Immunological Properties of Cephalexin-Induced Delayed Type Hypersensitivity Reaction in Guinea Pigs

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Immunological properties of delayed-type hypersensitivity (DTH) reaction induced by cephalexin (CEX) in ginea pigs were investigated. The animals were immunized with CEX using Freund's complete adjuvant. The time course of CEX-induced erythema showed some differences compared with that of classical DTH reaction. The erythema appeared at 6 h after intradermal administration of CEX, reached maximum size at 12 to 24 h and to be visible until 72 h. By enzyme-linked immunosorbent assay, anti-CEX antibody was detected in only one of 15 animals tested. Normal animals (recipients) which had received immune sera from CEX-sensitized animals (donors) showed no skin reaction to CEX. In contrast, reaction to CEX was observed in recipient animals which had received a local transfer of lymphocytes or T cells from CEX sensitized animals. In immunopharmacological study, cyclosporin A suppressed the skin reaction but cyclophosphamide did not. Administration of carrageenan, an inhibitor of macrophage function, had no effect on expression of the reaction. Post administration (1 or 15 h) of clemastine, an anti-histamine drug, did not affect the reaction. By histological examination, the infiltrating cell-types at the reaction site were mainly composed of mononuclear cells and neutrophils, but no basophils, indicating that CEX-induced DTH reaction is tuberculin-type DTH and not a cutaneous basophil hypersensitivity reaction.

Keywords beta-lactam; classical delayed-type hypersensitivity; cutaneous basophil hypersensitivity reaction; guinea pig

Introduction

Beta-lactam antibiotics are widely used because of their high bactericidal potency and low pharmacologic toxicity. Allergic reactions are a common side effect of these antibiotics and are generally divided into the two categories of immediate-type hypersensitivity, associated with humoral antibody-mediated immunity, and delayed-type hypersensitivity (DTH), associated with cell-mediated immunity.[1,2] It has been shown that cellular immunity plays an important role in allergic reactions induced by beta-lactams.[2-4]

The mechanisms of immune response of DTH reaction induced by beta-lactams are not clear. It is well known that the immune responses of guinea pig are similar to that of human in DTH. Therefore, the analysis of immunological properties of the DTH reaction induced by beta-lactam in guinea pig seems important in preventing the DTH reaction by beta-lactam in human. In this paper, we describe the immunological properties of DTH reaction caused by cephalexin (CEX), as beta-lactam, in guinea pig.

Materials and Methods

Animals Male outbred Hartley guinea pigs weighing 400—500 g were purchased from Japan SLC Co., (Hamamatsu) and female inbred JY-1 guinea pigs weighing 400—600 g were obtained from Funabashi Farm Co., (Funabashi) for study of the local transfer of lymphocytes.

Drugs Cephalexin (CEX) and Clemastine® fumarate were purchased from Wako Pure Chemical Industrials Co., (Osaka). Carageenan (Type IV), cyclophosphamide monohydrate and indomethacin were obtained from Sigma Chemicals Co., (St. Louis, MO., U.S.A.). Cyclosporin A (Sandumine®) was purchased from Sandoz Ltd. (Basel, Switzerland).

Immunization CEX was dissolved in physiological saline (10 or 20 mg/ml) and emulsified with an equal volume of Freund's complete adjuvant (FCA) (Difco Laboratories, Detroit, MI., U.S.A.). Each footpad of the guinea pigs was injected intradermally (i.d.) with a total of 0.5 ml of the emulsion on day 0. On day 14, each leg of the animals was injected intramuscularly (i.m.) with a total of 0.4 ml of the emulsion.

Skin Test On day 27, one-tenth ml of pyrogen-free saline solution of a non-irritating concentration (2 mg/ml) of CEX was injected i.d. into the shaved flank of the sensitized guinea pigs. Diameters of the erythematous area were measured with calipers 24 h after the challenge.

Sera Blood was collected by cardiac puncture under ether anesthesia on day 30 and allowed to clot at room temperature for 3 h. Serum was separated from the clot after overnight incubation at 4 °C and stored at −20 °C.

Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of CEX-Specific Antibodies ELISA was performed by the following method. Microplates of 96-wells (Falcon, No. 3915) were coated CEX-human serum albumin made by the alkaline method[5] overnight at 4 °C. Each well was washed with 0.05% Tween 20-Dulbecco's PBS and blocked with Block Ace® (Dai-nippon Pharmaceutical Co., Osaka) at room temperature for 1 h. The wells were washed, serial dilutions of sera were added to each well and incubated at 37 °C for 2 h. After incubation, the plate was washed and rabbit anti-guinea pig immunoglobulin M (IgM) (μ chain specific, ICN Immunological, Lisle, IL., U.S.A.) was added for IgM detection. After incubation at 37 °C for 2 h, the plates were washed and goat peroxidase-anti-rabbit IgG (Organon Teknika Co., West Chester, PA., U.S.A.) was added. After incubation at 37 °C for 2 h, the plates were washed and substrate solution was added. This solution was composed of 2 μl of 30% H_2O_2 and 0.2 ml of 52 mM 2,2′-azino-bis(3-ethyl-benzthiazolin-6-sulfonic acid) (ABTS) in 10 ml of 0.1 M citrate buffer (pH 4.2). The plates were incubated at room temperature for 1 h in the dark. The optical density was measured at 415 nm with an ELISA reader (Corona Electric Co., Tokyo). For IgG detection, rabbit peroxidase-anti-guinea pig IgG (heavy and light chain specific, Organon) was used.

Serum Transfer Study Serum transfer was carried out according to the method of Boerrigter *et al.*[6] with a slight modification. In brief, recipients received two 1 ml injections of donor serum into a vein of the hindleg at 18 and 2 h before the skin test with CEX.

Local Cell Transfer Study Local transfer of lymphocytes was performed according to the method of Jaffer *et al.*[7] JY-1 guinea pigs were immunized by CEX emulsified with Freund's complete adjuvant on days 0 and 14. On day 20, the sensitized donor animals were injected i.p. with 20 ml of liquid paraffin and peritoneal exudate cells were harvested after 4 d. The erythrocytes were lysed by 0.02 M Tris–HCl buffer (pH 7.9) containing 0.83% NH_4Cl. The cells were washed with RPMI-1640 medium and macrophages were removed by adhesion method.[8] Nonadherent cells were collected and separated into T and B cell fractions using petri dishes coated with anti-Ig Fab.[9] Nonadherent cells were designated the T cell fraction and adherent cells retained the B cell fraction. The cell fractions were washed with saline and mixed with an equal volume of CEX solution (20 mg/ml) immediately before transfer. Cell transfer was carried out by i.d. injection of a total volume of 0.1 ml.

Immunopharmacological Study Cyclosporin A (100 mg/kg), and carageenan (40 mg/kg) were injected i.p. at 2 h before challenge of CEX, respectively. Cyclophosphamide (150 mg/kg) was administered by i.p. injection 3 d before the challenge. Clemastine fumarate (40 mg/kg) was injected i.p. at 1 or 15 h after the challenge. Diameter of the erythematous areas was measured at 24 h after the challenge.

Histological Examination Pieces of full-thickness skin at the reaction sites were removed immediately after the measurement of 24 h reactions and fixed with formalin. Paraffin sections were stained with hematoxylin-eosin and Giemsa solution. Metacromasia was examined by toluidine blue

staining method.

Statistical Analysis Statistical differences were analyzed using Student's t test or unpaired Wilcoxon's test and p values of less than 0.05 were considered to be significant.

Results

Change of Skin Reaction after Intradermal Administration of CEX The change of erythema size induced by CEX in Hartley guinea pigs was measured (Fig. 1). The erythema was not observed till 3—6 h after i.d. injection of CEX. The reaction showed maximal size from 12 to 24 h and continued till 72 h after the challenge.

Degree of the Skin Reaction and the ELISA Titer of Anti-CEX Antibodies The relation between skin reaction and ELISA titer of anti-CEX IgG and IgM antibody was investigated (Table I). Unsensitized control animals did not experience erythema when injected i.d. with CEX. Mean diameter of the erythema was not correlated with the titers of anti-CEX antibodies. The antibodies were detected in only one out of 15 animals tested. The titer of IgG and IgM antibody in the animal was 160 and the diameter of erythema was 9.0 mm.

Transfer of CEX-Induced Skin Reaction When the immune sera or lymphocytes from sensitized animals (donors) were transfered into normal animals (recipients), the occurrence of skin reaction induced by CEX was examined (Tables II, III). The size of erythema induced by immune sera and CEX (0.2 or 1.0 mg/ml) was similar to that of the skin reaction induced by normal sera and the antigen, indicating that the transfer of immune sera did not cause the skin reaction in recipient animals.

In local transfer of sensitized lymphocytes, no skin reaction was observed after i.d. injection of B cells with antigen or saline. In contrast, reaction of almost the same intensity was induced by i.d. injection of the lymphocytes or T cells with antigen. The reactions were also recognized after transfer of the lymphocytes or T cells without antigen, but the intensity was weaker.

Immunopharmacological Effects on CEX-Induced Skin Reaction Effects of immunosuppressant, anti-histamine agent and inhibitor of macrophage function against immune-lymphocytes on CEX-induced skin reaction were examined

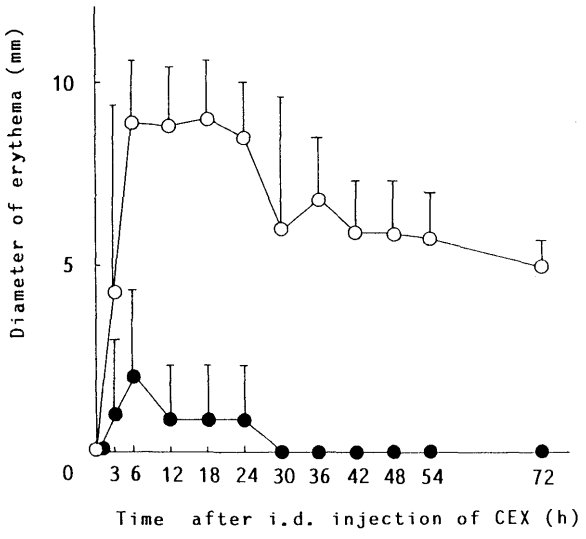

Fig. 1. Time Course of CEX-Induced Erythema in Hartley Guinea Pigs
—○—, CEX; —●—, saline.

TABLE I. Comparison of Degree of Skin Reaction and ELISA Titer of Anti-CEX Antibodies in Hartley Guinea Pig

No. of animals	Mean diameter of erythema (mm)	ELISA titer	
		IgG	IgM
1	8.5	<20	<20
2	7.0	<20	<20
3	7.5	<20	<20
4	10.5	<20	<20
5	10.5	<20	<20
6	12.0	<20	<20
7	11.0	<20	<20
8	6.0	<20	<20
9	9.5	<20	<20
10	10.0	<20	<20
11	11.0	<20	<20
12	8.0	<20	<20
13	9.0	160	160
14	10.0	<20	<20
15	13.0	<20	<20

TABLE II. Transfer of Immune Sera on Delayed-Type Skin Reaction of Hartley Guinea Pig

Antigen	Dose (mg/ml)	Mean diameter (mm) of erythema	
		Immunized sera transferred[a]	Normal sera transferred
CEX	1	2.4±1.5[b]	2.2±1.4
CEX	0.2	1.0±1.4	1.3±1.3
Saline		1.0±1.4	0.8±1.1

a) Anti-CEX antibody ELISA titer of the sera was 128. *b*) The mean±S.D. for each value was obtained from 5 recipients.

TABLE III. Local Transfer of Sensitized Lymphocytes on Delayed-Type Skin Reaction of JY-1 Guinea Pig

No. of cells[a]	Materials injected					
	Lymphocyte and CEX	T cell and CEX	B cell and CEX	Lymphocyte	T cell	B cell
1×10^6	5.3±0.4[b]	4.7±0.3	N.D.	1.5±2.1	3.2±2.6	N.D.
5×10^5	3.0	4.7±0.3[a]	N.D.	0.0	0.0	N.D.
2×10^5	N.D.	2.7±2.3	1.5±2.1	N.D.	1.2±2.0	0.0
1×10^5	N.D.	4.0±0.7[c]	1.3±2.3	N.D.	0.0	0.0
5×10^4	N.D.	4.5±0.0	0.0	N.D.	2.0±3.2	0.0

CEX (1 mg/ml) and saline did not cause the skin reaction. *a*) One-tenth ml of cell suspension was injected intradermally. *b*) Skin reactions in millimeters at 24 h. The mean±S.D. of each value was obtained from two or three sites. *c*) $p<0.05$ as CEX untreated control group by Student's t test. *d*) $p<0.001$ as CEX untreated control group by Student's t test. N.D.; Not done.

TABLE IV. Immunopharmacological Effects on Delayed-Type Skin Reaction of Hartley Guinea Pig

	Treatment[a]	Iinjection time (h)	Dose (mg/kg)	No. of animals	Skin reaction (mm) on day 28	
					0.2% CEX	Saline
Exp. 1	Cyclosporin A	−2	100	4	1.6±1.9[b]	0.6±1.3
	Saline			4	7.5±1.8	0.0
Exp. 2	Cyclophosphamide	−72	150	4	7.0±0.4	0.9±1.8
	Saline			4	8.8±1.9	1.4±1.6
Exp. 3	Carageenan	−2	40	5	10.4±1.5	0.0
	Saline			5	10.8±1.6	0.0
Exp. 4	Clemastine	1	40	5	8.5±1.9	0.0
	Saline	1		5	9.0±1.5	0.0
	Clemastine	15	40	5	9.1±0.5	0.0
	Saline	15		5	8.7±2.0	0.0

These animals showed about 8.1—9.8 mm of skin reaction with CEX on day 24. *a)* Drugs were administered i.p. into the animals. *b)* $p<0.05$ as saline treated group by unpaired Wilcoxon's test.

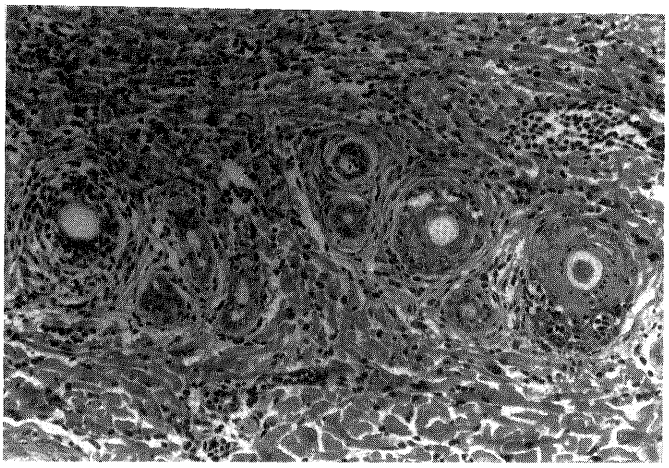

Fig. 2. Histological Feature of the Dermis in CEX-Induced Delayed Skin Hypersensitivity

DTH reaction in Hartley guinea pigs was induced by CEX. Infiltration of mononuclear cells into the reaction site was observed. Section was stained with hematoxylin–eosine solution. ×250.

(Table IV). An i.p. administration of cyclosporin A (100 mg/kg) at the eliciting phase significantly suppressed the expression of the skin reaction ($p<0.05$). However, an i.p. injection of cyclophosphamide (150 mg/kg) did not influence the reaction 3 d before the challenge. Post administration 1 or 15 h after the challenge of clemastine (40 mg/kg), an anti-histamine drug, did not affect the reaction. Similar intensity of the skin reaction was observed between the carageenan (40 mg/kg) administered group and control group.

Histological Study The reaction sites expressed the feature of tuberculin-type hypersensitivity. A cellular infiltration was detected in the dermis and the infiltrating cells were mainly composed of mononuclear cells and neutrophils (Fig. 2). Basophils were not detected by hematoxylin–eosin staining, Giemsa staining or methachromasy by toluidine blue staining.

Discussion

We previously investigated the cross-reactivities among beta-lactams in guinea pigs immunized by beta-lactams which caused DTH.[10] DTH reaction in guinea pig is of two types: tuberculin-type of classical DTH reaction and cutaneous basophil hypersensitivity (CBH) reaction.[11] CBH reaction is further classified into T cell dependent[12] and humoral antibody dependent reaction.[13] In guinea pigs which are immunized with protein antigen and complete Freund's adjuvant, CBH reaction is induced at the initial stage and this gradually changes to classical DTH reaction.[14] On the other hand, animals which are immunized with protein antigen and incomplete Freund's adjuvant, experience a CBH reaction.[11] There are few papers regarding the immunological property of DTH reaction induced by beta-lactams in guinea pig. Ikezawa and Nagai[15] examined the immunological characterization of DTH reaction which was induced by sulbenicillin or cephalothin. However, it has not been clarified whether beta-lactam-induced DTH reaction is classical DTH or CBH reaction.

Classical DTH reaction begins 6 h after administration of antigen, is maximal at 24 to 30 h, and continues up to 120 h. In contrast, CBH reaction appears 6 to 10 h after the challenge and is maximal at 24 h, but disappears within 48 h.[16] The time course of the hypersensitivity in this experiment was similar to that of classical DTH reaction. CBH reaction was not recognized in animals immunized with beta-lactam and incomplete Freund's adjuvant (data not shown).

In guinea pig, CBH reaction can be transferred by IgG_1[17] or IgE[18] hapten-specific antibodies which are obtained from hyperimmunized animals. In this study, no participation of serum antibodies in the hypersensitivity was observed. Anti-CEX IgG and IgM antibodies were scarcely detected by ELISA in sera from the animals tested, and the intensity of the skin reaction did not correlate with ELISA titer of the antibodies. The hypersensitivity was not transferable by immune sera. In contrast, local transfer of sensitized lymphocytes or T cells induced a reaction in donor animals, suggesting that the reaction is T cell dependent.

To investigate the role of immune-lymphocytes in the hypersensitivity, the immunosuppressive effects of two agents were examined. It is generally considered that cyclosporin A suppresses the function of helper T cell and cyclophosphamide inhibits that of B cell. At the eliciting phase, the administration of cyclosporin A suppressed CEX-induced reaction but cyclophosphamide had no effect. These results indicate that T cell plays an important role in DTH reaction induced by CEX at the eliciting phase.

With regard to CBH reaction in guinea pig[19] and DTH reaction in murine,[20,21] it has been reported that degranulation of mast cell and release of vasoactive amines participate in these reactions. Herrmann *et al.*[21] showed that administration of clemastine, an anti-histamine agent, 15 h after the transfer of cloned helper T cells inhibits DTH reaction in the murine model. In contrast, our results suggested that the histamine does not play an important role in the CEX-induced hypersensitivity and the reaction is not a CBH reaction. By histological study, the reaction sites expressed typical features of classical DTH reaction. The infiltrating cells were composed of mononuclear cells and neutrophils, but basophils were not detectable.

Richerson *et al.*[11] reported that administration of carageenan, an inhibitor of macrophage function, inhibits DTH reaction of tuberculin type in guinea pig. However, we have shown here that carageenan has no influence on the hypersensitivity reaction. The discrepancy between our experiments and Richerson's report[11] seems to be the difference in the type of carageenan used, or that of the typical tuberculin type and CEX-induced DTH.

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