Combined immunohistochemical and immunofluorescence method to determine the phenotype of proliferating cell populations

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Abstract

***Aims*: To determine the phenotype of proliferating cell populations.**

***Methods*: The double immunostaining technique combines the autofluorescent properties of alkaline phosphatase substrate naphthol/Fast Red with immunofluorescence using fluorescein. Fresh human tonsil and fresh atherosclerotic aortic aneurysm wall tissue were studied using a panel of monclonal antibodies including Ki-67, CD4, CD8, CD19, CD22, HLA-DR*a*, CD68 and CD31.**

***Results*: This double immunostaining method permitted simultaneous colocalisation of different markers on the same cell and could be used to identify HLA-DR positive cells as well as proliferation associated Ki-67 positive cells in human tonsil tissue and in chronic periaortitis associated with advanced atherosclerosis.**

***Conclusion*: This technique is simple and the results may be viewed using a single fluorescence filter. The Fast Red reaction product is stable and does not fade under storage. The staining works particularly well with markers for nuclear antigens in combination with markers for cytoplasmic or surface antigens.**

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Both experimental and diagnostic histopathologists have shown increasing interest in the detection of cellular proliferation markers, such as Ki67.[1] To determine the phenotype of the proliferating cell populations, double immunohistochemical and double immunofluorescence staining techniques are of great value. Double immunofluorescence in particular can give clear, sensitive results. But two filters need to be used for imaging these results.

In 1960 Burstone described the use of *a*-naphthol substrates in fluorescence techniques for alkaline phosphatase.[2] As a recent review of published findings has shown,[3] the use of Fast Red fluorescence, both singly and in combination with other immunofluorescence and immunohistochemical techniques, has been used to solve problems in cellular immunology.

In this paper we describe a simple double immunohistochemical/immunofluorescence technique. This technique makes use of the fact that Fast Red fluoresces red/orange at the same excitation wavelength as fluorescein fluoresces green. It is a double immunostaining technique that uses alkaline phosphatase anti-alkaline phosphatase (APAAP) and fluorescein isothiocyanate (FITC) conjugated antibodies. The results may be visualised using a single fluorescence filter. This technique is of particular value for showing two different phenotypic markers on the same cell.

We have a particular interest in the chronic inflammatory process which accompanies advanced atherosclerosis, a condition termed "chronic periaortitis".[4 5] This double staining technique is of value in showing the presence of proliferating lymphocytes in chronic periaortitis.

Methods

Fresh human tonsil and atherosclerotic abdominal aortic aneurysm wall tissue was obtained at surgery. Frozen tissue was embedded in OCT (Miles Laboratories Inc., Lab-Tek Div., Naperville, Illinois, USA) and then immersed in liquid nitrogen. Cryostat sections 6–9 μm were cut, air dried for 20 hours, and fixed in acetone for 10 minutes. Similar paraffin wax blocks were prepared by tissue fixation in 10% formalin, followed by routine embedding and sectioning procedures.

Activation and proliferation of cells were observed on serial cryostat and paraffin wax sections using a panel of monoclonal antibodies: Ki-67, HLA-Dr*a*, JC70 (CD31), CD68, CD4, CD8, CD22 and CD19 (table).

Single immunostaining was carried out on serial sections of frozen tissue using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique.[12]

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Double immunostaining was carried out to identify HLA-DR positive cells and to identify Ki-67 positive proliferating cells. The monoclonal antibody TAL.1B5 was used in conjunction with monoclonal antibodies to specific cellular markers: macrophage associated antigen (CD68:EBM 11), to the T cytotoxic/suppressor associated phenotype (CD8:Tü102), to the T helper associated phenotype (CD4:T3–10), to the B cell associated phenotype (CD19/22:HD37/4KB128) and to an endothelial cell marker (JC70:CD31). Ki-67 was used in conjunction with monoclonal antibodies to macrophage-associated antigen (CD68:EBM 11), to the T cytotoxic/suppressor associated phenotype

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Panel of monoclonal antibodies

Antibody	*Specificity*	*Cell association*	*Reference*
Ki-67	Nuclear antigen	Proliferating cells	6
TAL.IB5	HLA-DR α chain	Variety	7
JC70	CD31	Endothelial cells, some platelets, granulocytes and macrophages	8
EBM II	CD68	Macrophages	9
T3-10	CD4	T helper cells	10
Tü102	CD8	T cytotoxic cells	10
HD37	CD19	B cells	11
4KB128	CD22	B cells	11

(CD8:Tü102), to the T helper associated phenotype(CD4:T3–10) and to the B cell associated phenotype (CD19/22:HD37/4KB128).

Staining was carried out on serial cryostat sections which were air dried overnight and then fixed in acetone for 10 minutes at room temperature. The first monoclonal antibody (Ki-67, EBM 11, Tü102, T3-10, or HD37/4KB128) was applied as undiluted culture supernatant to dry tissue sections and incubated for one hour. Two subsequent 30 minute incubations were carried out with a 1 in 25 dilution of rabbit anti-mouse immunoglobulin (DAKO) followed by a 1 in 50 dilution of alkaline phosphatase anti-alkaline phosphatase monoclonal antibody (APAAP) (DAKO). The alkaline phosphatase substrate (0·2 mg/ml naphthol AS-MX phosphate free acid (Sigma), 0·5% dimethylformamide, 1 mM levamisole, 1 mg/ml Fast-Red TR salt (Sigma) in 0·1 M TRIS-HCl, pH8·2) was prepared, filtered, and immediately applied to the sections and was then allowed to develop for 20 minutes.

After washing thoroughly, the sections were incubated in 10% normal human serum (NHS; heat inactivated and filtered) for 15 minutes followed by the application of the second monoclonal antibody (all combinations with TAL.1B5 or Ki-67) for one hour. A 1 in 20 dilution of fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse immunoglobulin (Dako) in 10% NHS was then applied and incubated for 30 minutes. Two minute washes in TRIS-buffered saline (TBS; 0·05M TRIS-HCl pH7·6, 0·15M NaCl) were carried out between each step. All dilutions were made in TBS and all incubations were carried out at room temperature in a humid chamber.

Sections were mounted in the fluorescein preservative DABCO/glycerol mountant (90% glycerol, 10% TBS, 2·3% 1,4-diazabicyclo(2.2.2)octane (Sigma)) and observed under a Leitz Ortholux II fluorescence microscope with an excitation wavelength between 450 and 490 nm (fluorescein filter 3). Photographs were taken on Kodak Ektachrome 200 colour reversal film with an exposure of 60–90 seconds (figs 1 and 2).

Controls were carried out, using an irrelevant antibody (mouse anti-rabbit immunoglobulin (DAKO)), TBS, NHS and tissue culture media as substitutions at each stage, to exclude the possibility of cross–reaction. Optimal concentrations of secondary antibodies were determined in order to ensure saturating conditions, and single staining, and double staining using every combination of paired antibodies (for example, Ki-67 and EBM II) were carried out.

Results

Double staining was used to identify and characterise the cell types in the inflamed aortic adventitia and in tonsil. Double staining was also used to determine the phenotype of Ki-67 positive cells in these tissues.

In tonsil tissue, T- and B-lymphocyte populations could be distinguished. Mixed histocompatibility complex (MHC) class II molecule expression was seen in reactive lymphoid tissue and was found by double staining (yellow) to colocalise to B cells, T cells, and occasional endothelial cells. Ki-67 staining was seen in occasional B cells in germinal centres.

In the aortic adventitia, MHC class II expression was observed on various cell types, including on B cells and macrophages, which normally express this antigen, but also on over 50% of the T cells (mostly of the T-helper phenotype), on most non-lymphoid smooth muscle-like cells, and on most of the endothelial cells (fig 1).

When lymphoid follicles were present in the aortic adventitia Ki-67 positive B cells were present, predominantly in a circular pattern around germinal centres, with associated T cells with the T-helper phenotype (CD4) showing Ki-67 positivity (fig 2). No Ki-67 positivity was seen within intimal atheromatous plaques.

Discussion

A double immunohistochemical/immunofluorescence technique has been described that can be used for the simultaneous detection of different antigens on the same cell. The fluorescence properties of Fast Red have been reported before,[2 3] but as far as we know, this technique has not yet been widely used by diagnostic histopathologists. It is a simple method that uses widely available reagents and enhances the usefulness of the alkaline phosphatase technique. The results may be viewed using a single fluorescence filter. The Fast Red reaction product is also stable and does not fade under storage.

By examining the alkaline phosphatase reaction product under bright field and then switching to fluorescence conditions, we have identified Ki-67 positive cells as well as MHC class II positive cells using this method. This technique has been used successfully with monoclonal antibodies of the same species using blocking steps with normal human serum and using optimal antibody concentrations. Staining works particularly well with markers for nuclear antigens in conjunction with markers for cytoplasmic or surface antigens. The use of antibodies from different species would, of course, increase the staining possibilities and virtually eliminate the possibility of cross-reaction. It must be noted, however, that the Fast Red substrate gives a

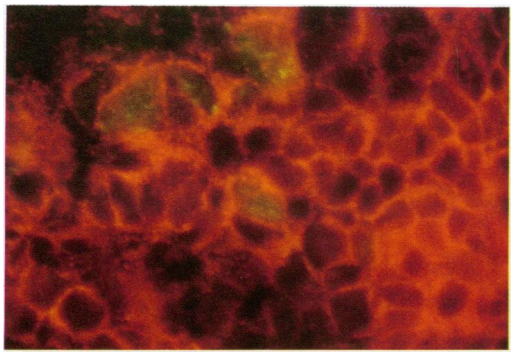

Figure 1 Section of an atherosclerotic abdominal aortic aneurysm showing the chronically inflamed adventitia ('chronic periaortitis'). Double immunostaining has been performed with TAL.1B5 (HLA-DRa) using the APAAP stain (red) and with JC70 (CD31) with indirect staining using FITC conjugated antibodies (green). In the centre of the figure is a vasa vasorum showing double staining (yellow) of the endothelium which is positive for both CD31 (green) and HLA-DRa (red).

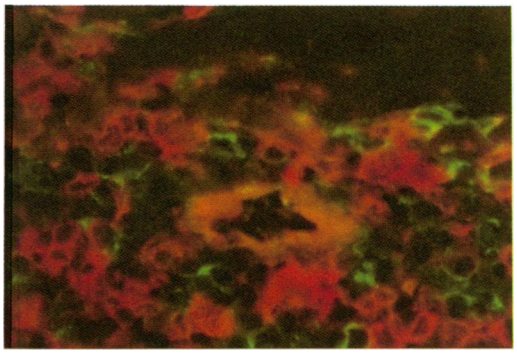

Figure 2 The periphery of a lymphoid follicle within the aortic adventitia from a section of an atherosclerotic aortic aneurysm. Double immunostaining has been performed with T3-10 (CD4) using the APAAP stain (red) and with Ki-67 (proliferation marker) with indirect staining using FITC conjugated antibodies (green). Ki-67 positive (green) staining is present within CD4 positive (red/orange) cells indicating that cells associated with the T-helper phenotype are proliferating.

variable colour from red to orange and controls must be used to ensure specificity of the reaction.

This double immunohistochemical and immunofluorescence technique combined with single immunohistochemical staining can demonstrate the presence of proliferating lymphocytes associated with advanced atherosclerosis. The combined immunohistochemical/immunofluorescence method permits the simultaneous detection of different phenotypic markers on the same cell and is particularly useful for the phenotypic identification of cell types associated with nuclear proliferation markers.

We have found this to be a particularly useful method for our work on chronic periaortitis. The findings using these techniques support the view that the inflammation associated with atherosclerosis is part of an ongoing immune reaction. This may explain why chronic periaortitis has the potential to manifest itself clinically, albeit rarely, as conditions variously termed 'idiopathic retroperitoneal fibrosis' and 'inflammatory aneurysm'.[4]

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