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Anti-complement Activities of 2,4-Bis(2-hydroxybenzamido)-benzoic Acid and Its Diacetylated Derivatives[1]

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Based on the report that 2,4-bis(2-hydroxybenzamido)benzoic acid (AB-23) inhibits immune hemolysis *in vitro*, the site of the action of this compound was studied. AB-23 had no effect on the binding of hemolysin to sheep erythrocytes (E). A high concentration of AB-23 was required for inhibition of higher dose C-induced hemolysis. E which escaped hemolysis in the presence of AB-23, were normally hemolyzed by readdition of fresh C after removal of the drug. Inhibition by AB-23 of C fixation to sensitized E was suggested from these results and confirmed by the finding that AB-23 interferes with immune adherence hemagglutination. The effect of AB-23 was apparently not due to chelation of Mg^{2+} or Ca^{2+}. AB-23, but neither ethylene diamine tetraacetic acid nor ethylene glycol tetraacetic acid showed inhibitory activity even when added 8 or 15 min after C addition. This suggests that AB-23 inhibits the sequence of later components of C from C3 to C9. This possibility was strongly supported by the following finding that AB-23 prevents the consumption of C activity by zymosan *via* an alternative pathway. Additionally, 2,4-bis(2-acetoxybenzamido)benzoic acid, which is easily changed to AB-23 in the body, inhibited the passive Arthus reaction in guinea-pigs and also prevented urinary protein excretion in rats after nephrotoxic serum injection in which complement is known to play an important role. These results suggest the anti-complement effect of AB-23 *in vivo*.

Keywords——anti-complementary agent; immune hemolysis; complement fixation; immune adherence hemagglutination; Arthus reaction; nephrotoxic serum nephritis

Introduction

It has been reported that 2,4-bis(2-hydroxybenzamido)benzoic acid (AB-23), newly synthesized in our laboratories, strongly inhibits the immune hemolytic reaction *in vitro*.[3] In this work, experiments were undertaken to determine the site of action of this compound. From the results of *in vitro* experiments, it was found that the inhibition of hemolysis by AB-23 was based upon its anti-complement activity.

It is well known that complement (C) plays an important role in some allergic reactions classified as types II and III by Coombs and Gell.[4] Consequently, it is considered that compounds which have anti-C activity should inhibit some allergic reactions. From this point of view, the experiments were designed to examine the effect of 2,4-bis(2-acetoxybenzamido)benzoic acid (AB-50), the diacetylated form of AB-23 which is easily converted to AB-23 in the body, on the Arthus reaction and nephrotoxic serum nephritis in which the action of C is essential. The results of these experiments are presented in this report.

Materials and Methods

I. Immune Hemolysis——Gelatin veronal buffer containing Ca^{2+} and Mg^{2+}(GVB^{2+}), prepared according to Mayer,[5] was used for solutions, dilutions and washings. Sheep erythrocytes, purchased from Toshiba

1) A part of this work was presented at the 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April, 1976.
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3) T. Mori, Y. Ohsugi, K. Furuno, S. Takaku, T. Matsuno, S. Suzuki, K. Koyama, M. Shindo, and Y. Takagaki, *Yakugaku Zasshi*, **95**, 1477 (1975).
4) R.R.A. Coombs and P.G.H. Gell, "Clinical Aspects of Immunology," 2nd ed., Blackwell Scientific Publications, Oxford, 1968, p. 575.
5) M.M. Mayer, A.G. Osler, O.G. Bier, and M. Heidelberger, *J. Exp. Med.*, **84**, 535 (1946).

Kagaku, were washed three times in GVB^{2+} and resuspended to a concentration of 1×10^9/ml(E). Anti-erythrocytes rabbit serum (hemolysin) was obtained from Kitasato Institute and 400-fold diluted with GVB^{2+}(A). Sensitized E(EA) was prepared by mixing E with the same volume of A containing twice the optimal dose. The source of C used was pooled serum obtained from five healthy guinea pigs by heart puncture and stored at −70° until use. Unless otherwise noted, the serum was diluted to 1: 400. The hemolytic reaction was performed according to Mayer's method[6] in principle. For estimation of hemolysis the reaction mixture was centrifuged and $OD_{541m\mu}$ in the supernatant was determined spectrophotometrically. Zymosan was obtained from the Sigma Chemical Company and boiled for 30 min before use.

Details are given in the legend to each Table and Figure.

II. Immune Adherence Hemagglutination (IAHA)——The method of the immune adherence hemagglutination test was as described by Nishioka.[7]

III. Passive Arthus Reaction——Antiserum against bovine serum albumin (BSA, Armour Pharmaceutical Company) was prepared as follows. Male albino rabbits (produced in our laboratories) weighing 2.5—3.0 kg were given injections into the foot pads of 3 mg of BSA emulsified in Freund's complete adjuvant. On the 33rd and 43rd day, the animals were intravenosuly injected with 1 mg of BSA emulsified in 1% potassium aluminum sulfate. Seven days after the last injection, blood was obtained from cardiac puncture and antiserum was separated.

Male guinea pigs weighing 250—300 g (Shizuoka Laboratory Animals, an Agricultural Cooperative Association) were given 1 ml of antiserum intravenously. One hour later 1 mg of antigen was injected intracutaneously into the flank.

IV. Nephrotoxic Serum Nephritis——The renal cortex of Wistar Imamichi rats (produced in our laboratories) was homogenized in sterile saline. Twenty per cent homogenate was mixed with a fourfold volume of 10% potassium aluminum sulfate and adjusted to pH 6.5. Rabbits were injected intramuscularly with 3 ml of the antigen emulsion thus prepared 4 times at weekly intervals. Fourteen days after the last injection animals were bled by cardiac puncture. Nephrotoxic serum (NTS) was separated and stored at −70° until use. For induction of nephritis, 0.1 ml of NTS was intravenously injected for 4 consecutive days into male rats weighing 130—180 g. Nephritic rats were kept individually in metabolic cages and the urine was collected from 5: 00 p.m. to 9: 00 a.m. the next day. Urinary protein was quantified by the method of Kingsbury-Clark.[8]

AB-50 was suspended in 1% aqueous arabic gum and administered orally 30 min before each injection of NTS.

Results

Effect of AB-23 on Immune Hemolysis

The effect of AB-23 on the binding of A to E was examined. As shown in Table I, when C was added to EA prepared in the presence of 5×10^{-4} M AB-23, hemolysis normally occurred. This result indicates that AB-23 does not inhibit binding of A to E.

Table I. Effect of AB-23 on Binding of Hemolysin (A) to Sheep Erythrocytes (E)

Hemolysin		Hemolysis (%)
1:400	control	31.6
	AB-23 5×10^{-4}M	34.3
1:200	control	53.8
	AB-23 5×10^{-4}M	54.1

A mixture of 0.2 ml of E and the same volume of A diluted to 1:200 or 1:400 was incubated for 30 min at 37° in the presence of 0.4 ml of 1×10^{-3}M AB-23 or GVB^{2+} alone (control). The cells were centrifuged, resuspended in 2 ml of GVB^{2+} and combined with 1 ml of C. The hemolytic reaction proceeded for 60 min at 37°.

To test the action of AB-23 on the interaction of EA and C, various concentrations of AB-23 were added to EA immediately before C addition. AB-23 inhibited hemolysis in a

6) M.M. Mayer, "Experimental Immunochemistry", 2nd ed., Charles C. Thomas, Springfield, Illinois, 1961.
7) K. Nishioka, *J. Immunol.*, **90**, 86 (1963).
8) F.B. Kingsbury, C.P. Clark, G. Williams, and A.L. Post, *J. Lab. Clin. Med.*, **11**, 981 (1926).

concentration dependent manner in a range of 1.25—5×10^{-4} M. No hemolysis occurred when unhemolyzed cells were resuspended after removal of the drug and incubated as they stood. However, hemolysis was induced by the addition of fresh C to the cells (Table II).

Table II. Inhibitory Effect of AB-23 on Complement (C) Fixation

AB-23 ($\times10^{-5}$ M)	Hemolysis (%)	Inhibition (%)	Addition of fresh C	Hemolysis (%)
50	2.9	96.1	−	2.3
			+	79.5
25	16.2	78.0	−	11.5
			+	86.8
12.5	44.1	40.0	−	7.0
			+	93.0
6.3	66.2	10.0	−	13.3
			+	93.0
0	73.5			

One ml of C was added to a mixture of 0.4 ml of EA and 1.6 ml of AB-23 solution or GVB^{2+} as a control. After incubation for 60 min at 37°, reaction mixtures were chilled and centrifuged. $OD_{541m\mu}$ was determined in the supernatant. Unhemolyzed cells were resuspended in 2 ml of GVB^{2+} and incubated for 60 min at 37° with or without 1 ml of fresh C.

In the next experiment, the relationship between inhibition by AB-23 and concentration of C was investigated. As shown in Fig. 1, hemolysis induced by the addition of 600-fold diluted C to EA was completely inhibited by AB-23 at 2.5—5×10^{-4} M, and even at 6.25×10^{-5} M, about 50% inhibition was obtained. The inhibitory effect was dependent on the dose of C *i.e.*, higher concentrations of AB-23 were required for inhibition of hemolysis by higher concentrations of C. Thus, AB-23 at 5×10^{-4} M could not inhibit hemolysis induced by 100-fold diluted C.

The possibility that AB-23 interferes with complement activation by chelating Mg^{2+} or Ca^{2+} was investigated. As indicated in Table III, addition of neither Mg^{2+} nor Ca^{2+} in molar excess over AB-23 reversed the inhibition of hemolysis. In contrast, inhibitory effects by ethylenediamine tetraacetic acid (EDTA) and ethylene glycol tetraacetic acid (EGTA) used as standards were abrogated by the addition of Mg^{2+} or Ca^{2+}.

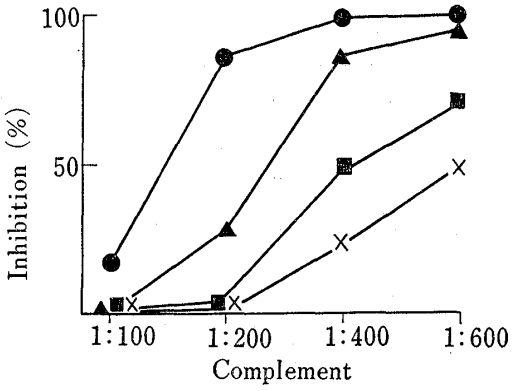

Fig. 1. Relationship between Inhibition of Hemolysis by AB-23 and Concentration of Complement

Immediately after the addition of 1.6 ml of AB-23 or GVB^{2+} to 0.4 ml of EA, 1 ml of C diluted to various concentrations was added. The hemolytic reaction proceeded for 60 min at 37°.
AB-23; (●): 5×10^{-4}M, (▲): 2.5×10^{-4}M, (■): 1.25×10^{-4}M, (×): 0.625×10^{-4}M

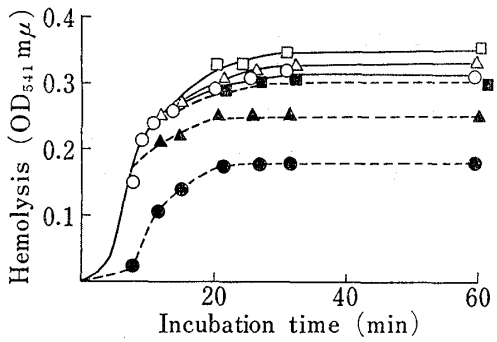

Fig. 2. Relationship between Addition Time of AB-23 and Inhibition of Immune Hemolysis

Samples of 0.3 ml of 1×10^{-3}M AB-23 (solid symbols connected with dotted lines) or GVB^{2+} (blank symbols connected with solid lines) was added to the reaction mixture consisting of 0.4 ml of EA, 1 ml of C and 1.6 ml of GVB^{2+}; at the same time as (circle), 8 min after (triangle), and 15 min after (square) the start of the hemolytic reaction.

Table III. Effect of Addition of Ca^{2+} and/or Mg^{2+} on Inhibition by AB-23 of Immune Hemolysis

Drug ($\times 10^{-4}$ M)		Inhibition(%) of immune hemolysis in the presence of; None	Mg^{2+} [a]	Ca^{2+} [b]	$Ca^{2+}+Mg^{2+}$ [c]
AB-23	5	99.7	99.7	98.1	99.3
	2.5	85.1	86.5	85.6	85.4
	1.25	51.4	53.7	53.5	52.4
	0.625	27.7	30.7	27.9	25.0
EDTA	10	94.5	83.9	57.1	—[d]
	5	97.9	39.7	25.0	19.5
	2.5	18.2	2.8	0	—
EGTA	10	99.3	99.7	56.7	—
	5	98.4	99.7	0	—
	2.5	95.1	96.5	0	—

a) 1×10^{-3}M $MgCl_2$, *b*) 1×10^{-3}M $CaCl_2$, *c*) 1×10^{-3}M $MgCl_2+1\times10^{-3}$M $CaCl_2$, *d*) not tested
Immune hemolysis was performed in the same manner as described in the legends of the preceding Table and Fig. Just before each drug addition, $CaCl_2$ and/or $MgCl_2$ was added at a final concentration of 1×10^{-3} M.

AB-23, EDTA or EGTA was added at various times after the addition of C, and the effect of each agent was investigated. The results of kinetic studies in the case of AB-23 are illustrated in Fig. 2. AB-23 inhibited hemolysis even when added 8 or 15 min after initiation of the reaction. The results of an experiment undertaken under conditions similar to those described in Fig. 2 are shown in Table IV. The degree of inhibition by AB-23 was less affected by the addition time. On the other hand, both EDTA and EGTA almost completely inhibited hemolysis when added at the start of the reaction, but no inhibition was observed when they were added 8 min after.

Table IV. Inhibition of Immune Hemolysis by AB-23, EGTA or EDTA Added at Various Time

		Inhibition (%)[a] Addition time (min) 0	8	15
AB-23	1×10^{-4}M	57.4	50.0	46.6
EGTA	2×10^{-4}M	91.9	3.0	0
EDTA	5×10^{-4}M	97.1	0	0

Experiments were performed as described in the legend of Fig 2.
a) % inhibition was calculated as follows:

$$\left[1-\frac{(\%\text{ hemolysis 60 min after in the tube containing drugs})-(\%\text{ hemolysis just before drug addition})}{(\%\text{ hemolysis in the control tube})-(\%\text{ hemolysis just before drug addition})}\right]\times 100$$

Effect on IAHA

EA was incubated with C in the presence or absence of AB-23, centrifuged, washed and resuspended in GVB^{2+}, and thereafter human erythrocytes (hu E) were added. As shown in Table V, AB-23 inhibited IAHA in a concentration-dependent manner (Exp. 1). However, AB-23 had no effect when added at the reaction stage in which EAC interacted with hu E (Exp. 2).

TABLE V. Effect of AB-23 on Immune Adherence Hemagglutination

Concentration of drugs ($\times 10^{-4}$ M)		Immune adherence pattern	
		Exp. 1	Exp. 2
Control		4	4
AB-23	5.0	0	4
	2.5	0	4
	1.25	2	4
	0.63	4	4
EDTA	5.0	0	4
	2.5	1	4
	1.25	4	4
	0.63	4	4

In Exp. 1, 0.1 ml of 0.5% EA was incubated with 0.3 ml of C diluted to 1:2500 for 30 min at 37° in the presence of 0.5 ml of AB-23, EDTA or GVB^{2+}. Cells were centrifuged, washed, resuspended with 0.9 ml of GVB^{2+}, and combined with 0.1 ml of 2% huE.

In Exp. 2, EA was incubated with C. After centrifugation and washing, cells were resuspended with 0.4 ml of GVB^{2+} and incubated with 0.1 ml of 2% huE in the presence of the drugs.

A grading of 0 to 4 was made depending on the intensity of IA pattern.

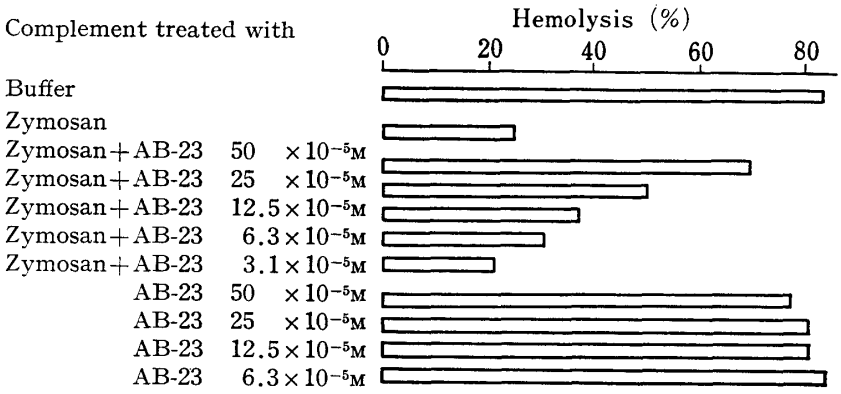

Fig. 3. Inhibitory Effect of AB-23 on Activation of Alternative Pathway by Zymosan

Samples of 0.1 ml of thirteen-fold diluted C was treated with 0.1 ml of zymosan (4 mg/ml) and/or 0.2 ml of AB-23 for 30 min at 37°. After that, the mixtures were centrifuged and 0.2 ml of supernatant was mixed with 0.4 ml of EA and 2.4 ml of GVB^{2+}. The hemolytic reaction proceeded for 30 min at 37°.

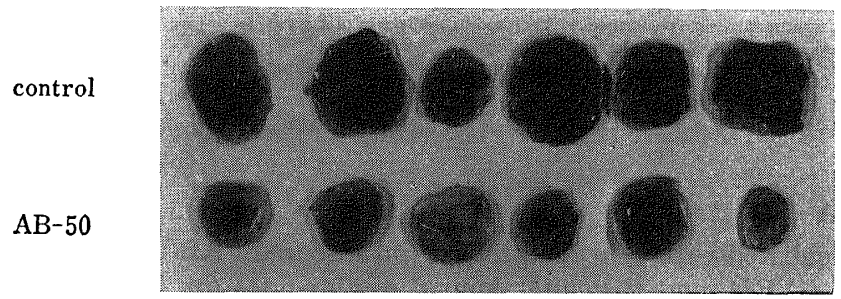

Fig. 4. Effect of AB-50 on Passive Arthus Reaction in Guinea Pigs

A dose of 50 mg/kg AB-50 was administered intraperitoneally 30 min before subcutaneous injection of antigen (1 mg). Photograph shows hemorrhage observed on the inside of the skin 5 hr after antigen injection.

Effect on Consumption of C by Zymosan

As shown in Fig. 3, zymosan caused a marked decrease in whole C activity in guinea pig serum. AB-23 prevented the consumption of C activity by zymosan with dependence on the drug concentration.

Incubation of C with 5×10^{-4} M AB-23 caused no significant decrease in C activity.

Inhibition of Passive Arthus Reaction

As shown in Fig. 4, an intraperitoneal injection of 50 mg/kg AB-50 30 min before antigen injection markedly inhibited the passive Arthus reaction in guinea pigs.

Effect on Nephrotoxic Serum Nephritis

As shown in Table VI, oral administration of AB-50 at doses of 200 or 500 mg/kg significantly prevented protein excretion in urine after NTS injection into rats. Urinary volume was not changed by treatment with AB-50.

Table VI. Inhibition by AB-50 of Protein Excretion in Urine after Nephrotoxic Serum Injection into Rats

Days after NTS injection	Dose (mg/kg)	No. of rats	Urinary volume (ml/16hr)	Urinary protein (mg/ml)	Urinary protein (mg/16hr)
3	0	15	8.1±1.0	12.1±2.0	94.1±18.4
	100	8	7.3±0.9	8.2±3.0	48.4±14.4
	200	16	6.1±0.5	7.6±1.8	45.3±10.5[a]
	500	12	6.9±1.1	4.6±1.1[b]	26.7± 5.9[b]
4	0	15	7.7±0.7	33.9±4.1	235.3±26.2
	100	8	6.1±0.5	27.5±3.9	164.3±23.1
	200	16	6.4±0.6	24.4±3.8	138.2±20.0[b]
	500	12	6.4±0.9	13.9±3.0[c]	84.8±17.0[c]

Statistical significance compared with control group;
a) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$

Discussion

It was previously reported that AB-23 strongly inhibited the immune hemolytic reaction.[3] In this paper the mechanisms of the action of AB-23 were studied. To study the site of action of AB-23, the hemolytic reaction was divided into two stages. The first stage was the binding of A to E and the second the C fixation to EA. The results revealed that AB-23 did not interfere in the first stage, but did interfere in the 2nd one. When unhemolyzed cells in the presence of AB-23 were harvested by centrifugation, resuspended and incubated, no hemolysis occurred. However, normal hemolysis was observed when fresh complement was added to this cell suspension. The inhibition of hemolysis by AB-23 depended on the concentration of the drug and C, *i.e.*, a high concentration of AB-23 was required for inhibition of hemolysis induced by a higher concentration of C. These results suggest that AB-23 inhibited the C fixation to EA. This idea was strongly supported by the finding that AB-23 also inhibited IAHA which was induced by the interaction between the third component of C (C3) fixed with EA and the C3 receptor on human erythrocytes. As the binding of C3 to the C3 receptor was not inhibited by the presence of high concentrations of AB-23, it is obvious that AB-23 inhibits the sequence from C1 to C3.

It is well known that compounds having phenolic hydroxyl groups usually form chelating complexes with metal ions. Therefore, an experiment was performed to determine if anti-C activity of AB-23 is due to the chelating effect against divalent cations such as Ca^{2+} and Mg^{2+} which were essential in C activation.[6] However, the inhibitory activity of AB-23

on hemolysis was not affected by the excess addition of divalent cations. In addition, inhibition of immune hemolysis by chelating agents such as EDTA and EGTA was observed only when the agents were added to the reaction mixture simultaneously with C, but it was not observed when they were added 8 min after C addition. In contarst, AB-23 inhibited the reaction even when added 15 min after, and the degree of the inhibition was not altered by the addition time. These results suggested that the C sequencial reaction from C1 to C2 which required divalent cations[6] was completed by 8 min after the start of the reaction. Therefore AB-23 probably inhibits the fixation stage of late components from C3 to C9. To assess this possibility, the effects of AB-23 on zymosan-induced C activation which is known to pass alternative pathway[9] was tested. It was found that AB-23 prevented the consumption of C activity by zymosan.

On the other hand, AB-50, the diacetylated form of AB-23 which is absorbable through the gastrointestinal tract and easily transformed into AB-23 in the body,[10] suppressed both the Arthus reaction in guinea pigs and nephrotoxic serum nephritis in rats, when administered intraperitoneally or orally. These experimental models belong to types II and III allergic reactions as classified by Coombs, in which C plays an important role.[4] These results suggested that AB-23 might prevent C activation following antibody-antigen interaction *in vivo*. This suggestion could not be clarified unfortunately because serum C level (CH_{50}) in nephritic rats did not decrease so strikingly as compared with normal rats.

As mentioned above, AB-23 was found to have an inhibitory activity against C both *in vitro* and *in vivo*. In the 6-month toxicity studies, rats tolerated well oral doses of up to 2500 mg/kg of AB-50 (unpublished data). These findings appear to suggest that AB-50 is useful as a chemotherapeutic agent for some allergic disorders in humans in which C might play a main role.

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10) Y. Ohsugi, S. Takaku, T. Mori, T. Matsuno, M. Shindo, and Y. Takagaki, *Yakugaku Zasshi*, **96**, 165 (1976).