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Studies on the Antigenicity of Glucagon

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Kasama, T., Aida, Y., Oshiro, K., Goto, M., Uchida, M. and Ohkuni, H. *Studies on the Antigenicity of Glucagon.* Tohoku J. exp. Med., 1983, **141** (4), 407–415 —— In order to test the safety of glucagon (GG), the immunogenicity of GG was studied in rabbits and guinea pigs. Any sensitization, as revealed by anaphylactic shock, Arthus reaction, passive cutaneous anaphylaxis (PCA) or radioimmunoassay, was not demonstrated in animals immunized with GG alone. The anti-GG antibodies were detected by PCA and radioimmunoassay in two of the five animals immunized with GG mixed with Freund's complete adjuvant. Anti-GG IgE antibody production in mice was found in one of the ten mice given 570 μg and threeof the six mice given 1,000 μg of GG with alminum hydroxide gel (Alum), but other doses of GG with Alum produced no IgE antibody. These results indicate that the antigenicity of GG is very weak. ———— glucagon; antiglucagon antibody; reaginic antibody; anaphylactic shock; passive cutaneous anaphylaxis

Glucagon (GG) is secreted by the cells of the islets of Langerhans in the pancreas. It is a polypeptide with a molecular weight of 3,485 daltons consisting of 29 amino acids. The effects of GG are well known to elevate the blood sugar level and to enhance the insulin and growth hormone secretion (Kimball and Murlin 1923; Samols et al. 1965; Crockford et al. 1966; Mitchell et al. 1969). Farah and Tuttle (1960) reported that continuous GG infusion affected the cardiac function.

The production of antibody against GG (anti-GG antibody) has recently been reported to occur by the sensitization of animals with GG and Freund's complete adjuvant (FCA) or by other methods (Unger et al. 1961; Sutherland et al. 1968), although it was considered unlikely that GG has an antigenicity because of its small molecular weight and the presence of GG-producing tissue in the body. In order to confirm the above results and demonstrate the safety of GG in clinical practice, the studies on antigenicity of GG were performed.

Materials and Methods

Antigens

Porcine glucagon preparation, a commercial product, containing 1.0 mg glucagon and 107.0 mg lactose (GG, Lot. No. 20 *4, 10 *6, Novo Industri A/s, Copenhagen, Den.)

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and bovine serum albumin (BSA, grade V, Wako Pure Chemical Industries, Osaka) were used as antigens.

Animals

Male albino rabbits weighing 2.5 to 3.5 kg (Nippon Bio-Supply Center, Tokyo), male Hartley guinea pigs of approximately 300 g body weight (Nippon Ikagaku Jikken Dobutsu, Tokyo), male A/He and BALB/c mice of 8 weeks old (Nippon Bio-Supply Center) were used in the present experiments. Female Wistar rats weighing 150 to 200 g (Nippon Bio-Supply Center) were used for the detection of reaginic antibody in mice against GG.

Immunization

In the group immunized with GG alone, 2 mg of GG dissolved in physiological saline was injected into the foot pads of the rabbits or 1 mg of GG was injected subcutaneously in the backs of the guinea pigs 3 times a week for 2 weeks.

In the group immunized with GG plus complete Freund's adjuvant (FCA, Difco Lab., Detroit, Mich.), a volume of GG (1 mg/ml) was mixed with an equal volume of FCA and 0.5 mg emulsion was injected into the foot pads of the rabbits or subcutaneously in the backs of the guinea pigs once a week for 3 weeks. The control group was injected with saline with FCA and the immunization was also carried out by using 1% BSA with FCA. Blood samples were obtained on the 10th day after the last immunization.

A/He and BALB/c mice were immunized by an intraperitoneal injection of 50, 200, 570 and 1,000 μg of GG added with 2.0 mg aluminum hydroxide gel (Alum). The mice were bled from the orbital sinus and serum specimens were taken subsequently. A booster injection of GG was given 3 weeks later. Similar immunization was carried out with Alum alone as the control.

Active systemic anaphylaxis

Anaphylactic shock was elicited in immunized animals by the administration of GG solution. To the immunized rabbits and guinea pigs, 1.0 ml of GG solution (1 mg/ml) was injected intravenously on the 10th day after the last immunization. To the rabbits and guinea pigs immunized with BSA, 1.0 ml of 1% BSA solution was injected intravenously to conduct the above test. Death by shock was regarded as the positive result.

Arthus reaction

The rabbits surviving the anaphylactic shock test were used again for the Arthus reaction which was carried out 10 days after the anaphylactic shock test. The backs of the immunized rabbits were shaved and 0.2 ml of GG (1 mg/ml) was injected intradermally. The result was judged based on the development of edema, erythema and/or hemorrhage at the site of injection 24 hr later. In the rabbits sensitized with BSA, the test was performed by injecting 0.2 ml of 1% BSA intradermally.

Passive cutaneous anaphylaxis (PCA)

Anti-GG antibody titers in the sera of GG-immunized rabbits were determined quantitatively by 3 hr-PCA in guinea pigs as described by Ovary (1953, 1958). After depilation of the side abdominal skin of guinea pigs, 0.1 ml aliquots of twofold dilutions of antiserum were injected intracutaneously. 3 hr later, the guinea pigs were injected intravenously with 0.5 ml of 2% Evans Blue (Wako Pure Chemical Industries, Osaka) saline solution containing 1.0 mg of GG. The animals were killed by heart puncture after 20 min.

Anti-GG reaginic antibody (anti-GG IgE antibody) titers in the sera of GG-immunized mice were determined by 48 hr-PCA in Wistar rats as described by Ovary et al. (1975). After depilation of the side abdominal skin of rats, each 0.1 ml aliquot of twofold dilution of antiserum was injected intracutaneously. 48 hr later, the animals were injected intravenously with 0.5 ml of 2% Evans Blue solution containing 1.0 mg of GG. The rats were killed after 30 min.

The diameters of the blue spot that appeared at the site of injection of the test antiserum specimen were measured; a spot with a diameter of 5 mm or more was regarded as inducating a positive response. The PCA titers of serum specimens were expressed as the highest serum dilutions giving a positive response.

Measurement of anti-GG antibody by radioimmunoassay

To a 0.4 ml aliquot of 10-fold dilution of GG-immunized rabbit serum were added 0.2 ml of phosphate buffer (pH 7.4), 0.1 ml of Trasylol (Bayer, Leverkusen) and 0.1 ml of ^{125}I-glucagon (20 pg). The mixture was incubated at 4°C for 48 hr, followed by adding 0.2 ml human serum and 1.0 ml of 25% polyethylene glycol as a carrier and stirred. Then, the mixture was centrifuged at 1,500×g for 20 min. The radioactivity of the precipitate was measured and the antibody titer was expressed as the % bound radioactivity of the total activity added.

Results

Anaphylactic shock test

The results of anaphylactic shock of GG-immunized rabbits are shown in Table 1. In neither the rabbits immunized by injection of GG alone nor those immunized with GG plus FAC, anaphylaxis occurred. Similarly no anaphylaxis was observed in guinea pigs.

As the positive control of anaphylaxis, rabbits and guinea pigs immunized by injection of BSA with FCA were elicited by the administration of BSA. Piloerection, tremor and a state of unrest occurred immediately and death due to dyspnea was rapidly caused. In the group immunized with BSA alone, piloerection, tremor, light dyspnea and gait disturbance were observed, but the animals never died (Table 1). Intravenous injections of the GG preparation in normal rabbits and guinea pigs did not cause the anaphylactic reaction at all.

Table 1. *Anaphylactic shock in rabbits and guinea pigs sensitized with glucagon or BSA*

Sensitizing antigen	Challenging antigen	Animal	
		Rabbit	Guinea pig
—	Glucagon	0/5*	0/5
Glucagon	Glucagon	0/5	0/5
Glucagon+adjuvant†	Glucagon	0/5	0/5
BSA	BSA	0/5	0/5
BSA+adjuvant	BSA	5/5	5/5

* Number of death/total number of animals.
† Freund's complete adjuvant.

Arthus reaction

As shown in Table 2, weak Arthus reaction was observed in the rabbit immunized by injection of GG with FCA. That is, three of five animals sensitized with GG and FCA showed edema and induration, but no hemorrhage. A mild erythema was observed even in animals immunized with GG alone. Since a similar degree of erythema was also induced in normal control rabbits, the reaction

TABLE 2. *Arthus reaction in rabbits sensitized with glucagon and BSA*

Sensitizing antigen	Antigen for skin test	Animal No.	Means of diameter of redness at 24 hr (mm)	Number of positives/ Total number of animals
	Lactose	1	2.5	0/5
		2	2.0	
		3	3.0	
		4	9.0	
		5	2.0	
	Glucagon	1	15.0	0/5
		2	3.0	
		3	15.0	
		4	13.0	
		5	11.5	
Glucagon	Glucagon	1	14.5	0/5
		2	11.5	
		3	13.0	
		4	13.0	
		5	11.0	
Glucagon+adjuvant	Glucagon	1	24.5	3/5
		2	21.5	
		3	13.0	
		4	15.5	
		5	27.5	
BSA	BSA	1	29.5	5/5
		2	33.5	
		3	33.0	
		4	39.0	
		5	31.0	
BSA+adjuvant	BSA	1	50.0	5/5
		2	53.0	
		3	45.5	
		4	48.5	
		5	50.5	

in the group immunized with GG alone appeared to be non-specific. In the animals immunized with BSA, especially those immunized with BSA emulsified with FCA an intense Arthus reaction exhibiting hemorrhagic spots was seen (Table 2).

PCA reaction

As shown in Table 3 and Fig. 1, the 3 hr-PCA reaction was positive in two of five animals immunized with GG mixed with FCA, and the antibody titer was 1:80. All the sera of animals immunized with GG alone were negative in this reaction. Anti-BSA antibody titers ranged from 1:10 to 1:80.

The production of anti-GG antibody was observed in the mice immunized with adsorbed Alum-GG by the 48 hr-PCA reaction. The result is shown in Table 4 and Fig. 2. On the 7th day after the second immunization (booster), two of six BALB/c mice which received 1,000 μg of GG showed a positive reaction, and the antibody titer was 1:10. On the 14th day after the booster, three of six animals receiving 1,000 μg of GG showed a positive reaction, and the antibody titers were 1:5 to 1;10. On the other hand, only one of the ten BALB/c mice receiving 570 μg of GG

showed a positive 48 hr-PCA reaction, and the antibody titer was 1:5. No IgE antibody was detected in any animals which received other doses. The A/He strain mice immunized with 570 μg of GG exhibited a negative reaction.

TABLE 3. *3 hr-PCA titers and antibody titers measured by radioimmunoassay in serum of rabbits sensitized with glucagon and BSA*

Sensitizing antigen	Animal No.	3 hr-PCA titer	% bound of ^{125}I-glucagon
Glucagon	1	—*	0
	2	—	2.5
	3	—	1.2
	4	—	1.4
	5	—	2.7
Glucagon+adjuvant	1	—	12.8
	2	80†	66.5
	3	—	9.9
	4	—	8.4
	5	80	53.4
BSA	1	10	ND
	2	80	ND
	3	80	ND
	4	80	ND
	5	80	ND
BSA+adjuvant	1	80	ND
	2	80	ND
	3	80	ND
	4	80	ND
	5	80	ND

* PCA reaction was negative (<5).

† The PCA titers of serum specimens were expressed as the reciprocals of the highest serum dilutions given a positive response.

ND, not done.

TABLE 4. *Production of reaginic antibodies in mice sensitized with glucagon mixtured with aluminum hydroxide gel as an adjuvant*

Sensitizing antigen (μg/mouse)		48 hr-PCA reaction (number of positives (>5)/total number of animals)				
		8	12	21	28	35 Days
Alum+saline		0/10	0/10	0/10	0/10	0/10
Alum+glucagon	50†	0/5	0/5	0/5	0/5	0/5
	200†	0/6	0/6	0/6	0/6	0/6
	570†	0/10	0/10	0/10	0/10	1/10(5)*
	570‡	0/10	0/10	0/10	0/10	0/10
	1000†	0/6	0/6	0/6	2/6(10)	3/6(5-20)

* The PCA titers of serum specimens were expressed as the reciprocals of the highest serum dilutions giving a positive response.

† BALB/c mice.

‡ A/He mice.

Alum, aluminum hydroxide gel.

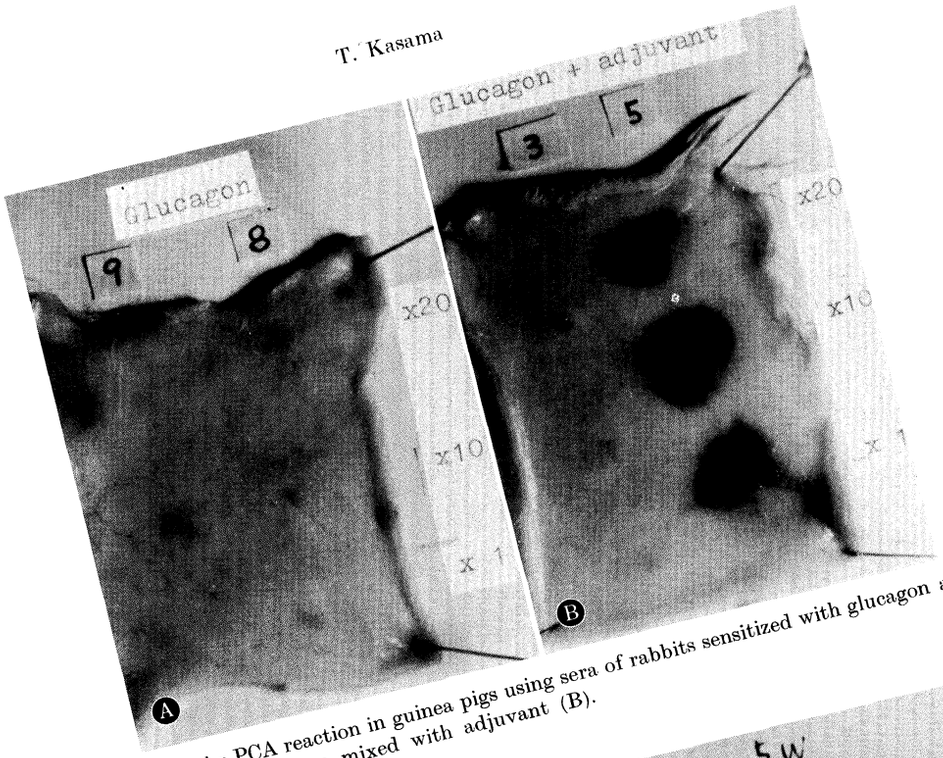

Fig. 1. 3 hr-PCA reaction in guinea pigs using sera of rabbits sensitized with glucagon alone (A) and glucagon mixed with adjuvant (B).

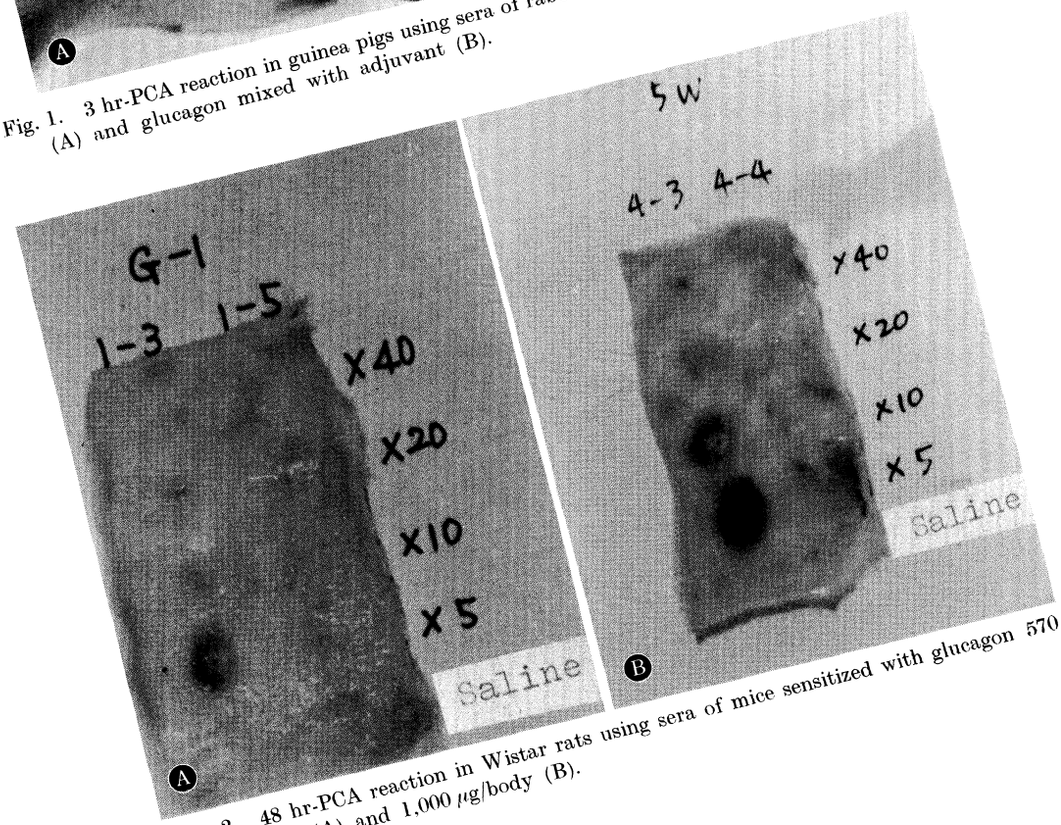

Fig. 2. 48 hr-PCA reaction in Wistar rats using sera of mice sensitized with glucagon 570 μg/body (A) and 1,000 μg/body (B).

Measurement of anti-GG antibody titer by radioimmunoassay

In two of five serum specimens from the rabbits immunized with GG and FCA, the anti-GG antibody was detected. The titers examined by radioimmunoassay 1:80 were identical with those obtained by 3 hr-PCA reaction (Table 3).

Discussion

Attention has recently been focused on the provocation of allergic reactions by administration of various drugs such as antibiotics, hormones and enzyme preparations. In order to test the safety of GG in clinical practice, the studies on antigenicity of GG were attempted.

It has been known that the production of anti-GG antibody is not readily induced by administration of GG to animals. However, Unger et al. (1961) reported the production of antibody against GG through immunization with adjuvant. Assan et al. (1965), on the other hand, found that it was difficult to produce a sufficient amount of antibodies by immunization with GG mixed with adjuvant. They reported that the addition of polyvinyl pyrrolidone facilitated the production of anti-GG antibody.

Through repeated immunization in the foot pad of rabbit with GG mixed with FCA, anti-GG antibody was detected by radioimmunoassay in two of the five animals, although the frequency of anti-GG antibody production was rather low. The 3 hr-PCA titer of these two serum specimens was 1:80. A weak Arthus reaction was observed not only in the two rabbits but also in another rabbit which exhibited negative 3 hr-PCA raction. A weak Arthus reaction was also observed in other animals in which the anti-GG antibody was not detected by radioimmunoassay. The discrepancy among the results of the 3 hr-PCA reaction, radioimmunoasay and the Arthus reaction may be due to differences in sensitivity among these tests.

An important role of reaginic antibody in allergic reaction, such as bronchial asthma and atopic dermatitis in human, has been elucidated. Aluminum hydroxide gel, *Bordetella pertussis* vaccine and gram-negative bacterial lipopolysaccharide have been found to be effective adjuvant-active substances for the production of reaginic antibody in experimental animals (Mota 1964; Prouvost-Danon et al. 1972; Perini and Mota 1973; Newburger et al. 1974; Danneman and Michael 1976). Concanavalin A has also been shown to have an adjuvant effect on reaginic antibody production in mice (Gollapudi and Kind 1975). In addition, helminth parasites have been reported to be effective potentiators of reaginic antibody formation in rats and mice (Orra and Blair 1969; Petillo and Smith 1973; Bradburg et al. 1974; Kojima and Ovary 1975). Then, Alum was used as an effective potentiator of reaginic antibody in the present experiments. BALB/c mice were immunized with GG added with Alum as an adjuvant. Anti-GG IgE antibody production was demonstrated in some animals though the 48 hr-PCA titer was low.

In all animals immunized with GG alone, anaphylactic shock and Arthus reaction were negative and antibodies were detected in none of them by 3 hr-PCA, 48 hr-PCA reaction or radioimmunoassay.

According to these experimental results, it is hardly possible to produce anti-GG antibody or anti-IgE antibody unless large doses of GG, i.e. 35 to 2,500 times as much as clinical dose, along with adjuvant, are used. Immunization with large doses of GG alone failed to produce anti-GG antibodies. So it is unlikely that the antibody against GG would be produced by the administration of GG preparation in human.

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