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Pharmacologic Dissociation of Immunologic Release of Histamine and Slow Reacting Substance of Anaphylaxis in Rats* (33437)

ROBERT P. ORANGE[1] AND K. FRANK AUSTEN

Department of Medicine, Harvard Medical School at the Robert B. Brigham Hospital, Boston, Massachusetts 02120

The immunologic pathways leading to the release of histamine and slow reacting substance of anaphylaxis (SRS-A^{rat}) in the rat can be dissociated in terms of the responsible homolgous immunoglobulins (1,2) and the participating target cells (3,4). Histamine release from rat mast cells is mediated by a heat labile (5), "mast cell sensitizing" (6) or homocytotropic (7) antibody; the immunoglobulin class of the homocytotropic antibody has not yet been established, but it appears to be distinct from the recognized classes, IgG, IgA, and IgM (8, 9). The formation and release of SRS-A^{rat} is mediated by a heat stable antibody of the IgGa class (2) and requires the presence of the polymorphonuclear (PMN) leukocyte (3) but not the peritoneal mast cell.

The present report reveals that these two distinct pathways can be selectively blocked *in vivo* by pharmacologic agents which act after antigen–antibody interaction but prior to the formation and release of the mediators. Diethylcarbamazine (3, 10) and certain structural analogs prevent the immunologic elaboration of SRS-A^{rat} but not of histamine and serotonin, whereas disodium cromoglycate (11) suppresses the antigen-induced release of the amines but does not inhibit the release of SRS-A^{rat}.

Materials. Diethylcarbamazine citrate (1-diethylcarbamyl-4-methylpiperazine) (Hetrazan, Lederle Laboratories, Pearl River, New York) was kindly supplied by Dr. H. G. Lockhard (Lederle Laboratories). Disodium cromoglycate (1, 3-bis[2-carboxychromon-5-yloxy]-2-hydroxypropane) (FPL-670, Fison's Pharmaceuticals, Loughborough, Leicestershire, England) was donated by Dr. J. S. G. Cox. Isonicotinic acid hydrazide and nicotinamide were obtained from Mann Research Laboratories, Inc. (New York). Isopropylisoniazid (Iproniazid) was donated by Hoffman-LaRoche, Inc. (Nutley, New Jersey), and monoethanolamine was acquired from Fisher Scientific Co. (Fairlawn, New Jersey). Cho-

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line chloride, benzamide, 1, 1-diethyl urea, *N*-amidinobenzamide, and benzhydrazide were purchased from Eastman Organic Chemicals (Rochester, New York). Piperazine, 2, 5-piperazinedione, *dl*-pipecolic acid, and pipecolamide were obtained from J. T. Baker Chemical Co. (Phillipsburg, New Jersey). Mepyramine maleate (Neo-Antergan) and methysergide (UML-491) were supplied by Merck, Sharp and Dohme (Rahway, New Jersey).

Bovine serum albumin (BSA) and hen egg albumin (Ea) were purchased from Pentex Inc. Kankakee, Ill. The Bacillus pertussis cultures were donated by Dr. Irving Millman of Merck, Sharp and Dohme.

Rat antisera against BSA were prepared as described (3) and contained 2.8–4.2 mg of specific antibody protein/ml as determined by precipitin analysis (12). Rat antisera against dinitrophenyl bovine gamma globulin (DNP-BGG) containing 3.4 mg of hapten-specific antibody/ml was prepared as described in Refs. (2) and (9). Antisera containing homocytotropic antibody activity against Ea were obtained as previously described (3).

Rabbit antisera directed against rat mast cells (Ra anti-RMC) were prepared as described in Ref. (13).

Methods. Study of the viability of rat PMN leukocytes. Peritoneal exudates consisting of greater than 70% PMN leukocytes were induced in 3 rats by the intraperitoneal (i.p.) injection of 20 ml of 0.1% oyster glycogen (Baker Chemical Co., Phillipsburg, N. J.) in 0.15 *M* saline. Sixteen hr later, the animals were stunned, exsanguinated, and injected i.p. with 5.0 ml of Tyrode's solution containing heparin, 50μg/ml. Following gentle massage of the abdomen for 1–2 min, the peritoneal cells were harvested using siliconized Pasteur pipettes. Fluorescein diacetate (FDA, Eastman Organic Chemicals, Rochester, New York) was dissolved in acetone and then diluted with distilled water to a final concentration of 10^{-6} *M*. One ml of the pooled cell suspension (44,000 cells/mm^3, 77% PMN leukocytes) was incubated at 37° for 10 min with 1.0 ml of Tyrode's solution ± a 20 m*M* concentration of diethylcarbamazine or its analogs (10 m*M* final concentration). One drop of each cell suspension was placed on a microscope slide with one drop of fluorescein diacetate (10^{-6} *M*), and the cells were examined with a fluorescent microscope for viability (14).

Preparation of rats for the antigen-induced release of SRS-A^{rat}. Male Sprague-Dawley rats weighing 225–300 g were prepared for the antigen-induced release of SRS-A^{rat} by the i.p. injection of 1.0 ml of a 1:3 to 1:4 dilution of rat antisera sufficient to mediate the release of about 150 units of SRS-A^{rat} per rat. Two hr later, the animals were challenged i.v. with 10 mg of specific antigen in 1.0 ml of 0.15 *M* saline. 5.0 ml of Tyrode's solution containing heparin, 50μg/ml, were immediately injected i.p., and exactly 5 min later, the animals were stunned, exsanguinated, and the peritoneal fluid was recovered using siliconized Pasteur pipettes. The peritoneal fluid was centrifuged at 150*g* for 4 min at 4° and the supernatants were collected in iced polypropylene tubes. The presence of SRS-A^{rat} was determined by bioassay on the isolated guinea pig ileum as described (1). One unit of SRS-A^{rat} was arbitrarily defined as the concentration required to produce a contraction of the isolated guinea pig ileum equivalent in amplitude to that produced by 5.0 mμg of histamine base in that assay.

Compounds tested for their ability to inhibit the antigen-induced release of SRS-A^{rat} were dissolved in 0.15 *M* saline and injected i.v. 30 sec before challenge with specific antigen in a dose of 20 mg/kg. In a typical experiment, each compound was tested in 3 rats of comparable weight and the mean SRS-A^{rat} release (units/rat) was compared with the mean release for control animals in that experiment; the results were expressed as percentage inhibition of SRS-A^{rat} release with inhibition of greater than 25% being considered significant. Each compound was tested in at least 3 separate experiments. Each experiment included effective and ineffective inhibitors and a dose–response effect for inhibition of SRS-A^{rat} release was established for some of the effective analogs.

Preparation of rats for the antigen-induced release of histamine. Male Sprague-Dawley

TABLE I. Inhibition of the Antigen-Induced Release of SRS-A^{rat} with Diethylcarbamazine and Its Analogs.

Ring structure	Noncarboxamide derivatives	% Inhibition SRS-A^{rat} release	Carboxamide derivatives	% Inhibition SRS-A^{rat} release
Piperazine	Piperazine	0	Diethylcarbamazine	66 ± 12.5
	2, 5-Piperazinedione	0		
Piperidine	*dl*-Pipecolic acid	12	Pipecolamide	82
Pyridine	—	—	Nicotinamide	31
			Isonicotinic acid hydrazide	67
			Isopropylisoniazid	6
Benzene	—	—	Benzamide	30
			Benzhydrazide	12
			N-Amidinobenzamide	44
—	Ethanolamine	0	—	
	Choline chloride	10	—	
	1, 1-Diethylurea	0	—	

rats weighing 200–300 g were prepared for the intraperitoneal release of histamine by the i.p. injection of 5.0 ml of Tyrode's solution containing a 1:20–1:50 dilution of antisera with homocytotropic antibody activity against Ea. Four hr later, the animals were challenged i.p. with 2.0 mg of Ea in 5.0 ml of Tyrode's solution containing heparin, 50μg/ml. Exactly 5 min later, the animals were stunned, exsanguinated, and the peritoneal fluid was harvested in the usual fashion. The presence of histamine was determined by bioassay on the isolated guinea pig ileum (1). Compounds tested for their ability to inhibit the homocytotropic antibody-mediated release of histamine were injected i.p. in a dose of 30 mg/kg 30 sec before challenge with specific antigen. Each compound was tested in 3 rats of comparable weight in an experiment and each experiment was repeated 3 times.

Passive cutaneous anaphylaxis (PCA). The PCA reactions were carried out in male Sprague-Dawley rats weighing 225–275 g as described (5, 15). Various dilutions of Ra anti-RMC in 0.15 *M* saline were also injected intradermally (i.d.) in 0.1-ml volumes immediately before challenge with antigen in Evans blue dye (16).

Results. Selective inhibition of the antigen-induced release of SRS-A^{rat} by diethylcarbamazine and its analogs. Structural analogs of 1-diethylcarbamyl-4-methylpiperazine (diethylcarbamazine) involving substitutions in the piperazine ring and at the carboxamide grouping were examined for their ability to inhibit the antigen-induced release of SRS-A^{rat} (Table I). The mean result for 8 separate experiments with diethylcarbamazine is included in Table I for comparative purposes. The ring substitutions studied included the piperidine, pyridine, and benzene rings. Only the carboxamide derivatives of these various rings demonstrated significant inhibitory activity. Ethanolamine, choline, and a substituted urea (1,1-diethylurea) were ineffective inhibitors of SRS-A^{rat} release.

The possibility that diethylcarbamazine and its effective analogs inhibited SRS-A^{rat} release by interfering with leukocyte viability was examined *in vitro*. Rat PMN leukocytes, suspended in Tyrode's solution con-

TABLE II. Viability of Rat PMN Leukocytes in the Presence of Diethylcarbamazine and Its Analogs.

Compound	No. of cells examined	Fluorescent (%)
None	133	95
Diethylcarbamazine	96	88
Pipecolamide	198	85
Isonicotinic acid hydrazide	74	89
N-Amidinobenzamide	123	83

TABLE III. Effects of Diethylcarbamazine and Its Analogs on the Homocytotropic Antibody-Mediated Release of Histamine in the Rat.

Compound	Peripheral blood				Mean histamine release (μg/rat)
	Total (WBC[a]/mm³)	Differential			
		Poly	Lymph	Mono	
None	12,900	26	73	1	2.07
Diethylcarbamazine	12,300	24	75	1	1.84[b]
Pipecolamide	10,670	23	74	3	1.58
Isonicotinic acid hydrazide	11,600	26	71	3	2.23
N-Amidinobenzamide	10,100	20	78	2	3.29

[a] WBC, white blood cell count.

[b] Corrected for antihistamine activity in bioassay.

taining 10 m*M* concentrations of diethylcarbamazine and its analogs, retained their viability as demonstrated by their ability to hydrolyze FDA and accumulate fluorescein intracellularly (14) (Table II).

Rats prepared for the antigen-induced release of histamine by the intraperitoneal injection of homocytotropic antibody against Ea were pretreated with diethylcarbamazine and its effective analogs (Table III). The dosage of inhibitor used was capable of producing a marked suppression of the immunologic release of SRS-A^{rat} but none of these agents significantly inhibited the homocytotropic antibody-mediated release of histamine.

Selective inhibition of the antigen-induced release of histamine in the rat by disodium cromoglycate. Rats prepared for the antigen-induced release of histamine by the i.p. injection of homocytotropic antibody demonstrated a marked suppression of histamine release when pretreated with disodium cromoglycate but not with diethylcarbamazine (Table IV). In contrast, when rats were prepared for the immunologic release of SRS-A^{rat} by the i.p. injection of homologous, hyperimmune antisera, no suppression of SRS-A^{rat} release was observed in rats pretreated with disodium cromoglycate but a comparable dose of diethylcarbamazine effected a greater than 60% suppression of SRS-A^{rat} release (Table V). Since the route of administration of the inhibitor may influence its activity, the compounds were compared both intravenously and intraperitoneally.

TABLE IV. Effects of Disodium Cromoglycate and Diethylcarbamazine on the Homocytotropic Antibody-Mediated Release of Histamine in the Rat.

Compound	Mean histamine release (μg/rat)
—	1.94
Disodium cromoglycate	0.07
Diethylcarbamazine	1.67

TABLE V. Effects of Disodium Cromoglycate and Diethylcarbamazine on the Antigen-Induced Release of SRS-A^{rat} in the Rat.[a]

Expt.	Compound	Mean SRS-A^{rat} release (units/rat)
A	—	148
	Disodium cromoglycate	149
	Diethylcarbamazine	42
B	—	637
	Disodium cromoglycate	593
	Diethylcarbamazine	230

[a] In Expt. A, compounds injected i.v. in a dose of 20 mg/kg 30 sec before antigen i.v. In Expt. B, compounds injected i.p. in a dose of 30 mg/kg 30 sec before antigen i.p.

Since disodium cromoglycate inhibited the intraperitoneal release of histamine mediated by rat homocytotropic antibody, an experiment was carried out comparing the ability of this agent to inhibit two mast cell-dependent cutaneous reactions, the 48-hr PCA reaction, and the blueing reaction elicited by the i.d.

TABLE VI. Homocytotropic Antibody-Mediated PCA in the Rat in the Presence of Various Inhibitors.[a]

	PCA (Dilutions of rat antiserum containing homocytotropic antibody)										Ra anti-RMC (Dilutions of Ra anti-RMC)			
Pretreatment procedure	1:10		1:20		1:40		1:80		1:100		1:25		1:50	
None	4+	14	4+	12	3+	10	3+	8	2+	5	4+	12	4+	10
Diethylcarbamazine	4+	13	3+	11	3+	9	3+	8	1+	5	4+	11	4+	9
Disodium cromoglycate	Tr	5	Tr	2	0	0	0	0	0	0	4+	10	3+	10
Mepyramine maleate + methysergide	0	0	0	0	0	0	0	0	0	0	Tr	4	Tr	2

[a] Diethylcarbamazine, 20 mg/kg i.v., and disodium cromoglycate, 50 mg/rat i.v., were injected 30 sec before challenge with specific antigen. Mepyramine maleate, 50 mg/kg i.p., and methysergide, 4 mg/kg i.p., were administered 30 min before challenge.

injection of Ra anti-RMC (Table VI). Disodium cromoglycate effectively suppressed the 48-hr PCA reaction without inhibiting the i.d. cytotoxic release of histamine. Pretreatment with the combination of an antihistamine (mepyramine maleate) and a potent serotonin antagonist (methysergide) virtually abolished both reactions. Diethylcarbamazine did not significantly inhibit either reaction.

Discussion. Specificity of the inhibitory activity of diethylcarbamazine and its analogs. A study of the chemical analogs of diethylcarbamazine was undertaken in an attempt to determine the subgroups within the molecule required for optimal inhibition of the antigen-induced release of SRS-A^{rat}. Since carboxamide derivatives of the piperidine, pyridine and, to a lesser extent, benzene rings demonstrated inhibitory activity, the piperazine ring itself does not appear to be essential for optimal inhibitory activity. However, the carboxamide grouping does appear to be required for inhibition of SRS-A^{rat} release since none of the ring structures alone were active (Table I). Inhibition of the antigen-induced release of SRS-A^{gp} *in vitro* has been observed with ethanolamine and choline (17); neither of these agents was active in inhibiting SRS-A^{rat} release. A substituted urea, 1,1-diethylurea, was also ineffective. It thus appears that the structural requirements for inhibition of the antigen-induced release of SRS-A^{rat} include the carboxamide grouping and a saturated or unsaturated ring containing nitrogen.

Studies concerned with the site of the inhibitory action of diethylcarbamazine have revealed that this agent does not block the end-organ activity of SRS-A^{rat}, and it does not interfere with antigen–antibody interaction *in vitro* or *in vivo* (3, 10). The viability of rat PMN leukocytes is not altered *in vitro* by high concentrations of diethylcarbamazine or its effective analogs (Table II), and it is unlikely that inhibition of SRS-A^{rat} release *in vivo* is due to some nonspecific toxic effect of these agents on this cell population. Diethylcarbamazine appears to act at some point subsequent to antigen–antibody interaction and prior to the formation and release of SRS-A^{rat}. The inhibition is selective in that diethylcarbamazine does not suppress the antigen-induced intraperitoneal (Table IV) or intracutaneous (Table VI) homocytotropic antibody-mediated release of histamine and serotonin.

Specificity of the inhibitory activity of disodium cromoglycate. The present study confirms the finding (11) that disodium cromoglycate blocks the 48-hr PCA reaction in the rat and indicates that it does so by selectively suppressing the homocytotropic antibody-mediated pathway to release of the vasoactive amines. Disodium cromoglycate failed to prevent the skin lesion elicited by the cytotoxic release of histamine and serotonin from mast cells following intradermal injection of Ra anti-RMC (Table VI). Since the combination of an antihistamine and an antiserotonin agent effectively blocked both

the 48 hour PCA reaction and the lesion elicited by Ra anti-RMC, it follows that disodium cromoglycate has a selective effect on the homocytotropic antibody-mediated pathway leading to the release of these vasoactive amines. The site of action was further delineated by the finding that disodium cromoglycate prevented the homocytotropic antibody-mediated release of histamine from the rat peritoneal cavity (Table IV), while failing to suppress the immunologic release of SRS-A^{rat} (Table V).

Dissociation of the immunologic release of SRS-A^{rat} and histamine by pharmacologic inhibition. The immunologic pathways leading to the antigen-induced release of SRS-A^{rat} and histamine can now be distinguished in terms of the immunoglobulins involved, the cellular participants and the effective pharmacologic inhibitors. SRS-A^{rat} release requires rat IgGa (2) and the PMN leukocyte (3, 4) whereas histamine release may be elicited with rat homocytotropic antibody sensitizing homologous mast cells (6, 7). Diethylcarbamazine effectively inhibits the antigen-induced release of SRS-A^{rat} (Table V) without suppressing the homocytotropic antibody-mediated release of histamine (Table IV). Conversely, at comparable doses and routes of administration, disodium cromoglycate suppresses the homocytotropic antibody-mediated release of histamine (Table IV) but does not inhibit the antigen-induced release of SRS-A^{rat} (Table V). Neither chemical interferes with the pharmacologic action of histamine or SRS-A^{rat}. These drugs represent the first examples of nontoxic agents which selectively suppress *in vivo* the specific immunologic pathways leading to the release of the chemical mediators of immediate hypersensitivity.

Summary. A study of the structural analogs of diethylcarbamazine reveals the requirement for a carboxamide grouping and a saturated or unsaturated ring containing nitrogen for optimal inhibition of SRS-A^{rat} release. Diethylcarbamazine and its analogs do not act by interfering with PMN leukocyte viability and they do not inhibit the homocytotropic antibody-mediated release of histamine and serotonin. Conversely, disodium cromoglycate suppresses the latter pathway without inhibiting the antigen-induced release of SRS-A^{rat}. Neither diethylcarbamazine nor disodium cromoglycate antagonizes the pharmacologic activity of histamine or SRS-A^{rat}. The use of these agents permits further dissociation of the pathways leading to the immunologic release of SRS-A^{rat} and histamine in the rat by selective pharmacologic inhibition.

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