Mechanism of Action of an Anti-allergic Compound; 2,4-Bis(2-acetoxybenzamido)benzoic Acid (AB-50): Inhibition of Anaphylactic and Non-anaphylactic Histamine Release from Rat Peritoneal Mast Cells[1]

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The mechanism of the inhibitory effect of the newly synthesized compounds, 2,4-bis(2-acetoxybenzamido)benzoic acid (AB-50) and 2,4-bis(2-hydroxybenzamido)benzoic acid (AB-23, an active form of AB-50) on IgE-like antibody-mediated passive cutaneous anaphylaxis (PCA) was studied in comparison with that of disodium cromoglycate (DSCG). AB-50 had no anti-inflammatory activity in carrageenin edema assay, nor did it inhibit dermal reactions elicited by local injection of histamine, serotonin, or bradykinin. AB-23 markedly inhibited rat mast cell degranulation (RMCD) *in vitro* following IgE-anti-IgE interaction and antigen-induced histamine release in the rat peritoneal cavity. It was concluded that the inhibition of IgE-mediated PCA by AB-50 was based on prevention of degranulation of and thus release of chemical mediators from mast cells without direct antagonism against chemical mediators of PCA. AB-23 also inhibited non-anaphylactic histamine release from rat peritoneal mast cells induced by Compound 48/80 or dextran. While all of these activities were also shared by DSCG, AB-23 was found to be more active than DSCG with respect to inhibition of RMCD and histamine release induced by Compound 48/80.

Keywords――――anti-allergic agent; histamine release; mast cell; homocytotropic antibody; passive cutaneous anaphylaxis

Introduction

The advance in the study of the immediate (Type I) hypersensitivity is closely associated with the discovery of IgE by Ishizaka, Ishizaka, and Hornbrook.[3] It is known that following the interaction of antigen with IgE or IgE-like antibody fixed on the surface of the membrane of tissue mast cells or blood basophils, chemical mediators such as histamine, serotonin, and SRS-A are liberated from these cells and induce allergic symptoms.[4] Bronchial asthma has been known as one of typical disorders caused by this type of allergic reaction.[5]

Disodium cromoglycate (DSCG), introduced by Cox[6] and clinically applied by Howell and Altounyan[7] as a prophylactic drug for treatment of bronchial asthma in man, has been shown to inhibit immediate hypersensitivity reactions *in vivo*[8] and *in vitro*.[9] These studies have demonstrated that activity of the compound lies in the inhibition of the liberation of chemical mediators from mast cells following antigen-antibody reaction. DSCG has also

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been indicated for exercise-induced asthma based on the finding that it inhibits non-anaphylactic histamine release from mast cells.[10] However, DSCG given by mouth was unable to inhibit the reactions because of its poor absorptivity from the alimentary tract.[11]

While investigating the biological activities of a new series of aminobenzoic acid derivatives, we have found that some of bis(salicylamido)benzoic acids are effective in inhibiting passive cutaneous anaphylaxis (PCA) induced by the homocytotropic IgE-like (HTA) antibody in rats.[12] This is a particularly pertinent experimental model as there is now little doubt that human IgE plays a major role in the pathogenesis of certain types of asthma and hay fever.[5] Among the compounds studied, 2,4-bis(2-acetoxybenzamido)benzoic acid, named AB-50, was found to inhibit PCA in rats even when administered orally.[12] AB-50 is readily converted into its deacetylated form, 2,4-bis(2-hydroxybenzamido)benzoic acid, named AB-23, after incubation of the compound with serum or liver homogenate of the rat. This conversion of AB-50 to AB-23 also occurs *in vivo*. Thus AB-23 is considered as an active form in the body. In this paper, we describe the properties of the new compounds, AB-23 and AB-50, with respect to their inhibitory activities on allergic reactions in comparison with DSCG.

Method

Animals——Male Wistar-Imamichi rats, weighing 150—300 g, maintained in our research laboratories were used in all the experiments.

Passive Cutaneous Anaphylaxis (PCA)——Rat sera rich in heat-labile HTA against egg albumin (Nakarai Chemicals, Ltd., Kyoto) were prepared according to the method of Mota,[4] using *Bordetella pertussis* vaccine (The Chemo-sero-therapeutic Research Institute, Kumamoto) as an adjuvant. The presence of HTA was established by the persistence of PCA activity after a latent period in excess of 7 days, as well as by the lability to heat. PCA with a 48 hr latency was performed as described by Mota.[4] AB-50 was administered intravenously 5 min before antigenic challenge. Pyribenzamine was administered orally 30 min before the challenge.

Skin Reactions Induced by Permeability-increasing Substances——Histamine (Wako Pure Chemical Industries, Ltd., Tokyo), serotonin (Tokyo Kasei Kogyo Co., Ltd., Tokyo), bradykinin (Institute for Protein Research, Osaka University, Osaka), and Compound 48/80 (Wellcome Research Laboratories, Beckenham, England) were employed as the permeability-increasing substances. Immediately after the intravenous injection of 1% Evans Blue solution (5 ml/kg body weight), rats were intradermally given 0.1 ml of a vasoactive substance dissolved in saline. After 20 min, the animals were exsanguinated and locally accumulated dye at the treated sites was extracted and estimated quantitatively according to the method of Harada, *et al.*[13] AB-50 was intravenously administered 5 min before injection of the dye and a vasoactive substance. Acetylsalicylic acid was orally administered 30 min before the injection as a standard drug. When administered locally, AB-23 and DSCG mixed with the Compound 48/80 solution were given intradermally.

Rat Mast Cell Degranulation (RMCD) Technique——Peritoneal cells of rats were isolated by the method of Uvnäs and Thon[14] using Ficoll (mol. wt., around 400000, Pharmacia Fine Chemicals, Sweden) density gradient. Isolated mast cells were suspended in a buffer salt solution (NaCl 0.9%, KCl 0.02%, CaCl$_2$ 0.01%, and Sörensen phosphate buffer, 10%, pH 7.0) containing 0.2% glucose and 2% cat serum.

The procedure of RMCD technique was carried out following the method of Korotzer, *et al.*[15] Twenty microliters of drug solution in saline with appropriate concentration was added to 20 µl of rat mast cell suspension (2×10^7 cells/ml) and the mixture was incubated for 10 min at 37°. Thereafter, 20 µl of human IgE (Pharmacia Fine Chemicals, Sweden) diluted to 250 U/ml and equal volume of rabbit anti-human IgE serum (Pharmacia Fine Chemicals, Sweden) diluted 2-fold were added and the mixture was incubated for 3 min. Twenty microliters of the reaction mixture was placed on a siliconized slide glass and covered with a

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dried coverslip previously dipped in 1% Neutral Red. One hundred mast cells over several randomly distributed microscopic fields were counted.

Antigen-induced Release of Histamine from Mast Cells in the Rat Peritoneal Cavity——Sera rich in HTA were prepared in rats as described above and were titrated by PCA in rats after a 48 hr latency. Dilution of the serum yielding a diameter of approximately 5 mm was 1: 40. Intraperitoneal release of histamine was estimated according to the method of Stechschulte and Austen.[16] Rats were injected intraperitoneally with 0.5 ml of antiserum and, 2 hr later, 2 mg of antigen was injected intraperitoneally in 5 ml of Tris-CM buffer (25 mM Tris buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 0.6 mM $CaCl_2$, and 1 mM $MgCl_2$) containing 250 μg of heparin, with or without drugs. Exactly 5 min after the antigenic challenge, the rats were stunned and exsanguinated, and the peritoneal fluid was removed. The peritoneal cells were separated by centrifugation at $175 \times g$ for 10 min at 4°. Histamine in the supernatant fluid was quantitated by fluorometry by the modification[17] of the method of Shore, Burkhalter, and Cohn.[18] Presence of drugs at the concentration used in this experiment did not interfere in the quantitative determination of histamine.

Histamine Release from Rat Peritoneal Mast Cells induced by Compound 48/80——Rats were exsanguinated and 7 ml of saline containing heparin (50 μg/ml) was introduced into the peritoneal cavity. After a gentle massage for 2 min, the peritoneal fluid was collected with a siliconized pipette into a polyethylene tube. The peritoneal fluid was centrifuged at $300 \times g$ for 5 min, the cell pellet was resuspended in 15—30 ml of Tris-CM buffer ($1—2 \times 10^4$ mast cells/ml), and divided into 0.5 ml aliquots in tubes. After preincubation of each tube for 5 min at 37°, 0.4 ml of the drug solution and 0.1 ml of the solution of Compound 48/80 were added at the same time. The reaction mixture was incubated at 37° for 15 min, and the reaction was stopped by the addition of 4 ml of chilled physiological saline. After centrifugation at $300 \times g$ for 5 min, supernatant and cell residue were analyzed for histamine content.

Dextran-induced Histamine Release from Rat Peritoneal Mast Cells——The procedure was carried out by the method of Garland and Mongar.[19] Rat peritoneal cells collected as described above were suspended in Tris-CM buffer with a higher concentration of $CaCl_2$ (1.2 mM). An aliquot of 0.4 ml of the cell suspension was placed in a siliconized tube and preincubated for 5 min at 37°. To each tube, 0.4 ml of the drug solution, 0.1 ml of phosphatidylserine (Tokyo Kasei Kogyo Co., Ltd., Tokyo) at a concentration of 100 μg/ml, and 0.1 ml of dextran (mol. wt. 250000, Pharmacia Fine Chemicals, Sweden) at a concentration of 50 mg/ml were added. After incubation of the mixture for 15 min at 37°, the reaction was stopped by the addition of 4 ml of cold saline. Histamine content was determined by the same procedure as mentioned before. Drugs, phosphatidylserine and dextran, were dissolved in Tris buffer.

Carrageenin-induced Paw Edema——The method of Winter, Risley, and Nuss[20] was used to induce paw edema in rats.

Results

PCA and Skin Reactions Induced by Permeability-increasing Substances

Both PCA and blueing reaction by histamine were performed on the same individual rats at the same time. After 48-hr latency following sensitization of skin with intradermal injection of HTA-rich serum, rats were challenged with the antigen and the dye. Immediately after injection of the antigen and the dye, histamine was injected intradermally at different sites from those of antiserum injection. Blueing reaction was observed 20 min after the injection of antigen and histamine.

As shown in Table I, intravenously administered AB-50 at a dose of 20 mg/kg almost completely inhibited PCA, but not the blueing reaction provoked by histamine. In contrast, pyribenzamine, a potent antagonist against histamine, showed a marked inhibition of both reactions at an oral dose of 125 mg/kg.

As shown in Table II, AB-50 at a dose of 20 mg/kg did not inhibit the increased vascular permeability responses induced by histamine, serotonin, or bradykinin. Acetylsalicylic acid effectively inhibited these reactions at an oral dose of 200 mg/kg.

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TABLE I. Effect of AB–50 on PCA Induced by Homocytotropic IgE-like Antibody and
Dermal Blueing Reaction Induced by Histamine in the Same Individual Rats

| Treatment | Dose (mg/kg) | Route of admini- stration | Diameter (mm) of blueing spots[a] | | | | | |
| | | | PCA | | | Histamine | | |
			1:4[b]	1:8	1:16	10[c]	2.5	1
Control			9.9 ± 0.4	7.8 ± 0.3	8.1 ± 0.9	8.1 ± 0.4	5.5 ± 0.2	4.5 ± 0.3
AB–50	20	i.v.	$1.4\pm1.1^{d)}$	0	0	8.8 ± 0.3	6.8 ± 0.7	5.8 ± 0.4
Pyribenzamine	125	p.o.	$1.3\pm0.9^{d)}$	0	0	$2.0\pm0.3^{d)}$	Not tested	$2.7\pm0.4^{e)}$

a) Value represents a mean±S.E. of four animals per group.
b) Dilution of antiserum.
c) Dose (μg) of histamine injected per site.
d) Significantly different from controls, $p<0.001$.
e) Significantly different from controls, $p<0.025$.

TABLE II. Influence of Intravenous Administration of AB–50 on Blueing
Reaction Provoked by Permeability-increasing Substances

| Treatment | Dose (mg/kg) | Route of administration | Diameters (mm) of blueing spots[a] | | |
			Bradykinin (10 μg)[b]	Serotonin (1 μg)	Histamine (5 μg)
Control			12.3 ± 0.25	13.5 ± 0.35	10.3 ± 0.14
AB–50	20	i.v.	11.7 ± 0.32	12.8 ± 0.25	9.8 ± 0.32
Acetylsalicylic acid	200	p.o.	$10.2\pm0.12^{c)}$	$10.4\pm0.24^{c)}$	$9.0\pm0.20^{d)}$

a) Value represents a mean±S.E. of four animals per group.
b) Dose injected per site.
c) Significantly different from controls, $p<0.001$.
d) Significantly different from controls, $p<0.005$.

Carrageenin-induced Paw Edema

Oral administration of 500 or 1000 mg/kg of AB–50 30 min before carrageenin injection into foot pad produced no inhibition against carrageenin-induced paw edema in rats.

Rat Mast Cell Degranulation

Results are illustrated in Fig. 1. Degranulation was observed in 48% of the mast cells in the control tube. Both AB–23 and DSCG inhibited degranulation almost completely over a range of concentration of 10^{-4} to 10^{-6} M. At 10^{-7} M, AB–23 and DSCG showed 80% and 30% inhibition, respectively.

TABLE III. Inhibitory Effect of AB–23 and DSCG on Antigen-induced Histamine
Release in the Rat Peritoneal Cavity

Drugs	Dose (M)	No. of rats	Histamine released[a] (μg/rat)
Control		26	4.00 ± 0.23
AB–23	1×10^{-5}	5	$2.30\pm0.24^{b)}$
AB–23	5×10^{-5}	9	$1.55\pm0.23^{b)}$
AB–23	1×10^{-4}	5	$0.82\pm0.35^{b)}$
DSCG	5×10^{-5}	3	$0.60\pm0.15^{b)}$
Spontaneous release[c]		14	1.31 ± 0.18

a) Value represents a mean±S.E.
b) Significantly different from controls, $p<0.005$.
c) Either antibody or antigen was substituted for by buffer.

Antigen-induced Histamine Release in the Peritoneal Cavity

As shown in Table III, inhibition of antigen-induced release of histamine in rat perito-neal cavity by AB-23 was found at doses between 1×10^{-5} and 1×10^{-4} M in a dose-dependent manner. DSCG also produced marked inhibition at 5×10^{-5} M.

Compound 48/80-induced Histamine Release from Rat Peritoneal Mast Cells

Compound 48/80 at a concentration of 0.5 µg/ml was capable of inducing release of 73.4% of total histamine. As illustrated in Fig. 2, the reaction was effectively inhibited by both
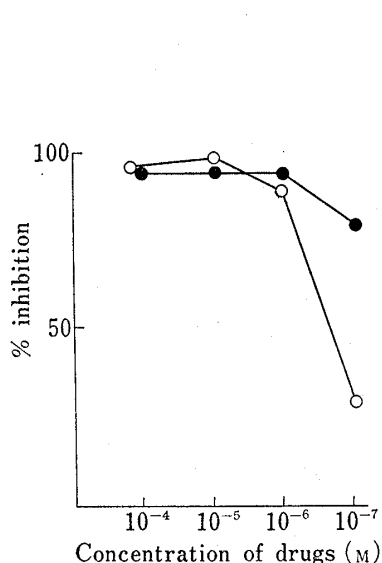
Fig. 1. Inhibitory Effect of AB-23 and DSCG on IgE-anti-IgE-Mediated Rat Mast Cell Degranulation
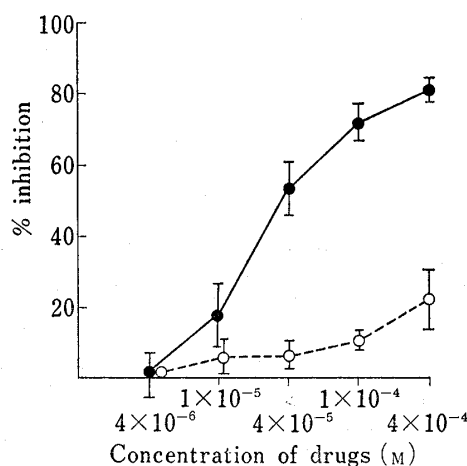
AB-23 (●); DSCG (○).

Fig. 2. Effect of AB-23 and DSCG on Histamine Release Induced by Compound 48/80 from Rat Peri-toneal Mast Cells

Each point indicates a mean ± S.E. of triplicate experiments. Final concentration of Compound 48/80 was 0.5 µg/ml. A total of 73.4% of hista-mine was released in control. AB-23 (●); DSCG (○).

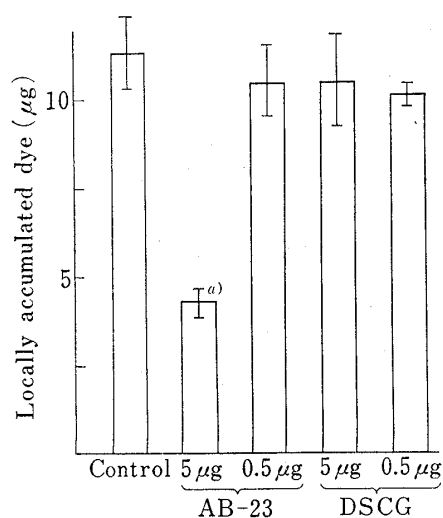| Drugs	Concentration (M)	Histamine released (%)			
		0	20	40	60
Control					
AB-23	1×10^{-4}			a)	
AB-23	4×10^{-4}			a)	
DSCG	1×10^{-4}			a)	
Spontaneous release					

Fig. 3. Inhibition of Blueing Reac-tions Provoked by Compound 48/80 by Intradermal AB-23 and DSCG

Compound 48/80 in a dose of 0.25 µg was injected per site. Height of bars and vertical lines indicate the mean ± S.E. of three animals per group.

a) Significantly different from controls, $p <$ 0.005.

Fig. 4. Effect of AB-23 and DSCG on Hista-mine Release Induced by Dextran and Pho-sphatidylserine from Rat Peritoneal Mast Cells

Height of columns and bars are mean ± S.E. of triplicate experiments.

a) Significantly different from control, $p < 0.005$.

AB–23 and DSCG in a concentration-dependent manner, but AB–23 was more potent than DSCG.

Compound 48/80-induced Blueing Reactions

As shown in Fig. 3, AB–23 when administered intradermally mixed with Compound 48/80 (0.25 μg) inhibited the blueing reaction at a dose of 5 μg/site but not at 0.5 μg/site. In contrast, DSCG at dose levels of 0.5 and 5 μg/site was ineffective.

Dextran-induced Histamine Release from Rat Peritoneal Mast Cells

As shown in Fig. 4, AB–23, when present at a concentration of 1 or 4×10^{-4}M, inhibited histamine liberation induced by dextran in the presence of phosphatidylserine from rat peritoneal mast cells. Protective potency of DSCG against this reaction at a concentration of 1×10^{-4} M was comparable to that of AB–23 at a concentration of 4×10^{-4} M.

Discussion

In a previous report, we described that both AB–23 and AB–50 inhibited PCA provoked by HTA in the rat, and that AB–50 was effective by the oral route of administration.[12] The mechanism of this action was examined in the present study and comparisons were made, both qualitatively and quantitatively, with DSCG.

Since anti-inflammatory drugs are known to suppress PCA provoked by IgE-like antibody,[21] the possibility was first tested using carrageenin edema assay whether AB–50 had an anti-inflammatory activity. This was proved to be negative. It is interesting that DSCG is also known to lack this activity.[6] The lack of anti-inflammatory activity in AB–50 may be related to the finding in the present work that this compound, unlike acetylsalicylic acid, did not inhibit the dermal reaction elicited directly by local injection of histamine, serotonin, or bradykinin which are known as chemical mediators of PCA following interaction of IgE-like antibody and antigen.[4] These results suggested a mode of anti-allergic action for AB–50 which is similar to DSCG, i.e., prevention of the release of chemical mediators from sensitized cells. Evidence supporting this suggestion was obtained from the findings that AB–23 inhibited rat mast cell degranulation *in vitro* induced by IgE-anti-IgE interaction and also reduced the amount of histamine released from mast cells in the peritoneal cavity following interaction between HTA and antigen.

The similarities between AB–50 and DSCG extend to inhibitory effect of non-anaphylactic, as opposed to anaphylactic, histamine release from mast cells. Thus both compounds reduced the histamine release from mast cells induced by Compound 48/80 or dextran.

A membrane-stabilizing effect has been implicated in the mechanism of action of DSCG on mast cells. Therefore, we have conducted several experiments to see whether AB–23 interacts with membranes of erythrocyte, lysosomes, and platelets that are known to be non-specifically stabilized by many non-steroidal anti-inflammatory drugs.[22] Except for heat-induced hemolysis which was strongly inhibited by AB–23,[23] there was no inhibition of spontaneous enzyme release from rat liver lysosomes and collagen-induced platelet aggregation (unpublished). The interaction of AB–23 thus appears to be limited to a particular type or types of cell membranes, i.e., those of mast cells and erythrocytes, and this gives an additional evidence indicating distinction between AB–50 and non-steroidal antiinflammatory drugs.

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While the results of this study demonstrate the qualitative similarities between AB–50 and DSCG in the mode of anti-allergic action, certain quantitative differences should be pointed out. First, a much higher degree of inhibition of IgE-anti-IgE-mediated mast cell degranulation was obtained at a low concentration of 10^{-7} M with AB–23 than with DSCG. Second, non-anaphylactic histamine release from mast cells induced by Compound 48/80 was inhibited by AB–23 at much lower concentrations than by DSCG. This *in vitro* difference was well reflected in the corresponding *in vivo* system of blueing reactions provoked by locally injected Compound 48/80, and 5 μg of AB–23 mixed with Compound 48/80 was capable of inhibiting the blueing reactions while the same amount of DSCG had no effect. Whether the difference between AB–23 and DSCG in the degree of the inhibition of histamine release from mast cells induced by dextran and phosphatidylserine is significant or not cannot be determined at the moment.