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Use of monoclonal antibodies in formol-paraffin sections

A study of normal and neoplastic haemopoietic tissues

T. Magyarlaki and G. Kelényi
Department of Pathology, University Medical School of Pécs, Pécs, Hungary

Summary. Thirteen monoclonal antibodies (the "VI-series") reactive with B- and T-lymphocytes, monocytes, granulocytes and erythrocytes were tested in sections fixed with formalin, formalin-sublimate or formalin-acetic acid. After fixation and embedding, most of the antigens were not detectable. However, VIE-G 4, a monoclonal antibody selective for glycophorin A, produced a strong reaction with erythroid cells in formalin fixed sections. The binding of the monoclonal antibodies VIM-D 5, VIM-2 and VIM-13 to myeloid, myelomonocytic and monocytic cells became either more intense or was inhibited by preincubation of the sections with pronase, trypsin, papain or neuraminidase. Following enzyme digestion, the monocyte-specific antibody VIM-13 reacted selectively with some of the germinal centre cells.

Key words: Monoclonal antibodies – Formol-paraffin sections – Enzyme pretreatment – Centroblast reaction

Introduction

With the advent of hybridoma technology, more and more tissue antigens can be detected by immunohistochemical methods by using monoclonal antibodies (MoAbs). However, in conventional formol-paraffin sections, many cell surface and intracellular antigens are either destroyed or become masked. Various attempts have therefore also been made to visualize tissue antigens in paraffin sections by changing the fixation and embedding methods (Dorsett et al. 1978; Giddings et al. 1982; Mullink et al. 1985; Rasztela et al. 1985) and by introducing enzyme pretreatment procedures (Mason et al. 1980; Murata et al. 1984). By means of such procedures, a number of cellular antigens can be demonstrated with MoAbs (Collings et al. 1984; Hancock et al. 1982; Naritoku et al. 1982). However, most of the surface antigens of human leukocytes still remain undetectable in formol-paraffin sections.

We tested thirteen MoAbs directed against T- and B-lymphocytes, monocyte, granulocyte and erythrocyte surface antigens for reactivity on formol-, formol-sublimate or formol-acetic acid fixed and paraffin-embedded sections pretreated by various enzymes. Although most of the antibodies proved to be non-reactive, four of them were found to react without any pretreatment or only after enzyme pretreatment. Three of them showed unchanged specificity; however, after pretreatment with pronase, one directed against monocytes (VIM-13) reacted with some cells of B cell origin.

Offprint requests to: G. Kelényi

Material and methods

Fifteen non-neoplastic, reactive lymph nodes from biopsies were fixed at room temperature for 24 h in the following fixatives: phosphate-buffered saline, pH 7.2 (PBS) with 4% formaldehyde, acetic acid-formalin (4 ml glacial acetic acid/100 ml buffered 4% formaldehyde solution) and formol-sublimate (4 ml glacial acetic acid and 6 g $HgCl_2$/100 ml buffered 4% formaldehyde solution, Mason et al. 1980). After dehydration through graded alcohols and xylene tissue blocks were embedded in paraffin.

Formol-paraffin blocks from twenty-three B cell malignant lymphoma cases from surgical specimens (10 centroblastic, 11 centroblastic-centrocytic and 2 centrocytic malignant lymphomas) were also studied. The diagnoses were based on the Kiel classification. Frozen sections of reactive lymph nodes and cell suspensions of lymphocytes isolated by Ficoll-Paque® (Pharmacia Fine Chemicals) were used to test the specificity of the MoAbs.

Pronase pretreatment was carried out by the method of Shishi et al. (1986): briefly 1 mg Pronase P (Protease from

Table 1. Monoclonal antibodies used in this study

Cluster of differentiation	Antibody designation	Target cell	Reference
T Cells			
CD 1	VIT 6	Cortical thymocytes and epidermal Langerhans cells	Amiot et al. (1966)
CD 3	VIT 3b	Mature T lymphocytes	Holter et al. (1986)
B Cells			
CD 10	VIL-A 1	CALLA, B precursors	Knapp et al. (1982)
CD 24	VIB-C 5	Pan B cell	Knapp et al. (1983)
Myeloid Cells			
CD 11	VIM 12	Monocytes, granulocytes, NK (C 3b receptor)	
–	VIM-D 2	Monocytes	
CD 14	VIM 13	Monocytes	Knapp et al. (1984)
CD 15	VIM-D 5	Granulocytes (X-hapten)	Majdic et al. (1981)
–	VIM 2	Granulocytes, monocytes	Majdic et al. (1984)
Erythrocytes			
–	VIE-G 4	Erythroid cells (glycophorin A)	Liszka et al. (1983)
Blasts and Precursors			
CD 38	VIP 2b	Blasts, activates lymphocytes and plasma cells	Holter et al. (1985a)
–	VID 1	HLA-DR	Köller et al. (1985)
–	VIP 1	Transferrin receptor	Holter et al. (1985b)

Streptomyces griseus, SERVA) dissolved in 2 ml of 0.02 M Tris-buffered saline, pH 7.4. The sections were treated for 20 min at 37° C.

Digestion with trypsin was performed by the method of Giddings et al. (1982). 2 mg Trypsin 2000 E/g (Merck, Darmstadt, FRG) in 1 ml of 0.02 M Tris-buffered saline, pH 7.4 for 20 min at 37° C was used.

Neuraminidase pretreatment was carried out by the method of Hsu et al. (1986): 2 U/ml neuraminidase (from Vibrio cholerae, SERVA, Heidelberg, FRG) in 0.1 M acetate buffer, pH 5.3 was used for 20 min at 37° C.

Papain pretreatment: 1 g papain/lyophilized, (Reanal, Budapest, Hungary) in 5 ml 0.02 M Tris-buffered saline, pH 7.4 for 20 min.

Lysis of 0- or N-glycosidic linked oligosaccharies of the cell surface proteins by potassium hydroxide (KOH) or β-glycosidase were carried out according to Ono et al. (1983) and to Murata et al. (1984).

Conventional unlabelled goat antibodies to human kappa and lambda light chains, to IgM, IgD, IgG and IgE heavy chains (Heintel, Vienna, Austria) were used with a peroxidase-antiperoxidase (PAP)-system (Giddings et al. 1982).

MoAbs (Table 1, dilution 1:40 to 1:100) raised by W. Knapp (Department of Immunology, University of Vienna, Austria) were visualized by a three-step PAP-procedure (Heintel, Vienna, Austria) as described by Naritoku et al. (1982). The bridging-antibody (goat) was diluted in the range of 1:40 to 1:80 while the mouse PAP-complex was diluted up to 1:400. In some experiments a four-step PAP-procedure was employed (Hancock et al. 1982).

Normal goat serum (diluted 1:20) was used to block the background staining (Fc-receptor binding) before the first step. 3,3′-diaminobenzidine tetrahydrochloride (Polysciences, Warrington, USA) was employed as a chromogen developed by hydrogen peroxide. The slides were counterstained with Meyer's haematoxylin for 10 min. Controls were carried out by omitting the MoAbs.

Table 2. Reaction of monoclonal antibodies in cryostat and formol-paraffin sections

	Reactive lymph nodes	
Antibody designation	Cryostat section	Formol-paraffin section
VIE-G 4	+	+
VIM 2	+	+
VIM-D 5	+	+[a]
VIM 13	+[c]	+[b]
VIM 12	+	–
VIM-D 2	+	–
VIT 6	+	–
VIT 3b	+	–
VIL-A 1	+	–
VIB-C 5	+	–
VIP 2b	+	–
VID 1	+	–
VIP 1	+	–

+ or –: positive or negative reaction

[a] only after pretreatment with neuraminidase

[b] only after pretreatment with pronase (or other proteases), cells stained by VIM 13 are centroblasts

[c] without any pretreatment – only macrophages were stained, after mild pronase pretreatment centroblasts also reacted

Results

Thirteen MoAbs were tested in formol-paraffin, formol-sublimate or formol-acetic acid fixed and paraffin-embedded tissues. Four of the MoAbs

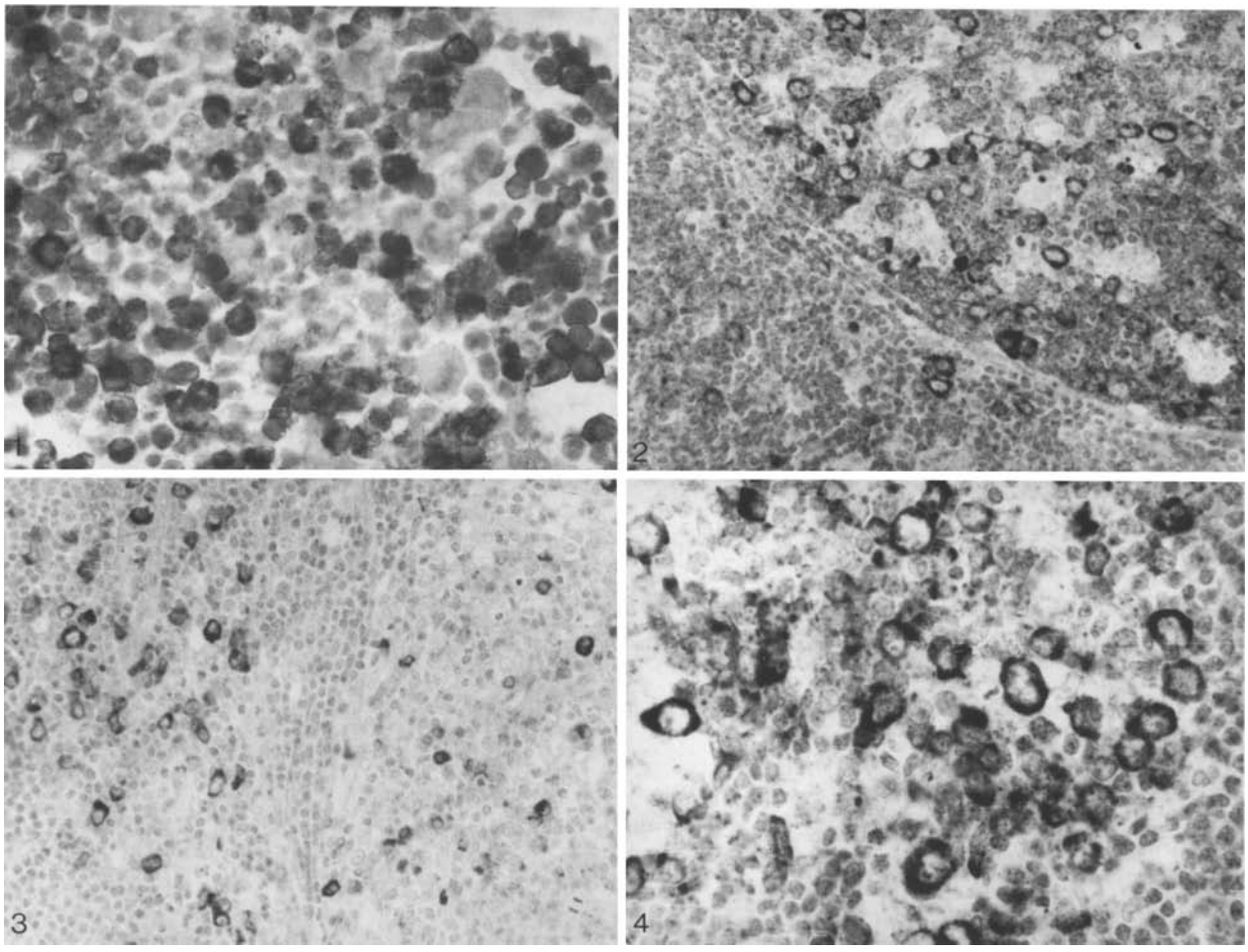

Fig. 1. Bone marrow, myeloid hyperplasia, VIM-2, Giemsa. Strongly positive myeloid cells. Erythroid cells megakaryocytes and other bone marrow cells are negative. × 390

Fig. 2. Lymph node, pronase pretreatment, VIM-13, Giemsa. Many positive centroblasts in, and a few around the germinal centre. × 182

Fig. 3. Lymph node, immunoglobulin light chain reaction ($\kappa + \lambda$) after pronase pretreatment, Giemsa. Reaction of plasma cells and some germinal centre cells in reaction adjacent to that shown in Fig. 2. × 182

Fig. 4. Lymph node, pronase pretreatment, VIM-13, Giemsa. Number of centroblasts and a few plasma cells in the germinal centre. × 390

reacted in formol-paraffin sections either after enzyme pretreatment or without it. No reaction was seen in formol-sublimate- or in formol acetic acid-paraffin sections (Table 2). No specific reactions were seen in the controls.

Two of the MoAbs, VIE-G 4 and VIM-2, detecting glycophorin A and a myeloid differentiation antigen of the granulocytes were positive in formol-paraffin sections without any pretreatment. Figure 1 shows the positivity of the myeloid cells with VIM-2 in the bone marrow. The binding of VIE-G 4 was inhibited by pretreatment with neuraminidase, β-glycosidase (N-oligosaccharide glycopeptidase) and KOH (O-glycosidically linked glycoprotein lysis). The proteases (pronase, trypsin, papain) showed partial inhibition in VIE-G 4 binding. Increased positivity of VIM-2 was observed after pretreatment with proteases (optimal results with pronase), while neuraminidase and β-glycosidase had no effect, and mild alkaline lysis with KOH inhibited the binding of VIM-2 (Table 3).

Positive reaction with the two other MoAbs were seen in formol-paraffin sections only after enzyme pretreatment. Binding of VIM-D 5 to myeloid cell membranes in formol-paraffin sections could be visualized only after neuraminidase pretreatment. The MoAbs VIM-13, directed against cells of monocytic origin, gave no reaction in for-

Table 3. Reaction of monoclonal antibodies after enzyme predigestion of KOH pretreatment in formol-paraffin sections

	Reactive lymph nodes						
Enzymes Monoclonal Antibodies	Pronase	Trypsin	Papain	Neuraminidase	KOH	Beta glycosidase	No enzyme pretreatment
VIE-G 4	+	+	+	−	−	−	+++
VIM 2	+++	+++	+++	++	−	++	++
↗ monocytes VIM 13	−	−	−	−	−	−	−
↘ centroblasts	+++	++	++	−	−	−	−
VIM-D 5	−	−	−	+++	−	−	−

− = negative reaction
+ − + + + = positive reactions of increasing intensity

Table 4. VIM 13 and immunoglobulin reactivity after pronase in formol-paraffin sections

Tissues[a]	Reactive lymph nodes ($n=15$)		Centroblastic ML ($n=10$)		CB-CC($n=11$)		Centrocytic ML ($n=2$)	
Cells[b]	pc	cb	pc	cb	pc	cb	pc	cb
Antibodies[c]								
VIM 13	9	7	6	3	4	2	1	0
Kappa	10	6	5^+	2^+	2	0	1	0
Lambda	10	3	5^+	2^+	2	0	1	0
IgM	7	3	−	−	1	0	−	−
IgG	7	2						
IgD	4	2						
IgA	3	1						
IgE	1	0						

[+] Monoclonal cell populations

[a] Formol-paraffin sections. ML = malignant lymphoma, CB-CC = centroblastic-centrocytic ML, follicular
[b] Cell type stained with antibodies: pc = plasma cells; cb = centroblasts
[c] Number of cases stained with the antibodies listed

mol-paraffin sections (Table 3). However, after pretreatment with pronase some germinal centre cells and plasma cells showed a strong reaction (Fig. 2). In Fig. 3 (section adjacent to that shown in Fig. 2), the reaction of plasma cells with antihuman light chain antibodies after pretreatment with pronase is presented. After pronase, VIM-13 stains mainly the centroblasts of the germinal centres and only a few plasma cells, whereas anti-Ig antibodies react with many plasma cells and only a few centroblasts. Trypsin and papain pretreatment gave similar results, but the reaction was less intense. Incubation of the sections with neuraminidase, KOH and β-glycosidase in a second step after pronase did not change the reactivity pattern. Figure 4 shows a high power field of the follicle depicted in Figs. 2, 3. The reactivity of VIM-13 and that of antibodies to immunoglobulins after pronase pretreatment in reactive lymph nodes and in 23 malignant lymphomas of follicular origin are shown in Table 4. No correlation between the number of cells reacting with VIM-13 and those reacting with antihuman immunoglobulin antibodies was seen. Tumor cells of 3 out of 10 centroblastic and 2 out of 11 centroblastic-centrocytic malignant lymphomas stained with VIM-13 only after pronase pretreatment.

Discussion

In our studies MoAbs of VI-series were used (Table 1). Most of the antigens detected by these antibodies in cell suspensions or frozen sections were areactive in formol-paraffin sections; however, VIE-G 4, VIM-2, VIM-D 5 and VIM-13 gave positive results. Formol-sublimate or formol-acetic acid fixed and paraffin-embedded tissues were not useful for immunostaining (Table 2). VIE-G 4, a

MoAb selective for glycophorin A (as a sialoglycoprotein marker of erythroid cells, Ekblom et al. 1983; Liszka et al. 1983) gave a strong reaction with erythrocytes of the bone marrow. The sialic acid bound to this antigen must be important in its immunoreactivity: neuraminidase pretreatment inhibited the binding of VIE-G 4. Pretreatment with N-oligosaccharide glycopeptidase (β-glycosidase) or mild alkaline hydrolysis of 0-glycosidically linked glycoproteins (KOH) also resulted in inhibition of binding. Proteases (pronase, trypsin, papain) have little effect on the receptor (Table 3).

The antigen detected by the MoAb VIM-2 is a class C differentiation antigen (glycoprotein) of myelo-monocytic cells (Gooi et al. 1985). Reaction with this MoAb was greatly increased by treatment of the sections with pronase. Neuraminidase and β-glycosidase had no effect, while KOH lysis resulted in inhibition of VIM-2 binding (Table 3). These findings are compatible with the glycoprotein nature of the receptor antigen visualized by VIM-2 (Majdic et al. 1984).

VIM-D 5 (CD 15) detects another myeloid differentiation antigen, a class A glycoprotein (Majdic et al. 1981), also in formol-paraffin sections but only after neuraminidase pretreatment. The antigen bears a vicinal oligosaccharide structure, Lacto-N-fucopentose III (X-hapten), which, in myelo-monocytic and also in lymphoid cells can be masked by sialic acid (Tettero et al. 1984). A similar structure is present in human milk fat globule membranes, and MoAbs directed against these membrane glycoproteins may be used as differentiation antigen markers of tumours (Helle et al. 1986). The X-hapten structure is similar to the antigen called SSEA-1, present in undifferentiated teratocarcinomas and in some normal and leukaemic human haemopoietic cells. These antigens can also be masked by sialization (Tabilio et al. 1984). A similar masking effect by sialic acid was seen when using Leu M 1 (CD 15) MoAb detecting X-hapten glycoprotein of cell membranes in the lymphocytic-histiocytic Sternberg-Reed cell variants of lymphocyte predominance Hodgkin's disease (Hsu et al. 1986).

The MoAb VIM-13 (CD 14), specific in cell suspension and cryostat sections for monocytes, reacted with some germinal centre cells in formol-paraffin sections after pronase pretreatment (Fig. 2). After pronase, VIM-13 (pron-VIM-13) stained mainly the centroblasts in the germinal centres and only a few plasma cells (Fig. 2), whereas anti-Ig antibodies reacted with many plasma cells and only few centroblasts (Fig. 3). There was no VIM-13 reaction without pronase pretreatment or with neuraminidase, KOH or β-glycosidase pretreatment.

In our experiments preincubating the pronase-pretreated sections with various unlabelled rabbit antihuman immunoglobulin antibodies against heavy or light chains did not inhibit the binding of VIM-13. In reactive tissues with hyperplastic germinal centres pron-VIM-13 reacted in adjacent sections in 7 cases with both centroblasts and plasma cells and in 2 cases only with plasma cells (Table 4). No correlation was found between the number of cells bearing different types of immunoglobulins and the reaction of plasma cells or centroblasts with pron-VIM-13. The cells of 3 out of 10 centroblastic malignant lymphomas reacted with pron-VIM-13.

In centroblastic-centrocytic malignant lymphomas a reaction with pron-VIM-13 of centroblasts was seen in 2 out of 11 cases. No correlation was found in these two lymphoma types between the number of cells bearing immunoglobulins and pron-VIM-13 positivity. In the cases studied the centrocytes were completely negative for pron-VIM-13.

In conclusion, the reaction of centroblasts with pron-VIM-13 seems to be independent of their bearing immunoglobulins, that is the receptor for CD 14 on centroblasts may be a differentiation marker. To prove this assumption further examinations are needed.

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