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Antigenicity Study of CFA-001, Cefazolin, a Cephalosperin Derivative Produced by an Enzymatic Semisynthesis

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Abstract – The antigenic potential of CFA-001, cefazolin, a cephalosporin derivative produced by an enzymatic semisynthesis, was determined in Hartley guinea pigs. A battery of tests employed consisted of active systemic anaphylaxis (ASA), passive cutaneous anaphylaxis (PCA), and indirect hemagglutination test (IHA). The results were as follows: 1) In ASA, no signs attributable to anaphylaxis was observed in guinea pigs sensitized with CFA-001, whereas OVA-sensitized animals induced severe anaphylactic symptoms; 2) guinea pigs did not produce antibodies against CFA-001 when sensitized with or without Freund's complete adjuvant (FCA) in homologous PCA tests. Meanwhile, antibodies against ovalbumin (OVA) were clearly detected; 3) No CFA-001-specific hemagglutination was observed in the IHA using sera obtained from CFA-001-sensitized guinea pigs. These results suggest that CFA-001 has no antigenicity potential in guinea pigs.

Keywords □ CFA-001, antigenicity tests, PCA, ASA, IHA, guinea pigs

Cephalosporins comprise about 38% of the world market size of antibiotics. Many of the recent cephalosporins are synthesized by conjugating distinct side chains to 7- and/or 3-positions of 7-aminocephalosporanic acid, which in turn had been produced by chemical or enzymatic hydrolysis of cephalosporin C, the natural fermentative product (Nishida *et al.*, 1970). For the conjugation step, chemical approaches inevitably require a large quantity of organic solvents and produce numerous undesirable environmental toxicants; utilization of microorganism-derived conjugating enzymes has been very successful in synthesizing most of the cephlosporins at an economic scale without problems associated with the chemical synthesis. Cheil Jedang Inc. recently succeeded in synthesizing cefazolin, coded CFA-001, with an enzymatic semisynthesis approach in which the enzyme was prepared by immobilizing penicillin amidase extracted from *E. coli* CFC-04717, a mutant strain of *E. coli* ATCC 9637.

As a part of toxicological research on CFA-001, antigenicity tests in guinea pigs were undertaken. All tests were done according to KGLP and were inspected by the QAU of Screening and Toxicology Center, Korea Research Institute of Chemical Technology, Taejon, Korea.

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METERAILS AND METHODS

Test Substance

CFA-001 (cefazolin Na salt) was supplied from the Bioprocess Team, R & D Center, Cheil Jedang, as white crystalline powder. The compound was stable at least 3 days after dissolution at 25-250 mg/ml in saline if preserved under refrigeration. Physiological saline (Choongwae Pharmaceutical) was used to dissolve the test compound and positive control. Ovalbumin (OVA) for the positive control group (Ogita and Mizushima, 1977) and Evans blue were obtained from Sigma Chemical Co. (Saint Louis, MO, USA).

Adjuvant

Freund's complete adjuvant (CFA, Difco Laboratories, Detroit, MI, USA) was used as an adjuvant.

Animals and Animal Room Conditions

Male Hartley guinea pigs, weighing 339-415 g at 7

weeks of age from which time the administration of test material was started, were purchased at 5 weeks of age from Sam-yuk Experimental Animal Breeding Center (77-1, Surangdong, Osan, Kyunggido, Korea). They were fed with solid diet (Purina Korea Co.) and tap water *ad libitum*, and were kept in rooms of 23±3 ℃, 50± 10% relative humidity, and 7:00 AM-7:00 PM lighting cycle. Animal identification was made with picric acid for actively immunized animals and those for passive immunization with oil colour paints. Animals were randomly assigned to each group on the basis of their body weights at the start of dosing.

Sensitization of Animals

Sensitization schedule is shown in Table I. CFA-001 (50-500 mg/kg) was dissolved in sterile saline at 250 mg/ml and diluted to appropriate concentrations. For co-administration of FCA, FCA suspension was mixed with an equal volume of the drug solution after calculation of inocula size based on the body weight (1-3 ml/kg). Three dosage groups (low, high, and high+FCA) were assigned on the basis of expected clinical doses. All test substances were injected subcutaneously into the animals' back skin. Sensitization was repeated a total of 9 times (3 times a week, for 3 weeks) except for groups III (high dose+FCA) and IV (positive control) in which a total 3 injections (3 weeks apart for 6 weeks) were given. Twelve days after the final sensitization, blood samples were collected from the retro-orbital venous plexus of the animals under ether anesthesia, and obtained antisera were stored at -70 ℃ until use. The same animals served for active systemic anaphylaxis (ASA) after recovery.

Active Systemic Anaphylaxis (ASA) Test

Two weeks after the final sensitization, CFA-001 (500 mg/kg) or OVA (1.67 mg/kg) was injected into the leg vein of the animals. Signs of anaphylaxis were evaluated according to the following criteria:

[−]: Asymptomatic

[±]: Mild; urination, evacuation

[+]: Moderate; above, coughing, sneezing

[++]: Severe; above, piloerection, salivation, nostril discharge, lacrimation, nasal bleeding, convulsion, dyspnea, staggering gait, rhonchus, cyanosis, side position, flattening

[+++]: Death

Homologous passive cutaneous anaphylaxis (PCA) Test

This test was performed according to the method of Ovary (1958). Each 0.1 ml of the guiea pig sera diluted from 10 to 5,120-fold was injected intradermally into the back of guinea pigs which had been clipped their back hair short. Four hours after the inoculation, 1 ml of 1:1 mixture of CFA-001 (500 mg/kg) or OVA (1.67 mg/kg) solution and 1% solution of Evans blue was injected into the hind leg vein. Thiry minutes after the antigen challenge, guinea pigs were bleed to death, and leakage of the dye at the serum-injected site was examined to determine PCA titers. The end point of the positive PCA reaction was defined as a diameter of 5 mm or more (major diameter+minor diameter)/2 (Ovary, 1964).

Indirect Hemagglutination (IHA) Test

The guinea pig antisera prepared as described above were used. Fresh whole sheep erythrocytes containing 0.005% tannic acid were incubated for 1 hr at 37 ℃, and then they were diluted to 4%. The erythocytes thus prepared were coated with antigen (CFA-001 or OVA) by incubating with equal volume of (6 mg/ml) CFA-001 or OVA, followed by washing 3 times with centrifugation for 15 minutes each at 700 rpm. This erythrocyte solution was finally diluted to 1% for IHA test. Guinea pig antisera diluted to 4-8,192 times were put into 96-well microplates in 50 μl, and sheep erythrocyte solution of 50 μl was added to each well. The presence of hemagglutination reaction was visually determined after incubation for 2 hr at room temperature. The tests were performed duplicately for each serum.

Statistics

Changes in body weight were compared among groups

Table I. Sensitization schedule of guinea pigs

Group	Substance	Dose (mg/kg)	No. of animals	No. of treatment	Route
I	CFA-001 (low dose)	0	5	9[a]	s.c.
II	CFA-001 (high dose)	500	5	9	s.c.
III	CFA-001 (high)+FCA	500	5	3[b]	s.c.
IV	OVA+FCA (positive)	2.5	5	3	s.c.
V	Saline	0	5	9	s.c.

[a]three times in a week every other day. [b]once in three weeks. s.c.: subcutaneously.

throughout the experiment. And their difference was declared significant when p-values were less than 0.05 in Kruskal-Wallis test.

RESULTS AND DISCUSSION

Cefazolin is a semisynthetic cephalosporin containing unique side chains at 7 and 9 position on the 7-aminocephalosporanic acid (Nishida *et al.*, 1970). Due to the hapten-forming property of β-lactam antibiotics in the body, the potential of antigenicity of cephalosporins have long been the concern for clinicians (Petz, 1978). Although chemically processed cefazolin does not seem to cause any antigenicity problem (Mine and Nishida, 1970) in experimental animals or humans, it was necessary to evaluate antigenicity property of CFA-001 which could be caused by possibly contaminating protein(s) because the present cefazolin was synthesized through an enzymatic procedure, i.e., acylation to 7 position with penicillin amidase derived from microorganisms.

In this experiment, the three most frequently utilized antigenicity tests were employed: active systemic ana-

Table II. Active systemic anaphylaxis in guinea pigs

Sensitization	CFA-001 (50 mg/kg)					CFA-001 (500 mg/kg)					CFA-001+FCA (500 mg/kg)					OVA+FCA (2.5 mg/kg)					Saline (1 ml/kg)				
Challenge	CFA-001 (500 mg/kg)					CFA-001 (500 mg/kg)					CFA-001 (500 mg/kg)					OVA (1.67 mg/kg)					CFA-001 (500 mg/kg)				
Sensitization Period	3 times/wk ×3					3 times/wk ×3					once/3 wk ×3					once/3 wk ×3					3 times/wk ×3				
Animal No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Symptoms on Challenge																									
1. Restlessness	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-		-	-	-	-	-	-
2. Piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-		-	-	-	-	-	-
3. Tremor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+		+	-	-	-	-	-
4. Licking nose	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	-		-	-	-	-	-	-
5. Sneezing	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-		+	-	-	-	-	-
6. Coughing	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-		-	-	-	-	-	-
7. Increased respiration	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+		-	-	-	-	-	-
8. Urination	-	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+		+	+	+	+	-	-
9. Evacuation	-	-	+	-	-	-	-	+	-	-	+	-	-	-	+	-	+	-		+	-	-	-	-	-
10. Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-
11. Dyspnea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-		-	-	-	-	-	-
12. Vocalization	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+		-	-	-	-	-	-
13. Cyanosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-
14. Staggering gait	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		+	-	-	-	-	-
15. Jumping	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-
16. Gasping	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+		-	-	-	-	-	-
17. Convulsion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+		+	-	-	-	-	-
18. Side position	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		+	-	-	-	-	-
19. Cheyne-Stokes respiration	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		+	-	-	-	-	-
20. Death	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-
Evaluation of the intensity	[-]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[++]	[++]	[++]		[++]	[+]	[+]	[+]	[-]	[-]

[-]: Asymptomatic
[±]: Mild : symptoms of 1 to 4
[+]: Moderate : symptoms of 1 to 10
[++]: Severe : symptoms of 1 to 19
[+++]: Death

In the OVA+FCA group one animal (#19) died on day 42 of the sensitization period.

phylaxis (ASA) test, homologous passive cutaneous anaphylaxis (PCA) test and indirect hemmaglutination (IHA) test in guinea pigs. Overall there was no CFA-001 treatment-related hypersensitive responses in these tests.

No apparent abnormal clinical sign was observed in all experimental groups during the sensitization period except in the positive control group in which one animal died on day 42 after intermittent hindleg paralysis between days 28 and 41. This death was not considered as an OVA-associated response since the hind leg had possibly been once trapped in the steel mesh of the suspension cage in which animals were housed. There was a slight but significant retardation in body weight gain from the first week of administration of the 500 mg/kg group ($p<0.05$, data not shown). This change seemed to be attributable to the high dose of CFA-001, approximately 10 times the projected clinial dose.

The test protocol of ASA and its results are summarized in Table II. When the animals treated with 50 mg/kg of CFA-001 were challenged with CFA-001 as the antigen, symptoms such as urination, evacuation, nose- rubbibng and -licking were observed in 4 out of 5. In the 500 mg/kg group, all 5 animals exhibited urination and evacuation, and 1 animal showed nose-rubbing and -licking. In contrast, severe symptoms were identified in all 4 animals treated with OVA+FCA of restlessness, piloerection, tremor, nose-rubbing and -licking, coughing, vocalization, gasping, convulsuion, side position and Cheyne-Strokes respiration. In the vehicle control, urination in 3 out of 5 was detected. In the terminal necropsy of OVA+FCA group, congestion or hemorrhagic foci were found in 3 out of 4 animals in the trachea, heart, lung. Similar congestive or hemorrhagic foci were detected in 500 mg/kg of CFA-001 group. Although urination and evacuation were also observed in this group, it was concluded that CFA-001 may not have antigenic potential because these findings have normally been observed in many ASA tests. In summary of the ASA test results, there seems to be no CFA-001-induced anaphylactic response in a resonable dose range while severe anaphylaxis responses were identified in all positive control animals. The mild symptoms such as urination and evacuation observed in CFA-001 treated groups may be part of i.v. injection-related responses rather than to CFC-001 dosing inasmuch as similar symptoms have commonly been observed in vehicle treated groups as also evident in the present study.

Table III. Four-hour homologous passive cutaneous anaphylaxis test in guinea pigs with sera of sensitized guinea pigs

Group	Sensitizing antigen	Challenging antigen	PCA titer	Positive ratio
I	CFA-001 (50 mg/kg)	CFA-001 (500 mg/kg)	-	0/10
II	CFA-001 (500 mg/kg)	CFA-001 (500 mg/kg)	-	0/10
III	CFA-001 (500 mg/kg)+FCA	CFA-001 (500 mg/kg)	-	0/10
IV	OVA (2.5 mg/kg)	OVA (1.67 mg/kg)	640x-1,280x	8/8
V	Saline (1 ml/kg)	CFA-001 (500 mg/kg)	-	0/10

All animals were injected intradermally with antisera on the clipped back of each recipient guinea pig. Four hours later 0.5-1.0 ml/kg of the challenging antigen was injected intravenously along with equal volume of Evans blue. At 30 min, the animal's back skin was removed for measuring the sizes of the blue spot caused by extravasation of Evans blue.

Passive cutaneous anaphylaxis (PCA) is to elicit an antigen-antibody reaction following passive intradermal immunization of recipient animals with sensitized serum. In the reaction both mast cells and basophils will be sensitized, and on antigen challenge the cells will release an array of chemical mediators such as histamine, leukotrienes, thromboxane A_2, serotonin, etc. (Mutschler and Derendorf, 1995). In the PCA using homologous guinea pig-guinea pig system, no evidence of anaphylaxis by CFA-001 was found with sera diluted to 10 to 5,120 times (Table III). In contrast the positive control group treated with OVA/FCA produced clear positive responses with sera diluted up to 1,280 times. Therefore, the PCA test results also indicate that CFA-001 might not be anaphylactogenic.

Indirect hemagglutination is primarily used to detect specific IgM antibodies to a particular antigen by coating erythrocytes with that antigen and reacting with sera (Boyden, 1951). This method is often utilized to identify drug-induced antibody production since IgM is a larger molecule than IgG and thus facilitates erythrocyte agglutination because the distance among erythrocytes are relatively long (Hudson and Hay, 1989). In the present test, we used the guinea pig serum from 50 and 500 mg/kg CFA-001-treated animals. Hemagglutination was observed when the low dilution sera from both CFA-001 of 50 and 500 mg/kg groups were reacted with uncoated or CFA-001-coated sheep erythrocytes (Table IV). The reaction was considered as a false positive stemmed prob-

Table IV. Indirect hemagglutination test of guinea pig sera with sheep red blood cells.

Group	Sensitizing antigen	Sheep erythrocyte coated with	IHA titer[a]	Positive ratio
I	CFA-001 (50 mg/kg)	none	16x	1/5
		CFA-001	16x	1/5
		OVA	16x	1/5
II	CFA-001 (500 mg/kg)	none	8-32x	3/5
		CFA-001	8-16x	3/5
		OVA	8-32x	4/5
III	CFA-001+FCA (500 mg/kg)	none	-[b]	0/5
		CFA-001	-	0/5
		OVA	-	0/5
IV	OVA+FCA (2.5 mg/kg)	none	-	0/4
		CFA-001	-	0/4
		OVA	256-2,048x	4/4
V	Saline (1 ml/kg)	none	-	0/5
		CFA-001	-	0/5
		OVA	-	0/5

[a]IHA titer represents the maximum dilution of original serum which showed the positive hemagglutination.
[b]Specific antibodies were not detected in 4-fold dilution of original serum.

ably from the reaction of nonspecific substances present in the guinea serum with sheep erythrocytes. In fact a similar false positive reaction was recognized (Hudson and Hay, 1989). This interpretation was further supported by the fact that OVA-coated erythrocytes precipitated CFA-001-sensitized sera at low dilutions. In contrast the sera from OVA+FCA group at dilutions of 256-2,048-fold agglutinated OVA-coated erythrocytes. In summary of the IHA result, CFA-001 does not seem to be antigenic especially compared to OVA.

Based on the three present antigenicity tests, we concluded that CFA-001 does not have antigenic potential. Consequently it is not likely that any substance of protein nature had been incorporated into the final CFA-001 preparation.

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