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Combined immunohistochemical staining for surface IgD and T-lymphocyte subsets with monoclonal antibodies in human tonsils

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Summary

The aim of the present paper is to detect two different antigens simultaneously in a single slide. In cryostat sections of human tonsils, B-lymphocytes of follicle mantle-bearing surface IgD were immunostained with the alkaline phosphatase method using monoclonal anti IgD. The subsequent staining for T-lymphocyte subsets (T-helper and T-suppressor lymphocytes) was performed again with the alkaline phosphatase method using one of the monoclonal antibodies OKT 4, OKT 8, Leu 3a, Leu 2a. The best results with the alkaline phosphatase method were achieved using naphthol AS phosphate and Fast Blue BB for the revelation of the first antigen and naphthol AS-BI phosphate and diazotized New Fuchsin for the second.

Introduction

Immunohistochemical monitoring of tissues antigens has led to a considerable enlargement of our knowledge on the distribution pattern of tissue antigens using monoclonal antibodies (Janossy *et al.*, 1980; Stein *et al.*, 1980; McMillan *et al.*, 1981; Poppema *et al.*, 1981). In contrast to heteroantisera (Mason *et al.*, 1981) raised in different species, there is no technique available for the simultaneous immunohistochemical staining of different antigenic sites with monoclonal antibodies generated in the usual mouse system. Looking for the tissue distribution of different lymphocyte subsets, in the present paper we describe a new technique for a simultaneous immunohistochemical staining with two different monoclonal antibodies applied to a single section.

Materials and methods

All studies were performed on freshly ectomized human tonsils with follicular hyperplasia (n = 10). 8 μm thick cryostat sections were fixed in acetone for 10 min at room temperature and air dried.

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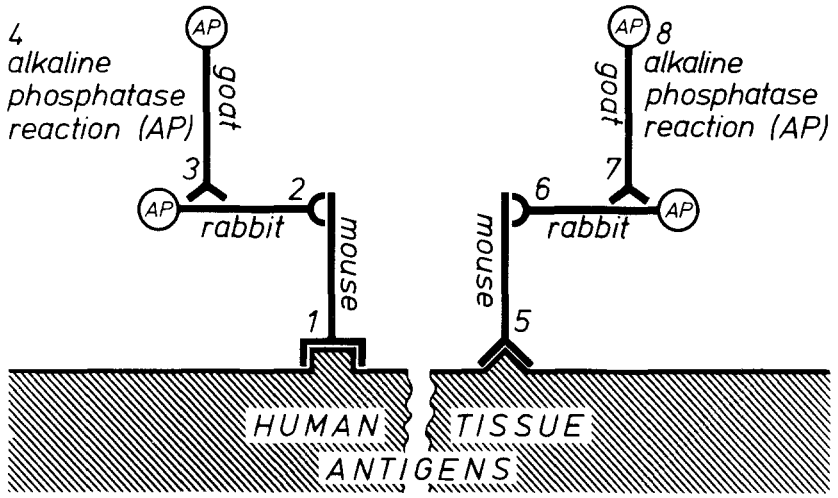

Fig. 1. Combined immunostaining of two different antigenic sites with two monoclonal antibodies using exogenous alkaline phosphatase. 1-8 indicate the sequence of the staining steps.

The primary antibodies used were: (a) OKT 4 (Ortho Diagnostic, Heidelberg, Germany), diluted 1 : 100; (b) OKT 8 (Ortho Diagnostic, Heidelberg, Germany), diluted 1 : 200; (c) Leu 3a (Becton Dickinson, Rödermark, Germany), diluted 1 : 1000; (d) Leu 2a (Becton Dickinson, Rödermark, Germany), diluted 1 : 1000; and (e) anti IgD (BRL, Neu-Isenburg, Germany), diluted 1 : 1000. All antibodies were diluted with 0.1 M phosphate-buffered saline (PBS), pH 7.4 (McMillan *et al.*, 1982).

The staining procedure was performed in the following order (Fig. 1): (1) slides were mounted with one of the primary antibodies for 30 min in a humified chamber at 37° C and rinsed twice in

Table 1. Enzyme histochemical results achieved by a combination of different substrates and couplers for the staining of exogenous alkaline phosphatase after inhibition of endogenous enzyme activity. For immunohistochemical staining, cryostat sections were incubated with monoclonal mouse anti-human antibodies (OKT 4, 8, Leu 3a, Leu 2a or anti-IgD) and bovine intestine alkaline phosphatase-conjugated rabbit anti-mouse and goat anti-rabbit IgG. Results were judged on the basis of the tinctorial intensity (+ to + + +) and diffusion artefacts in sections stained for one and two antigenic sites.

Stain	*Naphthol AS phosphate*	*Naphthol AS-MX phosphate, sodium salt*	*Naphthol AS-MX phosphate, alkaline solution*	*Naphthol AS-BI phosphate*
New Fuchsin	++	++	+	+++
Fast Red TR	+	++	+	+++
Fast Blue RR	+	+	+	++
Fast Blue B	+	+	+	+
Fast Blue BB	+++	++	+	++
Variamine Blue RT	+	+	+	+

PBS; (2) slides were mounted with rabbit anti-mouse IgG as secondary antibody conjugated with alkaline phosphatase (Sigma, München, Germany) diluted 1 : 10 with PBS for 20 min and rinsed twice; (3) slides were mounted with goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, München, FRG) diluted 1 : 10 with PBS and rinsed twice; (4) slides were then subjected to the alkaline phosphatase reaction which was performed in a modified version of the method described by Kaplow (1968) and Lojda *et al.* (1976).

Each of the substrates listed in Table 1 were dissolved in *N,N*-dimethylformamide (DMF; Merck, Darmstadt, Germany) (50 mg/ml). One of the six couplers listed in Table 1 was dissolved in DMF (50 mg/ml). The substrates were diluted with 50 ml 0.05 M propandiol buffer pH 9.75 and added to the coupler. The final pH was adjusted to pH 8.7. The incubation media were supplemented with 1 mM levamisole (Sigma, München, Germany) for the inhibition of the endogenous tissue enzyme activity (Ponder & Wilkinson, 1981) and filtered before use. The slides were incubated for 10 min at room temperature. The reaction was stopped by rinsing the slides in PBS.

For the immune staining of a second antigen the same procedure as detailed under steps 1–4 was repeated and the slides were again developed for the alkaline phosphatase at pH 9.4 (steps 5–8 in Fig. 1). The first reaction for staining alkaline phosphatase was performed with one of the couplers; Fast Blue BB, Variamine Blue RT, Fast Blue RR or Fast Blue B (Sigma, München, Germany). For the second reaction, couplers giving a bright red colour were utilized. These were Fast Red TR (Sigma, München, Germany) or New Fuchsin (Merck, Darmstadt, Germany). Nuclear counterstaining was omitted. Sections were mounted with glycerin-gelatine (Merck, Darmstadt, Germany).

Results

Systematic evaluation of the quality of the slides subjected to the immune alkaline phosphatase reaction using only a single monoclonal antibody revealed best results with the combination of naphthol AS phosphate and Fast Blue BB for a blue and naphthol AS-BI phosphate and Fast Red TR for a red-coloured staining (Table 1). These combinations were chosen due to the high staining intensity, the least diffusion artefacts and the best discernment when used for double staining. Alkaline phosphatase conjugate used with the linking rabbit anti-mouse IgG as well as with the secondary goat anti-rabbit antisera, considerably intensified the dye development as compared with the procedures utilizing one single enzyme conjugate.

Simultaneous immune staining with OKT 4 or Leu 3a and OKT 8 or Leu 2a showed the identical distribution pattern of T-helper and T-suppressor lymphocytes as known from studies using a single antibody (McMillan *et al.*, 1981; Poppema *et al.*, 1981). OKT 4- or Leu 3a-positive T(-helper) lymphocytes were mainly localized in the interfollicular region. A far smaller number of positive cells were observed within the follicle mantle and the germinal centre. These antibodies showed a cross-reactivity with macrophages. OKT 8- or Leu 2a-positive T(-suppressor) lymphocytes were also confined to the interfollicular region. They were considerably fewer in number than OKT 4- or Leu 3a-positive cells (Fig. 2). In the follicle mantle, a small number and in the germinal centre some solitary reactive lymphocytes could be observed. In our hands, no evident difference was detectable in the distribution pattern of OKT 4- and Leu 3a- or OKT 8-

and Leu 2a- positive cells. The reaction intensity, however, was considerably higher using Leu 3a as compared with OKT 4. The regularly observed cross-reactivity of OKT 4 with macrophages was higher than that of Leu 3a.

In combined reactions using either OKT 4, OKT 8, Leu 3a or Leu 2a together with anti IgD, the corresponding T-cell subsets were clearly discernible from the IgD-positive B-lymphocytes. Identical results were achieved using Leu 3a or Leu 2a instead of OKT antibodies. Follicle mantle lymphocytes stained with anti-IgD but germinal centre cells remained unstained (Fig. 3). In addition, a considerable number of interfollicular B-lymphocytes were also IgD-positive. A positive reaction could never be observed in one and the same cell for OKT 4, OKT 8, and anti IgD or Leu 3a, Leu 2a and anti IgD.

Structures known to have endogenous alkaline phosphatase activity, such as neutrophil granulocytes and capillary endothelia, invariably became negative on treatment with an enzyme inhibitor. Cells positive to the monoclonal antibodies used showed regularly a negative reaction in control slides omitting the primary or secondary antibody. Levamisole did not inhibit or reduce the activity of the exogenous alkaline phosphatase as tested in parallel slides.

Discussion

The distribution pattern of lymphocytes positive for OKT 4 or Leu 3a and OKT 8 or Leu 2a in human lymphatic tissue, for example, lymph nodes and tonsils, has been observed previously by others (McMillan *et al.*, 1981; Poppema *et al.*, 1981) using the immunoperoxidase technique. Also IgD as a surface immunoglobulin has been the subject of at least two previous investigations with the indirect immunoperoxidase method (Stein *et al.*, 1980; Poppema *et al.*, 1981).

No efforts, so far, have been made to achieve a simultaneous monitoring of these antigens in tissue sections. The use of alkaline phosphatase for immunohistochemical staining shows several advantages as compared with the immunoperoxidase method. Alkaline phosphatase reaction requires less toxic agents (Sheibani *et al.*, 1981). The endogenous enzyme activity is completely inhibited without any loss to the reactive antigens. The staining can be performed in at least four different colours: light blue (naphthol AS phosphate and Fast Blue BB), bright red (naphthol AS-BI phosphate and

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Plate 1. Immune alkaline phosphatase staining of T-helper lymphocytes with Leu 3a using naphthol AS phosphate and Fast Blue BB (blue) and T-suppressor lymphocytes with OKT 8 using naphthol AS-BI phosphate and New Fuchsin (red) in the interfollicular zone. Human tonsil; 8 μm thick cryostat section. × 350

Plate 2. Immune alkaline phosphatase staining of follicle mantle lymphocytes with monoclonal anti-IgD (red) using naphthol AS-BI phosphate and New Fuchsin. Simultaneous immunostaining of T-suppressor lymphocytes with OKT 8 using naphthol AS phosphate and Fast Blue BB (blue) mainly localized in the interfollicular zone. Germinal centres contain only a small number of OKT 8-positive cells. Human tonsil; 8 μm thick cryostat section. × 140.

Fast Red TR), dark blue (naphthol AS-MX phosphate and Fast Blue BB), dark red (naphthol AS-BI phosphate and diazotized New Fuchsin). The last possibility enables a clear visualization of at least two different antigens in one and the same section. The more common immunoperoxidase reaction, in contrast, has the shortcoming that the endogenous enzyme cannot be completely inhibited without causing a loss in its sensitivity to the tissue antigens. In comparative studies it could be ascertained that the reaction intensity and the number of positive cells remained unaltered using a combination of two different antibodies. In none of the slides was a cross-reactivity encountered between the combined monoclonal antibodies. This is very probably due to an exhaustive saturation of the reactive sites of the first monoclonal with the rabbit anti-mouse IgG and of the latter with goat anti-rabbit IgG.

To our knowledge, monoclonal antibodies have not been used in a combined application using immunoalkaline or immunoperoxidase techniques so far. This is mainly due to the fact that the overwhelming majority of monoclonals in use have been raised in mice. In contrast, heteroantisera, raised in different animals have been more readily used in a combined procedure, which allows for the application of linking antisera from different species. This issue has to be observed in performing the subsequent staining steps described above.

The combined application of the immunoalkaline phosphatase technique opens new perspectives: Using antibodies directed against antigens which are exprimed or reprimed along the maturation line of a given cell type, this technique would enable a clear-cut definition of different maturity and/or differentiation stages. In addition, monoclonal cell cohorts can be stained selectively and thus separated from the normal or reactive (polyclonal) counterparts on one and the same slide.

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