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Glutaraldehyde (GA)-Hapten Adducts, but without a Carrier Protein, for Use in a Specificity Study on an Antibody against a GA-Conjugated Hapten Compound: Histamine Monoclonal Antibody (AHA-2) as a Model

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In our recent study on monoclonal antibodies (mAbs AHA-1-5) against glutaraldehyde (GA)-conjugated histamine (HA), we identified one mAb (AHA-2) which can detect neuronal HA in the rat brain with an immunocytochemistry method (ICC) [Fujiwara *et al.* (1999) *J. Biochem.* 126, 503-509]. In the present study the specificity of AHA-2 mAb for use for ICC has been examined by means of competitive experiments involving HA and analogs, all of which had been allowed to react with GA followed by sodium borohydride, but not allowed to couple with the carrier protein. It was demonstrated that the antibody distinguished alterations in the chemical structure of the molecule, showing decreased immunoreactivity with all the GA-adducts of (*R*)-(−)-α-methylhistamine, 1- and 3-methylhistamine, L-histidine, and 1- and 3-methyl-L-histidine. On the other hand, AHA-1 mAb only reacted with GA-adducts of 3-MeHA (3-MeHA-GA) and HA (HA-GA), to almost the same degree, in relatively high concentration ranges. AHA-3, 4, and 5 mAbs reacted about 10- times more strongly with 1-MeHA-GA than with HA-GA, but reacted very little or not at all with the other analogs. These results may suggest that AHA-2 mAb recognized both the non-substituted imidazole and α-methine groups of a HA molecule in addition to the conjugation site of GA including the part(s) reduced with $NaBH_4$, and especially the imidazole group more strictly than the other mAbs. This may partly explain why AHA-2, among the five AHA mAbs, can detect neuronal HA with an ICC method. The present ELISA method for GA-hapten adducts should be applicable to other antibodies against GA-conjugated biologically active amines or amino acids, thus allowing the study of antibody specificity for ICC more easily and accurately than was previously possible with hapten-protein conjugates as antigens.

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

Immunocytochemistry (ICC) methods for biologically active amines and amino acids have been used extensively for studies on their localization in cells and tissues involving antibodies produced against these hapten compounds conjugated with proteins, the methods mainly involving carbodiimide (*1-3*), formaldehyde (*4-6*) and glutaraldehyde (GA) (*7-16*) as coupling agents. Among them, it recently became evident that antisera or mAbs against GA-conjugated GABA (*11, 12*), dopamine (*7, 9*), serotonin (*4-6*), glutamate or aspartate (*3, 8, 13*), histamine (*1, 2, 15*), polyamines (*14*), and glycine (*16*) are extremely useful for ICC studies (*6, 9, 10, 17, 18*), especially for immunoelectron microscopic studies, in which a strong tissue fixative, GA, is needed for the preservation of histological ultrastructures (*17-25*). Evaluation of the specificity of such antibodies could lead to their use in ICC studies. In general, antibody specificity has been examined by ELISA and immunoblotting tests involving hapten-protein conjugates *via* GA, since such hapten antibodies in general recognize the hapten molecule itself in addition to the conjugation site(s) of GA (*1-9, 11-16*). However, these methods might be inadequate for the quantitative analysis of antigen-antibody reactions, especially for studying the cross-reactivity with antigen analogs, since it is difficult to accurately measure the concentrations of antigen sites in hapten-

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Abbreviations: HA, histamine; GA, glutaraldehyde; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; ICC, immunocytochemistry; BSA, HSA, bovine (human) serum albumin; mAb, monoclonal antibody; HA-GA-HSA, histamine-glutaraldehyde-human serum albumin conjugate; 1- and 3-MeHA, 1- and 3-methylhistamine; 1- and 3-MeHis, 1- and 3-methyl-L-histidine.

protein conjugates by means of routine biochemical procedures, although the numbers of hapten incorporation have been determined roughly using isotopically labeled hapten compounds used for conjugation (*7, 9, 11*). The ELISA binding test developed in our previous study (*26*), which simulates the ICC of tissue sections, still has drawbacks for complete quantification.

Histamine (HA) plays a variety of biologically important roles such as in the regulation of gastric acid secretion, as the main mediator of allergic reactions, and as one of the neurotransmitters. Also recently reported was that HA is associated with cell growth and differentiation (*27-29*). We recently developed mouse monoclonal antibodies (AHA-1 to 5 mAb) against GA-conjugated HA, and identified one mAb (AHA-2) which can detect neuronal HA in the rat brain with a high degree of specificity, although all the other mAbs, except for AHA-5, reacted with HA in the enterochromaffin-like cells (ECL cells) of the rat stomach (*15, 17, 30*). In these studies, the antibody specificity of AHA-2 was evaluated using HA or amino acids, free or conjugated with a protein *via* GA, and it was found that AHA-2 mAb reacted specifically with a HA-protein conjugate, but not at all with free HA. However, the reaction of the antibody with HA analogs has not been examined in detail (*15, 17, 30*).

Since AHA-2 mAb was prepared with an HA-human serum albumin (HSA) conjugate *via* GA, the present study on the antibody specificity of AHA-2 was undertaken using HA and analogs, which had been allowed to react with GA followed by sodium borohydride, but without a carrier protein.

MATERIALS AND METHODS

Chemicals—Histamine 2HCl (HA), L-histidine 2HCl (His), 1- and 3-methylhistamine (1- and 3-MeHA), (*R*)-(−)-α-methylhistamine (α-MeHA), 1- and 3-methyl-L-histidine (1- and 3-MeHis), glycine HCl (Gly), β-alanine HCl (β-Ala), taurine HCl (Tau), glutaraldehyde (GA, 25% in water), sodium borohydride, bovine (human) serum albumin, and *o*-phenylenediamine were purchased from Nacalai Tesque (Kyoto). Goat anti-mouse gamma-globulin labeled with horseradish peroxidase (HRP) was purchased from Cappel (West Chester, PA, USA). The amino acids used in this study were all of the L-configuration, except for 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 batch as previously prepared against HA conjugated with bovine serum albumin (BSA) using GA and $NaBH_4$ (*15*). HA was conjugated to HSA using glutaraldehyde (GA) and $NaBH_4$ according to our previous methods (*15*), and the resulting HA-GA-HSA conjugate was used as a solid-phase antigen in the enzyme-linked immunosorbent assay (ELISA) for HA.

Preparation of GA-addition products of HA, analogs and amino acids: HA, 1- and 3-MeHA, α-MeHA, His, 1- and 3-MeHis, and the other amino acids were each dissolved at 33 mM in 0.5 ml of a solution of 1 M sodium acetate, and then incubated with 0.5 ml of 100 mM GA solution, with stirring for 90 min at room temperature, during which the pH of the solution was adjusted to 6.4. Then 5.0 mg of $NaBH_4$ was added to the reaction, and the mixture was incubated for 30 min at room temperature in the dark and used without further purification.

ELISA Method—The wells of microtiter plates (Nunc F Immunoplates 1; Nunc, Roskilde, Denmark) were coated by loading 100 μl of HA-GA-HSA conjugate (10 μg/ml) in 10 mM Tris-HCl buffer, pH 8.5, containing 10 mM NaCl and 10 mM NaN_3, and then left for 20 min at 37°C. After rinsing with 50 mM Tris-HCl buffer, pH 7.4, the wells were blocked with 1% skimmed milk for 1 h at 37°C. The wells were then filled with 50 μl of AHA-2 mAb, and 50 μl of HA or an analog sample specimen in amounts ranging from 10 nM to 1000 μM, followed by 3 h incubation at room temperature. After rinsing with PBST, the plates were incubated with 50 μl of goat anti-mouse IgG labeled with horseradish peroxidase (HRP, 1:2,000) for 1 h at 25°C. The amount of enzyme conjugate bound to each well was determined with *o*-phenylenediamine as the substrate, and the absorbance at 492 nm was read with an automatic ELISA analyzer (SLT-Labinstruments, Salzburg, Austria).

RESULTS AND DISCUSSION

In this study, HA, the main biologically metabolized products 1- and 3-MeHA, the H_3 histamine receptor agonist α-MeHA (*31*), His, 1- and 3-MeHis, and the other amino acids, all of which had been allowed to react with GA but not to couple with the carrier protein, were used to evaluate the specificity of AHA-2 mAb. Optimization of the reaction was first performed by means of experiments in which HA was allowed to react with GA under various conditions: *i.e.* pH levels, GA concentrations, and incubation periods, and then was incubated with $NaBH_4$ for 30 min at room temperature in order to remove the residual active aldehyde groups of GA. It was found that the best results were obtained at pH 6.4, with a molar ratio of HA and GA of 1 to 3, and the reaction of HA with GA was completed in 90 min at room temperature (Fig. 1). A similar reaction of HA with GA, but at pH 8.4 in borate buffer, however, proceeded rather slowly, and the reaction rate was about one-third that of the reaction at pH 6.4. Thus, the reaction of HA analogs or amino acids with GA was undertaken under the same conditions as those found to be optimizal for HA. Under these conditions, the primary amino groups of HA and of the analogs were all found to react nearly completely with GA, judging from the reaction with trinitrobenzene sulfonic acid (*32*). In the ELISA systems, the specificity was determined as to the competition between the analytes and a fixed amount of HA-GA-HSA coated on ELISA plates for the limited number of binding sites on the mAb. Calibration curves were plotted showing the relationship between the concentrations of the analytes and the percentage of bound mAb (Fig. 2). It was shown that AHA-2 mAb exhibited the highest immunoreactivity with HA linked to GA and then reduced with $NaBH_4$ (HA-GA) (Fig. 2). The antibody also showed low immunoreactivity with α-MeHA-GA, 3-MeHA-GA, non-reduced HA-GA (HA=GA), His-GA, 1-MeHA-GA, 3-MeHis-GA, and 1-MeHis-GA, with EC 50% cross-reaction values of 0.83, 0.71, 0.12, 0.10, 0.06, 0.03, and 0.01%, respectively. However, no cross-reaction occurred with free HA or the other amino acids, such as His, Gly, β-Ala, GABA, lysine, ornithine, glutamate, glutamine, aspartate, and taurine, tested even at concentrations of less than 1 μM (data not shown).

Evidence for the antibody recognition sites of AHA-2

mAb prepared using HA was provided by the competition for antibody recognition between GA-addition products of HA analogs and the HA-GA-HSA conjugate coated on the solid phase. Among the analogs tested (Fig. 3), substitution of a methyl group at either the N^3-position (in the case of 3-MeHA-GA), the α-carbon (α-MeHA-GA), or the N^1-position (1-MeHA-GA) on the HA molecule effectively eliminated the antibody recognition. The location of a carboxyl group at the α-carbon, producing His, and further alteration through substitution of a methyl group at either the N^3- or N^1-position, producing 3-MeHis or 1-MeHis, all led to much lower reactivity with the antibody. Also non-reduction of HA-GA (HA=GA) caused a marked decrease in the efficiency of competition for AHA-2 mAb. The lack of binding of HA with GA (free HA) completely abolished the immunoreactivity with the antibody. Furthermore, no antibody binding was seen with GA-HSA or HSA, as reported previously (*15*), demonstrating a lack of recognition. All these results strongly suggest that the antibody, which was produced against HA conjugated with HSA *via* GA and then reduced with $NaBH_4$ in our previous study (*15, 30*), strictly recognizes both the imidazole and α-methine groups of the HA molecule in addition to the conjugation site of GA including the part(s) reduced with $NaBH_4$.

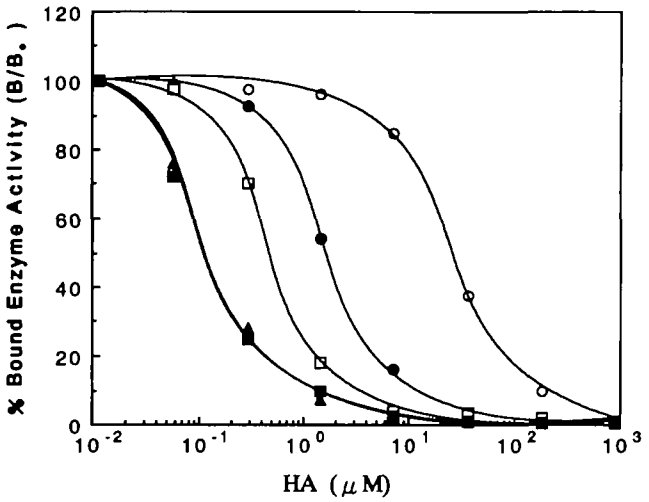


Fig. 1. **Time dependency of the production of the HA-GA adduct as assayed by the ELISA inhibition test.** The reaction of HA with GA was carried out as described under "MATERIALS AND METHODS," and at different intervals an aliquot of the reaction mixture was withdrawn and incubated with $NaBH_4$ to stop the reaction, and the samples serially diluted were assayed for the immunoreactivity of the HA-GA adduct by means of an ELISA system with AHA-2 mAb and an HA-GA-HSA conjugate-coated solid phase. ○, 2 min; ●, 10 min; □, 30 min; △, 90 min; ■, 180 min.

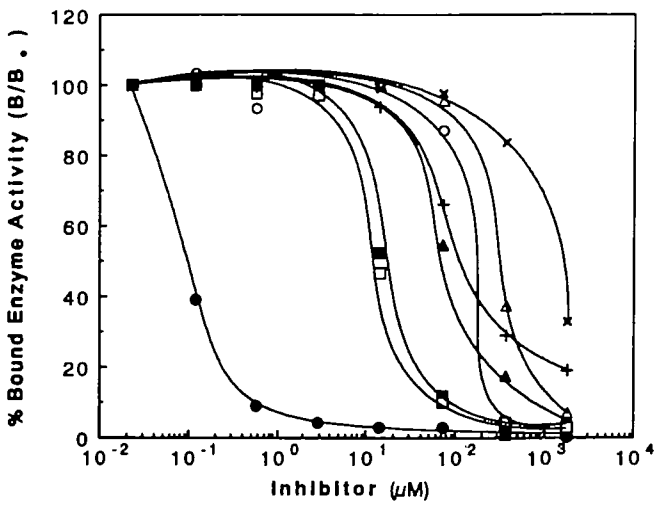
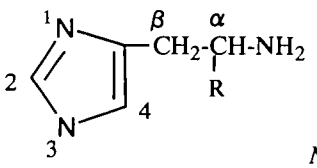


Fig. 2. **Cross-reactivity of some HA analogs with AHA-2 mAb.** The curves show the amount (%) of bound enzyme immunoreactivity for various doses of HA or its analogs (B) as a ratio of that bound using the horseradish peroxidase-labeled goat anti-mouse IgG alone (B^*). The concentrations of inhibitors on the abscissas represent those of the compounds themselves, not those of the compound-GA-adducts. The samples used were: ●, HA-GA; □, α-MeHA-GA; ■, 3-MeHA-GA; +, HA=GA; ▲, His-GA; ○, 1-MeHA-GA; △, 3-HeHis-GA; and ×, 1-MeHis-GA.

The immunoreactivity of AHA-1, 3, 4, and 5 mAbs with GA-adducts of HA and analogs was also examined by means of the ELISA with the HA-GA-HSA conjugate as the solid phase antigen, and the drug concentrations required for 50% inhibition for antibody binding were compared with those for AHA-2 mAb (Table I). Strikingly different immunoreactivity was observed among the five mAbs: AHA-1 mAb reacted with HA-GA to almost the same degree as 3-MeHA-GA in drug concentration ranges higher than



	R	N^1-position	N^3-position	EC50 % cross-reaction value of GA-adduct
histamine	H		H	100
1-methylhistamine	H	CH_3		0.06
3-methylhistamine	H		CH_3	0.71
(R)-(-)-α-methylhistamine	CH_3		H	0.83
L-histidine	COOH		H	0.10
1-methyl-L-histidine	COOH	CH_3		0.01
3-methyl-L-histidine	COOH		CH_3	0.03

Fig. 3. **Structures of HA and some histamine analogs.**

TABLE I. **EC_{50} values of GA-adducts of histamine analogs determined by ELISA using the histamine-GA-HSA conjugate as the solid-phase antigen.**

Analyte	AHA mAb 1	2	3	4	5
HA-GA	10	0.1	1.2	1.5	1.5
1-MeHA-GA	>	160	0.11	0.14	0.2
3-MeHA-GA	12	14	600	1000	>
α-MeHA-GA	>	12	140	160	210
HA=GA	80	83	240	420	320
His-GA	>	100	700	1000	>
1-MeHis-GA	>	1000	>	>	>
3-MeHis-GA	>	330	720	>	>

Each value is of the order of μM. >, value greater than 1,000 μM.

those for AHA-2 mAb, but weakly with GA=HA. Almost no immunoreactivity was seen with 1-MeHA-GA, α-MeHA-GA, His-GA, or 1- or 3-Me-His-GA at concentrations less than 1 mM. AHA-3, 4, and 5 mAbs showed almost the same immunoreactivity with all the GA adducts of HA and analogs (Table I), reacting to the highest degree with 1-MeHA-GA, followed by HA-GA with a reaction level of a tenth of that with 1-MeHA-GA, but very slightly with α-MeHA-GA and HA=GA. Little or no immunoreactivity was observed with 3-MeHA-GA, His-GA, or 1- or 3-MeHis-GA at concentrations less than 1 mM. The reason why AHA-3, 4, and 5 mAbs reacted more strongly with 1-MeHA-GA than with HA-GA, and also AHA-1 mAb reacted with 3-MeHA-GA almost equally to HA-GA, is not known at present, but it is suggested that HA mAbs were raised against different HA antigen structures, which might have been produced through the chemical reaction between the N^1- or N^3-nitrogen of the imidazole group of the HA molecule and GA, which occurred during the HA coupling reaction with HSA, and the mAbs cross-reacted rather strongly with 1- or 3-MeHA-GA. In other words, the HA antigen HA-GA-HSA conjugate, in which N^1- and N^3-positions of the imidazole group of the HA molecule were substituted by GA, might produce AHA-3, 4, or 5 mAbs, and AHA-1 mAb, respectively. It was therefore found that AHA-2 mAb has the ability to recognize the epitope structure of the non-substituted imidazole group of a HA molecule more strictly than the other mAbs, and such a characteristic might partly explain why AHA-2, among the five AHA mAbs, can detect neuronal HA with the ICC method (*30*).

In a HA ICC study, the HA-GA adduct was demonstrated to be very useful as to the antibody specificity of AHA-2 mAb, since the immunoreactivity of AHA-2 mAb for the HA neurons in the tuberomammillary nucleus of the rat posterior hypothalamus was completely abolished on the pre-absorption of AHA-2 mAb with GA-HA at the low concentration of 0.05 μg/ml (data not shown). This dose was much less than that of the HA-GA-HSA conjugate (2 μg/ml, protein concentration) used for the pre-absorption control, as previously reported (*15*).

Also of note regarding the present ELISA assay is that the residual GA and $NaBH_4$, which might be present in the reaction mixture of analytes, did not significantly affect either the enzyme activity of HRP or the immunoreactivity of the antibody used. This may thus suggest the possible development of an ELISA for sensitive quantification of HA in biological fluids such as urine and blood, the latter being reacted with GA and $NaBH_4$ prior to the ELISA assay. Actually, it has been reported that HA was measured by means of radioimmunoassays and enzyme immunoassays, after being led to the chemical derivatives with succinyl glycinamide *N*-hydroxysuccinimide ester (*33*), and 1,4-benzoquinone (*34, 35*) respectively, which were the cross-linkers used for HA coupling with a carrier protein in the preparation of each HA antigen.

In conclusion, it was clearly demonstrated that the GA-addition products of HA and analogs were extremely useful for evaluating the antibody specificity of AHA-2 mAb by means of the ELISA method, allowing quantitative analysis of the recognition site(s) for the mAb. The present ELISA method for GA-hapten adducts should be applicable to other antibodies against GA-conjugated biologically active amines or amino acids (*1–16*), thus allowing the study of the antibody specificity for ICC more easily and accurately than was previously possible with hapten-protein conjugates as antigens.

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