A new simplified catalyzed signal amplification system for minimizing non-specific staining in tissues with supersensitive immunohistochemistry*

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Summary. We investigated non-specific staining in a catalyzed reporter deposition (CARD) reaction and improved its blocking methods in supersensitive immunohistochemistry, based on our simplified catalyzed signal amplification (CSA) system (Hasui *et al.* 2002). In the CARD reaction using biotinyl tyramide, non-specific staining could be reduced by pretreatment with a casein solution or 3% bovine serum albumin (BSA)-phosphate buffer saline (PBS) with 0.1% Tween 20. In the CARD reaction using FITC-labeled tyramide, non-specific staining could be blocked by pretreatment with 0.3% BSA-PBS with 0.1% Tween 20 or 3% polyethylene glycol-PBS with 0.1% Tween 20. Thus, our new simplified CSA system features: 1) destruction of the endogenous peroxidase activity; 2) blocking of the non-specific reaction of the primary antibody; 3) a primary antibody reaction; 4) blocking of the non-specific reaction of the polymer reagent by casein treatment; 5) a polymer reaction; 6) blocking of the non-specific reaction of CARD reaction by casein treatment; 7)

a CARD reaction; and 8) detection of deposited tyramide. This new system proved useful for detecting an extremely low amount of antigen in the endogenous biotin-rich tissues such as the gastrointestinal tract and liver. By this method, the Ki67 antigen in the G1 phase cell cycle could be detected and a metabolic disorder of the Ki67 antigen was implicated in a carcinoid tumor in the stomach. We believe that this new simplified CSA system represents a new standard of supersensitive immunohistochemistry for use in light-microscopic investigation.

Introduction

The continuing importance of improving the sensitivity of immunohistochemistry has led to the development of supersensitive immunohistochemistry (Adams, 1992), which is also known as the ImmunoMax method by Merz *et al.* (1995) or the catalyzed signal amplification (CSA) system (Hashizume *et al.*, 2001). This technique basically comprises the following steps: 1) antigen retrieval; 2) the streptavidin-biotin complex (sABC) method; 3) the catalyzed reporter deposition (CARD) reaction of biotinylated tyramide (Bobrow *et al.*, 1989, 1991); and 4) visualization of the CARD reaction. In the course of investigation on the differentiation of adult T-cell leukemia/lymphoma (ATLL) and the other T-cell malignant lymphomas, we encountered the need for an immunohistochemical protocol that would enable the detection of extremely small amounts of human T-cell lymphotropic virus type 1 (HTLV-1)-related proteins on routinely formalin-fixed and paraffin-embedded tissue sections. We therefore introduced a modification of the ImmunoMax method for reducing non-specific endogenous biotin staining, and succeeded in detecting HTLV-1-related

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proteins in many cases of ATLL (Hasui *et al.*, 1997a, b, 2003b; Marin *et al.*, 2002).

However, even when using our modified ImmunoMax method, a high level of non-specific labeling of endogenous biotin was observed, especially in specimens of extranodal tissues (Kim *et al.*, 2002). We therefore developed a protocol (simplified CSA system, Hasui *et al.*, 2002) in which the sABC method was replaced with a polymer method (Sano *et al.*, 1996, Sabattini *et al.*, 1998). Our simplified CSA system proved useful in an automated supersensitive immunohistochemistry system because of its reproducibility with a minimized non-specific reaction of endogenous biotin, although some other systems (e.g. CSA II system comprising the HRP-labeled secondary antibody method and the CARD reaction employing fluorescein isothiocyanate (FITC)-labeled tyramide) were commercially supplied for the same purpose.

Nevertheless, we noticed that some residual non-specific staining was still present especially in specimens of the gastrointestinal tract and in tiny biopsy samples even with the simplified CSA method. On the other hand, we recently noticed a difference in the level and distribution of the non-specific staining between the protocol employing biotinyl tyramide and that employing FITC-labeled tyramide. The reason why the intensity of non-specific staining has not yet been reported in the protocol employing FITC-labeled tyramide (Shindler and Roth, 1996; van Gijlswijk *et al.*, 1997; van Heusden *et al.*, 1997; Reed *et al.*, 1998; van de Corput *et al.*, 1998; Zaidi *et al.*, 2000) was probably that a fluorescence decay of FITC masks the non specific reaction. We considered that this difference in the non-specific staining between the two protocols was attributable to the different substances used to label the tyramine ($C_8H_{11}NO$, molecular weight: 137.2) since the molecular weight of biotin ($C_{12}H_{16}N_2O_3S$, MW: 244.3) is smaller than that of FITC ($C_{21}H_{17}NO_5S$, MW: 389.4).

In this study, we introduced a process in which non-specific staining is blocked by treating sections with casein, non-fat milk, bovine serum albumin, or polyethylene glycol, as a step performed before implementing the CARD reaction in the simplified CSA system. We describe how our new simplified CSA system has been established with the intention of reducing non-specific staining in tissues processed using supersensitive immunohistochemical protocols.

Materials and Methods

Paraffin sections

Sections of 10% formalin-fixed and paraffin-embedded

tissues of the lymph node, the tongue with invasive squamous cell carcinoma (SCC), the liver with hepatocellular carcinoma (HCC), the stomach with carcinoid tumor or invasive adenocarcinoma, and the vermiform appendix were used for this study. The experiment was performed under the consent of the Ethical Committee for Clinical Studies, Kagoshima University Hospital.

Treatment of paraffin sections

After baking the sections at 60°C for 30 min, they were deparaffinized by three rinses in both xylene and then 100% ethanol for 10 min each.

Endogenous peroxidase activity was destroyed by incubating the sections in 0.3% hydrogen peroxide-methanol for 20 min. The sections were then hydrated in phosphate buffered saline (PBS) containing 0.15 M sodium chloride.

As an antigen retrieval protocol, the sections were heated in a 0.01 M citrate buffer pH 6.0 (Target Retrieval Solution, S1700, DakoCytomation, Kyoto) at 121°C for 5 min by an autoclave (Shin *et al.*, 1991; Bankfalvi *et al.*, 1994; Shi *et al.*, 2001). They were cooled and rinsed three times in PBS.

The sections were then set in an autostainer (DAKO autostainer, DakoCytomation). After incubation with each of the appropriate reagents (see below), the sections were rinsed with a solution consisting of a 0.05 M Tris HCl buffer, 0.18 M sodium chloride, and 0.1% Tween 20 (TBST solution), and were warmed to 35°C in a hot water bath (Hasui *et al.*, 1997a, b, 2002, 2003a).

After staining, the sections were removed from the autostainer and dehydrated in 100% ethanol three times for 10 min, incubated with xylene three times for 10 min, and then mounted in a plastic medium.

Detection of Ki67 antigen by means of the polymer method or the horse-radish peroxidase (HRP)-labeled antibody method

Either the polymer method (Dako ChemMate Envision, K5007, DakoCytomation) (Sano *et al.*, 1996; Sabattini *et al.*, 1998) in the simplified CSA system (Hasui *et al.*, 2002) or the HRP-labeled antibody method in CSA II^R (K1497, DakoCytomation) was used to detect Ki67 antigen; this protocol was performed by the autostainer as follows: After deparaffinized, the sections were treated for antigen retrieval and destruction of endogenous peroxidase activity as described above, and were set in the autostainer. The non-specific binding of the primary antibody was blocked by incubating sections for 5 min in a 0.25% casein solution (Non-Specific Staining Blocking

Reagent, X0909, DakoCytomation), followed by blowing off the casein solution. The sections were incubated with the primary antibody (anti-Ki67 antigen antibody, MB-1, M7240, 1:50 dilution, DakoCytomation) for 1 h and then rinsed three times with the TBST solution. The bound primary antibody was visualized by treating it with either horse radish peroxidase (HRP)- and a secondary antibody-labeled polymer reagent (ChemMate Envision, K5027, DakoCytomation) or a HRP-labeled secondary antibody reagent (CSAII, K1497, DakoCytomation), rinsing, and reacting it with a hydrogen peroxide-diaminobenzidine tetrahydrochloride (DAB) solution (DAB+ Liquid System, K3468, DakoCytomation). Nuclear counterstaining was carried out by incubating sections in a hematoxylin solution (Hematoxylin, DAKO ChemMate S2020, DakoCytomation) for 1 min.

Intensity of non-specific staining in tissues processed with the simplified CSA system or the CSA II system, on sections incubated with and without the primary antibody

The simplified CSA system protocol has been introduced previously (Hasui *et al.*, 2002). Briefly, the sections after the above-mentioned pretreatment were set in the autostainer and processed as follows: Residual endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide-PBS (no. 1 bottle in the CSA System, K1500, DakoCytomation) for 5 min. Non-specific binding of the primary antibody was blocked by incubating sections in a 0.25% casein solution (no. 2 bottle in CSA System K1500, DakoCytomation or X0909, DakoCytomation) for 5 min. The sections were then incubated for 15 min or 30 min with either the appropriately diluted (Antibody Diluent, Dako ChemMate, S2022, DakoCytomation) primary antibody anti-Ki67 antigen (MB-1, M7240, 1:50 dilution, DakoCytomation), or in the diluent alone (i.e. no primary antibody). Blocking of non-specific secondary antibody-related reagent binding was performed by incubation with the 0.25% casein solution (X0909, DakoCytomation) for 5 min. Either a polymer reaction (Dako ChemMate Envision, K5027, DakoCytomation) or a reaction of HRP-labeled secondary antibody reagent (CSA II kit, K1497, DakoCytomation), which takes 15 min, was then carried out, followed by the CARD reaction of biotinyl tyramide (no. 8 bottle in CSA System K1500 kit, DakoCytomation) or FITC-labeled tyramide (CSAII kit, K1497, DakoCytomation or Dako GenPoint system, K0618, DakoCytomation), which also takes 15 min. Deposited biotinyl tyramide or FITC-labeled tyramide was detected by incubating the sections with a solution of streptavidin-HRP (no. 9 bottle in CSA System

kit K1500, DakoCytomation) or the HRP-labeled anti-FITC antibody (the solution in Dako GenPoint system kit, K0618, or CSA II kit, K1497, DakoCytomation) for 15 min. Visualization of the HRP was carried out using the hydrogen peroxide-DAB reaction (DAB Liquid System, K3466, DakoCytomation), and subsequent nuclear counterstaining was carried out by incubating the sections in a solution of hematoxylin (ChemMate Hematoxylin, S2020, DakoCytomation).

Detection of endogenous biotin in the simplified CSA system

The simplified CSA system includes a biotin-streptavidin binding reaction (No. 9 bottle in CSA System K1500, DakoCytomation), which allows the detection of deposited biotinylated tyramide after the CARD reaction (Hasui *et al.*, 2002). The endogenous biotin in the simplified CSA system was detected by processes performed after the CARD reaction in the autostainer. The sections were removed from the autostainer after nuclear staining in hematoxylin (ChemMate Hematoxylin, S2020, DakoCytomation).

The new simplified CSA system, we proposed in this study

As shown in Table 1, the new simplified CSA system includes an additional treatment step before the CARD reaction for blocking the non-specific deposition of biotinyl or FITC-labeled tyramide apart from the site of the CARD reaction.

In the case of the system employing biotinyl tyramide, 0.25% casein (X0909, DakoCytomation) was applied to sections before the CARD reaction. In the new simplified system for the anti-Ki67 antigen antibody, the blocking effect on the non-specific staining in the CARD reaction was examined in the following pre-CARD reaction blocking solutions: 0.25% casein solution (no. 2 bottle in CSA System K1500, or Dako Protein Block, X0909, DakoCytomation), 3% bovine serum albumin (BSA)-PBS, 0.25% casein-PBS, 0.25% casein in 1% BSA-PBS, Block Ace (Snow Brand Milk Product Co., Tokyo), Block Ace diluted four times with deionized water, and the above-mentioned solutions with 0.1% Tween 20.

In the case of FITC-labeled tyramide, application of the 0.25% casein solution (X0909, DakoCytomation) was followed by two rinses in the TBST solution before commencement of the CARD reaction. The pretreatment before the CARD reaction with incubating sections in one of the following solutions for 1 min in the new simplified CSA system of the anti-Ki67 antigen antibody, blocking the effect on the non-specific staining in the CARD reaction

Table 1. Protocol of the new simplified CSA system, applied to an autostainer

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- 1) Deparaffinization by incubating sections in xylene (3, 10 min) and in 100% ethanol (3, 10 min);
 - 2) First destruction of endogenous peroxidase activity by incubating sections in 0.3% hydrogen peroxide methanol solution (20 min);
 - 3) Hydration in PBS;
 - 4) Antigen retrieval;
 - 5) Rinsing of sections in PBS and place them in an autostainer;
 - 6) Second destruction of endogenous peroxidase activity by incubating sections in 3% hydrogen peroxide PBS (5 min), then three rinses with TBST warmed to 35°C;
 - 7) Protein blocking to eliminate non-specific binding of the primary antibody by incubating sections in a solution of casein^{#1} and then, evaporating this off; no rinsing is required;
 - 8) Primary antibody reaction for 15 min to 1 h, and three rinses with warmed TBST;
 - 9) Protein blocking to eliminate non-specific binding of the polymer reagent by incubating sections in a solution of casein^{#1}, and then, evaporating it off; no rinsing is required;
 - 10) Polymer reaction (15 min) and three rinses with warmed TBST;
 - 11) Protein blocking to eliminate non-specific staining of the CARD reaction by incubating sections in a solution of casein^{#2}, and then, evaporating it off; no rinsing is required for the CARD reaction with biotinyl tyramide, and two rinses with warmed TBST are required for the CARD reaction with FITC-labeled tyramide;
 - 12) CARD reaction (15 min for biotinyl tyramide or 15 to 30 min for FITC-labeled tyramide), and two rinses by warmed TBST;
 - 13) Streptavidin-horse radish peroxidase (HRP) complex reaction for 15 min or HRP-labeled anti-FITC antibody reaction for 15 to 30 min, and two rinses by warmed TBST;
 - 14) Visualization of the CARD deposition labeled in (13) using hydrogen peroxide and DAB reaction, followed by rinsing with water;
 - 15) Nuclear counterstaining with hematoxylin, one rinse with water, and then removal from the autostainer;
 - 16) One rinse in running water;
 - 17) Dehydration and mounting in a plastic medium.
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Polymer in the 10th process: Secondary antibody- and HRP-labeled polymer agent (Dako ChemMate Envision). The 11th process (Protein blocking) was added to the simplified CSA system. Processes from 6 to 15th were performed by an autostainer.

#1: Non-Specific Staining Blocking reagent, DakoCytomation, or a solution of 3% BSA 0.1% Tween 20. #2: In the case employing FITC-labeled tyramide, the solution can be 0.3% BSA-0.1% Tween 20 -PBS or 3% PEG#20000-0.1% Tween 20-PBS without rinsing.

employing FITC-tyramide was evaluated. One was the solution mentioned above and the other was the solution 10 times diluted with 0.1% Tween 20 in PBS, 0.3% Glycerin-0.1% Tween 20-PBS, 0.3% polyethylene Glycol (PEG)#400-0.1% Tween20-PBS, 3% PEG#1500-0.1% Tween 20-PBS, and 3%PEG#20000-0.1% Tween 20-PBS.

Photomicrographs

Photomicrographs of the specimens shown in the figures were taken with a microscope (BX-50, Olympus) equipped with a digital camera (Fuji HC-300). The exact length of the long axis of the photomicrographs is 430 μm in those taken with a $\times 20$ objective, and 215 μm in those with a $\times 40$ objective.

Fig. 1. Non-specific labeling in sections of the vermiform appendix that had been processed using the simplified CSA system without the primary antibody reaction. These sections were 10% formalin-fixed and paraffin-embedded tissue. ($\times 20$ original objective, $\times 186$ in print). **a:** Tissue processed using biotinyl tyramide in the CARD reaction. **b:** Tissue processed using FITC-labeled tyramide in the CARD reaction. Less non-specific staining is seen in the sections processed using biotinyl-tyramide than in those processed using FITC-labeled tyramide.

Results

Intensity of non-specific staining in the simplified CSA system

In the simplified CSA system without any primary antibody reaction, non-specific staining was evident in tissues of the vermiform appendix (Fig. 1). There was a greater amount of non-specific staining on nuclei, cytoplasm, and other areas in the sections treated with biotinyl tyramide (Fig. 1a) than in those treated with FITC-labeled tyramide (Fig. 1b).

The incubation time of the anti-Ki67 antigen antibody solution was 1 h in the usual immunohistochemistry, when employing the streptavidin-biotin complex method or the polymer method. When the incubation time was 15 min, the simplified CSA system for Ki67 did not yield any obvious non-specific staining in tissues such as the well fixed lymph node, as reported previously (Hasui *et al.*, 2002). Extension of the incubation time of the anti-Ki67 antigen antibody solution to 30 min produced obvious granular or diffuse non-specific labeling even in such tissues.

Reaction of endogenous biotin in the simplified CSA system

The simplified CSA system carries the risk that endogenous

biotin could be detected by the streptavidin-HRP complex reaction that is used to detect deposited biotinyl tyramide. However, the visualization of endogenous biotin detected by the streptavidin-HRP complex reaction was quite low, as can be seen in Figure 2II. Only in the normal areas (Fig. 2IIc) surrounding the hepatocellular carcinoma in the liver biopsy specimen was any background staining of endogenous biotin even somewhat visible in the cytoplasm of the hepatocytes.

Detection of the Ki67 antigen by the polymer method, the simplified CSA system, and the new simplified CSA system

With the exception of the lymph node tissue, a relatively large number of nuclei were labeled with the anti-Ki67 antigen antibody when using the polymer method (Fig. 2I). In the lymph node, only larger nuclei, such as those of the centroblasts in the germinal center, were labeled.

Immunolabeling with the simplified CSA system (Fig. 2III) resulted in the staining of many nuclei, and there was a high level of background stain in all of the sections when the incubation time of the primary antibody reaction lasted 30 min. Particularly in the lymph node were many nuclei of the small lymphocytes located around the germinal center labeled.

Fig. 2. Legend on the opposite page.

Fig. 2. Non-specific labeling other than that of endogenous biotin is observed in tissue processed using the simplified CSA system, and no non-specific labeling is observed in that processed using the new simplified CSA system. ($\times 40$ original objective, $\times 186$ in print). **a:** Lymph node. **b:** Squamous cell carcinoma in the tongue. **c:** Liver, an almost normal area around a hepatocellular carcinoma. **d:** Liver, hepatocellular carcinoma. **e:** Carcinoid tumor in the stomach. **f:** Adenocarcinoma in the stomach. **I:** Polymer method: Detection of the Ki67 antigen by the polymer method, which was incorporated into both the simplified and new simplified CSA systems. The sections were incubated with the primary antibody for 1 h. Nuclei of some small lymphocytes in the germinal center are not labeled with the anti-Ki67 antibody (**a**), whereas relatively many nuclei of carcinoma cells are labeled (**b**, **d** and **f**). **II:** Endogenous biotin: Detection of endogenous biotin by means of the streptavidin-HRP complex, which was incorporated into both the simplified and new simplified CSA systems. Faint staining of endogenous biotin can be noted only in almost normal hepatocytes around the hepatocellular carcinoma (**c**). **III:** Simplified CSA system: Reported in Histochem. J. 34:215 by Hasui *et al.* (2002). Marked excess and non-specific staining is seen. **IV:** New simplified CSA system: The new supersensitive immunohistochemistry reported in this study, employing biotinylated tyramide. In the carcinoid tumor, cytoplasmic and nuclear staining of the anti-Ki67 antigen antibody is noted. **V:** New simplified CSA system employing FITC-labeled tyramide: The pretreatment before the CARD reaction employing FITC-labeled tyramide was the incubation in the protein block solution with two rinses. A remarkable decrease in the immunostain is seen in comparison with that employing biotinyl tyramide (**IV**).

With respect to the new simplified CSA system, more nuclei were labeled in the protocol employing biotinyl tyramide (Fig. 2IV) than in that utilizing FITC-labeled tyramide (Fig. 2V), although the primary antibody incubation time was only 15 min in both systems. In the lymph node sections, the nuclei of small lymphocytes located around the germinal center were not labeled. In the tongue and liver sections, heterochromatin or tiny nucleoli in some of the large nuclei of the squamous cell carcinoma and hepatocellular carcinoma were labeled in tissues processed with the system employing FITC-labeled tyramide, whereas dense labeling of nuclei was found in tissues processed with the system employing biotinyl tyramide. In the sections of the carcinoid tumor of the stomach, weak immunostaining in the cytoplasm was observed in the new simplified CSA system with biotinyl tyramide but not in that with FITC-labeled tyramide (Fig. 2IVe).

Comparison of the CSA II and the new simplified CSA system

The difference in antigen-detecting sensitivity between the CSA II and the new simplified CSA system was also investigated, as shown in Figure 3.

There were fewer nuclei labeled in tissues treated with the HRP-labeled antibody method in the CSA II than in those with the polymer method in the new simplified CSA system (Fig. 3, HRP-labeled antibody method and HRP- and antibody-labeled polymer method) although more labeled nuclei were observed in wider areas with the polymer method than with the HRP-labeled antibody method.

In the tissues treated with the CARD reaction employing biotinyl tyramide and protein blocking for 5 min, after either the HRP-labeled antibody method or the polymer method (Fig. 3, biotinyl tyramide-CARD with protein block), dense and tan-shaded immunostaining of nuclei was obtained in the CSA II, whereas a uniformly dense immunostaining of nuclei was observed in the new simplified CSA system.

In the tissues treated with the CARD reaction employing FITC-labeled tyramide and no protein blocking before the CARD reaction (Fig. 3, FITC tyramide-CARD without protein block), labeled nuclei decreased more in the CSA II than in the new simplified CSA system, when the immunostained nuclei appeared to be those labeled strongly in tissues treated with the CARD reaction employing biotinyl tyramide in the CSA II.

The polymer method was shown to be more sensitive in detecting antigens than the HRP-labeled antibody method. The signal amplification was higher in the CARD reaction

employing biotinyl tyramide than that employing FITC-labeled tyramide. The antigen-detecting sensitivity of the CSA II and the new simplified CSA system was shown to be high in the order of the new simplified CSA system employing biotinyl tyramide, the new simplified CSA system employing FITC-labeled tyramide, the CSA II employing biotinyl tyramide, and the CSA II employing FITC-labeled tyramide.

Blocking effects of non-specific staining in the CARD reaction employing biotinyl tyramide

The following solutions were used as blocking reagents; Dako Protein Block, 3% bovine serum albumin (BSA)-PBS, 0.25% Casein-PBS, 0.25% Casein-1% BSA-PBS, Block Ace, and Block Ace diluted 4 times with deionized water. Blocking effects of the non-specific staining in the new simplified CSA system employing biotinyl tyramide was compared among applications of the solutions for 5 min with or without 0.1% Tween 20, or for 15 min with 0.1% Tween 20, as the pretreatment of the primary antibody reaction, the polymer method, and the CARD reaction.

In the case of 5 min without 0.1% Tween 20 (Fig. 4a), only Dako Protein Block (Fig. 4aI) could suppress the non-specific reaction of the new simplified CSA system.

In the case of 5 min with 0.1% Tween 20 (Fig. 4b), Dako Protein Block slightly suppressed the specific staining (Fig. 4bI), and 3% BSA-PBS could suppress the non-specific staining (Fig. 4bVI), whereas the other solutions could not suppress the latter.

In the case of 15 min with 0.1% Tween 20 (Fig. 4c), Dako Protein Block greatly suppressed the specific staining (Fig. 4cI), 3% BSA-PBS slightly suppressed the specific staining (Fig. 4cVI), and 0.25% Casein-1% BSA-PBS and Block Ace incompletely suppressed the non-specific staining (Fig. 4cII, V), while 0.25% Casein-PBS and Block Ace diluted 4 times with deionized water could not suppress the non-specific staining in the new simplified CSA system (Fig. 4cIII, IV).

Casein was shown to mask the antigen dependently in the incubation time, whereas BSA had less effect on the antigen than casein. In the protocol of the new simplified CSA system employing biotinyl tyramide, the casein solution (Dako Protein Block) and 3% BSA-0.1% Tween 20-PBS could be used as the protein block solution.

Optimal blocking of the non-specific staining in the new simplified CSA system employing FITC-labeled tyramide

There was non-specific staining in the simplified CSA system employing FITC-labeled tyramide as shown in

Fig. 3. Comparison of the CSA II from DakoCytomation and the simplified CSA system (Hasui *et al.*, 2002). ($\times 40$ original objective, $\times 186$ in print). The CSA II comprises an originally HRP-labeled antibody method and the CARD reaction employing FITC-labeled tyramide. The staining in the germinal center is denser in the HRP-labeled antibody method than the polymer method, while the areas of the positive staining are wider in the polymer method than the HRP-labeled antibody method. The shading labeling in the CSA II with the CARD reaction employing biotinyl tyramide with the pretreatment of a solution of casein suggests that signal amplification in the CSA II does not parallel the antigen labeling by the primary antibody in comparison with that in the simplified CSA system (=the new simplified CSA system). This was shown in the cases of the CARD reaction employing FITC-labeled tyramide without the pretreatment of a solution of casein.

Fig. 4 (p.10, 11). Blocking effects of various solutions on the non-specific labeling in the tissues (lymph node with necrotizing lymphadenitis) treated with the new simplified CSA system employing biotinyl tyramide. ($\times 40$ original objective, $\times 186$ in print). The primary antibody was the anti-Ki67 antigen antibody (MIB-1). **I:** Dako Protein Block. **II:** Block Ace. **III:** Block Ace, 4 times diluted with deionized water. **IV:** 0.25% casein-PBS. **V:** 0.25% casein-1% BSA-PBS. **VI:** 3% BSA-PBS. **a:** The pretreatment by the non-specific reaction-blocking solution for 5 min was performed before the primary antibody, the polymer, and the CARD reactions. **b:** The pretreatment by the non-specific reaction-blocking solution containing 0.1% Tween 20 for 5 min was performed before the primary antibody, the polymer, and the CARD reactions. **c:** The pretreatment by the non-specific reaction-blocking solution containing 0.1% Tween 20 for 15 min was performed before the primary antibody, the polymer, and the CARD reactions. **1:** Cortical areas without obvious degeneration in the lymph node. **2:** An area with obvious degeneration in the lymph node. The non-specific labeling can be recognized as granular deposition in the cytoplasm of enlarged macrophages in the figures (2). It was shown that Dako Protein Block (**Ia**) and 3% BSA 0.1% Tween 20 (**VIb**) can be employed as the protein block solution in the new simplified CSA system. A solution of casein including Dako Protein Block (**Ib** and **Ic**) and 0.25% casein (**Vc**) suppresses the specific labeling, depending on the incubation time, whereas BSA does not show any obvious suppression of the specific labeling (**VIb**).

Fig. 4. I-III. Legend on the page 9.

Fig. 4. IV–VI. Legend on the page 9.

Fig. 5. The blocking effects of various solutions on the staining in tissues treated with the new simplified CSA system employing FITC-labeled tyramide. The incubation of the various solutions was for 1 min before the CARD reaction with no associated rinse. The primary antibody was the anti-Ki67 antigen antibody (MIB-1). ($\times 40$ original objective, $\times 186$ in print). The incubation with PBS and TBS (TRIS) removes the residual Tween 20, but there is a smaller amount of the non-specific labeling in the case of TBS than in the case of PBS. 0.025% casein suppresses the specific staining of MIB-1, whereas 0.3% BSA suppresses only the non-specific labeling. In the solutions of chemically stable substances, glycerin, PEG#400, PEG#1500 and PEG#20000, a solution of PEG#1500 suppresses the specific labeling and that of PEG#20000 suppresses the non-specific staining.

Figure 1 although the signal amplification in the CARD reaction employing FITC-labeled tyramide was lower than that employing biotinyl tyramide, as shown above and in Figure 3.

When the incubation time of one of the protein block solutions (Dako Protein Block, 3% BSA-PBS, 0.25% Casein-PBS, 0.25% Casein-1% BSA-PBS, Block Ace, and Block Ace diluted 4 times with deionized water) was reduced to 1 min before the CARD reaction employing FITC-labeled tyramide, the new simplified CSA system of anti-Ki67 antigen antibody did not yield either the specific staining on nuclei or the non-specific staining in the background.

When removing residual Tween 20 by incubating sections in either PBS or TBS for 1 min before the CARD reaction employing FITC-labeled tyramide, there was a specific and smaller amount of non-specific staining in tissues treated with TBS than those with PBS in the new

simplified CSA system, as shown in Figure 5.

When the protein block solutions mentioned above were diluted 10 times with 0.1% Tween 20-PBS and applied for 1 min before the CARD reaction employing FITC-labeled tyramide, only the 0.3% BSA-0.1% Tween 20-PBS suppressed the non-specific staining and did not suppress the specific staining of anti-Ki67 antigen antibody in the new simplified CSA system (Fig. 5, 0.3% BSA-0.1% Tween 20-PBS). The 0.025% casein-0.1% Tween 20 suppressed the specific and non-specific staining (Fig. 5, 0.025% casein-0.1% Tween 20-PBS). The Block Ace diluted 10 times could not suppress the non-specific staining in the tissues treated with the new simplified CSA system employing FITC-labeled tyramide.

Instead of the protein block solutions, we applied a solution of chemically stable substances such as 0.3% Glycerin-0.1% Tween 20-PBS, 0.3% PEG#400-0.1% Tween 20-PBS, 3% PEG#1500-0.1% Tween 20-PBS,

and 3% PEG #20000-0.1% Tween 20-PBS to the protein block before the CARD reaction employing FITC-labeled tyramide in the new simplified CSA system. Among these, 3%PEG#20000-0.1% Tween 20-PBS suppressed the non-specific staining (Fig. 5, 3% PEG #20000-0.1% Tween 20-PBS), and 3% PEG#1500-0.1% Tween 20-PBS suppressed non-specific staining and the specific staining of the anti-Ki67 antigen antibody (Fig. 5, 3% PEG#1500-0.1% Tween 20-PBS), while both the 0.3% Glycerin-0.1% Tween 20-PBS and 0.3% PEG#400-0.1% Tween 20-PBS did not show any effects on the specific and non-specific staining in the new simplified CSA system employing FITC-labeled tyramide.

Discussion

In the present study, we have investigated a method of supersensitive immunohistochemistry to minimize non-specific staining in tissues to propose a new simplified CSA system.

The supersensitive immunohistochemistry protocol with the CARD reaction employing biotinyl tyramide is 1,000 times more sensitive than the sABC method (Merz *et al.*, 1995; Sanno *et al.*, 1996), and has been applied to detect a quite small amount of antigen in such tissues as the pituitary gland, adrenal gland and lymph node, where a non-specific reaction is not induced in supersensitive immunohistochemistry after antigen retrieval (Merz *et al.*, 1995; Sanno *et al.*, 1996; Gresik *et al.*, 1997; Hasui *et al.*, 1997a, b; Malisius *et al.*, 1997; Katano *et al.*, 1999; Tajima *et al.*, 1999; Fayyazi *et al.*, 2000; Hoshino *et al.*, 2000; Lehner *et al.*, 2000; Katano *et al.*, 2001; Oertel *et al.*, 2002). On the other hand, supersensitive immunohistochemistry with a CARD reaction employing fluorochrome-labeled tyramide has been introduced for double immunostaining, by which one antigen can be detected by a routine immunohistochemistry and the other can be visualized by the supersensitive immunohistochemistry (Hunyady *et al.*, 1996; Shindler and Roth, 1996), and for the *in-situ* hybridization since its signal is quite low (van Gijlswijk *et al.*, 1997; van de Corput *et al.*, 1998; Zaidi *et al.*, 2000). It has been also applied to the immunohistochemical detection of lectin (Kressel, 1998) and to the field of electron microscope immunohistochemistry (Mayer and Bendayan, 1997). However, application of supersensitive immunohistochemistry employing the CARD reaction seems to be limited because of the presence of non-specific staining in those tissues treated with it (von Wasielewski *et al.*, 1997).

The ImmunoMax method is a protocol for supersensitive immunohistochemistry (Merz *et al.*, 1995) and was

introduced in 1995 in our laboratory by Hasui in collaboration with the Institute of Pathology (Prof. A.C. Feller and Dr. H. Merz) at Leubeck Medical University. This original ImmunoMax method employed animal serum to block the non-specific binding of antibodies, while a serum-free casein solution followed by washing in TBST with a high salt concentration is advised in the standard CSA system. In the course of minimizing the non-specific staining in the ImmunoMax method, we noticed the following phenomena: 1) Non-specific staining varies with the type of antigen retrieval solution (Hasui *et al.*, 1995) and is amplified by two times treatment of peroxidase reaction and biotin-streptavidin binding reaction in the protocol (Hasui *et al.*, 1997a); 2) the post-reaction wash in TBST at 37°C diminishes CARD reaction products, while that in PBS at room temperature does not (Hasui *et al.*, 1997a). Thus, we introduced a two-step treatment for destroying endogenous peroxidase activity before and after the antigen retrieval, endogenous biotin masking, and a post-CARD reaction wash in PBS in a modified ImmunoMax method (Hasui *et al.*, 1997a). However, the endogenous biotin masking in this modified method was not usually effective for diminishing the non-specific staining. It is true that Kim *et al.* (2003) reported the effectiveness of various solutions for blocking non-specific labeling in the CSA system, but they did not try to replace the sABC method in the CSA system with a Zymed non-biotin amplification system (NBA™ Kit, Zymed Laboratories, CA 94080, USA), which comprises a FITC-labeled secondary antibody reaction and an HRP-labeled anti-FITC antibody reaction. Thus, we replaced the sABC method in the protocol with the polymer method using a HRP- and secondary antibody-labeled polymer (as a simplified CSA system, Hasui *et al.*, 2002) and succeeded in reducing the non-specific labeling of endogenous biotin. However, in spite of the reduced non-specific labeling of endogenous biotin, we still encountered strong non-specific staining in the simplified CSA system, especially in the biopsy specimens, although this was not common in well-fixed biopsy specimens (Hasui *et al.*, 2002). To overcome this problem, the present study has compared the simplified CSA system employing biotinyl-tyramide with that employing FITC-labeled tyramide and found that the former yields a larger amount of non-specific labeling than the latter.

Products of the CARD reaction are deposited near the reaction site where HRP catalyzes tyramide. It should be noted that the post-reaction wash was performed with PBS at room temperature in the modified ImmunoMax method (Hasui *et al.*, 1997a), while sections were only rinsed twice in warmed TBST in the simplified CSA system (Hasui *et al.*, 2002); we thought that the non-specific labeling might appear on tissues treated with the complete post-reaction

wash in the simplified CSA system because of the removal of the casein and other substances that bind catalyzed biotinyl or FITC-labeled tyramide. We therefore introduced a casein solution treatment before the CARD reaction in a simplified CSA system (a new simplified CSA system), and succeeded in removing the non-specific staining in the protocol using biotinyl tyramide. However, this treatment suppressed completely the deposition of catalyzed FITC-labeled tyramide. Double rinses with TBST after the casein treatment before the CARD reaction resulted in positive immunostaining in the new simplified CSA system employing FITC-labeled tyramide although detection of the antigen was still less sensitive than in the new simplified CSA system with biotinyl tyramide.

In the present study, we also examined the applicability of protein blocking solutions other than a casein solution (Dako Protein Block) to the new simplified CSA system employing biotinyl tyramide. Our results indicated that 3% BSA-0.1% Tween 20-PBS can be useful in the new simplified CSA system as well as Dako Protein Block. The others could not be applied to the new simplified CSA system although they were known to be effective in routine immunohistochemistry. The present study further revealed that the protein blocking ability of a casein solution is dependent on the incubation time and can suppress the specific binding of the antibody in a long incubation, whereas BSA does not.

Signal amplification of the CARD reaction with FITC-labeled tyramide is lower than that with biotinyl tyramide. While the pretreatment of casein with a double rinse of TBST does not suppress the CARD reaction with FITC-labeled tyramide, there is a possibility that the HRP- and antibody-labeled polymers that react with primary antibodies are partially washed out by the double rinse of TBST. We thus examined the effects of incubation solutions on the new simplified CSA system employing FITC-labeled tyramide, by replacing the protein block before the CARD reaction with a 1-min incubation of various solutions. The results showed that the incubation of tissues in TBS prevents the non-specific labeling more efficiently than in PBS in the new simplified CSA system employing FITC-labeled tyramide. The solutions diluted 10 times with 0.1% Tween 20-PBS, 0.3% BSA-0.1% Tween 20-PBS suppressed only the non-specific labeling, while 0.025% casein-0.1% Tween 20-PBS also suppressed the specific labeling of the primary antibody. This suggests that casein suppresses not only the non-specific labeling but also the specific binding of an antibody. Deposition of the catalyzed tyramide is non-specific rather than specific with tyrosine and tyrosine-like substances in tissues, so that the molecular size of substances in the solvent affects the deposition of the catalyzed tyramide. Because tyramine

is catalyzed by HRP to yield a homo- or heterodimer with tyrosine and to bind related compound (Gross and Sizer, 1959), the molecular weight of the deposited tyramide is larger in the FITC-labeled tyramide (about $1053 = 2 \times (137.2 + 389.4)$) than in the biotinyl tyramide (about $763 = 2 \times (137.2 + 244.3)$). Since solutions of chemically stable substances differ in molecular weight from each other, we prepared 0.1% Tween 20-PBS solutions containing either 0.3% glycerin (Molecular weight: 92.1), 0.3% PEG#400 (Molecular weight: about 400), 3% PEG#1500 (Molecular weight: about 1500) or 3% PEG#20000 (Molecular weight: about 20000). Our findings indicate that deposition of the catalyzed FITC-labeled tyramide is competitively disturbed in the solutions of substances, whose molecular weight is about 1500.

The non-specific staining that results from the combination of the sABC method and the CARD reaction was eliminated in this new simplified CSA system. The new simplified CSA system with biotinyl tyramide maintained the supersensitive antigen detection that was reported in the ImmunoMax method by Merz *et al.* (1995). Although both the sABC method with the CARD reaction employing FITC-labeled tyramide and the CSA II supplied from DakoCytomation are also free from the non-specific staining, their antigen detection is less supersensitive than the new simplified CSA system for the following reasons: 1) The polymer method is as sensitive as the sABC method (Hasui *et al.*, 2002). 2) As shown in the present study, the HRP-labeled antibody method in the CSA II is less sensitive than the polymer method although the former produces a higher contrast of staining than the latter; the binding ratio of the labeled HRP to the secondary antibody is lower in the HRP-labeled antibody method than in the polymer method. 3) The signal amplification in the CARD reaction with FITC-labeled tyramide is lower than that in the protocol using biotinyl tyramide, as mentioned above. 4) The sABC with the CARD reaction using FITC-labeled tyramide might be free from the non-specific staining in the CARD reaction because of the low signal amplification in the CARD reaction using FITC-labeled tyramide. Thus, we consider that the newly simplified CSA system is currently the most supersensitive indirect immunohistochemistry free of the non-specific labeling that derives from the methodological problems with a light microscope.

There remains non-specific labeling that comes from the antigen retrieval, the incubation time of the primary antibody reaction, and the dilution of the primary antibody solution. To be exact, optimal antigen retrieval must be investigated for each antigen (Shi *et al.*, 2001). When a usual dilution of the primary antibody solution is applied to the new simplified CSA system, the incubation time of the primary antibody reaction is only 15 min in the case

of anti-Ki67 antigen antibody. In the other words, in the case of setting the incubation time constantly at 1 h in the newly simplified CSA system, the optimal dilution of the primary antibody must be determined (von Wasielewski *et al.*, 1997).

The protocol of the new simplified CSA system proposed in this study is applicable to the autostainer. We consider this system capable of opening a new field of light-microscope-based supersensitive immunohistochemistry. As shown in this study, Ki67 immunostaining was revealed in cytoplasm of carcinoid tumor cells in the stomach after processing with our new system, something which was never seen with the previous polymer method. We believe that the presence of the Ki67 antigen in the cytoplasm reflects a metabolic disorder in these cells although further studies are waited to confirm this hypothesis. Thus, the new simplified CSA system is expected to reveal abnormalities in the proliferation signal transduction system, detecting the abnormal expression of a small amount of the molecules involved in it, as has been reported for cyclin D1 (Korin *et al.*, 2000; Oertel *et al.*, 2002).

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