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A Rapid, Sensitive and Simple One-stage Immunostaining Procedure for Detection of CA19-9

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SAMMARY

A rapid, sensitive and simple one-stage immunostaining procedure for the detection of antigen such as CA19-9 in the tissues was established in our laboratory. This method was performed by using a mixture of biotin-labelled antibody and avidin-labelled peroxidase. The staining intensity of CA19-9 was increased by mixing these two reagents, resulting in a more sensitive immunostaining procedure than the conventional ABC method.

Key words: CA19-9, Immunostaining, Avidin-biotin

INTRODUCTION

In immunoperoxidase techniques, indirect methods such as peroxidase-anti peroxidase (PAP)(1) or avidin-biotin-peroxidase complex (ABC)(2) are generally used. Various monoclonal antibodies (mAb) including several human mAbs have been established and immunostaining of the tissues using these antibodies are performed extensively in various research fields. Among these mAbs, mAb 19-9(3) has been already adapted in clinical examinations to detect pancreas cancers or other gastrointestinal malignancies both in the sera and in the tissues. In the present study, we have developed a new, sensitive, one-step immunohistochemical staining procedure employing a mixed solution of biotin labelled antibody with horseradish peroxidase-avidin (BAM method) for the detection of an antigen using mAb
19-9 as an example and we have run a comparison with conventional or the two-step ABC(2, 4) method.

MATERIALS AND METHODS

Reagents

Horse biotinylated anti-mouse IgG (heavy chain and light chain), horseradish peroxidase-avidin (HRP-avidin) and ABC reagent were obtained from Vector Lab. N-Hydroxysuccinimide biotin (NHS-biotin) was purchased from Pierce Chemical Co. 3,3'-diaminobenzidine tetrahydrochloride (DAB) was obtained from Sigma Chemical Co., USA.

Biotinylation of mAb 19-9 and mouse IgG

A mAb 19-9 (IgG1), was kindly supplied by Toray-Fuji, Bionics, Inc., Tokyo, Japan and mouse IgG (Cappel, USA) were biotinylated as described by Bayer *et al.*(5). Briefly, 1 mg of mAb 19-9 or mouse IgG in 1 m*l* of 0.1 M $NaHCO_3$ (pH 8.0) was incubated with 60 μl of NHS-biotin solution (1 mg/m*l*) dissolved in dimethyl sulfoxide (DMSO, Merk, West Germany) for 4 h at room temperature and dialyzed against 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS).

Tissue sections

Serial 4 μm-thick paraffin sections of formalin-fixed human pancreas and stomach carcinoma tissues were used for immunohistochemical stainings with mAb 19-9.

Immunostaining procedures

In all methods, the sections were hydrated, treated with 0.3% H_2O_2 in methanol for 20 min and were then incubated with 10% normal bovine serum in PBS for 20 min. After washing by PBS, each method was performed as follows.

In the one-step BAM method, the sections were directly incubated with BAM solution consisting of 2 μg/m*l* of biotin-labelled 19-9 (19-9-biotin) and 2.5 μg/m*l* of HRP-avidin in PBS containing 10% fetal calf serum (FCS) for 90 min. The sections were washed three times for 5 min each in PBS, followed by color development which was achieved by incubating the sections with 0.01% hydrogen peroxide and 0.05% DAB in 0.05 M Tris HCl buffer (pH 7.5) for 10 min.

In the conventional ABC method, the sections were first incubated with 2 μg/m*l* of mAb 19-9 for 90 min, washed, and secondly incubated with 7.5 μg/m*l* of biotinylated anti-mouse IgG in PBS containing 10% FCS for 60 min. Then they were washed and incubated with ABC solution (1 : 100 dilution, as per manufacturer's directions) for 30 min, followed by color development under the same condition as the BAM method. In the two-step ABC method, the sections were incubat-

ed with 2 μg/m*l* of 19-9-biotin for 90 min, washed, incubated with ABC solution for 30 min, and then they were washed and followed by color development under the same condition. All sections were counterstained with hematoxylin, dehydrated and mounted in "Eukitt™" (O. Kindler, West Germany).

RESULTS

Optimization of staining conditions of the BAM methods

In order to obtain the optimum contents of BAM solution, serial sections were stained by BAM solution containing 2, 4 or 8 μg/m*l* 19-9-biotin and 1.25, 2.5, 5, 10, 20 or 40 μg/m*l* of HRP-avidin. The results were summarized in Table 1. When using 8 μg/m*l* of 19-9-biotin, the optimum condition was obtained by mixing 20 μg/m*l* of HRP-avidin. Two μg/m*l* of 19-9-biotin in conjunction with 2.5 or 5 μg/m*l* of HRP-avidin can also be used.

Comparison of the staining results between the BAM and the ABC methods

Serial stomach carcinoma sections were stained by the BAM and ABC methods as shown in Fig. 1. These sections contained both cancerous and non-cancerous areas. In the cancerous area the BAM method gave the strongest staining and the two-step ABC method the weakest. The results of staining intensities in cancerous area of 1 pancreas carcinoma and 5 stomach carcinomas are summarized in Table 2. The BAM method showed the highest sensitive staining in both cancerous tissues among these three methods, and the staining intensity by the two-step ABC methods was the weakest. No nonspecific staining was observed, when 2 μg/m*l* of biotinlyated mouse IgG instead of 19-9-biotin in the BAM and two-step ABC methods, and 2 μg/m*l* of mouse IgG instead of mAb 19-9 in the conventional ABC method were used, respectively.

DISCUSSION

The hybridoma technique has made possible a continuous large supply of

Table 1 *Effect of Contents of BAM Solution.*

Concentration of 19-9-biotin (μg/m*l*)	Concentration of HRP-avidin (μg/m*l*)					
	1.25	2.5	5	10	20	40
2	+*	++	++	+	nd**	nd
4	nd	nd	+++	+++	++	nd
8	nd	nd	nd	+++	++++	+

* Staining intensity graded semiquantitatively as <3% staining of tumor cells (−), 3-30% (+), 30-70% (++), 70-90%(+++), 90< (++++).

** nd: not done

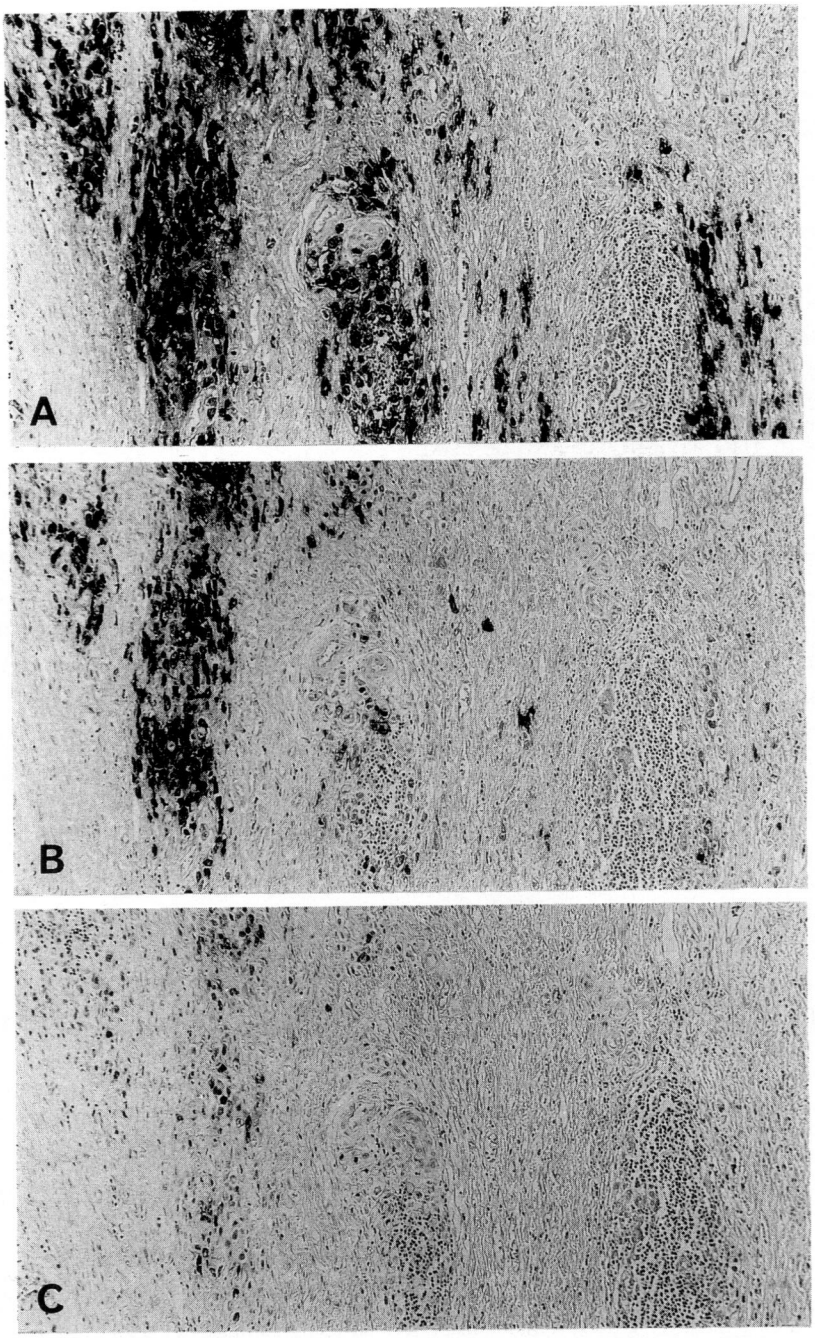

Fig. 1 Serial paraffin sections of a gastric carcinoma stained by BAM (A), conventional ABC (B) and two-step ABC (C) methods. Hematoxylin counterstain, ×25.

Table 2 *Staining Results Using mAb 19-9 by the BAM and ABC Methods.*

Method	Pancreas carcinoma (1)[a]	Stomach carcinoma (5)[a]				
BAM	++[b]	++	+	++	−	−
conventional ABC	+	±	±	+	−	−
two-step ABC	±	−	−	±	−	−

[a] No. of cases tested

[b] Symbols for staining intensity are: −, not stained; ±, staining of <5% of tumor cells; +, 5-50%; ++, >50%

antibodies, which are useful as primary antibodies in immunohistochemical studies. However, the conventional indirect immunoperoxidase methods can not be easily applied to the staining of human tissues with human monoclonal antibodies, since anti-human immunoglobulin antiserum also reacts with immunoglobulins in the tissue of the section. Although this problem can be solved if the human monoclonal antibody can be directly labelled with peroxidase, a large amount of antibodies in a purified form is required which renders it time consuming. Therefore, the development of a sensitive direct-immunoperoxidase methods is urgently needed.

The present report introduces a new one-step immunoperoxidase technique using monoclonal antibody 19-9-biotin as an example. The advantages of this method are as follows:

First, the procedures, including biotinylation of antibodies, are very easy. Second, the BAM method requires only a one step incubation of 90 min, whereas an additional 105 min is required in the case of the conventional ABC method. Third, the staining by the BAM method is more intensive than the conventional ABC method. In addition, this method has a potential use for application to other mAbs (6).

The difference of staining intensities in the ABC methods and the BAM method, was observed, although the same concentration of mAb 19-9 was used in all methods, suggesting that the BAM method can detect CA19-9 which is poorly expressed by the conventional ABC method.

In conclusion, the BAM method is a new one-step immunohistochemical staining, which is easily performed and economic. In addition, it can be useful for detecting CA19-9 in tumor research as well as in clinical applications.

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