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Postembedding Immunohistochemical Demonstration of Antigen in Experimental Polyarthritis Using Plastic Embedded Whole Joints*

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Summary. A method is presented for the immunohistochemical demonstration of antigens in whole undecalcified joints of small laboratory animals. With this method of tissue preparation, involving embedding in a medium mainly based on 2-hydroxyethyl methacrylate, preservation of antigenicity is satisfactory. Antigens can be demonstrated in 2 µm sections by either immunofluorescence or immunoperoxidase and an indirect technique. Therefore in addition to the morphological analysis of joint alterations in experimental polyarthritis, there is now an opportunity to trace the inciting antigen and to study in parallel the enzymatic equipment of the cells involved, using consecutive sections from a single block of tissue.

Introduction

Recently we have described the application of enzyme histochemistry to problems of experimental chronic polyarthritis, using plastic embedded whole rat joints (Hermanns and Schulz 1981). Use of the water miscible plastic 2-hydroxyethyl-methacrylate (glycol methacrylate – GMA) allows also postembedding immunohistochemical demonstration of the inciting antigen in joints and other tissues and its correlation with the results of enzyme histochemistry and pathomorphology.

In past years methods have been reported for the immunohistochemical demonstration of antigens in soft tissues such as that of Diesfeld et al. (1973), using an embedding schedule developed by Ruddell (1967). Other workers have used UV light-induced polymerization of plastic (Hoshino and Kobayashi 1972; Arnold et al. 1974) or prepolymerized plastic (Spaur et al. 1975; Osamura et al. 1980).

We describe here a postembedding method for the immunohistochemical demonstration of various antigens in routinely plastic embedded joints and organs of rats.

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Materials and Methods

Experimental animals were Han: Sprague-Dawley rats (Zentralinstitut für Versuchstiere, Hannover) with 180–200 g body weight, which were inoculated either subcutaneously with 2×10^7 living bacteria of *Erysipelothrix rhusiopathiae* strain T 28 (Böhm 1971) or intravenously with 5 ml of a suspension of heat-killed bacteria or sonicated bacteria of the same strain, containing 470 µg nitrogen/ml each. Specimens of knee joints and local lymph nodes were taken five days after the inoculation.

Processing of Tissue. The longitudinally split joints and the lymph nodes were fixed for 24 h with 4% formalin (freshly prepared from paraformaldehyde) in 0.1 M cacodylate buffer containing 5 mM calcium chloride pH 7.4 and then washed in 0.1 M cacodylate buffer with 7% sucrose and 5 mM calcium chloride pH 7.4 for another 24 h. These and the following steps were carried out at 4° C with gentle stirring of the fluid. Tissues were dehydrated in a graded series of ethanols for 8 h and impregnated as a first step by equal parts of methyl methacrylate, without stabilisator (WIV, Schwetzingen), and 2-hydroxyethyl methacrylate (Serva, Heidelberg) for 16 h. For the second step of impregnation a mixture of 2-hydroxyethyl methacrylate, methyl methacrylate, ethylene glycol monobutyl ether and polyethylene glycol 400 (80:20:16:2), containing 270 mg/dl benzoylperoxide as catalyst, was used. The same freshly prepared mixture with 0.1 ml/dl N,N-dimethylaniline as accelerator served as embedding medium. The specimens were embedded in polyethylene moulds, polymerization at 4° C was complete within 24 h. Sections of 2 µm were cut with a heavy microtome (Autocut 1140, Jung, Heidelberg), floated on water at 37° C, mounted on clean slides, and dried at 50° C. Slides were stored at 4° C until used.

Antisera. Serotype specific antiserum against *Erysipelothrix rhusiopathiae* strain T 28 (kind gift from Dr. G. Kerlen) was prepared in rabbits according to Seidler et al. (1971); peroxidase-conjugated goat-anti-rabbit IgG (heavy and light chains) was purchased from Nordic (Tilburg, The Netherlands) and FITC-labeled swine anti-rabbit Ig was purchased from DAKO (Copenhagen, Denmark). Normal goat serum was obtained from conventionally raised animals. The normal goat serum was used for both dilution of antisera and as a blocking step following the enzymatic treatment of the sections.

Buffers. To test the effect of pH on background staining in addition to the 0.1 M TRIS-HCl buffer pH 7.6 a citrate-phosphate buffer pH 6.0 was used, both thoroughly degassed under vacuum to avoid air bubbles beneath the sections.

Enzymatic Treatment. To enhance the sensitivity of the immunohistochemical reaction (Vogt et al. 1976; Denk et al. 1977) sections were treated for 60 min at 4° C with pronase (protease type V, Sigma, München), 2 mg/ml TRIS-HCl buffer and then washed for 30 min in three changes of the same buffer.

Blocking of Endogenous Peroxidase. In sections for the immunoenzymatic demonstration of antigen endogenous peroxidase was blocked with 1% H_2O_2 in methanol for 30 min at room temperature (Streefkerk 1972). The reaction was stopped by washing with TRIS-HCl buffer for 30 min.

Immunohistochemistry. Normal goat serum for 30 min was used to block unspecific binding sites; this and the following incubation steps were carried out at room temperature in a moist atmosphere. Sections were then incubated with the first antibody (rabbit anti-*Erysipelothrix rhusiopathiae*) for 30 min, washed in TRIS-HCl or citrate-phosphate buffer for immunofluorescence or immunoperoxidase respectively, and then incubated with the second antibody (FITC-labeled swine anti-rabbit Ig or peroxidase-conjugated goat anti-rabbit IgG) for another 30 min. Optimal antisera dilutions were tested by block titration and ranged between 1:20 and 1:80 for the first antibody and for the second were 1:20 (FITC-labeled antiserum) or 1:80 (peroxidase-conjugated antiserum).

To demonstrate peroxidatic activity sections were incubated 5 min in 0.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Fluka, Neu-Ulm) in citrate-phosphate buffer with 0.01% H_2O_2 (Graham and Karnovsky 1966). After thorough washing sections were counterstained with hematoxylin, dried in air and mounted with Canada balsam.

Unstained sections for immunofluorescence were mounted with glycerine-PBS.

Controls. To test the specificity of staining, the following procedures were used: Incubation without the first antibody, absorption of the first antibody with living cells of *Erysipelothrix rhusiopathiae,* incubation of specimens from uninoculated animals, and incubation with rabbit anti-*Mycoplasma arthritidis* strain ISR1 (kind gift from Dr. J. Heitmann).

Photography. Immunofluorescence was examined with a Zeiss standard microscope (darkfield transillumination) with a HBO 200 mercury lamp and with a 490 nm exitation and a 500 nm barrier filter. Observations were done with an apochromat 40/1.0 Oil; photographs were taken on Ilford HP 5 (400 ASA) films. Immunoperoxidase sections were recorded with a Zeiss photomicroscope III and oil immersion objectives (40/1.0 and 63/1.4) and photographed with an Agfapan 25 professional (25 ASA) film.

Results

Preparation of Tissue Sections

Fixation with buffered 4% formalin, freshly prepared from paraformaldehyde in combination with a washing step of the same duration, produces good morphology and the antigenicity of several antigens is sufficiently retained to demonstrate them with an indirect immunohistochemical technique (in preliminary experiments we demonstrate IgG-containing plasma cells in gut submucosa and lysozyme in rat pneumocytes). The same holds true for embedding in water miscible plastic. Sections of 2 μm from whole undecalcified rat joints can readily be cut and stretched on a water bath (Fig. 1). A critical step is to avoid air

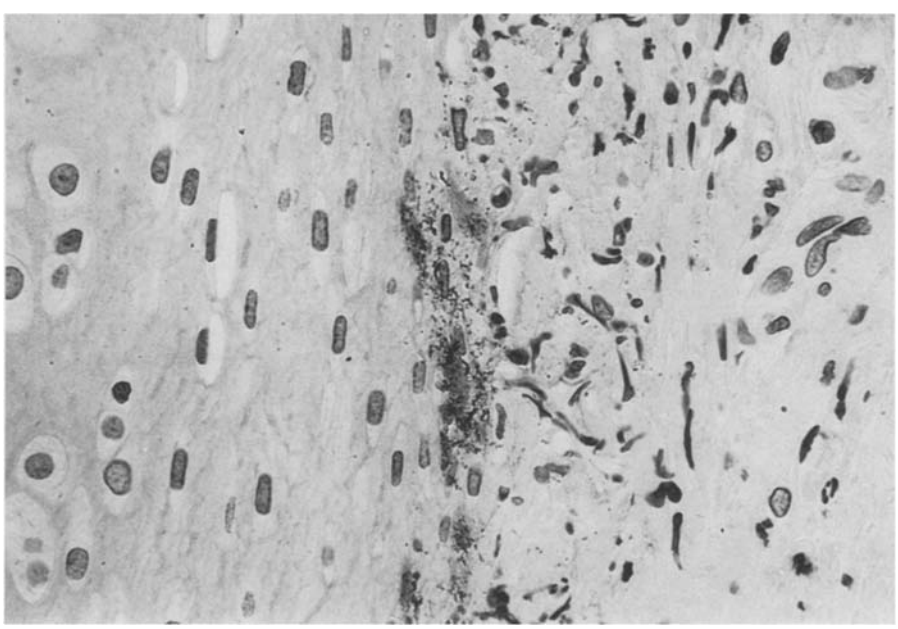

Fig. 1. Immunoenzymatic demonstration of *Erysipelothrix rhusiopathiae* antigen in a knee joint 5 days post infection. Antigen is laying in the vicinity of the epiphyseal cartilage with an acute type of inflammatory reaction in the surrounding connective tissue. Orig × 160

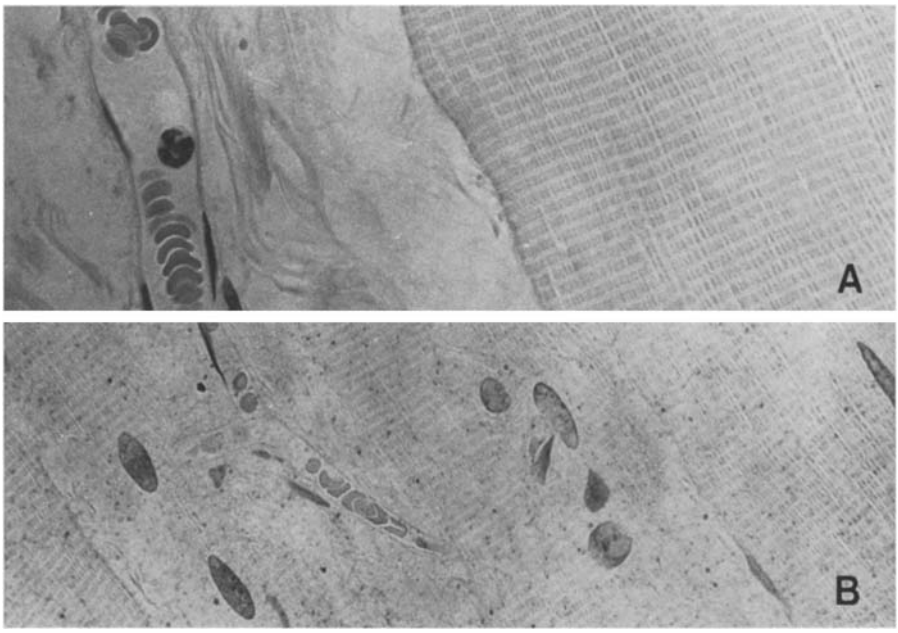

Fig. 2A, B. Periarticular tissue without detectable antigen to demonstrate the influence of buffer pH on unspecific binding of antibody. In sections treated with citrate-phosphate buffer pH 6.0 no unspecific binding is visible (**A**) whereas the use of TRIS-HCl buffer pH 7.6 results in marked unspecific binding (**B**). Orig ×252

bubbles beneath the sections leading either to retention of antiserum in this area with consecutive staining artefacts or to the detachment of the section. Therefore in addition to exact mounting of the sections, and drying by higher temperature, the use of degassed buffers is recommended.

Immunohistochemistry

Enzymatic treatment with pronase results in an increased sensitivity of antigen demonstration using either FITC- or peroxidase-labeled antisera, that is to say an intensified fluorescence or a clearer enzymatic color reaction. The blocking method used for endogenous peroxidatic activity prevents any reaction of neutrophils and erythrocytes. The use of citrate-phosphate buffer with its lower pH reduces to a great extent unspecific binding of peroxidase-conjugated antibody (Fig. 2), without any deleterious effect on antigen demonstration or enzyme color reaction. Both immunofluorescence and immunoperoxidase lead to corresponding results in antigen demonstration (Figs. 3 and 4), with immunofluorescence showing the greater sensitivity and immunoperoxidase allowing a more precise localization of antigen in the tissue.

The specificity of the reaction is proved by the negative controls; some autofluorescence exists in erythrocytes and to a lesser extent in the internal elastic lamina of arteries.

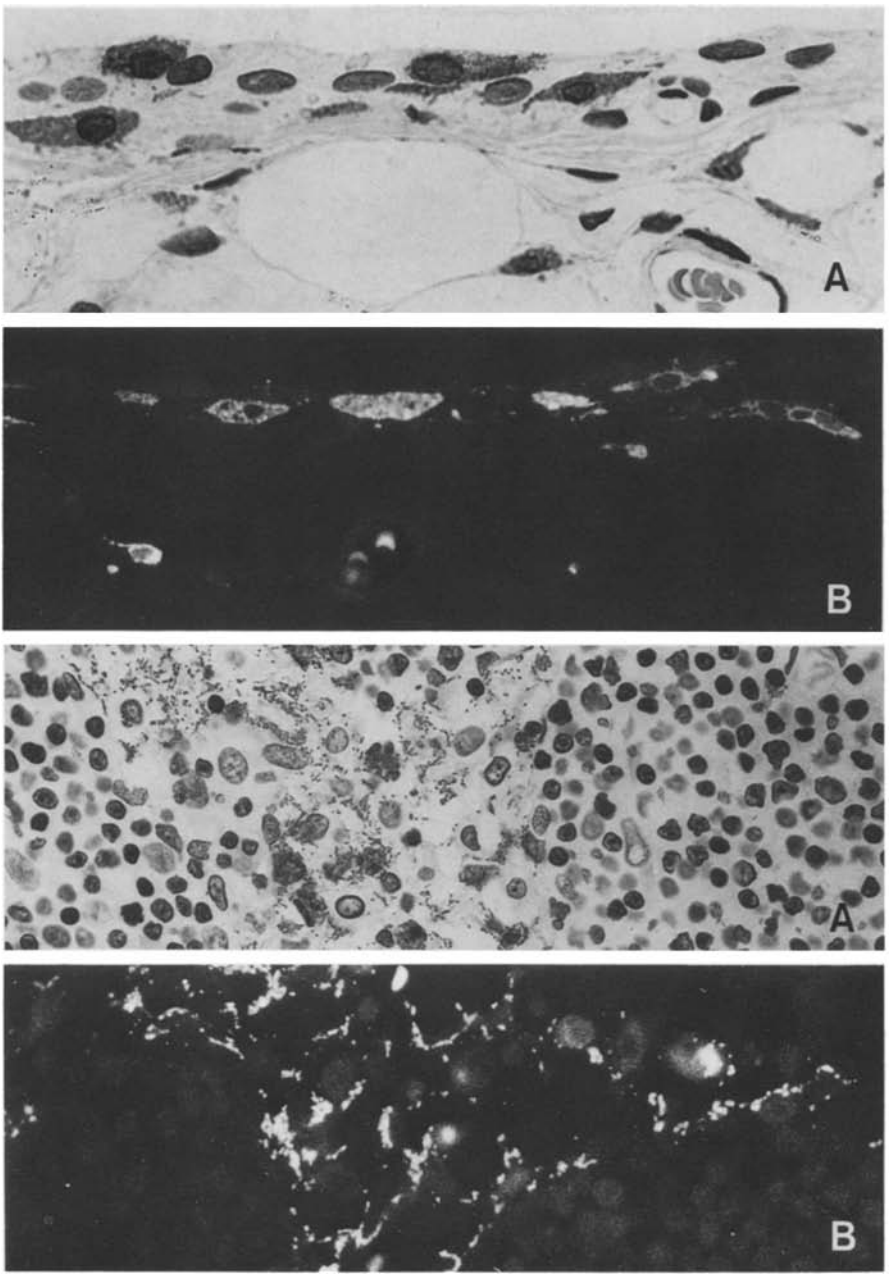

Fig. 3A, B. Synovial lining cells and subsynovial tissue 5 days after the application of sonicated cells of *Erysipelothrix rhusiopathiae*. Storage of bacterial antigen in lining cells and some mononuclear cells in the subsynovial layer demonstrated by the indirect immunoperoxidase (**A**) and immunofluorescence (**B**) technique. Orig ×252

Fig. 4A, B. Extracellulary and phagocytosed heat-killed bacteria can be seen in the medullary sinus of a lymph node, shown by immunoperoxidase (**A**) and immunofluorescence (**B**), respectively. Orig ×252

Discussion

One of the advantages of experimentally induced chronic polyarthritis models is the known causative agent or antigen. It is therefore of pathogenetic interest to investigate the distribution of the antigen in the joint in relation to time and localization. Most polyarthritis models employ small rodents (rat, mice) and in these animals precise sampling of joint tissues (cartilage, menisci, intraarticular ligaments) is difficult, as is the further processing of these tissues for conventional antigen demonstration.

We therefore established an embedding method that allows not only enzyme histochemical techniques (Hermanns and Schulz 1981), but also the demonstration of several antigens in undecalcified whole rat joints.

The suitability of low concentrations of buffered formaldehyde, freshly prepared from paraformaldehyde, combined with a subsequent washing step as used for enzyme histochemical techniques (Holt 1959), was also reported by Takamiya et al. (1979). Dehydration with ethanol does not influence the reactivity of the antigens tested so far and provides a better morphology by comparison with dehydration by acetone. The applicability of water miscible 2-hydroxyethyl methacrylate to immunohistochemical studies in general was first shown by Hoshino and Kobayashi (1972). Compared with other methods the embedding procedure described above, using chemically induced polymerization by means of benzoylperoxide and N,N-dimethylaniline (Ruddell 1967), seems easier than polymerization induced by UV light (Hoshino and Kobayashi 1972; Arnold et al. 1974) and has a less deleterious effect on proteins (von Mayersbach and Höpfel-Kreiner 1978). For demonstration of antigens only by light microscopy a prepolymerization of the plastic (Spaur et al. 1975; Osamura et al. 1980) can be abandoned. Compared with commercially available embedding kits based upon 2-hydroxyethyl methacrylate (Diesfeld et al. 1973; Franklin and Martin 1980) the embedding method described offers a chance to vary the composition of the embedding mixture and is less expensive as compared with the GMA-Quetol method (Takamiya et al. 1979). In our embedding mixture we have combined 2-hydroxyethyl with methyl methacrylate to improve tissue penetration and sectioning quality.

With postembedding immunohistochemistry several antigens (for demonstration of immune complexes e.g.) can be demonstrated using consecutive sections, whereas preembedding (Franklin and Martin 1980) shows one antigen in only a small tissue block. The indirect immunohistochemical techniques applied (preliminary experiments with the peroxidase-antiperoxidase (PAP) method showed no advantages) correspond to those in common use. As with paraffin embedded material a sufficient blocking of unspecific binding sites of tissue and embedding material is important. Unspecific binding is more obvious in the immunoperoxidase method and in this case, besides the application of undiluted normal serum for blocking and the dilution of antisera, lowering of the pH also has a positive effect. That a lower pH does not influence the antigen antibody reaction was stated by Bergroth et al. (1980) and it should also enhance the enzymatic color reaction (Weir et al. 1974). Unlike tissues embedded in epoxy

no enhancing pretreatment (etching) of the sections (Erlandsen et al. 1979) is necessary.

In *summary,* it may be said that the method described for postembedding staining of antigens in whole undecalcified joints offers some advantages. The method works with easily obtainable reagents, is inexpensive, and can be used as a routine procedure. Besides the morphological analysis of joint alterations in experimental polyarthritis there is a chance to follow up the antigen responsible and to study the enzyme pattern of cells involved. Furthermore, the embedding of whole joints gives an opportunity to investigate joint locations or joint tissues otherwise accessible only with difficulty.

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