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Enzyme and immunohistochemistry on undecalcified bone and bone marrow biopsies after embedding in plastic: a new embedding method for routine application*

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Summary. A simplified method of low temperature methyl and butyl methacrylate embedding (up −20° to −15° C) is demonstrated using a proper redox system of benzoyl peroxide and aromatic amine. This method combines the morphological superiority of plastic-embedded bone tissue and bone marrow sections with the advantages of specific enzyme histochemical and immunochemical markers. The method permits good preservation of morphological details, the survival of antigenic determinants and the retention of enzyme activities. The specimens were fixed in 1.6% formaldehyde and 5% sucrose in 0.02 M phosphate buffer at pH 7.4, washed in 0.02 M phosphate buffer and 5% sucrose, dehydrated with acetone and impregnated with monomers of embedding medium. All these steps were carried out at +4° C. The method presented is especially suitable for enzyme histological and immunohistological diagnosis of primary and secondary bone tumours, soft tissue tumours, as well as myelo- and lymphoproliferative disorders of bone marrow biopsies. Examples are demonstrated with mono- and polyclonal antibodies and reaction products of hydrolytic enzymes.

Key words: Methyl methacrylate embedding method – Low temperature embedding – Bone tumours – Bone marrow disorders – Enzyme and immunohistochemistry

Introduction

In the past, most enzyme histological and immunohistological studies were performed on cryostat sections. This technique is difficult on undecalcified bone material (Stein 1983). A further problem is that the frozen sections do not provide the tissue preservation required for optimal antigen and enzyme detection as well as localization. Antigen survival and retention of enzyme activities in specially fixed, EDTA-decalcified, paraffin-embedded bone and bone marrow tissue using the method described by Schaefer (1984) have been limited to relatively small biopsies such as the iliac crest biopsies of Jamshidi and Swaim. The results obtained were improved if fixation and dehydration of tissue specimens were carried out at a low temperature (Curran and Gregory 1978; Stein et al. 1984; Sato et al. 1986; Liu 1987; Hantschick et al. 1988). The advantages of using fixed paraffin-embedded sections lie in the well-preserved morphology provided by adequate fixation of tissues, the possibility of cutting thinner sections to reveal more histological details, and the easier storage of samples over a long period (Seifert et al. 1984). A further advantage of this technique when compared with paraffin embedding is the use of methacrylate since it is easy to obtain particularly thin and semi-thin sections. In addition, tissue shrinkage is reduced compared with paraffin embedding or other media. The conventional methyl-methacrylate embedding method (MMA), mainly in combination with *n*-butyl-methacrylate (BMA) or plastoid-N (nonylphenol polyglycol ether acetate), is still the most widely used embedding method for the sectioning of undecalcified bone as well as non-mineralized tissues (Burkhardt 1966, 1971; Delling 1972; Te Velde et al. 1977; Wolf and Pompe 1980a). Usually, the polymerization process starts by a thermal or UV-light initiated decomposition of the initiator (catalyst) benzoyl peroxide (BPO). Since the polymerization of MMA is exothermic, the temperature during this process may become too high and inactivate all enzymatic systems or denature protein antigenic structures. Because of fast polymerization in the embedding medium a high concentration of free radicals reacts with protein structures and may change the conformation of secondary and tertiary structures, resulting in a loss of specific antibody binding to epitopes. Traditional staining methods have been widely adapted to methacrylate embedding (Burkhardt 1971; Delling 1980; Schenk et al. 1984; Hahn et al. 1991). During the last decade several investigators have

* Dedicated to Prof. Dr. Gerhard Seifert on the occasion of his 70th birthday

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demonstrated that certain histo- and immunohistochemical procedures may be applied to plastic sections if the conditions of polymerization and methacrylate components were adapted (Beckstead and Bainton 1980; Westen et al. 1981; Blazek and Georgii 1983; Bianco et al. 1984; Beckstead 1985; Hermanns et al. 1986; Schröder and Delling 1986; Chappard et al. 1987; Liu 1987; Liu et al. 1987; Wolf et al. 1988; Burgio et al. 1991). Some of these authors used chemical polymerization at a low temperature for methacrylate embedding with BPO as the initiator and an aliphatic or aromatic amine such as *N,N*-dimethylaniline as the accelerator (so-called redox system). The main components were water soluble methacrylate with low penetration properties, for larger bone specimens. For this reason, we decided to use MMA as the basic embedding medium in our investigations.

The aim was to develop an embedding procedure combining both conventional histological staining methods and enzyme and immunohistological methods on larger bone tissue specimens. We report on a method which enables us to conduct these investigations on bone tumours with a higher content of mineralized bone and and on larger bone marrow cylinders from the iliac crest for the diagnosis of metabolic bone diseases and haematological disorders.

Table 1. Steps of preparation and methacrylate embedding

Preparation	Time (h)
1. Fixation	24
2. Washing	Overnight
3. Dehydratation	
Acetone 70%	1
Acetone 90%	1
Acetone 100%	3 × 1
4. Intermedium and impregnation	
a) xylene	1
b) xylene/MMA	1
c) MMA, BMA, PEG-400 (ratio of 40:60:2 parts) +1.5% BPO	Overnight
5. Embedding medium and polymerization (−15° C to −20° C)	
Stock embedding medium A (4° C): MMA (unstabilized) +2 g % BPO	
Stock embedding medium B (4° C): BMA (unstabilized) + 2 Vol.% PEG-400 +1 Vol. % *N,N*-dimethyl-*p*-toluidine	
Mixture: 2 parts medium A +3 parts medium B	

MMA, Methyl-methacylate; BMA, *n*-butyl-methacylate; PEG, polyethylene glycol; BAO, benzoyl peroxide

Materials and methods

Freshly obtained human bone tumour tissues and bone marrow cylinders from the iliac crest according to Jamshidi and Swaim (1971) or Burkhardt (1971), 2–5 mm in diameter, which had been kept in Ringer's solution, were processed as follows.

Thin (3–4 mm) tissue specimens of bone tumours up to 15 × 15 mm and bone marrow cylinders up to 20 mm length were fixed immediately for 24 h at 4° C in 1.6% paraformaldehyde in 0.02 M phosphate buffer with 5% sucrose pH 7.4, freshly prepared from cold stock solutions (1 part 8% paraformaldehyde solution, 1 part 0.04 M phosphate buffer and 10% sucrose solution at pH 7.4 mixed with 2 parts double distilled water). After fixation, tissues were washed overnight at 4° C in 0.02 M phosphate buffer and 5% sucrose at pH 7.4. The specimens were dehydrated at 4° C in 70%, 90%, and 3 × 100% pure acetone and 2 × xylene was used as intermedium. The time of procedure for each step was 1 h.

The samples were impregnated with equal parts of stabilized MMA and xylene for 1 h at 4° C. For the second step of immersion a mixture of unstabilized MMA, BMA and polyethylene glycol 400 (PEG-400) in a ratio of 40:60:2 parts was used containing 1.5% dried BPO as catalyst.

The embedding medium was made of pure MMA and BMA. In order to obtain uniformly reproducible results, highly purified and dried components of resin from which the hydrochinon had been removed (added polymerization inhibitor in commercial products) were used. The embedding medium contains 2 g of the catalyst (initiator) dried BPO mixed with 100 ml of unstabilized MMA (stock embedding medium A), 150 ml unstabilized BMA (plastecizer) mixed with 3 ml PEG-400 and 1.5 ml *N,N*-dimethyl-*p*-toluidine, accelerator of the redox system (stock embedding medium B). Both stock embedding mixtures were stored at +4° C. Immediately before used for polymerization, both cold stock embedding solutions were mixed (pending: P 4039 716.5 FRG, see Table 1).

For polymerization each specimen was transferred into a gelatine capsule (23 mm in diameter and 50 mm in length), filled with a fresh mixture (4° C) of 2 parts stock embedding medium A and 3 parts stock embedding medium B, capped and stored at −20° to −15° C. Polymerization was completed within 24 h. The polymerized blocks were allowed to warm up to +4° C over 2 h and then stored at room temperature.

The procedure for cutting and section preparation was carried out according to the previously described method established in our laboratory (Delling 1972, 1980; Hahn et al. 1991). All the specimen sections were routinely stained by toluidine, Giemsa, Goldner's trichrome and Movat's silver impregnation.

For enzyme histochemistry and immunostaining, sections on Burkhardt's chromalaun gelatine-coated slides were used. Sections should dry overnight or longer at 40° C to ensure adhesion throughout the entire immuno- and enzyme histochemical procedures. The complete removal of resin (deacrylation) was achieved by using 2 × xylene for 20 min and 1 × methyl glycol acetate for 20 min. The rehydration of sections was carried out by using 2 × acetone for 2 min and 2 × distilled water for 5 min. Rehydrated sections were transferred to a 0.1 M phosphate buffer for enzyme histological and to a 0.01 M phosphate buffer for immunohistological procedures. The enzyme histological investigations were conducted by well-known methods (Lojda et al. 1970; Rath 1981) modified for our embedding technique (Table 2). For immunostaining we used sensitive systems corresponding to ABC (avidin biotin peroxidase complex)/HRP (horse-radish peroxidase) staining procedure, PAP (peroxidase-anti-peroxidase) staining procedure and APAAP (alkaline phosphatase-anti-alkaline phosphatase) immuno-alkaline phosphatase labelling technique (Dako, Hamburg, FRG) according to investigations on deparaffinized tissue sections. Digestion with proteases was not necessary. Immunohistological procedures were performed in the following sequence:

1. Incubation with 10% normal serum (same species as secondary antibody) and 4% BSA (bovine serum albumin) in 0.01 M phosphate buffer at pH 7.4 for 30 min at room temperature to reduce the non-specific background.
2. Incubation with the primary monoclonal or polyspecific antibody overnight at 4° C. All dilutions were made in 0.01 M phosphate buffer and 4% BSA at pH 7.4.
3. Inhibition of endogene peroxidase with hydrogen peroxide (80% methanol with 0.06% hydrogen peroxide for 30 min) for ABC/HPR and PAP technique.

Table 2. Parameters for enzyme histological investigations

Histochemical method	Substrate	Concentration of substrate	Buffer, pH	Coupling salt, reaction	References
1. Specific esterase	Alphanaphtol-AS-D-chloracetate	15 mg/50 ml	Phosphate pH 6.4	Hexazotized pararos-aniline	Leder (1964)
2. Acid non-specific esterase	Alpha-naphthyl acetate	15 mg/50 ml	Phosphate pH 6.4	Hexazotized pararos-aniline	Rath (1981)
3. Acid non-specific esterase	Alpha-naphthyl-butyrate esterase	50 mg/50 ml	Phosphate pH 6.3	Hexazotized pararos-aniline	Rath (1981) Jam et al. (1971)
4. Neutral non-specific esterase	Alpha-naphthyl acetate	15 mg/50 ml	Phosphate pH 7.4	Hexazotized pararos-aniline	Davis and Ornstein (1959)
5. Acid phosphatase	Naphthol-AS-BI-phosphate	15 mg/50 ml	Veronal-acetate pH 5.0	Hexazotized pararos-aniline	Bursten (1958)
6. Alkaline phosphatase	Naphthol-AS-TR-phosphate	25 mg/50 ml	TRIS pH 9.4	Fast blue BB salt	Kaplow (1955)
7. Myelo-peroxidase	Diaminobenzidine	40 mg/50 ml	0.05 m phosphate pH 7.2	30% Hydrogen peroxide	Graham and Karnowsky (1966)

4. Incubation with secondary antibody at room temperature for 30 min.
5. Staining procedures with strept-ABC/HRP (Dako, code no. K 377), PAP complex from rabbit or mouse (Dako, code No. 7113, rabbit and code no. P 855, mouse monoclonal), APAAP complex, monoclonal (Dako, code no. D651).

After incubation for 30 min the enzyme developments (peroxidase, alkaline phosphatase) were carried out by incubation with the corresponding substrate solution (DAB- peroxidase, naphthol-AS- BI- phosphate) in accordance with the producers' instructions. Positive and negative controls were processed for immunostainings in accordance with Bourne (1983). Finally the sections were counterstained with haematoxylin and mounted.

Results

The influence of formaldehyde fixation, dehydration and methacrylate embedding at low temperature was studied on the results of enzyme histochemical demonstration of hydrolases and immunohistological staining with mono- and polyclonal antibodies in bone tumours and haematological disorders.

Adequate fixation and dehydration were of great significance for the quality of the results. Different concentrations of paraformaldehyde ranging from 1% to 4% in different kinds of buffer with and without sucrose (ranging from 3% to 20%) were tested under various temperature conditions (0° C up to room temperature). Furthermore, the osmolarity of the fixation solution was very important, using the cold fixation method. The osmolarity in our fixative ranged from 550 to 680 mosmol kg^{-1}. Higher osmolarity resulted in a shrinkage of bone marrow cells in core biopsies. The effects of changes in osmolarity on tumour cells in bone and soft tissue tumours was not so evident. Paraformaldehyde fixatives led to a higher density of nuclear chromatin in Giemsa's staining compared to Schaffer's fixative. The nuclear density was evidently smaller if the concentration of paraformaldehyde in the fixative was not higher than 1.6%. In addition, the use of sucrose as a protective substance to avoid overfixation and washing the fixed samples with buffer and sucrose solution were important conditions to obtain good results in immunological and histochemical staining. The aim of testing these fixation and dehydration procedures was to obtain histological pictures closely resembling those obtained by Schaffer's fixation and the conventional MMA embedding method (Delling 1972, 1980) for bone and bone marrow cells. Attempts to minimize the dehydration time were made by using a wide range of conditions as described for fixation and using various organic solutions (different alcohols, glycol acrylates, ethylene glycol monobutylether, methyl glycol acetate and acetone, especially purified acetone for chromatography use). In conclusion, low concentrations of buffered paraformaldehyde at low temperature and dehydration with pure acetone in ascending concentrations yielded the best results with regard to the survival of antigenic properties and enzyme activities.

The monomer embedding media described above had very good penetration properties. The impregnation of samples was also tested under vacuum conditions. The impregnation time could be reduced under vacuum especially for large specimens. For this particular size of samples a vacuum procedure was not necessary. The combination of aromatic amine *N,N*-dimethyl-*p*-toluidine with benzoylperoxide in the concentrations used revealed the possibility of a slow chemical polymerization at −15° to −20° C. The concentrations of accelerator and initiator were so far minimized that no damage

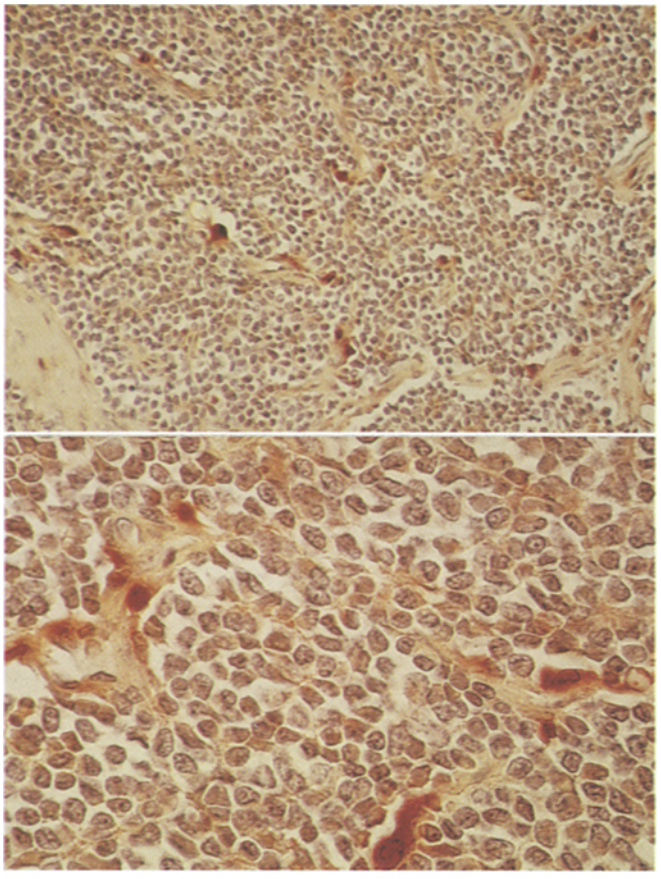


Fig. 1. Enzyme histochemistry of acid non-specific esterase in Ewing's sarcoma. The patient had no other tumour outside the bone. Strong cytoplasmatic reaction product (*brown*) in histiocytic cells. Tumour cells were negative. Upper part, ×160; lower part, ×400

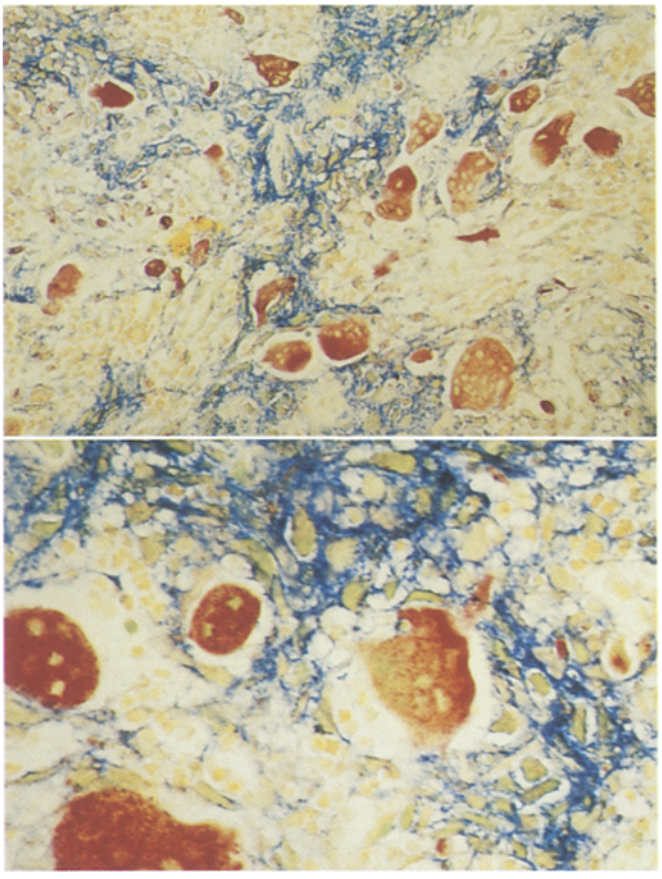


Fig. 2. Enzyme histochemistry of bone alkaline and acid phosphatase activities in osteoblastoma of femur. Simultaneous demonstration of blue (alkaline phosphatase) and red (acid phosphatase) reaction products in the tumour tissue. Nuclear counterstaining with methyl-green. Alkaline phosphatase activities in tumour osteoblasts and unmineralized bone matrix with fibres. Acid phosphatase activities in giant cells. Upper part, ×160; lower part, ×400

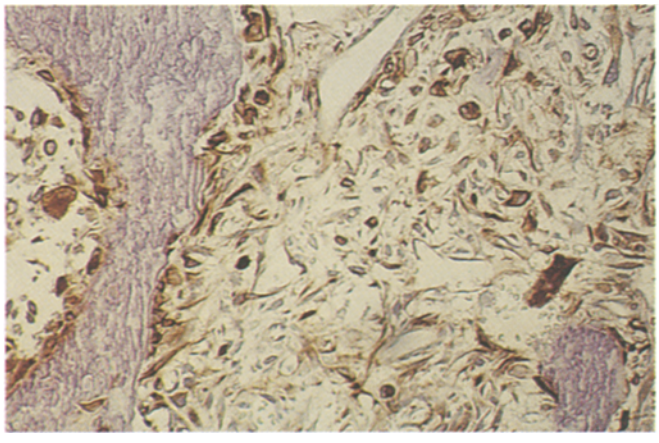


Fig. 3. Immunohistochemistry of vimentin in central sclerosing osteosarcoma of tibia bone. ABC method. Well-differentiated area of osteosarcoma. Osteoblasts and tumour cells showing positive reaction to vimentin in the cell cytoplasm. Anti-vimentin monoclonal antibody, (Dakopatts); titre, 1:100; ×400

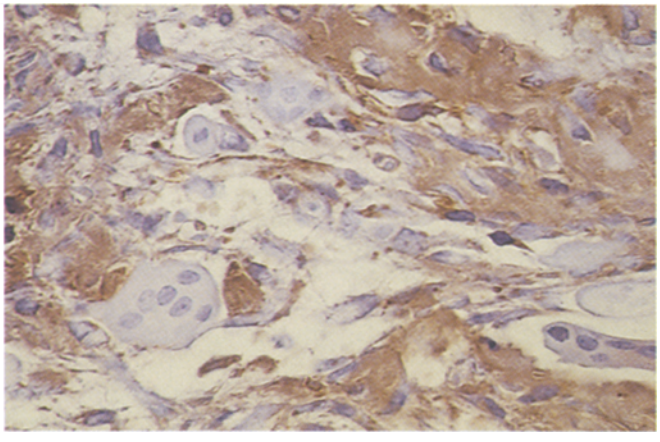


Fig. 4. Immunohistochemistry of osteonectin in osteoblastoma of femur, ABC method. Positive reaction in tumour cells of osteoblastic differentiation and in unmineralized bone matrix in tumour area. Giant cells were negative. Polyclonal anti-osteonectin antibody; titre, 1:4000; ×400

Table 3. Immunological markers: applications in the diagnosis of bone and haematopoetic tumours on methacrylate embedded sections (ABC method)

Antibody	Source	Titre
Epithelial cell types		
1. CK-low	Enzo	1:4000
2. CK-high	Enzo	1:3000
3. CK-KL1	Dianova	1: 100
4. CK-1	Dakopatts	1: 100
5. CK-MNF 116	Dakopatts	1: 200
6. CK-19	Dakopatts	1: 150
7. CK-BB	Dakopatts	1:5000
8. EMA	Dakopatts	1: 100
9. CEA	Bio-Genex	1: 10
10. AFP	Dakopatts	1: 150
11. PSA	Camon	1: 10
12. THG	Camon	1: 5
Mesenchymal cell types		
Vimentin	Dakopatts	1: 100
Desmin	Dakopatts	1: 500
Laminin	Dianova	1: 50
Myoglobin	Dakopatts	1:2000
Myosin	Bio-Genex	1: 200
Actin	Dianova	1: 50
Factor VIII (related antigen)	Dakopatts	1: 20
Fibronectin	Dianova	1:1000
Lysozyme	Dakopatts	1:4000
Alpha-1-Anti-chymotrypsin	Dakopatts	1:4000
Ki-M1	Behring	1:3000
Ki-M6	Behring	1: 200
Ki-M7	Behring	1: 300
Ki-M8	Behring	1:2000
Neuroendocrine cell types		
1. NSE	Dakopatts	1:2000
2. NSE	Dianova	1: 40
3. S-100protein	Dakopatts	1:2000
4. S-100 protein	Dianova	1: 40
5. Chromogranin	Camon	1: 10
6. Synaptophysin	Camon	1: 20
7. Neurofilament	Dianova	1:2000
Haematological cell types		
1. LCA (CD 45)	Dakopatts	1: 150
2. Myeloperoxidase	Dakopatts	1: 50
3. DF-T1 (CD 43)	Dakopatts	1:1000
4. L-45-R (CD 45 R)	Dakopatts	1: 500
5. L-45-RO (CD 45 RO)	Dakopatts	1:2000
6. L-26	Dakopatts	1:2000
7. THY 1a (CD 1a)	Dianova	1: 50
8. THY 1a (CD 1a; IOT6)	Dianova	1: 200
9. THY 1b (CD 1b)	Dianova	1: 100
10. T-12 (CD6)	Dakopatts	1: 200
11. Ki-1 (CD30)	Dakopatts	1: 100
12. Glycoprotein IIIa	Dakopatts	1: 10
13. Glycophorin A	Dianova	1: 150
Markers of cell proliferation		
1. HLA-DR	Dakopatts	1: 5
2. IL 2-receptor	Dakopatts	1: 10
3. PNCA	Dakopatts	1:1000

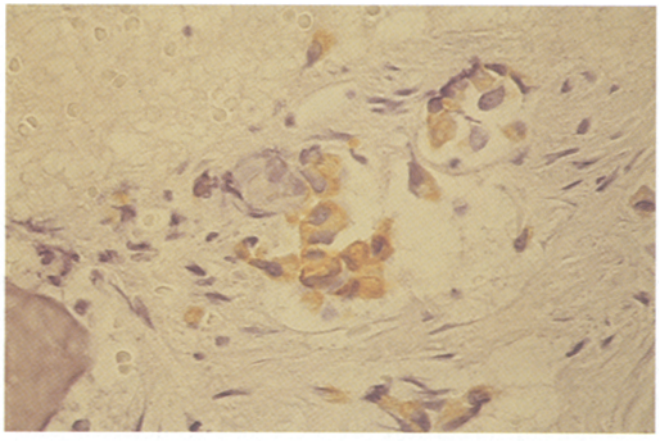
of enzyme activities in this low-temperature embedding procedure was noticeable. The polymerization was slow and evident temperature differences were not measured in samples under these conditions. At room temperature, the temperature within the samples (10 cm^3) rose after a latency period of 20 min to 60° C. The temperature peak was reached after 1 h and hardening of the block was complete after 2 h. The polymerized blocks were transparent and had good cutting properties for both steel and glass knives. The sections were mounted and spread well with mounting fluid (70% ethanol). The procedure of deacrylation effected the complete removal of resin from the sections but it was necessary to use non-aggressive organic solvents.

Using this procedure we were able to detect a wide range of enzyme reaction products accurately; even sensitive enzymes, such as alpha-naphthyl acetate esterase, acid phosphatase and alkaline phosphatase were in found accurate localization (Figs. 1, 2). The azo dyes employed for phosphatase and esterase reactions did not cause any non-specific precipitation. Diaminobenzidine was satisfactory for demonstrating myeloperoxidase (Table 2).

The immunohistological methods were suitable to localize a wide range of antigens (Table 3). Morphological preservation was found to be superior to optimally prepared cryostat and paraffin sections, and antigen localization was frequently localized to specific regions of an individual cell (Figs. 3–7). Control procedures showed no evidence of non-specific staining at the recommended dilutions. The endogenous peroxidase used here was completely blocked. It was necessary to conduct the blocking reaction after instead of before the first antibody incubation, especially if monoclonal antibodies were used. The sensitivity for antigen detection obtained by the APAAP method was far better than by the ABC and PAP methods. Our efforts to detect some special antibodies were just as unsuccessful as when paraffin sections were used. Antigens defined as membrane protein antigens of lymphocytes, for example helper and suppressor subsets of T-lymphocytes or the Ki 67 (Dako) proliferation marker, evaded detection. These antibodies are characterized by properties such as low affinity and avidity to their antigens. The cold fixation and dehydration conditions led to good survival of antigenic determinants. It was not necessary to "unmask" the antigens in methacrylate sections by proteolytic enzyme treatment such as pronase, trypsin or chymotrypsin.

Discussion

Recent investigations have shown that MMA combined with a water soluble glycolmethacrylate (GMA) polymerizes at a temperature of 4° C with BPO as initiator and an aliphatic or aromatic amine such as *N,N*-dimethylaniline or -amine as accelerator (Beckstead and Bainton 1980; Westen et al. 1981; Beckstead 1985; Bianco et al. 1984; Hermanns et al. 1986; Chappard et al. 1987; Liu 1987; Liu et al. 1987). These redox systems were



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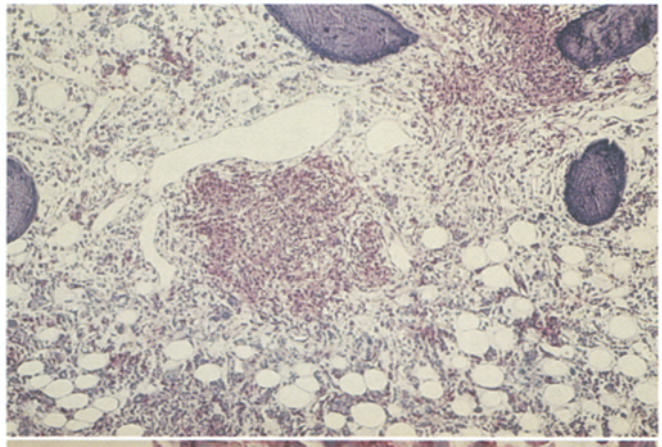

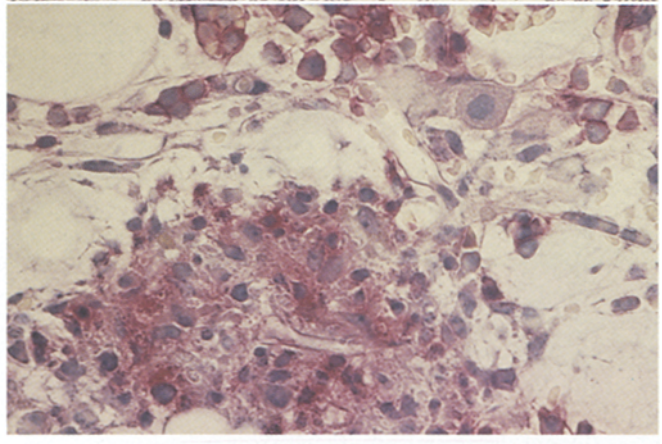

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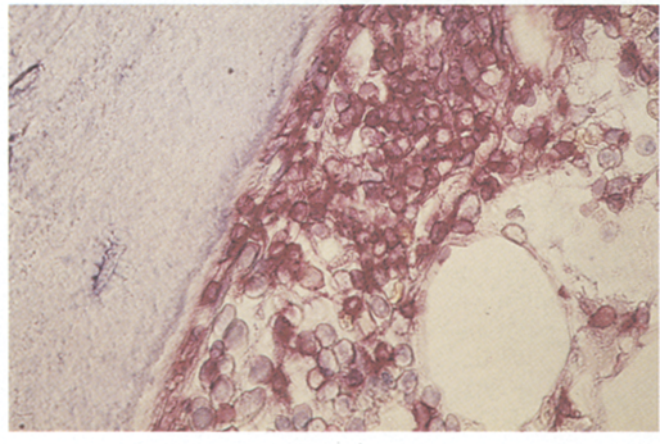

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not allowed to go through a slow polymerization at temperatures ranging from −20° to −10° C compared with our redox system. But these conditions were evidently necessary to protect the antigenicity and enzyme activities of the tissues. Proper tissue processing methods for antigen preservation in soft tissues are the AMeX (Sato et al. 1986) and freeze-drying in combination with paraffin embedding (Stein et al. 1984). However, these methods are not suitable for bone tissue processing since paraffin is an inert medium without reactive chemical groups. Most of the above embedding systems used MMA mainly in combination with water soluble methacrylates (GMA) and a wide range of softening agents. The main disadvantages of using GMA as an embedding medium are that the plastic cannot be dissolved out of the sections and there is difficulty of penetration of the monomer into larger pieces of undecalcified bone and soft tissue. GMA has the advantage over MMA in that it is water-soluble and less toxic (Meeuwsen 1986), but revertheless immunohisto- and enzyme histochemistry show severe limitations with GMA-embedding systems. GMA is a bifunctional methacrylate and forms cross-links during polymerization, which is one of the reasons why the polymer cannot be removed from tissue sections by non-aggressive organic solvents without doing such damage to the tissue that it becomes useless for microscopy. The complete removal of plastic from tissue sections is necessary to prevent a highly non-specific background in the immunohistological reaction. Many investigators have tried to develop modifications in order to apply enzymatic and immunohistological techniques to GMA-embedded tissues, because of their increased importance (Beckstead 1985). Immunohistological and enzyme histological stainings on GMA-embedded tissues were only possible on small samples like iliac crest biopsies, according to Jamshidi. The impregnation of larger tissue specimens was incomplete. Furthermore, antibodies appear to react primarily with antigens on the surface of the plastic sections. Variation in section thickness of GMA sections has only minimal effects on the amount of labelling observed. However, if the sections

Fig. 5. Immunohistochemistry of cytokeratin in bone metastases of breast cancer. ABC method. Prominent reaction to cytokeratin in tumour cells cytoplasm of an undifferentiated adenocarcinoma. Monoclonal anti-cytokeratin antibody KL1 (Dianova); titre, 1:100; ×400

Fig. 6. Immunohistochemistry of lymphocytic antibodies in bone marrow biopsy of iliac crest. APAAP method. Granulomatous myelitis in a patient with AIDS in differential diagnosis of T-cell lymphoma. *Upper part*: Lymphocytes in the granuloma with positive reaction to CD 45 antigen with monoclonal antibody L45-R0 (Dakopatts); titre, 1:4000; ×160. *Lower part*: Mononuclear cells with positive reaction to CD43 antigen with monoclonal antibody DF-T1 (Dakopatts); titre, 1:8000; ×400

Fig. 7. Immunohistochemistry of lymphocytic antibody in bone marrow biopsy of iliac crest. APAAP method. Acute lymphoblastic leukaemia (Ph +) in second relapse after bone marrow transplantation, with strong positive reaction of blast in the paratrabecular region with the monoclonal antibody LCA (Dakopatts); titre, 1:4000; ×400

are somewhat thicker the detection of the reaction products in the microscopic picture is improved, because of intensified staining. This possibility was exploited in sections with the resin removed, where the plastic yielded a higher non-specific background staining if not removed completely (Westen et al. 1981). There are few reports on MMA-embedding systems without GMA components, used for enzyme and immunohistological stainings with different enzyme and immunohistological results (Meeuwsen 1986; Wolf and Pompe 1980b; Wolf et al. 1988, 1990; Chappard 1987; Bernhards et al. 1990). These reports describe a simpler method with reproducible results that allow the demonstration of a wide range of antigens and hydrolytic enzymes in well-preserved MMA-embedded undecalcified bone tissue sections. We have systematically investigated a variety of mild fixations and dehydrations at low temperatures in combination with the MMA-embedding procedures with an initiator and accelerator system at $-10°$ to $-20°$ C for both large undecalcified bone and soft tissue biopsies. The results described come from our efforts to combine a careful fixation, washing with buffer and sucrose solution, dehydration with acetone, complete infiltration of specimens with monomeres of MMA and MMB and a slow chemical polymerization at low temperature using a proper redox system. This system of low temperature polymerization provides small free radicals for chemical polymerization of methacrylates as a condition for the survival of antigenic determinants and enzyme activity. Using this procedure, treatment with proteolytic enzymes prior to immunostaining is not necessary as for conventional paraffin-embedded tissues. MMA embedding is a necessary condition for undecalcified procedures of bone. A good retention of antigenic properties by decalcification with EDTA (Schaefer 1984) is only possible for small iliac crest biopsies with a short decalcification time. Some bone tumours have a high content of mineralized bone and the bone samples for that reason needed a long decalcification time, of up to 4 weeks for lesions such as central sclerosing osteosarcoma, low-grade central sarcoma and small cell osteosarcoma. Antigen and enzyme detections are poor under these conditions. Moreover, our method shows a greater range of antigen and enzyme detection when compared with paraffin embedding material. The method described combines the morphological superiority of plastic-embedded tissue sections with the advantage of histochemical detection of specific enzymes and proteins compared with paraffin-embedded decalcified bone tissues.

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