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Collagenase in the immunohistochemical demonstration of laminin, fibronectin and factor VIII/RAg in nervous tissue after fixation*

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Summary. The effects of collagenase on the immunohistochemical demonstrability of laminin, fibronectin and Factor VIII/RAg in human nervous tissue have been studied. The influence of this, and other proteolytic enzymes such as pepsin and trypsin, has been investigated in relation to different fixatives. Collagenase gave better results with Carnoy fixed material than after formalin fixation; unlike trypsin and pepsin, it did not produce tissue digestion.

Introduction

The tissue vasculature may be immunohistochemically studied with antisera against several antigens of the vessel wall, such as laminin (LM), fibronectin (FN) and factor VIII/related antigen (FVIII/RAg). LM is a glycoprotein present in the basement membranes of various animal tissues (Foidart et al. 1980; Laurie et al. 1982), and it has been isolated from cultures of a transplantable mouse sarcoma (EHS) which produces an extracellular matrix of basement membranes (Timpl et al. 1979). FN is a high molecular weight glycoprotein present in body fluids, soft connective tissue matrices and most basement membranes (Hynes and Yamada 1982). It is capable of interacting with extracellular substances such as sulfated proteoglycans, hyaluronic acid, collagen and fibrin (Ruoslahti et al. 1981, 1982; Hay 1981; Hynes and Yamada 1982); it is integrated into basement membranes with LM, collagen IV and sulfated proteoglycans (Laurie et al. 1982; Sanes 1982). FVIII/RAg is one of the three functional components of coagulation factor VIII. It is a specific marker for endothelial cells and megakaryocytes (Bloom et al. 1973; Mukai et al. 1980; Sehested and Hou-Jensen 1981; McComb et al. 1982a).

Unfortunately the immunohistochemical demonstration of these antigens in routinely fixed paraffin embedded tissues is often difficult, and therefore the possibility of using stored material for retrospective studies is greatly limited. Nevertheless, several works have shown that treatment with proteolytic enzymes, such as trypsin, pepsin and pronase, may restore the antigenicity of LM, FN and FVIII/RAg in formalin-fixed paraffin embedded tissues (Burns et al. 1980; Dixon et al. 1980). Hølund et al. (1981) pointed out that FN may be demonstrated with nearly identical results in frozen unfixed sections and paraffin sections of ethanol-acetic acid fixed tissues, without enzymatic digestion.

The aim of the present study was to verify the immunoperoxidase (PAP) demonstrability of LM, FN and FVIII/RAg in normal and pathological, fixed nervous tissue. We compared the effect of collagenase, an enzyme never used before in immunohistochemistry, with that of pepsin and trypsin, in two different conditions of fixation, that is formalin and Carnoy.

Materials and methods

Tissue and tissue preparation. The material consisted of 5 normal nervous tissue specimens and 20 brain tumors (5 astrocytomas, 5 glioblastomas, 5 mixed glio-mesodermic tumors, 5 hemangioblastomas), all surgically removed. Tissues were divided in small pieces and fixed in Carnoy at 0°–4° C or in 10% neutral buffered formalin for 24 h. New sections of 5 μm were cut from the paraffin embedded tissue blocks and mounted on glycerin coated glass slides.

Pretreatment of the sections with proteolytic enzymes. Deparaffinized sections brought to water were incubated for 5, 15, 30, 45, 60, 90, 120, 180 min at 37° C in the following proteolytic solutions:

a) 0.05%–0.5% Collagenase (Sigma type I, C0130) in 0.05%–0.5% $CaCl_2$, pH 7.4

b) 0.05%–0.1% Trypsin (GIBCO 1:250) in 0.05%–0.1% $CaCl_2$, pH 7.6

c) 0.4 Pepsin (Sigma, P7012) in 0.01 N HCl.

Staining procedure. The peroxidase-antiperoxidase (PAP) technique (Sternberger et al. 1970) was used. The procedure comprises the following steps: 1) inhibition of endogenous peroxidase activity with H_2O_2 0.3% in methanol for 30 min; 2) normal swine serum (Dako) 1:20 for 20 min; 3) rabbit immunoglobulins anti-laminin, anti-fibronectin, or anti-FVIII/RAg at variable dilution for 30 min; 4) swine antirabbit immunoglobulins (Dako) 1:20 for 20 min; 5) PAP complex (Dako) 1:50 for 20 min; 6) 0.05% diaminobenzidine tetrahydrochloride (DAB) (Bionetics) and 0.01% H_2O_2 in PBS pH 7.4 for 3–6 min.

The slides were carefully rinsed in PBS after each step. The sections were counterstained with hematoxylin, dehydrated and mounted in balsam. Rabbit anti-laminin serum prepared with LM purified from the EHS sarcoma, according to Timpl et al. (1979), was used at variable dilutions ranging from 1:200 to 1:2000. Rabbit antifibronectin serum prepared with highly purified human FN (Tarone et al. 1981) was used at variable dilutions ranging from

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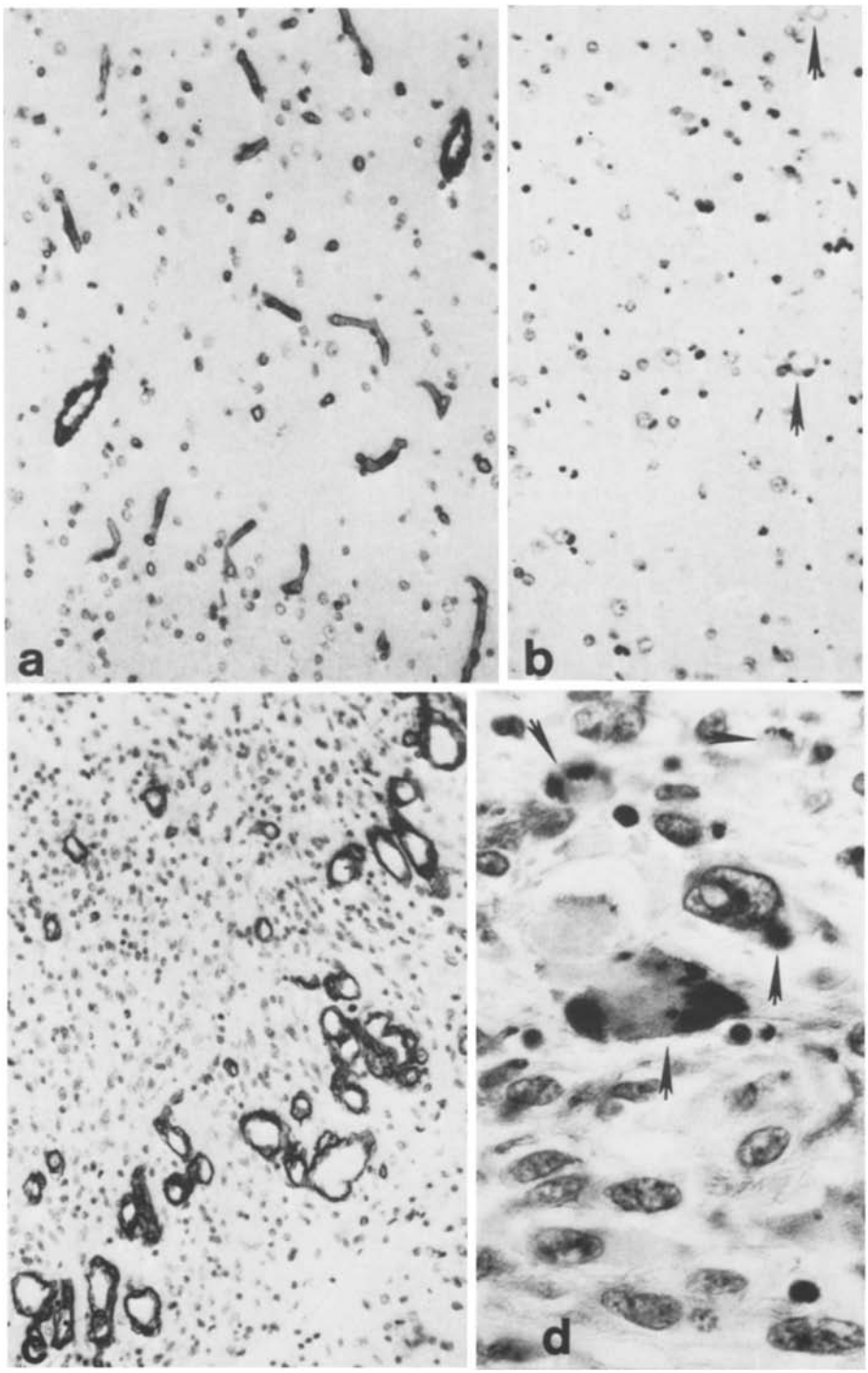

Fig. 1 a–d. Normal cortex: laminin **a** with and **b** without collagenase treatment. Carnoy ×200; **c** Glomeruli of a glioblastoma: laminin with collagenase treatment. Carnoy ×300; **d** Giant cell of a gliosarcoma: intracytoplasmic staining for laminin; no enzymatic treatment. Carnoy × 1000

1:100 to 1:1000. Rabbit anti-FVIII/RAg (Dako A082) was used at variable dilutions from 1:100 to 1:1000.

All antisera were diluted in PBS pH 7.4 containing 15 mM NaN_3.

Control stainings. Were performed by replacing the primary antiserum (step 3) with: a) Ig-fraction from nonimmunized rabbit; b) antisera adsorbed by corresponding purified antigens; PBS pH 7.4 with 1% BSA.

Evaluation. We recorded five features: staining intensity, background, sensitivity, morphological localization and tissue digestion using the following score system: a) staining intensity (referred to specific staining only), 0 = none; + = slight; + + = moderate; + + + = strong; + + + + = very strong; b) background, 0 = none; + = slight; + + = moderate; c) sensitivity (corresponding to the relative number of demonstrated vessels), 0 = none; + = < 50%; + + = 50–90%; + + + = > 90%; d) morphological localization (corresponding to the definition of the antigenic structure and to the local diffusion of the staining product), 0 = diffusion without relation to the structure of the antigen; + = slight definition and moderate diffusion; + + = good definition and slight local diffusion; + + + = optimal definition without any diffusion; e) tissue digestion, 0 = no digestion, morphology of sections perfectly main-

Table 1. Effects of the enzymatic treatments after fixation in formalin and Carnoy[a]

	Material fixed in carnoy				Material fixed in formalin			
	Undigested	Collagenase 0.05% × 30′	Trypsin 0.05% × 10′	Pepsin 0.4% × 10′	Undigested	Collagenase 0.1% × 60′	Trypsin 0.05% × 40′	Pepsin 0.4% × 60′
Laminin 1:800[b]								
Staining intensity	0/+	+++	++	++	0	+	++	++/+++
Background	0	0	+	0/+	0	0	+	0/+
Sensitivity	0/++	+++	+++	+++	0	+/++	+++	+++
Morphological localization	++	+++	+	+/++	0	++	++	++/+++
Tissue digestion	0	0	+++	++	0	0	+/++	+
Fibronectin 1:500[b]								
Staining intensity	0/+	+++	++	++	0/+	++	++	++/+++
Background	0	0	+	0/+	0	0	+	0/+
Sensitivity	0/++	+++	+++	+++	0/+	++	+++	+++
Morphological localization	++	+++	+	+/++	+	++	++	++
Tissue digestion	0	0	+++	++	0	0	+/++	+
F VIII/RAg 1:800[b]								
Staining intensity	0/++	+++	++	++	0/++	+++	+++	+++
Background	0	0	+	0/+	0	0	+	0/+
Sensitivity	+/++	+++	+++	+++	+/++	++/+++	+++	+++
Morphological localization	+++	+++	+	+/++	+++	+++	++	++/+++
Tissue digestion	0	0	+++	++	0	0	+/++	+/++

[a] The score system is explained in Material and methods [b] Optimal dilution of antiserum

tained; + = slight digestion; ++ = moderate digestion; +++ = tissue largely damaged.

Results

All controls, performed by replacing the primary antiserum, were completely negative. The pattern of LM distribution in normal CNS corresponded to the occurrence of basement membranes, as known from electron microscopy (Peters et al. 1976): LM surrounded endothelial cells (Fig. 1a), separated the neuropile from the wall of larger vessels (Fig. 2a), and lay between the pia mater and the limitans gliae. The final reaction product had a laminar appearance. In gliomas, LM was always present in the basement membranes of vessel walls (Fig. 1c) and in gliosarcomas it separated the glial from the mesodermic component. Intracytoplasmic LM was never observed. Only in the sarcomatous component of a gliosarcoma a few cells displayed a corpuscular-granular intracytoplasmic staining (Fig. 1d).

FN was present in the vessel walls of normal nervous tissue and cerebral tumors without a definite laminar pattern. The staining was diffuse in the endothelial proliferation of gliomas (Fig. 3b) and in the mesodermic areas of gliosarcomas.

The expression of FVIII/RAg was restricted to the cytoplasm of endothelial cells in normal vessels and in the proliferated vessels of gliomas (Fig. 3d). In the hemangioblastomas, only the cells lining the vascular lumina were positive. The final reaction product was granular.

The comparison of serial sections with and without enzymatic treatments and after fixation in formalin and Carnoy is shown in Table 1 and Figs. 1, 2, and 3.

Sections without prior enzymatic digestion

LM and FN were more easily detected after Carnoy than after formalin fixation. However, the number of positive blood vessels was low and unpredictable. An intracytoplasmic reation for LM, which occurred in one gliosarcoma, was clearly evident in sections not exposed to enzymatic digestion (Fig. 1d); in these, the staining of the basement membranes was poor. The staining for FVIII/RAg demonstrated a few vessels, with a slight difference between formalin and Carnoy fixation.

Treatment with collagenase

This markedly increased the staining intensity for the 3 antigens and the number of positive blood vessels when the tissue had been fixed in Carnoy (Figs. 1, 2, and 3). The best results were achieved by using 0.05% collagenase in 0.05% $CaCl_2$ for 30 min; in these conditions, no tissue digestion occurred. After longer exposure to the enzymes (Table 2), the staining intensity further increased, but at the same time a local diffusion of the reaction product took place and a background staining appeared; these effects occurred for the 3 antigens, being more evident for FVIII/RAg. Nevertheless, even if the concentration of the enzyme was up to ten fold the optimum, and the time of treatment was prolonged, no tissue digestion was observed.

On formalin fixed material, even if the concentration of the enzyme and the time of digestion were augmented, the effect of collagenase was less evident than on Carnoy fixed material. However, there was a definite enhancement of staining intensity and sensitivity obtained for FVIII/RAg demonstration.

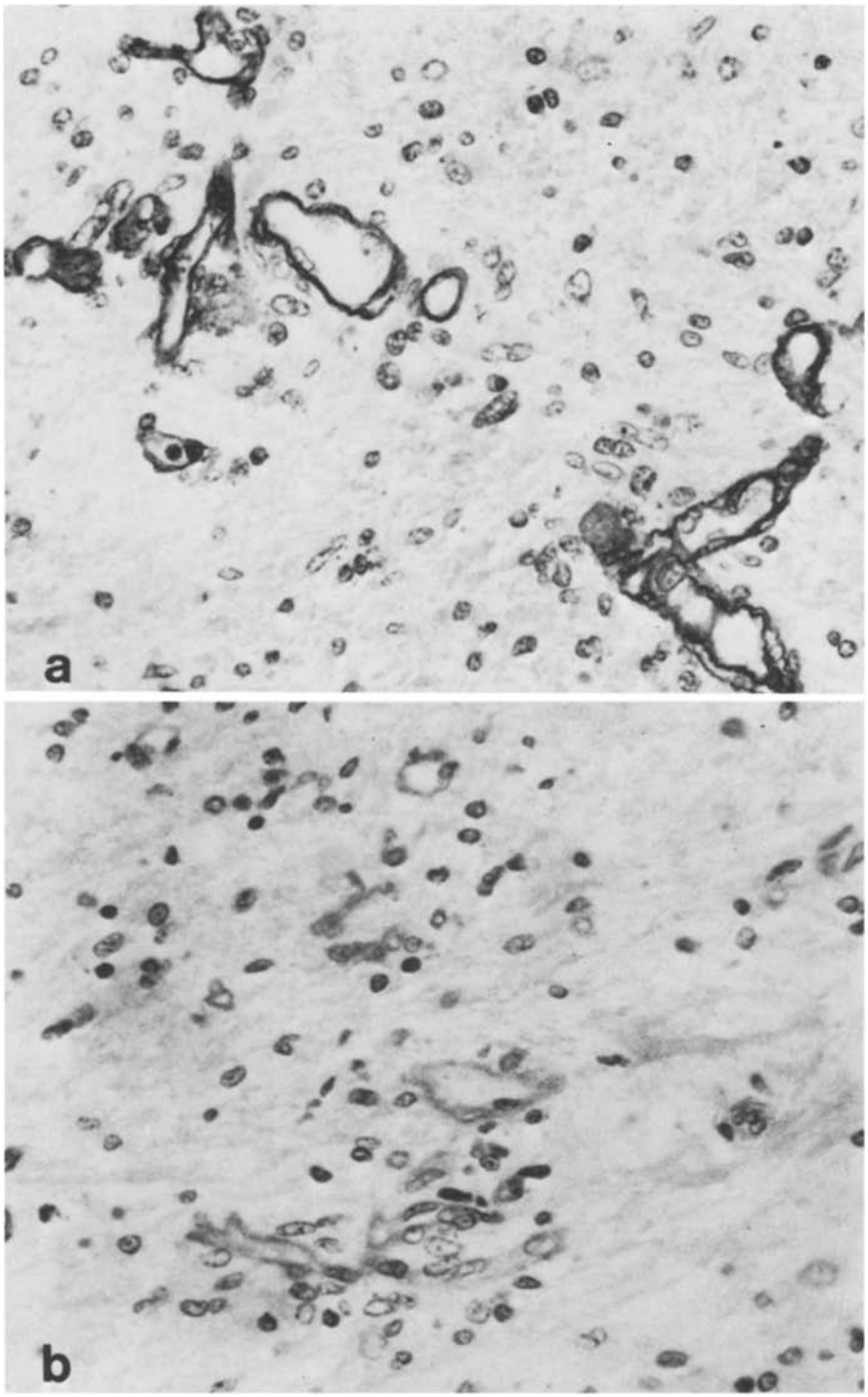

Fig. 2a, b. Vessels of a glioblastoma: laminin **a** with and **b** without collagenase treatment. Note in **a** the presence of two basement membranes around the vessels. Carnoy ×400

Table 2. Effect of various times of exposure to collagenase on laminin demonstration in Carnoy fixed material[a]

	5′	15′	30′	45′	60′	120′	180′
Staining intensity	+	+ +	+ + +	+ + +	+ + + +	+ + + +	+ + + +
Background	0	0	0	0	0/+	+/+ +	+ +
Sensitivity	+	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Morphological localization	+ +	+ + +	+ + +	+ + +	+ + /+ + +	+ +	+
Tissue digestion	0	0	0	0	0	+	+/+ +

[a] The score system is explained in Material and Methods

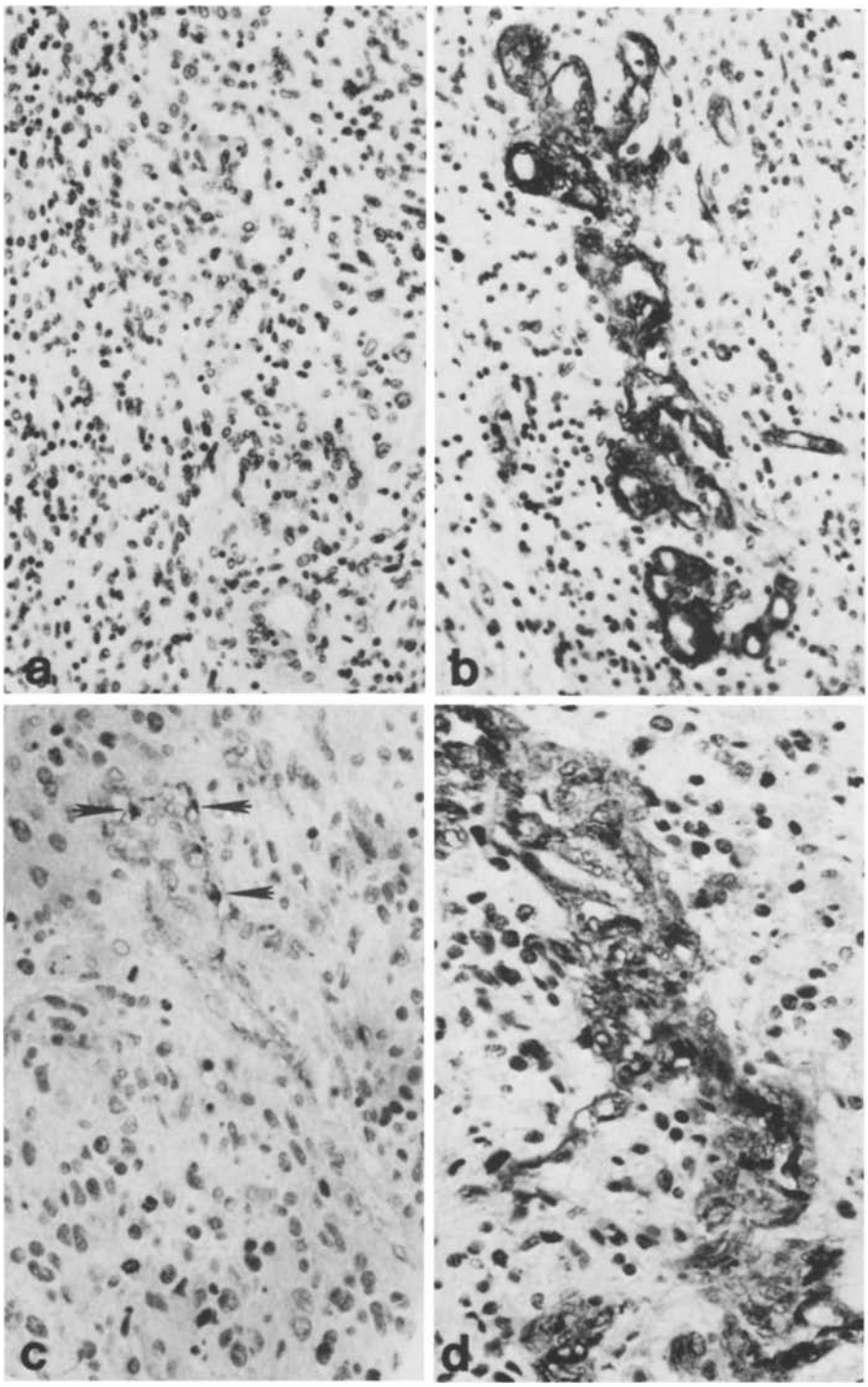

Fig. 3a–d. Glomeruli of a glioblastoma: fibronectin **a** without and **b** with collagenase treatment. Carnoy ×300; **c** and **d** Endothelial bud of a glioblastoma: Factor VIII/RAg **c** without and **d** with collagenase treatment. Carnoy ×300

Treatment with trypsin and pepsin

In Carnoy fixed sections both produced the same increase of sensitivity as collagenase, but caused a weaker staining intensity, an evident background and a local diffusion of the reaction product. On formalin fixed tissue, trypsin and pepsin were more effective than collagenase, expecially for LM demonstration; however, the morphological localization of the 3 antigens was unsatisfactory. Pepsin, and expecially trypsin produced a severe tissue digestion in Carnoy fixed sections, while the tissue fixed in formalin was more resistant.

Discussion

The usefulness of enzymatic pre-treatment for immunohistochemical studies of formalin fixed and paraffin embedded tissues is well recognized (Curran and Gregory 1977, 1978; Reading, 1977; Brozman 1978; Mepham et al. 1979; Mepham 1982; Hautzer et al. 1980; De Lellis 1981). The expo-

sure of sections to proteolytic enzymes, such as trypsin, pepsin and pronase, has proved to be helpful in the demonstration of LM (Ekblom et al. 1982), FN (Burns et al. 1980; Hølund et al. 1981; Hølund and Clemmensen 1982) and FVIII/RAg (Sehested and Hou-Jensen 1981; McComb et al. 1982a).

Our results show that in order to achieve a reliable immunohistochemical demonstration of LM, FN and FVIII/RAg, the treatment with collagenase of Carnoy fixed material is superior to that with trypsin and pepsin, as far as nervous tissue is concerned. As a matter of fact, Carnoy fixation preserves LM and FN better than formalin does, thus permitting partial detection of these two antigens even without enzymatic pre-treatment. On the other hand, collagenase significantly enhances the staining intensity and the sensitivity of immunostaining for LM, FN and FVIII/RAg, so that negative results in the absence of enzymatic digestion cannot be considered reliable.

Treatment with collagenase does not produce non specific staining if the enzyme concentration and the incubation time are optimal; under these conditions the antigens display the already known pattern of distribution in CNS: basement membranes for LM (Timpl et al. 1982), vessel walls and mesodermic areas of gliosarcomas for FN (Paetau et al. 1980; Jones et al. 1982; Kochi et al. 1983) and cytoplasm of endothelial cells in normal nervous tissue and brain tumors for FVIII/RAg (McComb et al. 1982b). It is to be stressed, moreover, that collagenase does not cause any damage to the morphology of the tissue.

We also found that the sensibility of tissues to enzymatic digestion depends on the fixative employed, as Mepham (1982) maintains. In fact, Carnoy fixed tissue is easily damaged by pepsin and trypsin, while formalin fixed tissue is more resistant; likewise a stronger collagenase treatment is necessary for formalin fixed material than for Carnoy-fixed, in order to obtain an increase of sensitivity and of staining intensity.

The target of the disclosing action of proteolytic enzymes on fixed tissue sections is unknown. However, since collagen is the specific substrate of collagenase (Seifter and Harper 1970; Peterkofsky 1982) our results suggest that collagen itself masks the antigenic sites of LM, FN and FVIII/RAg. The binding of FN, and also of LM, to collagen in extracellular matrix has been widely demonstrated (Hay 1981; Ruoslahti et al. 1981, 1982; Laurie et al. 1982; Sanes 1982; Timpl et al. 1982; Hynes and Yamada 1982). Moreover, FN is known to be bound to the collagen region that collagenase attacks (Dessau et al. 1978; Kleinman et al. 1978; Hay 1981). In extracellular matrix FN and LM are bound to other molecules as well, such as hyaluronic acid and sulphated proteoglycans (Hay 1981; Del Rosso et al. 1981; Ruoslahti et al. 1981, 1982; Hynes and Yamada 1982; Timpl et al. 1982); the masking action of these molecules is suggested by the disclosing effect of hyaluronidase treatment on FN immunohistochemical demonstration (Hølund and Clemmensen 1982). Indirect evidence of the masking action of collagen comes from the only case in which LM was observed inside the cytoplasm, and therefore it was presumably not linked to collagen; in this case, without previous collagenase digestion, intracytoplasmic LM was clearly evident, whereas the basement membranes of the vessels were not stained.

In the immunohistochemical study of fixed sections, collagenase acts in a specific way, different from that of pepsin and trypsin. In fact, it does not abolish the background staining which disturbs the immunohistochemical evidentiation of immunoglobulins (Reading 1977); it does not cause tissue digestion; it has no effect in unmasking antigens not linked to collagen, such as lysozyme and alfa-1-antitrypsin (personal observations), which require proteolytic digestion (Reitamo 1978; De Lellis 1981). Furthermore, from these considerations it can be deduced that the contaminating non specific proteolytic activities of the commercially available collagenase (Peterkofsky 1982) do not significantly affect our results.

The unmasking effect of collagenase on FVIII/RAg needs other interpretations, since in the endothelium this antigen is not linked to collagen. The permeability for antibodies of endothelial cells, which are coated by a collagen-rich basement membrane, might be increased by collagenase. Curran and Gregory (1978) suggested a similar mechanism for the action of trypsin in immunoglobulin demonstration.

Different mechanisms can limit the demonstrability of FN, LM and FVIII/RAg. From our results it appears that denaturation, and the consequent modification of antigens caused by fixatives, seem to have little importance. On the contrary, other factors produce a prominent masking effect. Formalin is known to link proteins by intermolecular bonds (Sabatini et al. 1963; Pearse 1968), rendering the antigenic sites temporarely inaccessible (Mepham 1982); in the extracellular matrix, a tight network could originate among collagen, FN, LM and proteoglycans. From our results Carnoy seems to cause a weaker cross-linking effect than formalin; ethanol-acetic acid (Hølund et al. 1981) seems to cause no cross-linking effect at all. Eventually, a large number of antigenic sites of LM and FN in the extracellular matrix are probably masked by bonds with collagen, independent of fixation.

Collagenase seems to unmask the antigenicity of LM, FN and FVIII/RAg without a direct action on the antigenic sites, contrary to what other enzymes probably do, lowering the specificity of the antigen-antibody reaction (Heyderman 1979). The use of collagenase may overcome this undesirable effect.

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