obtained from 22 children aged from eight months to 16.4 years, median 5.5 years, and 10 adults aged between 26 and 44 years. Data from adults and children were combined for analysis. The numbers of the different cell types were higher in skin puncture blood (4.8–6.4% higher for subsets other than CD3+ CD16+ CD56+ natural killer cells, which were 10.7% higher), the difference being statistically significant for all cell types except CD3+ CD8+ T lymphocytes (table). Significance was lost for all pairings when total lymphocytes and subpopulations were expressed in proportional terms (data not shown). Paired values were highly correlated ($r=0.90$ for CD3+ CD16+ CD56+ natural killer cells, and 0.96–0.98 for total white cells, lymphocytes and other subsets, $p<0.0001$ for all pairings). The differences between paired CD3+ CD4+ T cell counts is shown in the figure.

Discussion

Collection of blood by skin puncture provides a rapid and simple alternative to venepuncture when small volumes of blood are required, and is useful when dealing with small children and subjects with difficult veins. In this study no undue difficulty was found in obtaining the required volume of skin puncture blood; should problems arise it may be possible to reduce test volumes by 25–50%, at least when the total lymphocyte count is within normal limits.

Previous studies have shown clinically acceptable comparability in white cell and differential counts between the two samples types,^{2,3} although in the earlier study neutrophil values

were significantly higher in skin puncture blood. Similar results were obtained in the present study, the usually slightly higher total white cell count of skin puncture samples being reflected proportionally throughout the range of lymphocyte subsets. Although of borderline statistical significance for most subpopulations, the magnitude of the difference was small and unlikely to be of clinical importance.

The precision of both replicate haemoglobin and platelet estimations from skin puncture samples has been reported to be poorer than that from venous blood,^{4,5} although the latter claim could not be verified using the same sample collection method as that of the present study.² While we did not count lymphocytes in sequential paired samples there appears to be no reason why their numbers should fluctuate to a greater degree in skin puncture blood, a contention supported by the highly significant correlations found for each lymphocyte subpopulation between the two sample types.

Our results indicate that skin puncture blood can be safely used as an alternative to venous blood for the enumeration of CD4 and other lymphocyte subsets. It would be prudent when monitoring cell numbers over time, however, to use one sample type alone in order to minimise the variables affecting the results obtained.

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J Clin Pathol 1995;48:1138–1140

Detection of Ki67 antigen by a new sheep polyclonal antiserum

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Accepted for publication
1 June 1995

Abstract

This report describes the characterisation of a polyclonal sheep antiserum against the Ki67 antigen. On western blots, this antiserum recognises a pair of bands of high molecular weight identical with those

seen with another polyclonal Ki67 antiserum and the MIB 1 monoclonal antibody. The new antiserum showed nuclear staining of a proportion of cells in paraffin wax embedded tissue sections following antigen retrieval using a microwave oven

or pressure cooker. This staining pattern was blocked by incubating the serum with the peptide used as immunogen. The proportion and distribution of immunostained nuclei was identical with that seen with the alternative reagents that recognise the Ki67 antigen. The new reagent stained the same proportion of cells when used over a wide range of dilutions. There was no cross-reactivity with unrelated antigens sometimes detected by the monoclonal antibodies.

(*J Clin Pathol* 1995;48:1138–1140)

Keywords: Ki67 antigen, antiserum, immunohistochemistry, cell proliferation.

The Ki67 antigen is a nuclear protein expressed in all proliferating cells and is a useful means of estimating the growth fraction by immunohistochemistry.¹ Although the function of the protein remains unknown, the chromosomal location, nucleotide sequence and protein structure have been determined.^{1,2} The protein has DNA binding properties.¹

The antigen was first recognised by Gerdes *et al*³ in 1983 using the monoclonal antibody Ki67. Following the identification and cloning of the cDNA, further monoclonal antibodies have been produced using recombinant protein as immunogen.⁴ Of these, MIB 1 is used most widely. All of these monoclonal reagents recognise the same epitope.⁵ More recently, a polyclonal antiserum raised in rabbits has been produced using a synthetic peptide as the immunogen.⁶

This report describes the characterisation of a further polyclonal antiserum against the Ki67 antigen which has been raised in sheep (AU177, The Binding Site, Birmingham, UK). This antiserum adds to the choice of available reagents directed against the Ki67 antigen, particularly with regards to detection systems for sheep immunoglobulins and in double immunostaining procedures.

Methods

The immunogen used for the production of the sheep antiserum was a 23 amino acid synthetic peptide corresponding to residues 1212 to 1234 of the Ki67 antigen (QTPKEKAQALDLA GFKELFQTP) and encompassing the epitope recognised by the monoclonal antibody Ki67.² The peptide was synthesised in the form of a multiple antigenic peptide⁷ and injected with Freund's complete adjuvant. The antisera was affinity purified using an immobilised form of the same peptide.

The other anti-Ki67 reagents with which this sheep antiserum was compared on serial sections were the original monoclonal Ki67 antibody (M722, Dako, High Wycombe, UK) used at a 1 in 50 dilution, MIB 1 (0505, Immunotech, Marseille, France) used at a 1 in 100 dilution, and a commercially available polyclonal rabbit anti-Ki67 (A047, Dako), used at a dilution of 1 in 50. Immunostaining was carried out on formaldehyde fixed, paraffin wax embedded tissues using a Sequenza (Shandon, Runcorn, UK). Tris buffered saline (pH 7.6) was used for washes and for diluting the anti-

bodies with the exception of the primary antibodies, to which 10% normal swine serum was added. Sections were placed on coated slides (Fro-tissuer pen, The Binding Site) and after incubation and subsequent rehydration, were pretreated in a microwave oven according to standard protocols.⁸ Sections were immunostained using the Dako Duet Strept ABC HRP system (K492, Dako), but a biotinylated rabbit anti-goat secondary antibody (E466, Dako), at a 1 in 100 dilution, was applied for the detection of sheep polyclonal Ki67. All sections were visualised with diaminobenzidine (DAB) (X901, Sigma, Poole, UK).

Inhibition assays were performed with all Ki67 antibodies studied at their optimal dilutions using the synthetic peptide described earlier. The peptide was double diluted from a 1 in 1 dilution up to 1 in 1024, which represented a range of peptide concentrations between 2.4 and 0.002 mg/ml.

Double immunoenzymatic staining was performed, using an appropriate panel of controls, as described previously.⁹ The sheep antiserum was detected using donkey anti-sheep horseradish peroxidase antibody (AP184P, Chemicon, Temecula, California, USA) at a 1 in 25 dilution. For the mouse and rabbit antibodies, biotinylated donkey anti-mouse (AP192B, Chemicon) and rabbit (AP182B, Chemicon) antibodies at 1 in 200 and 1 in 50 dilutions, respectively, were used, followed by Strept ABC alkaline phosphatase (K391, Dako). The alkaline phosphatase activity was demonstrated using Naphthol AS-MX phosphate and Fast red,⁹ followed by DAB to demonstrate Ki67 antigen expression.

The western blot was produced according to the method described by Gerdes *et al*² and was probed with the sheep, rabbit and mouse antisera.

Results and Discussion

The sheep polyclonal antiserum gave similar reactivity to that of other anti-Ki67 reagents. Sections from 16 normal and nine tumour specimens were studied. Normal tissue included six tonsil, one oesophageal, two colon, three skin, one stomach, one ileum, and two kidney specimens. Tumour tissue sections were comprised of two benign (a basal cell papilloma and a colonic tubular adenoma) and seven malignant lesions (two squamous cell carcinomas, one basal cell carcinoma, two colon and one stomach adenocarcinoma, and a breast cancer). The only antibody found to give sub-optimal staining was the original Ki67. Using the sheep polyclonal antibody, staining was clean and confined to the nuclei of the proliferative compartment of the tissues (fig 1). No non-specific cytoplasmic staining was seen within squamous epithelial cells, which has been seen with the Ki67 antibody. Furthermore, no cross-reaction was observed with perivascular stroma or the brush borders of renal proximal tubules, which occurs with the MIB 1 monoclonal antibody. The sheep polyclonal antibody has since been used on over 50 routine surgical cases and cytological specimens.

Figure 1 Section of formalin fixed, paraffin wax embedded normal tonsil immunostained with the new sheep polyclonal antiserum directed against the Ki67 antigen. The nuclei of proliferating cells within the germinal centre are clearly labelled and the non-proliferating lymphocytes in the surrounding mantle zone are negative ($\times 420$).

Titration of the sheep polyclonal antibody showed strong staining with no background at concentrations between 0.0137 and 0.0034 mg/ml of immunoglobulin (corresponding to a 1 in 100 to a 1 in 400 dilution). The proportion of positive nuclei within a particular tonsil germinal centre was constant over a range of concentrations from 0.1096 to 0.0017 mg/ml (corresponding to a 1 in 12.5 to a 1 in 800 dilution).

In the inhibition assays staining of normal tonsil by the Ki67 and MIB 1 monoclonal antibodies and the sheep polyclonal antibody (at a concentration of 0.0069 mg/ml) was completely abolished following pre-incubation with the synthetic peptide at a concentration of up to 0.0094 mg/ml. Positive staining using the rabbit polyclonal was not affected by pre-incubation with the peptide at the same concentration.

Double immunostaining was achieved successfully using the sheep antiserum with both mouse and rabbit antibodies. No background staining or cross-reaction in the test sections or controls was seen.

The sheep, rabbit and mouse antisera revealed identical double bands with apparent molecular weights of around 300–400 kD on western blot (fig 2).

In conclusion, the sheep antiserum provides a useful tool for immunohistochemical studies of cell proliferation on archival histological material. It is comparable with the other anti-Ki67 antigen reagents presently available, producing clean, strong staining. It also has the added

Figure 2 Western blots stained with the new sheep polyclonal antiserum (lane A) and MIB 1 monoclonal antiserum (lane B). Both reagents react with identical bands consistent with the expected molecular weights of around 300–400 kD.

advantage of permitting double staining of other markers of interest, either monoclonal or polyclonal.

The authors would like to thank The Binding Site for providing the sheep polyclonal anti-Ki67 antiserum.

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