IgE ANTIBODIES FOR PENICILLINS AND CEPHALOSPORINS IN RATS. I

CHARACTERISTICS OF THE IgE ANTIBODIES FOR PENICILLINS AND CEPHALOSPORINS IN RATS

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Rats immunized with sulbenicillin-ovalbumin (SBPC-OvA) in combination with aluminum hydroxide (alum) and thimerosal-killed *Bordetella pertussis* produced high levels of anti-SBPC antibodies. Anti-SBPC antibodies were first detected on day 8, reaching the maximum titer on day 12 and rapidly declined thereafter. Anti-SBPC sera obtained on day 13 were sulfhydryl-labile and heat-labile. The optimal latent period in the passive cutaneous anaphylaxis (PCA) reaction was 60~72 hours. These results indicate that anti-SBPC antibodies were IgE antibodies. Sprague-Dawley (SD), Wister and F344 rats were equally productive of anti-SBPC antibodies, while SD rats were more productive of anti-cephaloridine (CER) antibodies than Wister and F344 rats did. In SD rats, the IgE antibodies for penicillin G (PCG), ampicillin (ABPC) and SBPC were more easily produced than the IgE antibodies for CER, cefazolin (CEZ) and cephacetrile (CEC).

Penicillins and cephalosporins are potentially capable of inducing all types of allergic reactions defined by COOMBS and GELL[1]. Types I, II and III allergies are thought to be elicited by the humoral antibodies, *i.e.* the IgG and IgE antibodies in type I allergy and the IgG and IgM antibodies in types II and III allergies. Type IV allergy is thought to be elicited by cell-mediated immunity. The properties of the IgG antibodies for penicillins and cephalosporins have been widely investigated in experimental animals. Usually, rabbits are used for the immunization with penicillin- or cephalosporin-protein conjugates[2~8]. However, the IgE antibodies and cell-mediated immunity for penicillins and cephalosporins have not been fully studied yet[9,10]. The present studies are, therefore, designed to investigate the production and characterization of the IgE antibodies for penicillins and cephalosporins in rats.

Materials and Methods

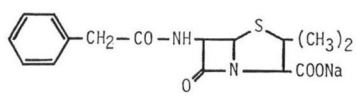
Animals

Male JCL: Spraque Dawley (SD, CLEA Japan Inc.), Wister/SLC (Shizuoka Agricultural Cooperatives Association for Laboratory Animals, Japan) and F344/DuCrj (Japan Charles River, Ltd.) rats were routinely used at 5 weeks of age for the immunization and at 8~10 weeks of age for the passive cutaneous anaphylaxis (PCA) tests.

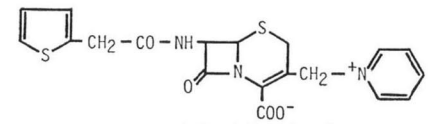
Chemicals

Penicillin G (PCG), ampicillin (ABPC) and sulbenicillin (SBPC) were obtained from commercial sources of Takeda Chemical Industries, Ltd., Osaka, Japan. Cephaloridine (CER) and cefazolin (CEZ) were purchased from Shionogi & Co., Ltd., Osaka, Japan and Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan, respectively. Cephacetrile (CEC) was supplied by CIBA-GEIGY Ltd., Basel, Switzerland. The chemical structures of these penicillins and cephalosporins are shown in Fig. 1. Ovalbumin (OvA), bovine gamma-globulin (BGG) and human serum albumin (HSA) were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A. Saline suspension of thimerosal-killed *Bordetella pertussis* containing 2×10^{10} cells per ml was prepared by Takeda Chemical Industries, Ltd.

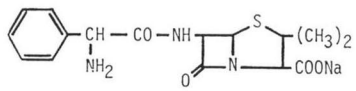
Fig. 1. Chemical structures of various penicillins and cephalosporins.



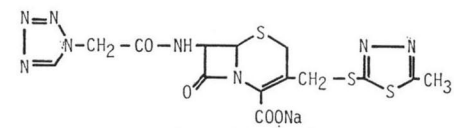
Penicillin G (PCG)



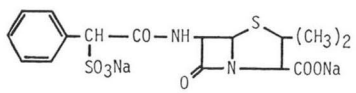
Cephaloridine (CER)



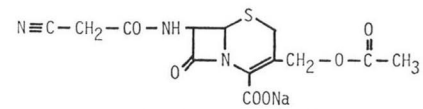
Ampicillin (ABPC)



Cefazolin (CEZ)



Sulbenicillin (SBPC)



Cephacetrile (CEC)

Hapten-protein conjugates

One hundred mg of OvA, BGG or HSA was dissolved in 10 ml of physiological saline, and penicillin or cephalosporin was added at a ratio of 50 to 2,000 M *vs.* 1 M protein. The reaction mixture was adjusted to pH 10 to 10.5 with 1 N NaOH. After 24 hours incubation at 37°C, the reaction mixture was dialysed at 4°C for 3 days continuously against 5 liters of physiological saline and then hapten-protein conjugates were chromatographed on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (3×100 cm). The final concentration of hapten-protein conjugates was adjusted to about 10 mg protein per ml. The epitope densities of haptens in penicillin- and cephalosporin-protein conjugates were measured by the penamaldate method[11] and the amino acid analysis method[12], respectively. Hapten-protein conjugates used in this study were $SBPC_{3,6,10,12}$-OvA, PCG_{20}-OvA, $ABPC_{12}$-OvA, CER_{21}-OvA, CEZ_{19}-OvA, CEC_{10}-OvA, $SBPC_{3,11,13,27,37}$-BGG, $ABPC_{56}$-BGG, CER_{86}-BGG, CEZ_{67}-BGG, CEC_{67}-BGG and $SBPC_{32}$-HSA.

Immunization

Following the method of TADA[13], rats were initially intramuscularly with 1 ml of a mixture of 5 mg of hapten-OvA and 5 mg of aluminum hydroxide (alum) and intraperitoneally with 1 ml of thimerosal-killed *B. pertussis* saline suspension. At day 5, secondary immunization was made by the intraperitoneal injection of the same dose of the mixture of hapten-OvA and alum. Blood specimens were obtained from the retro-orbital plexus at appropriate intervals or the abdominal aorta on day 13 after the first immunization. Anti-SBPC IgG sera were obtained by the method of BLOCK[14]. Rats were injected with 1 ml of a mixture of 1 mg of $SBPC_{32}$-HSA and FREUND's complete adjuvant (FCA; Difco Labs., Detroit, Michigan, U.S.A.) in four footpads, and the animals were bled from the heart 5 weeks later.

Passive cutaneous anaphylaxis (PCA) test

Assays of anti-hapten antibodies were performed by the rat homologous PCA reactions in SD rats. One-tenth ml of serial two-fold dilutions of antisera were intradermally injected in the back of rats, and 1 mg of hapten-BGG in 2 ml of 1% Evans blue saline solution was intravenously injected to the rats at various time after the sensitization. The rat 60-hour PCA reaction was routinely used for the characterization of antihapten sera. Rats were killed 30 minutes after the challenge and the degree of the reaction was estimated by measuring two perpendicular diameters of the blue spot on the underside of the skin. Blue spots with an average diameter of more than 5 mm were regarded as a positive PCA reaction. The tests were carried out in duplicate for each sample, and the PCA titers were expressed as the highest dilution which gave a positive PCA reaction.

Treatment by heat and 2-mercaptoethanol (2-ME)

Undiluted anti-SBPC-OvA serum was heated at 56°C for 30~120 minutes. The treatment of anti-SBPC-OvA serum with 2-ME was performed by the method of CRESTFIELD[15]. Anti-SBPC-OvA serum was treated with 0.01~0.3 M of 2-ME and then carboxymethylated by iodoacetamide.

Results

Induction of the Rat Homologus PCA Reaction

Rat anti-SBPC-OvA serum obtained on day 13 after the first immunization was used. The rats were sensitized by the intradermal injection of the antiserum, and the PCA reaction was elicited by the intravenous injection of SBPC-BGG at 60 hours after the sensitization (Table 1). The rats were sensitized with serial two-fold diluted antisera and challenged with various doses of $SBPC_{37}$-BGG. The same degree of the PCA reactions were elicited with 0.625 mg or more of SBPC-BGG per rat but 0.16 mg or less of SBPC-BGG per rat hardly elicited any PCA reactions. The dose of the challenging antigen used in following study was 1 mg/rat. The rats were sensitized with serial two-fold diluted antisera and challenged with SBPC-BGG which have various epitope densities. $SBPC_3$-BGG failed to elicit a significant PCA reaction, but $SBPC_{11,13,22,37}$-BGGs elicited almost the same degree of PCA reactions. In order to see whether the antisera would induce the PCA reaction specifically for hapten, 1 mg of BGG, SBPC or $SBPC_{37}$-BGG was administered to the rats sensitized with serial two-fold diluted antisera. Only SBPC-BGG elicited the PCA reaction but neither BGG nor SBPC elicited any PCA reactions. Normal serum did not react with SBPC-BGG.

Table 1. Effect of various factors on the 60-hour PCA reaction.[a]

Factor	Sensitization (Serum)	Challenge		PCA titer (Mean)
		Antigen	Dose (mg/rat)	
Dose	Anti-SBPC-OvA	$SBPC_{37}$-BGG	0.01	4 (n=2)
			0.04	4 (n=2)
			0.16	32 (n=2)
			0.625	128 (n=2)
			2.5	128 (n=2)
			10	128 (n=2)
Epitope density	Anti-SBPC-OvA	$SBPC_3$-BGG	1	8 (n=2)
		$SBPC_{11}$-BGG	1	256 (n=2)
		$SBPC_{13}$-BGG	1	128 (n=2)
		$SBPC_{22}$-BGG	1	128 (n=2)
		$SBPC_{37}$-BGG	1	256 (n=2)
Hapten specificity	Anti-SBPC-OvA	BGG	1	0 (n=3)
		SBPC	1	0 (n=3)
		$SBPC_{37}$-BGG	1	256 (n=3)
	Normal	$SBPC_{37}$-BGG	1	0 (n=3)

[a] Anti-SBPC-OvA serum was obtained on day 13 after the first immunization. Rat were sensitized with 0.1 ml of serial two-fold diluted anti-SBPC-OvA serum or normal serum, and challenged with SBPC-BGG, BGG or SBPC 60 hours after sensitization.

Table 2. Effect of various factors on the production of anti-SBPC-OvA antibodies.

Factors		Diameter of dye leakage (mm)[a] (Mean±S.D.)			PCA titer[c]
		7 days[b]	10 days	13 days	
Dose of immunization[d]	0.008 mg/rat (n=6)	0	0	0	0
	0.04 (n=6)	0	0.3±0.5	2.0±3.6	0
	0.2 (n=6)	0	0.8±2.0	8.0±8.2	11
	1 (n=6)	1.2±2.0	11.6±3.5	16.9±5.0	97
	5 (n=6)	4.4±4.1	14.7±3.2	16.9±2.0	84
Epitope density[e]	$SBPC_3$-OvA (n=10)	0.9±1.9	0	3.3±5.8	8
	$SBPC_6$-OvA (n=10)	4.1±4.1	6.7±2.3	12.7±7.5	84
	$SBPC_{10}$-OvA (n=10)	5.2±4.9	13.3±3.4	14.7±8.2	74
	$SBPC_{12}$-OvA (n=10)	2.9±4.0	11.6±2.5	17.0±1.4	97

a) Rats were sensitized with 0.1 ml of antisera, and challenged with 1 mg of $SBPC_{37}$-BGG 60 hours after sensitization.

b) Days after the first immunization.

c) PCA titers were measured on sera obtained on day 13 after the first immunization. Mean titer of 5 rats was represented.

d) Rats were injected with a mixture of $SBPC_{12}$-OvA and 5 ml of alum on day 0 and 5, and injected with 2×10^{10} cells of thimerosal-killed *B. pertussis* on day 0.

e) Rats were injected with a mixture of 5 mg of SBPC-OvA and 5 mg of alum on day 0 and 5, and injected with 2×10^{10} cells of thimerosal-killed *B. pertussis* on day 0.

Production of Anti-SBPC Antibodies in Rats

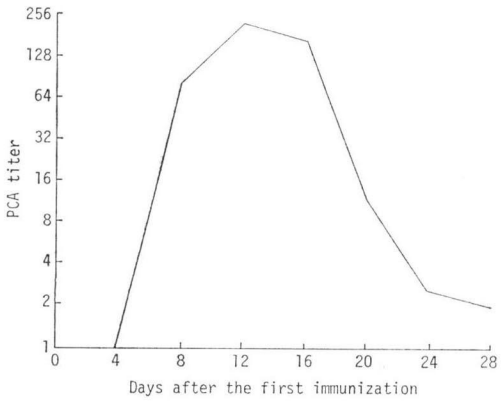
Rats were immunized with 0.008 ~ 5 mg of $SBPC_{12}$-OvA per animal (Table 2). The production of anti-SBPC antibodies by SBPC-OvA was dose-dependent, namely, 0.008 or 0.04 mg of SBPC-OvA did not produce any of the antibodies, 0.2 mg of SBPC-OvA produced only a low level, and 1 or 5 mg of SBPC-OvA produced high levels of anti-SBPC antibodies. Five mg of hapten-OvA per animals was routinely used for the immunization. The rats immunized with $SBPC_3$-OvA hardly produced any anti-SBPC antibodies, however, the rats immunized with $SBPC_{6,10,12}$-OvA produced anti-SBPC antibodies almost equally in these groups. Hapten-OvAs with more than 10 moles/mole of epitope densities were used for the immunization.

Characteristics of Anti-SBPC-OvA Serum

Anti-SBPC antibodies were detected on day 8 after the first immunization, reaching a maximal PCA titer on day 12 and rapidly declined thereafter (Fig. 2). The rats sensitized with serial two-fold dilutions of the antisera were challenged with $SBPC_{37}$-BGG at various time after sensitization (Fig. 3). The PCA reaction of anti-SBPC serum was observed for a 6-day period after the sensitization, and the maximum reaction was observed at 60 ~ 72 hours after the sensitization. The skin sensitizing activity of the serum disappeared completely after the heat treatment at 56°C for 90 minutes. On the other hand, the PCA reaction was observed only up to 12 hours after the sensitization in the rats sensitized with anti-SBPC IgG serum produced by injection with a mixture of SBPC-HSA and FREUND's complete adjuvant. The PCA activity of anti-SBPC sera was almost destroyed when the serum was treated with more than 0.05 M of 2-ME, and the anti-SBPC serum heated at 56°C for more than 60 minutes completely lost its ability to induce the PCA reaction (Table 3). These findings indicate that the anti-SBPC antibodies produced

Fig. 2. Time course of the production of anti-SBPC-OvA antibodies in rats.

Rats were injected with a mixture of 5 mg of $SBPC_{12}$-OvA and 5 mg of alum on day 0 and 5, and injected with 2×10^{10} cells of thimerosal-killed *B. pertussis* on day 0. Each point represents the mean of 5 rats.



by injection with SBPC-OvA in combination with alum and thimerosal-killed *B. pertussis* have the characteristics of IgE antibody.

Production of the IgE Antibodies

SD, Wister and F344 rats were immunized with $SBPC_{12}$-OvA and CER_{21}-OvA (Table 4). Three strains of rats were responsive equally to SBPC, however, SD rats were more responsive to CER than Wister and F344 rats. SD rats were immunized with several penicillin- and cephalosporin-OvA. The appearance of the IgE antibodies for PCG, ABPC and SBPC were the earliest, slightly later for CER and the latest for CEZ. The IgE antibody formation for CEC was not clearly observed. The PCA titers of the antisera for penicillins observed on day 13 were significantly higher than those of the antisera for cephalosporins. These results indicated that the IgE antibodies for penicillin were more easily produced than those for cephalosporins in rats.

Fig. 3. Time course of the skin sensitizing ability of rat anti-SBPC sera in rats.

Rats were sensitized with non-heated and heated anti-SBPC-OvA sera and non-heated anti-SBPC-HSA sera, and challenged with $SBPC_{37}$-BGG at various time after sensitization. Anti-SBPC-OvA sera were obtained from the rats immunized with $SBPC_{12}$-OvA, alum and thimerosal-killed *B. pertussis*.

Anti-SBPC-HSA sera were obtained from the rats immunized with $SBPC_{32}$-HSA and FREUND's complete adjuvant on day 35 after immunization.

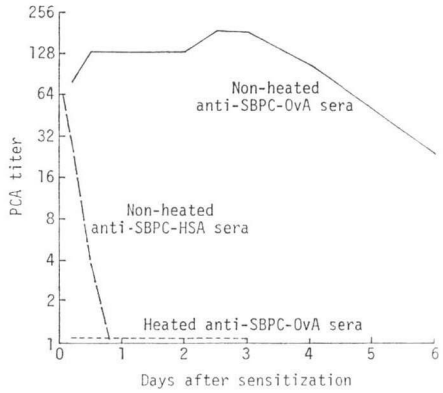


Table 3. 2-Mercaptoethanol and heat labilities of rat anti-SBPC-OvA serum.

Treatment[a]		PCA titer
2-Mercaptoethanol (Concentration; M)	0	128
	0.01	64
	0.05	2
	0.1	0
	0.3	0
Heating at 56°C (Time; minute)	0	128
	30	128
	60	0
	90	0
	120	0

[a] Rats were sensitized with 0.1 ml of serial two-fold diluted anti-$SBPC_{12}$-OvA serum, and challenged with 1 mg of $SBPC_{37}$-BGG 60 hours after sensitization.

Discussion

Rats produce reaginic antibodies which resemble the human IgE antibodies in some immunological properties as reported by BINAGHI[16], MURPHEY[17] and STECHSCHULTE[18]. The optimum conditions for

Table 4. Effect of various factors on the production of IgE antibody for various penicillins and cephalosporins.[a]

Factor		Diameter of dye leakage (mm)[b] (Mean±S.D.)			PCA titer[d]
Rat strain	Antigen	7 days[c]	10 days	13 days	
JCL: SD (n= 8)	$SBPC_{12}$-OvA	9.4±7.8	16.2±2.5	16.5±2.0	97
Wister/SLC (n= 9)	$SBPC_{12}$-OvA	4.3±3.8	11.7±5.2	16.4±9.4	84
F344/DuCrj (n= 9)	$SBPC_{12}$-OvA	5.2±4.1	13.1±2.3	17.1±2.9	111
JCL: SD (n=10)	CER_{21}-OvA	4.5±5.4	11.4±5.2	14.9±1.5	37
Wister/SLC (n= 5)	CER_{21}-OvA	0	5.7±3.6	12.8±1.3	28
F344/DuCrj (n= 5)	CER_{21}-OvA	0.6±1.2	2.7±1.4	5.5±3.5	3
JCL: SD (n= 9)	PCG_{20}-OvA	6.0±2.2	15.7±0.4	15.7±0.5	147
JCL: SD (n=10)	$ABPC_{12}$-OvA	11.3±2.2	15.8±0.4	13.4±1.7	239
JCL: SD (n= 7)	$SBPC_{12}$-OvA	9.4±1.4	16.5±0.4	16.7±0.4	97
JCL: SD (n=10)	CER_{21}-OvA	4.5±1.7	11.4±1.6	14.9±0.5	21
JCL: SD (n=10)	CEZ_{19}-OvA	0	0	13.9±1.0	28
JCL: SD (n=10)	CEC_{10}-OvA	4.8±2.1	0	0	0

a) Rats were injected with a mixture of 5 mg of hapten-OvA and 5 mg of alum on day 0 and 5, and injected with 2×10^{10} cells of thimerosal-killed *B. pertussis* on day 0.

b) Rats were sensitized with 0.1 ml of antisera, and challenged with 1 mg of hapten-BGG 60 hours after sensitization.

c) Days after the first immunization.

d) PCA titers were measured on sera obtained on day 13 after the first immunization. Mean titer of 5 rats was represented.

the production of the IgE antibodies for SBPC and the rat homologus PCA reaction specific for SBPC were studied in SD rats. Rat anti-SBPC serum showed several common properties with the IgE antibodies: (a) the time course of the antibody production was the same as reported by TADA[13], (b) the optimum sensitizing period in PCA reaction was 60~72 hours as reported by MURPHEY[17] and TADA[13], (c) anti-SBPC serum was sulfhydryl-labile and heat-labile as reported by ISHIZAKA[19] and STECHSCHULTE[18]. From these findings, the rat 60-hour PCA reaction was felt to be mediated by the IgE antibodies. The finding that monovalent hapten failed to elicit the PCA reaction is considered to be identified with the fact the multivalent hapten is necessary to release histamine from basophiles[20]. The difference in the production of the IgE antibodies for various penicillins and cephalosporins may be associated with the difference in the immunogenicity of the haptens since the epitope densities of hapten in penicillin- and cephalosporin-protein conjugates used in this study were not considered to affect the immunogenicities of hapten-OvA conjugates and the reactivities of hapten-BGG conjugates. JARRETT[21] reported that Hooted-Lister rats produced IgE antibody after a single injection of 1 μg per animal. SD and Wister rats required a 5 mg dose of hapten-OvA conjugate for production of a high titer of IgE antibody. The response of SD and Wister rats to IgE antibody production may be lower than that of Hooted-Lister rats.

The studies of the rat IgE antibodies for penicillins and cephalosporins may be useful for the analysis of the immunogenicity and the antigenic specificity of drugs.

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