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Histamine Monoclonal Antibody for Brain Immunocytochemistry

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Among five monoclonal antibodies (AHA-1 to 5 mAbs) prepared against glutaraldehyde (GA)-conjugated histamine (HA) in our previous study, only mAb AHA-2 was found to detect HA specifically in rat brain neurons by an immunocytochemistry method (ICC) using GA as a tissue fixative. All the other mAbs, except for AHA-5, reacted with HA in the enterochromaffin-like cells (ECL cells) of rat stomach [Fujiwara *et al.* (1997) *Histochem. Cell Biol.* 107, 39-45]. Enzyme-linked immunosorbent assay (ELISA) binding and inhibition tests demonstrated that AHA-2 is specific for HA, with almost no detectable cross-reaction with any other established or putative amino acid neurotransmitters, LH-RH, TRH, or peptides with N-terminal histidines. ELISA assays also suggested that the AHA-2 mAb recognizes a HA epitope structure different from the one recognized by the AHA-1 mAb. The immunostaining patterns with AHA-2 mAb, as seen in the five subgroups of the tuberomammillary nuclei in the rat posterior hypothalamus, were very similar to those described by Inagaki *et al.* [(1988) *Brain Res.* 439, 402-405; (1990) *Exp. Brain Res.* 80, 374-380] and Panula *et al.* [(1984) *Proc. Natl. Acad. Sci. USA* 81, 2572-2576; (1988) *J. Histochem. Cytochem.* 36, 259-269] using polyclonal anti-HA serum. However, it was also noted that moderate numbers of immunoreactive nerve fibers projected into the median eminence. The present HA ICC method using AHA-2 mAb allows highly sensitive HA detection in brain, and thus might permit detailed studies of HA localization hitherto impossible using previously available anti-HA polyclonal antibodies produced against carbodiimide-conjugated HA.

Key words: glutaraldehyde, histamine, immunocytochemistry, monoclonal antibody, neuron.

Histamine (HA) has been implicated in a variety of biologically important events such as the regulation of gastric acid secretion, the mediation of allergic reactions, and neurotransmission. Recent studies have reported that HA functions in association with cell growth and differentiation (*1-3*). Immunocytochemical (ICC) studies of HA will lead to a better understanding of these functional roles by revealing the exact cellular and subcellular localization of endogenous HA. Although many ICC studies of HA in the neural tissues of animals have been undertaken at the light microscopic level using polyclonal rabbit anti-HA serum produced against carbodiimide-conjugated HA (*4-10*), no immunoelectron microscopic study has been reported to date, except for studies of rat peritoneal mast cells and gastric enterochromaffin-like cells (ECL cells) (*11-13*). This is most likely due to the lack of an ultrastructural detection method for HA that can be applied generally. Recently, it has become evident that a monoclonal antibody produced against a glutaraldehyde (GA)-conjugated hapten molecule, is applicable to immunoelectron microscopic ICC studies of hapten, in which a strong tissue fixative, GA, is used for the preservation of excellent histological structures (*14-20*). This can be explained by the fact that in ICC studies of small hapten molecules, the antibody recognizes not only the antigen structure but also, in part, the cross-linking sites of GA with carrier proteins (*6, 14, 17, 19*).

In our previous studies we prepared mouse monoclonal antibodies (AHA-1 to 5 mAbs) against GA-conjugated HA, and identified two mAbs (AHA-1 and 2) which gave the best ICC results in the rat stomach ECL cells at both the light and electron microscopic levels (*11, 21*). In the present study, we tried to extend the applicability of the mAbs to brain ICC of HA, and found that only AHA-2, among the five mAbs, reacts specifically with neuronal HA in rat brain. Of note is the HA immunoreactivity found not only in the neurons of the tuberomammillary nuclei, known to contain HA (*4-7*), in the posterior hypothalamus, but also in nerve fibers in the median eminence of the rat brain. Berkenbosch and Steinbusch (*22*), however, have reported that antiserum raised against GA-conjugated HA cross-reacts with

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Abbreviations: HA, histamine; ICC, immunocytochemistry; GA, glutaraldehyde; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; BSA, HSA, bovine (human) serum albumin; mAb, monoclonal antibody; HA-GA-HSA, histamine-glutaraldehyde-human serum albumin conjugate; His, histidine; GABA, γ-aminobutyric acid; ECL cells, enterochromaffin-like cells.

luteinizing hormone-releasing hormone (LH-RH) in the rat median eminence, although the exact machanism for the cross-reaction of the antiserum with the hormone remains unclear. It would, therefore, be very important to evaluate the antibody specificity of AHA-2 mAb in order to apply it to brain ICC.

The present study demonstrates clearly that AHA-2 mAb is monospecific for HA, but does not cross-react with LH-RH or other tested peptides that contain a histidine residue at their amino termini. This may mean that an HA immunocytochemistry method using AHA-2 mAb provides a potential new tool, allowing detailed studies of HA localization in the brain hitherto impossible by previously available anti-HA polyclonal antibodies (*4-7*). Also, the usefulness of AHA-2 mAb is evident from the fact that anti-HA serum obtained from the same mouse used for hybridoma experiments for HA mAbs was shown to be of little use in the ICC study, because it showed only very slight immunoreaction with neuronal HA. On the other hand, the antiserum, at greatly diluted concentrations, specifically and strongly stains HA in ECL cells of the rat stomach.

MATERIALS AND METHODS

Chemicals—Histamine-2HCl (HA), L-histidine-2HCl (His), glycine-HCl (Gly), β-alanine-HCl (β-Ala), taurine-HCl (Tau), glutaraldehyde (GA, 25% in water), sodium borohydride, bovine (human) serum albumin (BSA or HSA), and *o*-phenylenediamine were purchased from Nacalai Tesque (Kyoto). Luteinizing hormone-releasing hormone (LH-RH), thyrotropin-releasing hormone, anorexigenic peptide, and peptides with N-terminal His such as His-Gly, His-Lys, His-Ser, and His-Gly-Gly, were from the Peptide Institute (Osaka). Goat anti-mouse gamma-globulin labeled with horseradish peroxidase (HRP) was purchased from Cappel (West Chester, PA, USA). Amino acids used in this study were all L-configuration except Gly, β-Ala, and γ-aminobutyric acid (GABA). The mouse monoclonal antibodies (AHA-1-5, all IgG_1 sub-isotype mAb) against HA used in this study were the same as previously prepared against HA conjugated with bovine serum albumin (BSA) using GA and $NaBH_4$ (*21*).

Purification and Biotinylation of AHA-1 and 2 mAbs—Protein A-binding IgG antibody was prepared from the hybridoma culture supernatant with Protein-A Sepharose CL-4B (Pharmacia Fine Chemicals, Sweden, 1×2 cm). For the preparation of biotinylated antibodies, purified AHA-1 or 2 mAb (1.0 mg) was dialyzed against 100 mM sodium bicarbonate buffer, pH 8.5, and then mixed with 0.1 mg of sulfosuccinimidyl-6-(biotinamido)hexanoate sodium salt (Vector Lab., CA, USA) at room temperature for 2 h according to the method of Yoshida *et al.* (*23*).

Synthesis of Conjugates—The following compounds, HA, His, β-Ala, GABA, Tau, glutamate (Glu), aspartate (Asp), and glutamine (Gln) were conjugated to HSA using GA and $NaBH_4$ according to our previous methods (*21*). LH-RH and TRH were conjugated to HSA using *N*-[β-(4-diazophenyl) ethyl]maleimide as a coupling agent according to our previous method (*24*).

Characterization of mAbs—The supernatants of AHA-1-5 were serially diluted for further characterization by ELISA on GA-HSA and a series of amino acid conjugates (HA-, His-, Gly-, β-Ala-, GABA-, Asp-, Glu-, Gln-, and Tau-HSA), which were used in a concentration range of 1.5 to 15 μg/ml. The mixtures were treated with 1% skimmed milk for 1 h to block the protein binding sites, and then incubated overnight at 4°C with mAb hybridoma culture supernatant (diluted 1:100). The samples were then incubated with HRP-labeled anti-mouse IgG (1:1,000) for 1 h. The bound enzyme activity was measured using 100 μl of 30 mM citrate buffer, pH 5.3, containing 0.5 mg/ml *o*-phenylenediamine and 0.012% H_2O_2 at 492 nm with an automatic ELISA analyzer (SLT-Lab Instruments, Salzburg, Austria).

ELISA Binding Test and Inhibition Test—These were carried out essentially according to our previous method using test compounds including HA, His, Gly, β-Ala, GABA, Tau, Glu, Asp, and Gln (*21, 25, 26*).

Tissue Samples—Healthy, adult male Wistar rats (300 g body weight) were either untreated or pretreated with colchicine (75 μg in 20 μl of normal saline injected into the lateral ventricle 2 days before killing). Rats were anesthetized with Nembutal (50 mg/kg body wt) and perfused intracardially with phosphate-buffered saline (PBS) at 50 ml/min for 2 min at RT, and then with a freshly prepared solution of 2.5 to 5% GA in 10 mM phosphate buffer, pH 7.2, for 6 min. The brains were quickly excised, immersed overnight at 4°C in the same fixative, and cut into 50-μm thick sections with a Microslicer (Dosaka EM, Kyoto).

Immunocytochemistry (ICC)—Sections were treated with 0.2% $NaBH_4$ for 10 min, and incubated at 4°C for 48 h with primary mAb AHA-1 or 2 at concentrations ranging from 10 to 50 ng/ml in 50 mM Tris-HCl buffer, pH 7.4, containing 0.86% NaCl (TBS) [the concentration of which was determined by the conventional "sandwich ELISA" using chromatographically purified mouse IgG (Zymed Lab.; San Francisco, CA) as a standard]. Sections were then incubated with goat anti-mouse IgG/Fab′ labeled with horseradish peroxidase (HRP) (MBL; Nagoya), 1:200, for 12 h at 4°C. After rinsing with TBS, the HRP was stained for 5-10 min with diaminobenzidine and H_2O_2. The HRP substrate consisted of 10 mg of 3,3′-diaminobenzidine tetrachloride (Sigma) dissolved in 20 ml of 50 mM Tris buffer, pH 7.4, supplemented with 20 μl of 30% H_2O_2 (*27*).

Control Experiments—In the HA immunocytochemistry study, the specificity of immunostaining was ascertained by incubating control sections with (a) the secondary antiserum alone, (b) type-matched mAb (IgG_1) anti-penicillin (30-80 ng/ml; Cosmo Biological, Tokyo), or (c) mAb AHA-2 preabsorbed with HA-GA-HSA at a concentration of 2 μg/ml.

RESULTS

In order to apply HA mAbs-1-5 (AHA-1-5) to brain immunocytochemistry studies, we examined an indirect immunoperoxidase method using 50-μm Microslicer sections of the posterior parts of rat hypothalamus, in which the tuberomammillary nucleus, known to contain the HA neurons, is contained. Interestingly, it was found that the neurons immunostained intensely with AHA-2 mAb at very low concentrations (10 ng/ml), but not at all with the other four HA mAbs even at 10 μg/ml, although ECL cells in rat gastric glands are immunostained with all the HA mAbs except AHA-5 (*21*). The HA antiserum obtained

from the mouse used to prepare hybridoma for mAbs was also tested for staining, but showed only very weak immunoreactivity with neuronal HA, even at concentrations less than 1 to 500. Thus, in the following experiments, the AHA-2 mAb was characterized and compared with the AHA-1 mAb reported in our previous study (*21*).

The AHA-2 mAb was studied using a marker index of the immunoreactive enzyme activity bound to a variety of HSA conjugates of hapten compounds including HA, His, Gly, β-Ala, GABA, Tau, Glu, Asp, LH-RH, or TRH employed as a solid-phase antigen in the ELISA. Among these conjugates, the mAb showed significant binding activity only to the HA-GA-HSA conjugate, although a negligibly small immunoreactivity was seen on GABA-solid phase at a rather high concentration of AHA-2 mAb (Fig. 1). No immunoreactivity was observed in the ELISA for the GA-HSA conjugate or the other amino-acid conjugates tested.

Evaluation of Antibody Specificity by ELISA Binding Test—This was done in the same manner as described for AHA-1 mAb (*21*). AHA-2 mAb bound only to HA, and not to the other amino acids, conjugated or free, listed above (data not shown). Type-matched control antibodies did not react with any compounds tested in the ELISA binding test (data not shown).

ELISA Inhibition Test—This was achieved by competition between a variety of compounds and a fixed amount of

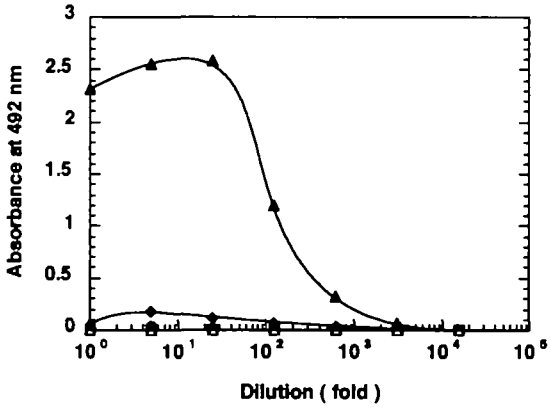

Fig. 1. **Immunoreactivity of AHA-2 mAb with HSA-conjugates in ELISA plates.** Serial dilution of hybridoma cell culture supernatants containing AHA-2. Amount of conjugates used as solid-phase antigens, 15 μg/ml; GA-conjugated HSA of HA (▲), GABA (◆), β-Ala (○), Gly (●), Asp (□), Glu (△), Gln (■), and Tau (◇); LH-RH (▼) and TRH (▽) conjugated to HSA *via* *N*-[β-(4-diazophenyl)ethyl]maleimide.

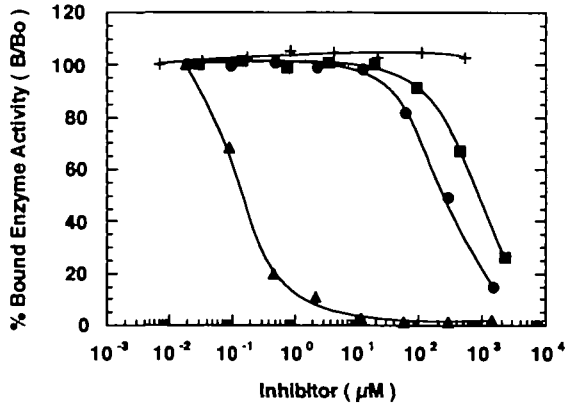

Fig. 2. **Reactivity of AHA-2 as measured by its immunoreactivity in the ELISA inhibition test.** The curves show the amount (percentage) of bound enzyme activity (B) at various doses of HA-GA-HSA conjugate (▲), His-GA-HSA conjugate (●), HA-GA-HSA conjugate (■), or free HA (+) as the ratio to that bound using the HRP-labeled second antibody alone (B*). The concentration of HA (or His) in the HA-GA-HSA conjugate (or His-GA-HSA conjugate) was photometrically calculated, assuming a molar extinction coefficient of HSA of 43,000 at 280 nm, and that 100 molecules of HA (or His) are incorporated into a molecule of HSA.

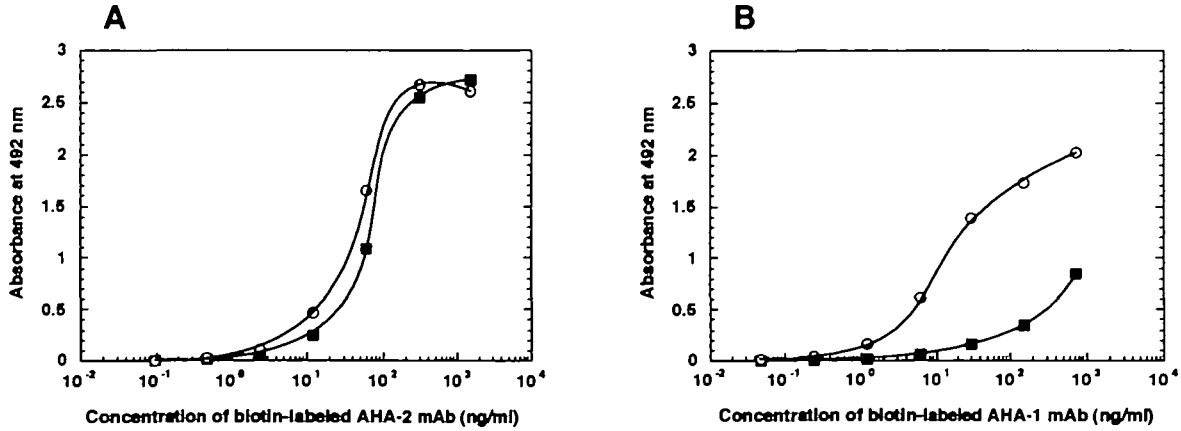

Fig. 3. **A and B: ELISA for the evaluation of the HA epitope structure recognized by AHA-2 mAb.** Wells of microtiter plates coated with 10 μg/ml of HA-GA-HSA conjugate were incubated with 5 μg/ml of AHA-1 (A) or AHA-2 mAb (B) at 25°C for 1 h, followed by incubation with biotin-labeled AHA-2 (A) or AHA-1 mAb (B) at various concentrations (0.045 ng/ml to 1.8 μg/ml) for 1 h. The wells were then allowed to react with streptavidin-HRP (diluted 1:3,000) for 30 min, and bound enzyme activity was measured by the method described in "MATERIALS AND METHODS." Note that the binding of labeled AHA-2 mAb to the HA-GA-HSA conjugate was inhibited only in part by AHA-1 mAb, which was bound to the conjugate at the saturation level (A), but the binding of labeled AHA-1 mAb to the conjugate was strongly inhibited by pre-bound AHA-2 mAb (B). The amounts of bound biotin-labeled AHA-2 mAb are shown in chart A for sample (■) and control (○) specimens; those of AHA-1 mAb are shown in the same way in chart B.

HA-GA-HSA for a limited number of binding sites on the mAb. Dose-dependent inhibition curves for AHA-2 mAb were obtained with HA-GA-HSA, His-GA-HSA, and non-reduced HA-GA-HSA conjugate (HA=GA=HSA), with EC50 values of 0.14, 300, and 1,000 μM, respectively (Fig. 2). No cross-reaction occurred with LH-RH, TRH, anorexigenic peptide, or peptides with N-terminal His, such as His-Gly, His-Lys, His-Ser, or His-Gly-Gly, or any amino acids, conjugated or free, tested, even at concentrations less than 1 μM (data not shown).

Evaluation of the Epitope Structure Recognized by AHA-2—As shown in our previous ELISA studies (*21*), analysis of the relationship between the concentration of AHA-1-5 mAbs and the immunoreactive enzyme activity bound to HA-GA-HSA conjugate coated on ELISA plates produced a saturation curve dependent on the concentration of each mAb. The bound enzyme activity of AHA-2 mAb at the saturated level was about 2-times that of the other four mAbs, each of which showed almost the same activity (*21*): This may mean that the AHA-2 mAb recognizes the HA

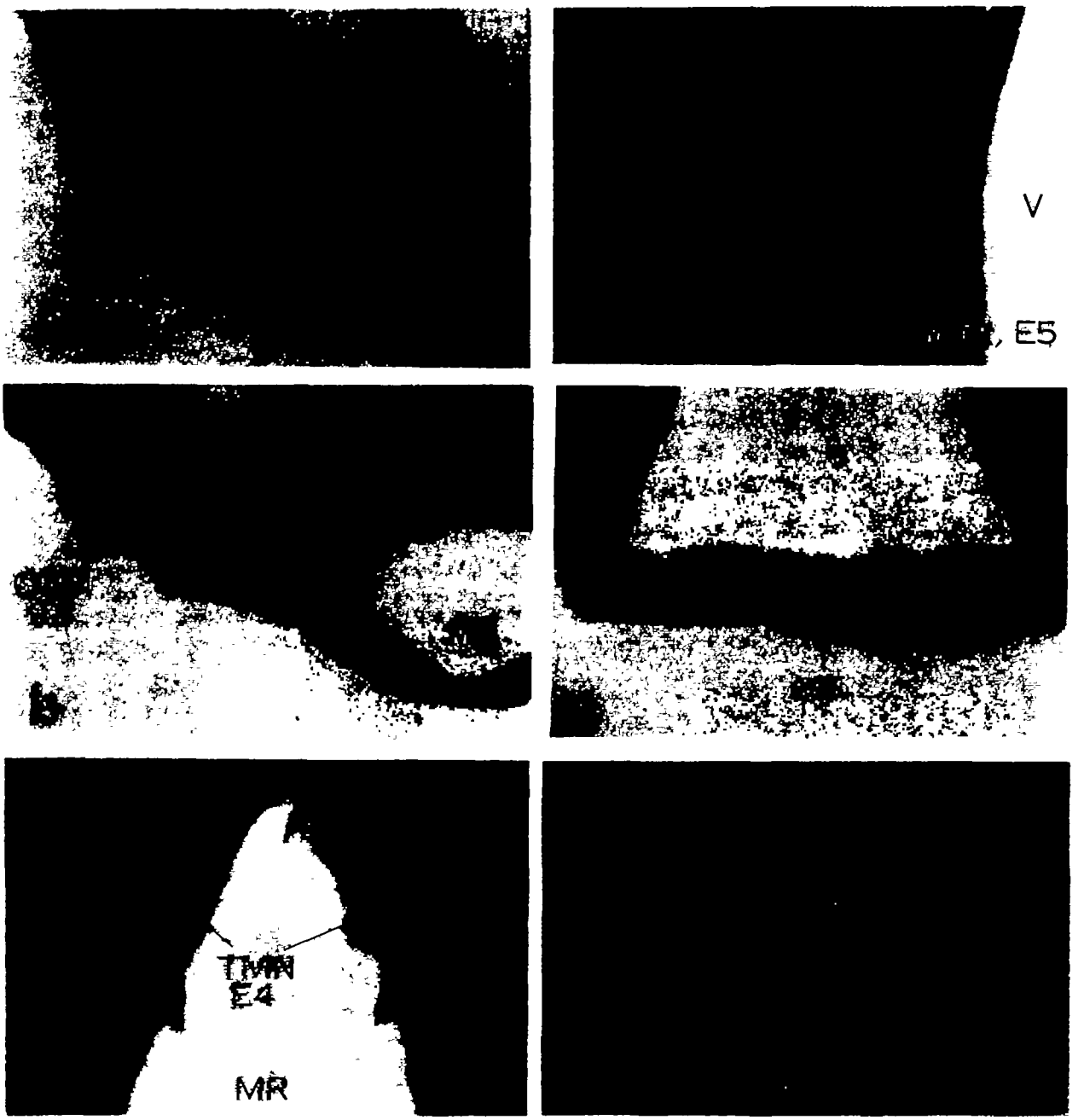

Fig. 4. **a-f: Different neuron types stained for HA in the posterior hypothalamic area by immunocytochemistry (indirect method) using AHA-2 mAb.** HA immunoreactive neurons were found in the postmammillary caudal magnocellular nucleus (PCMN, or the E1) (a), caudal magnocellular nucleus (CMN, or the E2), the E3 (b), tuberal tuberomammillary nucleus (TMN, or the E4) (c), and tuberomammillary diffuse parts (TM diff, or the E5) (d). Moderate numbers of HA-containing nerve fibers project into the median eminence (ME) (e). Staining (seen in e) was completely abolished by the absorption of the mAb with HA-GA-HSA conjugate (2 μg/ml) (f). MR, mammillary recessus; V, third ventricle; Frontal sections. (a, c, d, e, f) ×60; (b) ×32.

epitope structure in a different way from the other mAbs. In order to further confirm this, we examined here whether or not AHA-2 mAb binds to the HA-GA-HSA conjugate of the ELISA plate, on which the AHA-1 mAb was bound at a saturation level. As shown in Fig. 3A, the amounts of bound biotin-labeled AHA-2 mAb, expressed as the enzyme immunoreactivity of HRP-streptavidin bound to the ELISA plate, increased progressively with increasing concentration of the labeled AHA-2 mAb, nearly approaching the control level, in which the binding activity of the labeled AHA-2 mAb was assayed using non-pretreated well plates. On the other hand, the amounts of biotin-labeled AHA-1 mAb bound to the HA-GA-HSA conjugate of the ELISA plate, on which the AHA-2 mAb was bound at a saturation level, was only 40% that of the control value when compared at 70 ng/ml of labeled AHA-1 mAb (Fig. 3B). No binding occurred with a different mAb, ASPM-29, specific for polyamines (*26*) (data not shown).

Immunocytochemistry—Hypothalamic neurons exhibited immunoreactivity to HA by reaction with AHA-2 mAb only after colchicine pretreatment, while the other four mAbs showed no immunoreactivity at all. At the caudal level of the posterior hypothalamus, a cluster of HA immunoreactive cells were found in what Bleier *et al.* called the postmammillary caudal magnocellular nucleus (PCMN) (*28*), and what Inagaki *et al.* called E1 (*5*) (Fig. 4a). In the rostral parts, this cell group extends medially along the ventral surface, and then fuses with a large group of HA immunoreactive cells in the caudal magnocellular nucleus (CMN or the E2) (Fig. 4b). At the same level, these cells spread medially to the dorsal and ventral aspect of the ventral premammillary nucleus (the E3) (Fig. 4b). In the more rostral parts of the posterior hypothalamic nucleus, a smaller number of HA immunoreactive cells in the CMN (the E2) are present. Then another cluster of HA immunoreactive cells emerge in the tuberal magnocellular nucleus (TMN or the E4), which is dorsolateral to the mammillary recess (Fig. 4c). Here and at a slightly rostral level, HA immunoreactive cells appear in moderate numbers in the tuberomammillary diffuse parts (TM diff or the E5) (Fig. 4d), but few are evident in the lateral hypothalamus. No immunoreactive cells were observed in the median eminence (Fig. 4e). In the posterior hypothalamic areas, HA-containing fibers can be seen in the basal hypothalamus, median eminence, supraoptic and arcuate nuclei (Fig. 4, a–e).

In these tissue sections the conventional ICC staining controls (second level controls) were all negative. The immunostaining was completely abolished by pre-absorption of the mAb with HA-GA-HSA (2 μg/ml conjugate concentration) (Fig. 4f), while no inhibition occurred with LH-RH, TRH, anorexigenic peptide, His-GA-HSA, GABA-GA-HSA, glycine-GA-HSA, GA-HSA, on HSA, even at much higher concentrations (100 μg/ml) (data not shown). Nor was there cross-reactivity with free compounds such as HA, His, glycine, Asp, Glu, or GABA at a concentration of 200 μg/ml. Also, no reaction was observed with type-matched anti-penicillin mAb (IgG_1) (data not shown) (*26*).

DISCUSSION

The goal of the present study was to demonstrate that among five mAbs (AHA-1-5) prepared in this laboratory, only AHA-2 mAb can detect neuronal HA in the rat brain with a high degree of specificity, although our previous studies at the light and electron microscopic levels showed both AHA-1 and 2 mAbs to be immunoreactive with HA in the ECL cells of rat stomach (*11, 21*). Thus, the AHA-2 mAb was characterized in comparison with the AHA-1 mAb reported previously (*21*). AHA-2 mAb exhibited exclusive binding immunoreactivity to HA-GA-HSA conjugate in ELISA (Fig. 1), and to HA bound *via* GA to poly-L-lysine coated in ELISA the binding test. However, no binding immunoreactivity in these ELISA assays was seen with conjugates of other established or putative amino acid neurotransmitters likely to be present in neural tissues, such as Gly, β-Ala, GABA, Asp, Glu, Gln, Tau, LH-RH, and TRH (Fig. 1). Furthermore, AHA-2 mAb was demonstrated to be nearly monospecific to HA by the ELISA inhibition test, since antibody binding in the test was inhibited most by HA-GA-HSA, followed by His-GA-HSA and HA=GA=HSA, with cross-reaction values of 100, 0.04, and 0.014%, respectively (Fig. 2). In our previous study, however, no cross-reaction occurred between AHA-1 mAb and the His-GA-HSA conjugate (*21*). Also, no inhibition was evident with free HA, LH-RH, TRH, anorexigenic peptide, or peptides with N-terminal His, even at concentrations above 1 mM. No antibody binding was seen with GA-HSA or HSA, demonstrating a lack of recognition. All these ELISA results strongly suggest that AHA-2 mAb recognizes not only the HA molecule but also, in part, the carrier protein-conjugation site(s) of GA, including the part(s) reduced by $NaBH_4$, as was the case with AHA-1 mAb. Furthermore, in the previous ELISA assays with coated HA-GA-HSA conjugate, AHA-2 mAb showed a bound enzyme immunoreactivity 2-times higher than that of AHA-1 mAb at a saturated level (*21*). In the present ELISA study using HA-GA-HSA conjugate, the AHA-2 mAb recognized the HA epitope structure differently from AHA-1 such that the binding of AHA-2 mAb to the HA-GA-HSA conjugate was inhibited only in part by AHA-1 mAb bound to the conjugate at a saturated level, but the binding of AHA-1 mAb to the conjugate was strongly inhibited by pre-bound AHA-2 mAb. These results suggest that there may be at least two types of antibody binding sites in the HA-GA-HSA conjugate molecule: one recognized by both AHA-2 and, to a lesser degree, AHA-1 mAbs, and the other only by AHA-1 mAb (Fig. 3, A and B). In addition, Fig. 3, A and B, shows that labeled AHA-2 mAb at high concentrations binds almost completely at a saturated level to the AHA-1 mAb-saturated HA-GA-HSA conjugate (Fig. 3A). On the other hand, high concentrations of labeled AHA-1 mAb bind in part to the conjugate pre-saturated with AHA-2 mAb (Fig. 3B). These phenomena might be due to an exchange reaction by which labeled mAb displaces unlabeled mAb to bind to the HA-GA-HSA conjugate.

Previous ICC studies for HA in neural tissues have been undertaken using polyclonal rabbit anti-HA serum and a carbodiimide-based tissue fixative, but only at the light microscopic level (*4–10*). This method, however, is unlikely to be applicable to ICC at the ultrastructural level, since it produces rather poor morphology (*9, 11*). In the earlier immunocytochemical studies, however, carbodiimide was an unavoidable tissue fixative, since the HA antiserum was produced against the carbodiimide-conjugated HA (*9*). In general, in immunocytochemical studies of small-sized

molecules, the best results can be expected when the epitope of the hapten-protein conjugate used for immunization closely resembles the fixed tissue antigen (*6, 19, 25, 26, 29, 30*).

In ICC applications, borohydride treatment in combination with GA fixation, is absolutely necessary for antibody-antigen reaction, as also found for ICC reactions involving rat stomach ECL cells in our previous study (*11, 21*). Under such conditions, AHA-2 mAb stain certain neurons in the posterior hypothalamus specifically at very low concentrations, but none at all with other HA mAbs, even at concentrations higher than a thousand times that of AHA-2 mAb (Fig. 4, a-f). Also, the staining pattern of the neurons was much more specific as well as stronger with AHA-2 mAb than with the polyclonal anti-HA serum (obtained from the mouse used for hybridization), which stained only very slightly not allowing a specific HA immunoreaction to be detected in specimens (data not shown). The reason why HA immunoreactivity in neural tissues was detected only by AHA-2, and not by the other companion mAbs, nor by HA antiserum, is not known at present, but it is possible that endogenous HA is chemically modified by reaction with GA under the fixation conditions, producing a variety of HA derivatives *in situ* in the organ tissues, and in particular in the brain neurons. Our previous studies showed that even the perfusate for fixing the rat produces organ tissue specimens fixed at different levels, with neural tissues fixed more weakly than other tissues (*31*).

In the adult rat brain, HA immunoreactive cell clusters were observed in the tuberomammillary nuclei (TM) in the posterior hypothalamic area, and especially large groups of HA immunoreactive cells in the five subregions (Fig. 4, a-d) called E1, E2, E3, E4, and E5 by Inagaki *et al.* (*4, 5*). These staining patterns obtained with AHA-2 mAb were very similar to those described by Inagaki *et al.* (*4, 5*) and Panula *et al.* (*6, 7*) using polyclonal anti-HA serum, and also those obtained with antibodies against histidine decarboxylase, the HA-forming enzyme (*32, 33*). In the present study it was also found that moderate numbers of immunoreactive nerve fibers project into the median eminence (Fig. 4e). Berkenbosch and Steinbusch (*22*) have previously found that one of the two antisera produced against GA-conjugated HA showed HA immunoreactivity in nerve fibers in the median eminence, while the other did not. However, they concluded that the HA-positive reaction was a consequence of cross-reaction of the antiserum with LH-RH contained in the regions, although it was unclear whether or not the immunoreaction was inhibited by LH-RH, and occurred in neurons other than those in the median eminence, known to contain LH-RH. In the present study no effect of LH-RH on the immunoreaction with AHA-2 mAb was evident in the ELISA tests or control immunostaining experiments, indicating that the immunoreactivity found in the median eminence might be truely due to HA itself in the region, and not to LH-RH. Inagaki *et al.* (*4, 5*) previously reported a few HA fibers in the median eminence, immunoreactive with antibodies against HA and against histidine decarboxylase.

It is important to develop an immunoelectron microscopy method to localize HA neurons directly in the brains, although in previous HA localization studies, indirect methods to detect the HA-forming enzyme, L-histidine decarboxylase, instead of HA have been employed (*32, 33*). The former method for HA would be useful in combination with the latter method for the enzyme, especially for studies of the intracellular pathways of HA in the brain neurons at ultrastructural sites of synthesis, storage, and HA release. In this line, we recently succeeded in evaluating the mechanism of HA in ECL cells in rat stomach by immunoelectron microscopy studies using AHA-1 or 2 mAb (*11*).

In conclusion, the present HA ICC method with AHA-2 mAb provides highly sensitive HA detection in the brain, and thus might allow detailed studies of HA localization hitherto impossible using the previously available anti-HA polyclonal antibodies (*6, 7*). Since the present method detects HA neurons fixed with GA, it presents a potential new tool for immunoelectron microscopic studies of HA neurons in the brain.

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