Histochemistry (1986) 85:193–195

Histochemistry

Immunohistochemical detection of bromodeoxyuridine in formalin-fixed tissues

H. Sugihara *, T. Hattori, and M. Fukuda
Department of Pathology, Fukui Medical School, Matsuoka, Yoshida-gun, Fukui, 910-11 Japan

Received December 23, 1985 / Accepted March 1, 1986

Summary. Bromodeoxyuridine (BrdUrd) immunohistochemistry has recently been introduced for the visualization of DNA-synthesizing nuclei. In order to detect the BrdUrd incorporated into nuclear DNA in formalin-fixed, paraffin-embedded tissues, we tested several different pretreatment procedures including digestion with proteinase and hydrolysis with HCl, prior to immunoperoxidase staining. In order to determine the optimal conditions for detecting nuclear BrdUrd, mice were given BrdUrd and ^{3}H-thymidine simultaneously, and the autoradiographic and immunohistochemical results obtained in BrdUrd-stained sections were compared. It was found that digestion with 0.05% proteinase at 37° C for 20 min and hydrolysis with 1 *N* HCl at 37° C for 20 min was sufficient to detect BrdUrd immunoreactivity in ^{3}H-thymidine-labelled nuclei, the results being virtually unaffected by the orders in which the two pretreatments were performed. Our method extends the range of application for BrdUrd immunohistochemistry in cell-kinetic studies.

Introduction

Bromodeoxyuridine (BrdUrd) is a pyrimidine analogue which is incorporated into DNA-synthesizing nuclei. In immunohistochemical studies, the BrdUrd incorporated into DNA has been detected using antibodies against BrdUrd (Gratzner et al. 1976; Morstyn et al. 1983). This immunohistochemical method for the detection of DNA-synthesizing cells has recently been applied for cell-kinetic analysis instead of (or in conjunction with) ^{3}H-thymidine autoradiography (Dolbeare et al. 1983; Raza et al. 1985; Hamada 1985). In comparison with ^{3}H-thymidine autoradiography, this immunohistochemical method using BrdUrd has several advantages: firstly, labelled cells can be detected more quickly without the long delay required for the exposure of autoradiographs. Also, the immunoreaction products are visualized with a high resolution, without the background noise which is present in autoradiographs. Another advantage is that BrdUrd is used in anti-cancer therapy and may be utilized for the analysis of cell kinetics of human cancer tissues. However, previous studies have shown that, in order to detect BrdUrd immunohistochemically, it is necessory to fix tissues with dehydrating agents, i.e. methanol/acetic acid (Gratzner et al. 1976), acetone (Morstyn et al. 1983) or 70% ethanol (Hamada 1985). Therefore, this technique cannot be applied when tissues need to be fixed using aldehydes for electron microscopy or for the immunohistochemical detection of certain kinds of antigens, e.g. fibronectin (Kirkpatrick and D'Ardenne 1984).

In the present study, we found that the antigenicity of BrdUrd in formalin-fixed tissues can be unmasked by enzymatic digestion combined with acid hydrolysis. To test whether BrdUrd-positive cells represent all DNA-synthesizing cells, we administrated BrdUrd and ^{3}H-thymidine to mice, and the immunohistochemical and autoradiographic results were compared.

Materials and methods

Five ICR mice (Clea Japan, Tokyo) were given an intraperitoneal injection of 100 mg/g body weight BrdUrd (Sigma, St Louis, USA) and 10 μCi/g body weight ^{3}H-thymidine (sp. act., 5 Ci/m*M*; Amersham International, Amersham, U.K.). The mice were killed and the stomach was removed 30 min after the injection. One half of the stomach was fixed with 70% ethanol for 3 h, and the other half was fixed with 10% phosphate-buffered formalin for 24 h. The tissues were embedded in paraffin and cut into 4-μm-thick sections. Sections of formalin- and ethanol-fixed tissues were mounted on glass slides and were kept overnight at 60° C. After deparaffinization with xylene, the sections were rinsed in 0.01 *M* phosphate-buffered saline (PBS), and were hydrolysed with 1 *N* HCl and/or digested with 0.05% proteinase (type VII; Sigma) in PBS at 37° C according to the protocol shown in Table 1. The sections were then stained by the avidin biotin peroxidase complex method (Hsu et al. 1981) using a Vectastain ABC Kit (Vector Laboratories, Burlingame, USA). The sections were incubated successively in normal horse serum, in a 1:50 dilution of a monoclonal antibody against BrdUrd (Becton Dickinson Monoclonal Center, Mountain View, USA) overnight at 4° C, in biotinylated anti-mouse IgG (horse) at room temperature for 1 h, and finally in ABC at room temperature for 2 h. After each step, the sections were rinsed with PBS. The sections were then incubated in 0.05 *M* *Tris*/HCl buffer (pH 7.0) containing 0.03% 3,3′-diaminobenzidine (DAB) and 1% H_2O_2. BrdUrd-positive nuclei exhibited deposits of brown-coloured DAB precipitates. These sections were dipped in nuclear emulsion (Kodak NTB2) and were developed in FD 111 after 4 weeks of exposure at 4° C. In each autoradiograph, 200 cells labelled with ^{3}H-thymidine were randomly chosen and the percentage of BrdUrd-positive cells was determined.

Results

The murine tissues studied comprised the forestomach and the glandular stomach. The various pretreatments with pro-

* To whom offprint requests should be sent

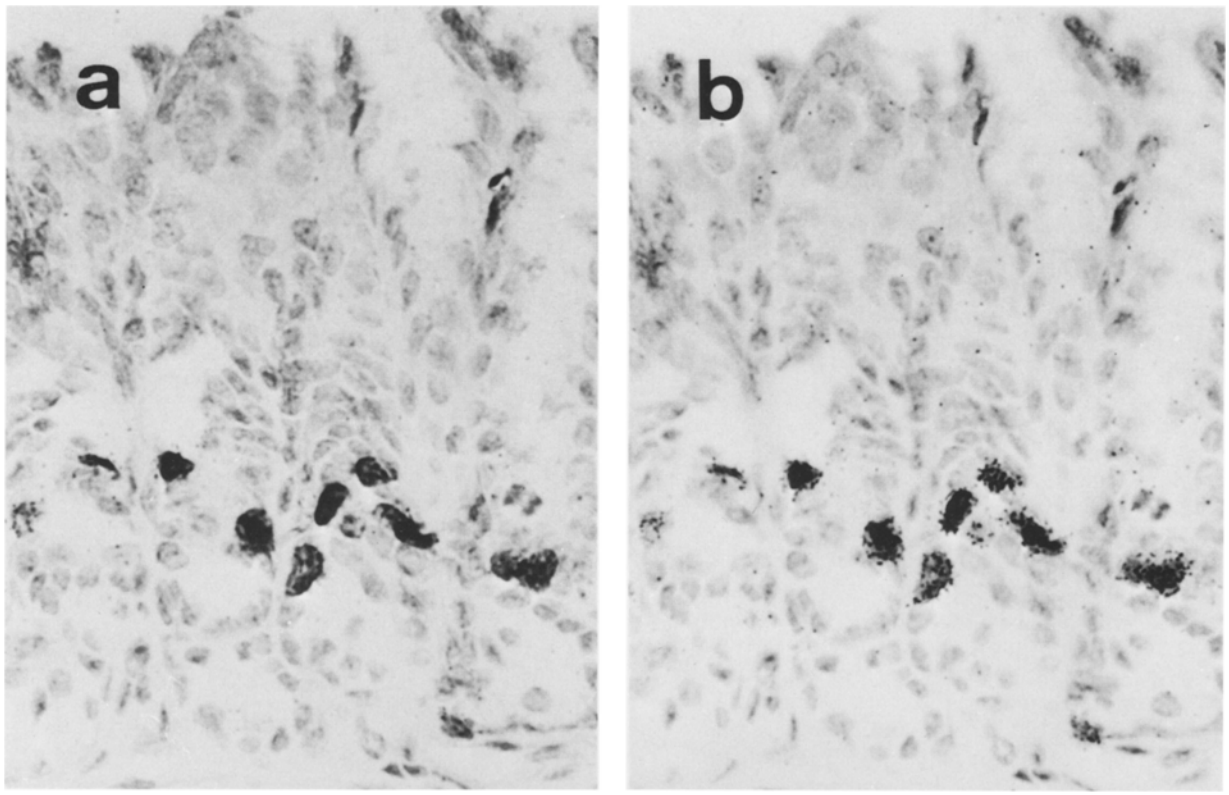

Fig. 1 a and b. Autoradiographs of a BrdUrd stained section of mouse pyloric mucosa that had been double labelled with ^{3}H-thymidine and BrdUrd and then fixed with formalin. Prior to immunoperoxidase procedures, the sections were pretreated with 1 *N* HCl for 20 min and then with 0.05% proteinase for 20 min. **a** Focused on BrdUrd staining; **b** focused on the silver grains of an autoradiograph of the same section. Note that all of the nuclei covered with silver grains are BrdUrd positive. The nuclei were counterstained with haematoxylin. ×200

Table 1. BrdUrd staining after various pretreatments with proteinase and/or HCl

Digestion with proteinase [min]	Hydrolysis with HCl [min]	Percentage of BrdUrd-positive nuclei in cells labelled with ^{3}H-thymidine	
		Formalin fixed	Ethanol fixed
0	0	0	0
0	10	0 (0)*	50–95
0	20	<50 (<50)*	95<
0	40	<50 (<50)*	95<
10	0	50–95 (0)	0*
20	0	50–95 (0)	0*
40	0	50–95 (0)	0*
10	10	50–95 (<50)	95<*
20	20	95< (95<)	95<*
40	40	95< (95<)	95<*

The percentage of BrdUrd-positive cells in the cells of pyloric mucosa labelled with ^{3}H-thymidine is given for both formalin-fixed and ethanol-fixed tissues. The percentage of positive cells in the fundic mucosa is given in parenthesis. *Asterisks* indicate sections with considerable breakdown or loss of tissue

teinase and HCl used as well as the percentage of BrdUrd-positive cells found in ^{3}H-thymidine-labelled cells are given in Table 1.

Without any pretreatment, neither the formalin-fixed tissue nor the ethanol-fixed tissue exhibited positive by immunohistochemical staining for BrdUrd. In ethanol-fixed tissues, hydrolysis for 10–40 min was sufficient to allow the detection of BrdUrd immunoreactivity in most of the nuclei labelled with ^{3}H-thymidine; however, in formalin-fixed tissues, even 40 min of hydrolysis with HCl revealed the BrdUrd-positive staining in less than 50% of the nuclei labelled with ^{3}H-thymidine. On the other hand, enzymatic digestion for even 40 min failed to reveal BrdUrd-positive cells in ethanol-fixed tissues; indeed, after only 10 min, this digestion resulted in severe damage of the ethanol-fixed tissues. In contrast, after digestion of formalin-fixed tissues, part of the pyloric mucosa was positively stained for BrdUrd, while the fundic mucosa and forestomach were not stained. When a combination of proteinase digestion and HCl hydrolysis was used, it was found that, in formalin-fixed tissues, 20 min of each treatment was sufficient to allow the detection of BrdUrd immunoreactivity in most of the nuclei labelled with ^{3}H-thymidine (Fig. 1). The orders in which the two treatments were applied had little effect on the results obtained.

Discussion

BrdUrd immunohistochemistry has recently been introduced for studies of cell kinetics. In combination with ^{3}H-thymidine autoradiography, this simple method allows reliable double labelling (Hamada 1985) and is useful for studying the cell kinetics of human tissues in vivo (Morstyn

et al. 1983). However, the tissue fixation remains a problem. It has been suggested that the antibody against BrdUrd fails to reach BrdUrd incorporated into double-stranded DNA unless some nuclear proteins are removed, and the DNA strands are uncoiled by hydrolysis with HCl (Freeman et al. 1971). To obtain this DNA denaturation, it is usual to fix tissues mildly using ethanol or other dehydrating agents. But, previous studies have not revealed whether BrdUrd is also detectable in formalin-fixed tissues.

In the present study, after double labelling with BrdUrd and ^{3}H-thymidine, gastric tissues were removed and fixed with 10% phosphate-buffered formalin or 70% ethanol. The sensitivity of BrdUrd staining for detection of DNA-synthesizing cells was checked by examining ^{3}H-thymidine autoradiographs of identical sections. It was found that, in ethanol-fixed sections, 10–40 min of hydrolysis with 1 *N* HCl before the application of immunoperoxidase procedures allowed the immunoreaction products of BrdUrd to be visualized in most of the nuclei labelled with ^{3}H-thymidine. However, in formalin-fixed sections, hydrolysis for even 40 min was insufficient to reveal BrdUrd immunoreactivity (Table 1). This may have been partially caused by nuclear proteins tightly fixed with formalin not being sufficiently removed by the HCl treatment. These proteins may hinder DNA denaturation and/or mask the antigenic sites of BrdUrd in nuclear DNA.

To unmask antigenic sites in formalin-fixed materials, enzymatic digestion is usually performed (Mepham et al. 1979; Sinclair et al. 1981). We applied this method in conjunction with HCl hydrolysis in order to investigate the antigenicity of BrdUrd in formalin-fixed tissues. It was found that enzymatic digestion alone revealed BrdUrd-positive cells in only part of the pyloric mucosa whereas hydrolysis for 20–40 min followed by digestion for 20–40 min resulted in a close similarity between BrdUrd-staining and ^{3}H-thymidine labelling. Moreover, performing the treatments in reverse order, i.e. the digestion followed by hydrolysis, produced similar results, although the BrdUrd staining was slightly less intense. It seems likely that the enzyme may reach and sufficiently unmask the antigenic sites of BrdUrd incorporated into double-stranded DNA without DNA denaturation using HCl, whereas the antibody against BrdUrd may not reach the antigenic sites unless the DNA is uncoiled. Thus, the molecular weight of proteinase must be much smaller than that of IgG.

After digestion alone, BrdUrd-positive cells were not detected in ethanol-fixed tissues. False negative results due to excessive digestion can be ruled out, because digestion followed by HCl hydrolysis revealed the presence of BrdUrd-positive cells. Therefore, further study is required to clarify this point.

References

Dolbeare F, Gratzner H, Pallavicini MG, Gray JW (1983) Flow cytometric measurement of total DNA content and incorporated bromodeoxyuridine. Proc Natl Acad Sci USA 80:5573–5577

Freeman MVR, Beiser SM, Erlanger BF, Miller OJ (1971) Reaction of antinucleoside antibodies with human cells in vitro. Exp Cell Res 69:345–355

Gratzner HG, Pollack A, Ingram DJ, Leif RC (1976) Deoxyribonucleic acid replication in single cells and chromosomes by immunologic techniques. J Histochem Cytochem 24:34–39

Hamada S (1985) A double labeling technique combining ^{3}H-thymidine autoradiography with BrdUrd immunocytochemistry. Acta Histochem Cytochem 18:267–270

Hsu S-M, Reine L, Fanger H (1981) Use of avidine-viotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J Histochem Cytochem 29:577–580

Kirkpatrick P, D'Ardenne AJ (1984) The effect of fixation and enzymatic digestion on the immunohistochemical demonstration of laminin and fibronectin in paraffin embedded tissue. J Clin Pathol 37:639–644

Mepham BL, Frater W, Mitchell BS (1979) The use of proteolytic enzymes to improve immunoglobulin staining by the PAP technique. Histochem J 11:345–357

Morstyn G, Hsu S-M, Kinsella T, Gratzner H, Russo A, Mitchell JB (1983) Bromodeoxyuridine in tumors and chromosomes detected with a monoclonal antibody. J Clin Invest 72:1844–1850

Raza A, Spiridonidis C, Ucar K, Mayers G, Bankert R, Prisler HD (1985) Double labeling of S-phase Murine cells with bromodeoxyuridine and a second DNA-specific Probe. Cancer Res 45:2283–2287

Sinclair RA, Burns J, Dunnill MS (1981) Immunoperoxidase staining of formalin-fixed, paraffin-embedded, human renal biopsies with a comparison of the peroxidase-antiperoxidase (PAP) and indirect methods. J Clin Pathol 34:859–865