New monoclonal antibody (AIC) identifies interstitial cells of Cajal in the musculature of the mouse gastrointestinal tract

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Abbreviations: ICC, Interstitial cells of Cajal; GI tract, gastrointestinal tract; AIC, anti-ICC antibody; ICC-MY, ICC surrounded myenteric plexus; ICC-IM, ICC in the muscularis; ICC-DMP, ICC at the deep muscular plexus; ICC-SM, ICC at the submucosal border; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin

Abstract

Interstitial cells of Cajal (ICC) are pacemaker cells for the spontaneous muscular contractions and neuromodulators that mediate neurotransmission from enteric neurons to smooth muscle cells in the gastrointestinal (GI) tract. They express c-Kit, and the antibody for c-Kit (especially ACK2) has been a useful tool for functional and morphological studies. ACK2, however, does not work on tissues fixed with paraformaldehyde and not all ICC express c-Kit in human. Therefore, in order to find a new marker of ICC and/or new antibody resisting aldehyde fixation we produced a new monoclonal antibody that identifies ICC and then investigated the properties of its antigen. Isolated ICC were used for immunization. Hybridomas fused with myeloma SP2 were screened by immunohistochemistry. ACK2 and each antibody were applied on serial sections, and the clone producing antibody AIC that stains ICC was established. The distribution of AIC immunopositive cells were examined in other organs and also GI muscles of *W/Wv* mice. The biochemical properties were studied using dot blot analysis. AIC recognized ICC, however, distribution of immunopositive cells in *W/Wv* mice and other organs was different from that of c-Kit. The immunoreactivity was stable for paraformaldehyde but was blocked by either Triton X-100 or SDS. In conclusion new antibody AIC recognized ICC but the antigen was not c-Kit, which confirms the existence of good markers of ICC besides c-Kit. Although the antigen has not been isolated, AIC is suitable for morphological study and useful for investigation of ICC in c-Kit mutants.

Keywords

Interstitial cells of Cajal, monoclonal antibody, c-Kit, gastrointestinal tract, c-Kit mutant

Introduction

Interstitial cells of Cajal (ICC) constitute a unique cell population in the musculature of the gastrointestinal (GI) tract and play important roles as the pacemaker cells of spontaneous muscle contractions and as the mediators of neurotransmission. A subpopulation of ICC generates slow waves and propagates them to smooth muscles. Others mediate and control neurotransmission from the enteric neurons to the smooth muscles (Sanders et al., 1999; Ward and Sanders, 2001). ICC are required for gastrointestinal motility, and any deficiency of ICC caused by pathological states, such as diabetes, hypertrophic pyloric stenosis, Chagastic megacolon, idiopathic gastroparesis or inflammation, renders pacemaking activity and neurotransmission dysfunctional as the contractions come for smooth muscle that are coordinated by ICC (Vanderwinden et al., 1996; Hagger et al., 2000; Ördög et al., 2000; Der et al., 2000; Camilleri, 2002; Wang et al., 2002; Zarate et al., 2003).

The investigation of ICC has made major advances since it was recognized that ICC express a proto-oncogene *c-kit* and its product c-Kit receptor. Most ICC differentiate using c-Kit signals. When c-Kit is blocked during differentiation, most ICC can not develop normally and disappear from the musculature (Ward et al., 1994; Torihashi et al., 1995; Huizinga et al., 1995). c-Kit is not only required for normal differentiation of ICC but it also serves as a useful marker for ICC. Thus, the immunohistochemistry for c-Kit has been a useful tool for identifying ICC. Among anti-c-Kit antibodies, ACK2 (anti-mouse c-Kit antibody) has contributed greatly to morphological and physiological studies of ICC. ACK2 blocks the development of ICC as a neutral antibody and then ICC disappear (Maeda et al., 1992; Torihashi et al., 1999b). In this state, the musculature of the GI tract loses both slow waves and spontaneous contractions. ACK2 has demonstrated physiological functions of ICC as

well as their morphology.

Although anti-c-Kit antibodies have advanced the investigation of ICC, it is still unclear whether all ICC express c-Kit equally. In fact, in the human small intestine, ICC at the level of the deep muscular plexus scarcely express c-Kit (Torihashi et al., 1999a; Vanderwinden and Rumessen, 1999). It is still unclear in c-Kit mutant animals whether ICC do not exist at all, or whether c-Kit-negative ICC exist (Klüppel et al., 1998; Torihashi et al., 1999b). To solve these questions we need another marker for ICC, and antibodies other than anti-c-Kit antibody are required. Production of the antibodies may also lead the finding of new molecules specifically expressed in ICC.

In this study we have raised a new monoclonal anti-ICC antibody, to which we have given acronym AIC, that specifically labels ICC in the musculature of the mouse GI tract, and we have described its properties. Although we could not identify the antigen of AIC, it is not c-Kit, and the distribution of ICC detected by AIC in *W/Wv* mice, was different from the immunohistochemistry of c-Kit. The identification of ICC by AIC in c-Kit mutants may enable us to better understand the fate of ICC precursors when the c-Kit signal is compromised. Furthermore, AIC is particularly suitable for morphological study compared to most anti-c-Kit antibodies because the antigenicity is retained even after paraformaldehyde fixation. These results indicate that this new monoclonal antibody, AIC, is useful in the investigation of ICC.

Materials and Methods

Production of hybridoma cells secreting AIC, a rat anti-mouse ICC monoclonal antibody

Cells were separated by 1.3 mg/mL collagenase (Wako, Osaka Japan) from the small intestines of 6- to 10-day-old BALB/c mice. c-Kit-positive cells were then collected by a magnetic micro bead-sorting system, miniMACS (Miltenyi Biotec, CA), using anti-c-Kit antibody ACK2 (gift from Dr. Nishi), and the beads labeled with anti-rat IgG. The clone establishment mainly followed the method reported by Kishiro (Kishiro et al., 1994). Briefly, c-Kit-positive cells were mixed with Freund's complete adjuvant and were injected into the toe pads of WKY/NCrj rats. After three weeks of immunization, lymphocytes from the iliac lymph nodes were collected and fused with myeloma cells (SP2/O-Ag14). Screening of hybridoma cells was performed by immunohistochemistry using frozen sections of the mouse GI tracts, and a hybridoma cell line producing AIC was isolated and cloned. Antibody AIC was collected from the ascites of nude mice into which hybridoma cells were injected, and was then concentrated. The subclass of antibody was examined and determined to be IgG2a by immunoblotting and immunohistochemistry using rat Mono AB ID/SP kit (Zymed Lab, CA).

Immunohistochemistry

Mouse tissues (BALB/c) were dissected after Zamboni's solution was perfused from the heart, and were fixed for 3 h in the same fixative. Fresh tissue of the GI tracts from BALB/c and *W/Wv* mutant mice were also dissected and fixed in ice-cold acetone for 5 min. Fixed samples were embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan), and frozen sections (6 μ m thickness) were stained with AIC or ACK2.

Serial sections of acetone-fixed GI tracts were stained with ACK2 (1:400) and AIC (1:4000), respectively. Negative controls were prepared by omitting primary antibodies. For whole mount preparations, muscle layers of the small intestine were separated, stretched, fixed with Zamboni's solution, and prepared for immunohistochemistry without any detergent (e.g., Triton X-100, etc). Primary antibodies were treated overnight at 4°C, and immunoreactivities were detected with Cy 3-conjugated affinity-purified goat anti-rat IgG (1:400, Jackson Immunoresearch Lab, PA), Alexa 594-conjugated affinity-purified goat anti-rat IgG (Molecular Probes, OR) for 1 h, or with a combination of biotin-conjugated anti-rat IgG (1:200, Vector, CA) for 1 h and treatment with avidine-conjugated Texas Red (1:200, Vector) for 1 h. Nuclear counterstaining by DAPI (0.1 μg/mL, ICN, CA) was also used on some sections. Confocal images and double staining of fluorescent micrographs were respectively obtained by LSM 5 PASCAL (Zeiss, Germany) and Axiophot2 (Zeiss, Germany) with a digital camera AxioCam (Zeiss, Germany).

Immunoelectron microscopy

Mouse small intestines were perfused for 5 min with Zamboni's solution containing 0.5% glutaraldehyde. They were dissected, cut into small pieces, and treated with another fixation for 30 min without glutaraldehyde. Samples were infused with a mixture of sucrose and polyvinylpyrrolidone (Sigma, MO) and frozen quickly with liquid nitrogen. Ultrathin cryo-sections were stained with AIC (1:2000) and processed for indirect immunostaining with 10 nm colloidal gold-conjugated goat anti-rat IgG (Amersham Bioscience, NJ). As a negative control, the primary antibody was omitted. Sections were fixed again with 2% glutaraldehyde for 10 min and then embedded in a mixture of 2% methylcellulose (Nacalai Tesque, Kyoto、 Japan) and 0.5% uranyl

acetate. Sections were examined with an electron microscope (Hitachi H-7100; Tokyo, Japan).

Chemical properties of antigen examined by dot blot analysis

To determine whether AIC can be used for western blottings, dot blot analysis was used. The homogenized tissue lysate (10 μg protein/mL in PBS) of mouse small intestine was treated with sodium dodecyl sulfate (SDS, Sigma), Triton-X100 (Yoneyama, Osaka, Japan) or CelLytic MT (Sigma) at 4°C or room temperature and blotted onto nitrocellulose membrane. Bovine serum albumin (BSA, Wako Osaka, Japan; 10 μg/mL in PBS) treated with Triton-X100 was also blotted as a control. They were probed with AIC or anti-BSA antibody (1:2000; Biomeda, CA), respectively. The blotted membranes were then incubated with peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Lab, PA) or goat anti-rabbit IgG (Amersham Bioscience, NJ) respectively. Reactions were visualized using Supersignal West Dura extended duration substrate (Pierce, IL). Negative controls were treated without primary antibodies (e.g., AIC and anti-BSA antibody).

Results

1. Immunoreactivity to ICC

Cells showing AIC immunoreactivity in the mouse GI tract were compared with c-Kit immunopositive cells stained with anti-c-Kit antibody (ACK2) in the serial sections to determine whether AIC recognized ICC. In the stomach, c-Kit immunopositive cells indicating ICC surrounded myenteric plexus (ICC-MY) at the pylorus and corpus, and positive cells were also distributed in the muscularis (ICC-IM) throughout the stomach. AIC immunopositive cells in the serial sections showed exactly

the same distribution as that of c-Kit immunopositive cells (Fig. 1A, B). In the small intestine ICC shown by c-Kit immunohistochemistry were located at both levels of the myenteric plexus and the deep muscular plexus. It was confirmed that AIC immunopositive cells were the same cells showing c-Kit immunoreactivity in serial sections (Fig. 1 C, D). ICC in the colon stained with ACK2 were also stained with AIC in their serial sections (data not shown). Faithful match of ACK2 and AIC immunopositive cells clearly showed that monoclonal antibody AIC recognized ICC.

It is well known that ICC demonstrate the unique three-dimensional features shown in whole mount preparations. AIC immunopositive cells in the small intestine displayed the typical shape of ICC in such whole mount preparations (Fig. 1E, F). At the myenteric plexus level, AIC positive cells were multi-polar in shape and formed a cellar network as demonstrated by c-Kit immunohistochemistry. ICC at the deep muscular plexus (ICC-DMP) ran parallel to neighboring smooth muscle cells, indicating a bipolar cell type. AIC immunopositive cells in the same area showed a shape similar to that of ICC-DMP (Fig. 1F).

We used both ACK2 and AIC to stain ICC in c-Kit mutant animals. As already described, the distribution of ICC indicated by ACK2 in c-Kit mutant *W/Wv* mice was different from that in wild type mice. In the small intestine, ICC-MY were greatly reduced in number, suggesting their eventual disappearance, while IC-DMP was not much different from the wild type (Fig. 2A). On the other hand, immunohistochemistry using AIC demonstrated a different distribution pattern from that shown by ACK2. Although fewer than in the wild type mice, AIC positive cells at the myenteric plexus exceeded that of c-Kit positive cells stained with ACK2 (Fig. 2B). By the same token, ICC-IM in both the stomach and colon of *W/Wv* animals disappeared from the tissues stained with anti-c-Kit antibody. AIC, however, demonstrated some ICC-IM in those

tissues (Fig. 2C and D). Our data suggest that some c-Kit negative ICC or cells that had failed to differentiate to ICC in *W/Wv* animals could be recognized with AIC.

2. Distribution of AIC immunopositive cells in the mouse GI tract

In the stomach most AIC positive cells were ICC located in the muscle layer as mentioned above. Besides ICC some epithelial cells showed weak immunoreactivity on their microvilli (Fig. 3A). On the other hand, the microvilli of columnar epithelial cells in the small intestine exhibited strong immunoreactivity as well as ICC in the muscle layer (Fig. 3B). In the colon some epithelial cells in the crypts showed AIC immunoreactivity on their microvilli in addition to ICC in the muscle layer (Fig. 3C). The immunoreactivity of AIC in ICC in the colon was more intense compared to ICC in other regions. Mast cells in the lamina propria, which were c-Kit immunopositive and prominent in GI tracts, were not labeled by AIC immunohistochemistry.

3. Distribution of AIC immunopositive cells in other organs and tissues

AIC immunoreactivities were demonstrated not only in GI tracts but also in several organs and tissues as summarized in Table 1. Although no immunoreactivity was detected in the pancreas, it was observed in hepatocytes in the liver. AIC stained hepatocytes at their surfaces along the bile canaliculi (Fig. 4A). Cells in the theca interna in the ovary showed AIC immunoreactivity (Fig. 4B). In the cortex of the thymus epithelial reticular cells were AIC immunopositive (Fig. 4C). The cortex of the kidney showed strong immunoreactivity in the renal corpuscle (glomerulus and glomerular capsule) and on the brush border of the proximal tube (Fig. 4D). The endothelium of the capillaries and arterioles in the brain as well as the endothelium of the central artery in the spleen showed immunoreactivity. Muscles cells, i.e., cardiac, skeletal and smooth muscles, were not stained with AIC.

In the testis, where c-Kit immunopositive cells are usually distributed, Leydig cells, some spermatogonia and spermatocytes were ACK2 immunopositive (Fig. 4E). On the other hand, AIC did not recognize any cells in the testis as shown in Fig. 4F. Thus, the immunoreactivity of AIC except for ICC was different from that of ACK2.

4. Intracellular localization of AIC immunoreactivity

Immunoelectron microscopy demonstrated a fine localization of AIC immunoreactivity in ICC and the epithelium of the GI tract. ICC with many mitochondria and gap junctions showed immunoreactivity on their cell membranes (Fig. 5A). Epithelia of the small intestine also showed strong immunoreactivity on their microvilli just outside the cell membrane (Fig. 5B and C). The distribution of the immunoreactivity indicated that antigens of AIC were located at the cell surface of ICC and microvilli in the GI tract epithelium.

5. Chemical properties of the antigen

AIC did not give positive labeling in western blotting. To analyze the chemical properties of the antigen, we performed labeling of dot blots on nitrocellulose membranes (10 μg protein / drop). The results are shown in Figure 6. Homogenized fresh epithelium of the small intestine (1 μg protein / μL in PBS) showed strong immunoreactivity. Even samples taken from the tissue fixed with 4% paraformaldehyde for 30 min showed strong immunoreactivity. However, samples mixed with 2% SDS lost immunoreactivity. Treatment with 1% SDS at 4°C did not retain immunoreactivity, indicating that SDS denatured the antigen structure and destroyed its immunoreactivity (Fig. 6A).

A non-ionic detergent, 1% TritonX-100, also abolished immunoreactivity of

AIC, and even 0.5% TritonX-100 decreased it. The same treatment did not interfere with labeling by anti-BSA antibody, indicating that the effect is specific to the antigen of AIC. A commercial solvent CelLytic MT, reduced immunoreactivity but did not abolish it completely. Samples solubilized by CelLytic MT, however, did not show immunoreactivity when SDS was added (Fig. 6B).

Discussion

AIC identifies ICC in the mouse GI tract

Since both AIC and anti-c-Kit antibody ACK2 are rat monoclonal antibodies, they could not be used for double staining to compare immunopositive cells. The immunoreactivities of the two antibodies, therefore, were examined by staining serial sections of the muscle layer of the GI tracts from the stomach to the colon. All the cells in the muscle layer labeled by the two antibodies matched faithfully each other. The whole mount preparations stained with AIC and ACK2 also demonstrated the same cellular network at the myenteric plexus level and in bipolar cells in the muscle layer (Ward et al., 1995, 2000). Based on these results, we concluded that AIC recognizes ICC in the muscle layer of the mouse GI tract.

On the other hand, ACK2 and AIC showed different immunoreactivities in ICC in the c-Kit mutant *W/Wv* mice (Huizinga et al., 1995; Burns et al., 1996; Seki and Komuro, 2002). AIC demonstrated more positive cells than ACk2, especially on ICC-MY in the small intestine and ICC-IM in the stomach and the colon. Although ICC did not express c-Kit immunoreactivity in the c-Kit mutant, some cells appeared to persist. These residual cells may be recognized by AIC, suggesting that AIC is useful to study the fate of ICC in c-Kit mutant animals.

AIC showed no immunoreactivity in the lamina propria where mast cells are

usually located. AIC, however, showed strong immunoreactivity in the microvilli in the small intestine. The differential distribution of immunoreactivity in the epithelium and lamina propria suggests that the epitope of AIC is different from c-Kit. For the investigation of ICC, AIC has an advantage over ACK2 in that it does not stain mast cells. Under pathological conditions mast cells sometimes migrate into the muscle layer, and their immunoreactivity for c-Kit can be confused with that of ICC (Malandrini et al., 2000; Demirbilek et al., 2001; Pinheiro et al., 2003). In this report, AIC clearly distinguished ICC from mast cells.

The other advantage of AIC is that it can be used for immunohistochemistry of aldehyde-fixed tissues. AIC give positive labeling in tissues fixed with either acetone or aldehyde fixatives including Zamboni and glutaraldehyde. Aldehyde is better to maintain fine structures than acetone and more suitable for morphological studies. This property enables us to exploit AIC for double staining with a variety of antibodies and for immunoelectron microscopy. In the present study, localization of AIC immunoreactivity was demonstrated by immunoelectron microscopy to be located on the cell surface. These properties showed that AIC is useful for morphological investigations of ICC both at the light and electron microscopic levels.

Immunoreactivity of AIC in other organs, and antigen properties

AIC showed positive labeling in several organs and tissues other than the GI tract, which are listed in Table 1. On the other hand, anti-c-Kit antibody labeled mast cells, hematopoietic stem cells, progenitors of spermatids, Leydig cells in the testis, melanocytes, and certain neurons in the brain (Nishikawa et al., 1991; Yoshinaga et al., 1991; Manova et al., 1992, 1993; Horie et al., 1993; Lammie et al., 1994). Obviously, AIC and anti-c-Kit antibody do not label the same cell population except for ICC. The

results strongly suggest that the antigen of AIC is not c-Kit and also that there is some ICC-specific antigen besides c-Kit. The intense AIC immunoreactivity in microvilli of the small intestine, the brush border of the renal tubes, the bile canaliculi in the liver, and its particular sub-cellular localization, together suggest that the antigen of AIC may be a membrane molecule.

Dot blot analysis showed that the antigenicity recognized by AIC is susceptible to SDS, Triton X-100, other non-ionic (Tween 20, Nonident P-40), and ionic (CHAPS) detergents (data not shown). This property may suggest that the epitope of AIC is based on a delicate three-dimensional structure of a molecule, which may be easily disrupted by any detergent treatment. It is also possible that AIC recognizes either lipid or a carbohydrate chain. But the persistence of reactivity in acetone-fixed tissue makes the former possibility unlikely. The latter possibility is also slim, because the reactivity was quite susceptible to heating (not shown). AIC proved unsuitable for biochemical analysis, and consequently we could not examine the molecular structure of the antigen.

In conclusion we developed a new monoclonal antibody, AIC, that recognized ICC in mice GI tracts. Although the antigen of AIC could not be determined, it is clear that the antigen is not c-Kit, suggesting that ICC contain a specific substance other than c-Kit. AIC is suitable for morphological studies especially for immunoelectron microscopy because its immunoreactivity is highly resistant to aldehyde fixatives. Therefore, AIC should advance ultra structural studies of ICC. This antibody is also valuable for the study of ICC in c-Kit mutant animals.

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Figure Legends

Fig. 1

Verification of AIC immunoreactivity on ICC.

A: In the pylorus many AIC immunopositive cells are located between the circular and longitudinal muscle layers, and some of them surround myenteric ganglia (Ga). Positive cells are also distributed in the muscle layers. Their distribution is compared to that in the serial sections stained with anti-c-Kit antibody shown in B. **B**: Cells stained with the anti-c-Kit antibody, ACK2, show a distribution similar to those in the serial section stained with AIC shown in A. Typical cells stained with both antibodies are indicated by arrows in A and B. **C**: In the small intestine, AIC positive cells are located at the level of both the myenteric plexus (My) and the deep muscular plexus (Dp). Their distribution is compared to that in the serial sections stained with anti-c-Kit antibody shown in D. **D**: Immunohistochemistry for ACK2 in the serial sections confirms that AIC positive cells are ICC. Arrows indicate cells in C and D clearly stained with both AIC and ACK2. **E**: The whole mount preparation of the muscle layer of the small intestine stained with AIC shows characteristic network of ICC-MY around the myenteric plexus. Positive cells are of the multi-polar cell type, and their branching processes contact each other to form a characteristic network. **F**: Cells at the deep muscular plexus of the whole mount preparation in the small intestine stained with AIC also show typical shapes of ICC-DMP. Most are bi-polar cell types that run parallel to surrounding circular muscles. **G**: Control sample treated without primary antibodies, showing no specific immunoreactivity. The scale bars in A and E apply to A-D and E-F, respectively.

Fig. 2

Distribution of AIC and ACK2 immunopositive cells in c-Kit mutants.

A: ICC in the small intestine of a *W/Wv* mouse stained with ACK2 are greatly reduced in number and only ICC-DMP (Dp, arrowheads) remain. B: Note that some but not all ICC-MY (My, arrows) are recognized with AIC in the small intestine of a *W/Wv* mouse. ICC-DMP (Dp, arrowheads) are also distributed in the same manner as those stained with ACK2 in A. C: In the pylorus ICC-MY (My) are normally stained with ACK2, ICC-IM distributed in the muscle layer have completely disappeared. D: AIC demonstrates not only ICC-MY (My) but also some ICC-IM (Im) in the muscle layer, which were not stained with ACK2 in the pylorus of a *W/Wv* animal (see C).

Fig. 3

Immunohistochemistry of AIC in the mouse GI tract.

A: In the pylorus immunoreactivity is demonstrated in ICC-MY (My) and ICC-IM (Im) of the muscle layer. Microvilli of some epithelial cells in the mucosae (Mu) also express immunoreactivity. **B**: In the small intestine the brush border of the epithelium in the mucosae (Mu) expresses strong immunoreactivity except for ICC-MY (My) and ICC-DMP (Dp) in the muscle layer. **C**: In the colon immunoreactivity is located in ICC (ICC-MY, My; ICC-IM, Im; ICC at the submucosal border (ICC-SM), Sm) in the muscle layer. In the mucosae (Mu), some microvilli of the epithelial cells are also labeled positively.

Fig. 4

Immunoreactivity for AIC in other organs.

A: In the liver, bile capillaries are labeled by AIC (red). Cytoplasms of hepatocytes along bile capillaries show AIC immunoreactivity. Nuclei are stained blue with DAPI. **B**: Ovarian follicles with oocytes (Oo) are surrounded by AIC immunoreactivity.

Positive cells are located in the theca interna. Nuclei are stained with DAPI. **C**: In the thymus, epithelial reticular cells of the cortex (Co) show immunoreactivity. The medulla (Me), on the other hand, shows no labeling. **D**: The cortex of the kidney is strongly stained with AIC. Bowman corpuscles (Bow) and proximal urinary tubules show immunoreactivity. The brush borders of proximal tubules are immunopositive. Arrowhead indicates a junction of the Bowman capsule and the proximal urinary tubule. **E**: In the testis, Leydig's cells (asterisk), the spermatogonium and some spermatocytes are stained with anti-c-Kit antibody ACK2, as already described. **F**: AIC shows no immunoreactivity in the testis. The scale bar in E also applies to F.

Fig. 5

Immunoelectron micrographs of ICC and microvilli in the small intestine stained by AIC.

A: ICC processes have numerous mitochondria (m) and are connected with each other by the gap junction (arrowheads). Gold particles indicating AIC immunoreactivity is closely attached to the plasma membrane. **B**: A longitudinal section of the brush border demonstrates strong immunoreactivity. Note that the cytoplasm shows no immunoreactivity. **C**: A cross section of microvilli also demonstrates AIC immunoreactivity on the extracellular surface of the cell membrane. **D**: Control section of microvilli treated without AIC. Few background labeling is seen, but no specific reaction is observed. The scale bar in C also applies to D.

Fig. 6

Biochemical properties of AIC antigen

AIC antigen was examined to determine whether AIC can be used for western blotting. Dot blots (10 μL each） of the homogenized tissue lysate (10 μg protein/mL in PBS) of mouse GI tracts or BSA (10 μg protein/mL in PBS) on nitrocellulose membranes were probed with AIC and anti-BSA antibody, respectively. Controls were treated with neither AIC nor anti-BSA antibody. The tissue lysate was treated with either fixative (4% paraformaldehyde), SDS (2%, 1%), Triton X-100 (1%, 0.5%) or CelLytic before blotting, and their effects on immunoreactivity were examined.

A: Although an unfixed fresh sample and the one fixed with 4% paraformaldehyde showed immunoreactivity, samples treated with SDS either at 90°C for 5 min or at 4°C for 12 h lost AIC immunoreactivity. **B**: Treatment of samples with 1% Triton X-100 abolished immunoreactivity. This inhibition was not caused by interference from sample absorption on the nitrocellulose membranes because BSA under the same condition retained reactivity. CelLytic dissolved the antigen only slightly, but SDS blocked immunoreactivity of the solubilized antigen.