

Passive Paw Anaphylaxis in the Rat

Optimum Conditions for Use in Studies on Immediate Hypersensitivity

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The difficulties involved in quantitating passive cutaneous anaphylaxis led us to examine the rat paw as a site for passive anaphylaxis and to define optimum conditions for passive paw anaphylaxis. Generation of homocytotropic antibodies in a number of different rat strains revealed that female Brown Norway rats were excellent producers of high titre antisera after only a single injection of antigen and *Bordetella pertussis*. Injection of ratpaws with diluted antisera followed by short (1-2 h) or long (72 h or more) sensitization periods showed that maximum paw swelling occurred 15 min after antigen challenge. Retention of tissue sensitizing capacity in the paw for greater than 35 days but loss of this capacity following heating of antiserum at 56°C, indicated that the rat homocytotropic antibodies involved in passive paw anaphylaxis belong to the IgE class. Experiments using mepyramine, methysergide and diethylcarbazine showed that 5-hydroxytryptamine is the most important mediator of passive paw anaphylaxis after both a short (2 h) and a long (72 h) sensitization procedure. Passive paw anaphylaxis in the rat is at least as easy to perform as passive cutaneous anaphylaxis, results can be obtained more rapidly and accurate measurement of paw thickness is not difficult. The procedure is a viable alternative to passive cutaneous anaphylaxis and may offer advantages over the latter method especially in the search for, and rapid assessment of, anti-allergic compounds.

Key words: anti-allergy screen; homocytotropic antibodies; 5-hydroxytryptamine; immediate hypersensitivity; passive anaphylaxis.

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Since its introduction into animal experiments over 20 years ago (17, 18), passive cutaneous anaphylaxis (PCA) has been of great value in *in vivo* studies on immediate hypersensitivity reactions. Although the skin of experimental animals is a convenient site for detecting and measuring local anaphylactic responses, other tissues and organs may be passively sensitized *in vivo* with antibody and used to assess immediate allergic reactions (4, 7, 8, 14, 20). For many laboratory studies on inflammation and hypersensitivity, the rat is a convenient animal to use since its small size allows large numbers to be easily housed and handled in a single experiment. In contrast to the mouse,

intravenous (i.v.) injection of rats is relatively easy to perform, the rat has a large area of skin on the back available for injection at multiple sites, the peritoneal cavity yields suitable volumes of extravasated fluid for analysis and the rat paws are large enough for the accurate measurement of changes in thickness. The difficulties involved in quantitating PCA responses while assessing inhibitory effects of drugs on immediate hypersensitivity reactions led us to examine the rat paw as a site for such reactions. In this communication, optimum conditions have been defined for obtaining anaphylaxis in the rat paw following passive sensitization of the paw with rat

homocytotropic antibody (1, 2, 12) and the advantages and disadvantages of passive paw anaphylaxis (PPA) are discussed.

MATERIAL AND METHODS

Rats. Male Fullinsdorf rats from stock originally supplied by Hoffmann La Roche and Company, Basel, Switzerland were bred in the animal house, Roche Research Institute, Dee Why. Male and female Oxford Hooded, Albany, August and Brown Norway (BN) rats were obtained from the Medical School animal house, Blackburn Building, University of Sydney.

Antisera. Sera containing homocytotropic antibodies for use in passive anaphylaxis experiments were raised in 200–250 g BN rats. The same injection procedure was used for the other rat strains listed above. Each rat was injected intraperitoneally (i.p.) with 0.5 ml of *Bordetella pertussis* vaccine (Commonwealth Serum Laboratories, Melbourne) containing 2×10^{10} organisms and intramuscularly (i.m.) with 2 mg of crystalline chicken egg albumin (OA) (Sigma, St Louis, grade VI, lot 67C–8035) in 0.5 ml of physiological saline (11). Eleven days later, animals were bled by decapitation and the sera recovered.

Heat treatment of sera. Rat anti-OA sera prepared in BN rats and used in passive anaphylaxis experiments were placed in 6-ml Medical Plastics Serology Tubes, capped and heated in a Grant model SS30 shaking water bath at a temperature of 56°C ($\pm 0.2^\circ\text{C}$) for periods of up to 4 h (19).

Compounds. Diethylcarbamazine citrate was supplied by Hoffmann La Roche and Company, Basel. Mepyramine maleate was obtained from May and Baker Ltd, West Footscray, Victoria, methysergide bimaleate from Sandoz A.G. Basel and indomethacin from Merck, Sharp and Dohme (Aust.) Pty Ltd, Sydney.

PCA. This was carried out as described by Ovary (18). Male Fullinsdorf or Oxford Hooded rats of approximately 200 g were

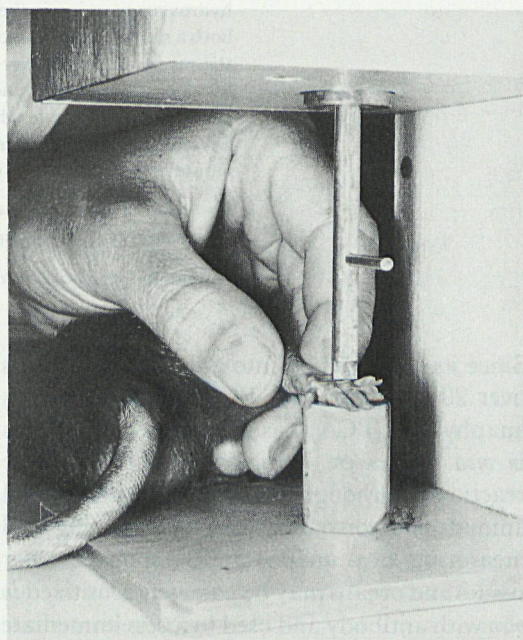
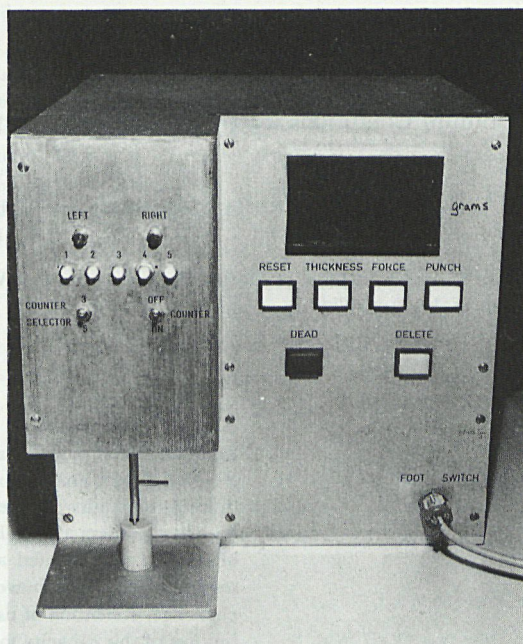


Fig. 1. (a) Thickness measuring transducer apparatus AASE (Hoffmann La Roche and Company, Basel) used for rat paw thickness measurements. (b) Close-up of paw measurement procedure. Rat hind paw with plantar surface uppermost is placed between the anvil and the free-moving pin. Paw thickness in mm is displayed on a digital panel meter.

Table 1.
Mean PCA titres of anti-ovalbumin antisera^a produced in different rat strains

Strain	Sex	Mean PCA titre ^{b, c, d}	No. rats examined
Albany	M	7±3	6
Albany	F	15±5	7
August	M	0	6
August	F	1±1	6
Brown Norway	M	616±159	35
Brown Norway	F	1272±218	33
Fullinsdorf	M	1±0	83
Oxford Hooded	M	38±8	52
Oxford Hooded	F	48±20	6

^a Sera containing homocytotropic antibodies produced by injection of ovalbumin and *Bordetella pertussis* according to the method of Mota (11).

^b Titres expressed to the nearest whole number.

^c The PCA titre was taken as the last dilution giving a blue spot at least 5 mm in diameter.

^d PCA titre ranges for the different strains were: Albany 4–32, August 0–2, Brown Norway 128–8192, Fullinsdorf 0–8, Oxford Hooded 4–256.

shaved on the back and injected intradermally at four sites with dilutions of rat anti-OA sera in physiological saline. Seventy-two hours later the animals were challenged i.v. with a mixture of 0.3 ml of OA 1% w/v and 0.4 ml of Gurr Pontamine Sky Blue (Searle Diagnostics, High Wycombe) 5% w/v in physiological saline. Rats were killed after 20 min, skinned and the diameters of the blue wheals at the injection sites were measured on the inner skin surfaces. The PCA titre of a serum was taken as the last dilution giving a blue spot at least 5 mm in diameter.

Measurement of paw thickness. Rat paw thickness was measured using the thickness measuring transducer, AASE (Anti-inflammatory Screening Equipment) manufactured by Hoffmann La Roche and Company, Basel (Fig. 1). The transducer consists of a motor-driven leadscrew connected to a precision potentiometer from which an electrical voltage proportional to paw thickness is extracted. This voltage is displayed on a digital panel meter calibrated to read paw thickness directly

in mm. The measuring specifications of the instrument are: Range 0 to 9.99 mm, resolution 0.01 mm, accuracy ± 0.05 mm, reproducibility ± 0.02 mm. Measuring cycles are controlled by a foot switch. With the machine in reset mode, the rat paw is put upside down between the anvil and the free-moving pin (Fig. 1b). Depression of the foot switch activates the internal mechanism to generate a thickness measurement. Depressing the foot switch a second time resets the machine for another measurement. Foot control of the transducer permits one man operation, enabling the worker to handle conscious rats with both hands. If desired, the transducer may also be used to apply a squeeze-load (force) to the paw for assessment of analgesic activity and the readings of both thickness and force may be recorded on paper tape. This simple device enables a single operator to measure the hind paw thicknesses of approximately 100–120 rats per hour with an impressive degree of resolution, accuracy and reproducibility.

PPA. Groups of six to eight rats of approximately 200 g were used in all experiments. Following measurement of the thicknesses of both hind paws, Oxford Hooded or Fullinsdorf rats were given subplantar injections in each paw of 0.05 ml of a suitable dilution of rat anti-OA serum. After the desired sensitization period, animals were injected i.v. in the tail or dorsal vein of the penis with 0.3 ml of a 1% w/v solution of OA in physiological saline. Intravenous and paw injections were administered via 27 gauge needles. At the desired time after challenge, the ensuing swelling in the paws was measured using the anvil and pin AASE thickness measuring transducer. Paws of control animals were injected with diluted antiserum but these rats were not challenged with antigen before paw thicknesses were read. The mean increase in paw thickness in the rats challenged with OA after a 2-h sensitization procedure was obtained after deducting the mean figure obtained from control animals sensitized for 2 h with diluted antiserum. This procedure

Table 2.
Effect of serum dilution^a on rat paw thickness in passive paw anaphylaxis

Dilution of antiserum injected ^b (reciprocals)	Mean increase in paw thickness (mm) in	
	Fullinsdorf rats ^c	Oxford Hooded rats ^c
10	3.0±0.1	3.8±0.3
20	2.2±0.2	3.2±0.1
50	1.3±0.1	1.9±0.2
100	0.5±0.1	0.7±0.2
200	0.4±0.1	0.2±0.1
400	0	0.1±0.1

^a Brown Norway rat anti-ovalbumin serum with PCA titre of 1600.

^b 50 µl of diluted serum injected into each hind paw.

^c Seven animals per group.

avoided artificially high paw thicknesses in the animals sensitized for 2 h due to the presence in the paw of unabsorbed fluid following the injection of antiserum. Mean paw size in rats sensitized for 24 h but not challenged with OA was used as a control for animals challenged after 24 or more hours. Three other groups of control animals used in PPA studies were injected in the hind paws with saline, serum from unimmunized rats or serum from rats injected with OA and *B. pertussis* but which gave a PCA titre of 0.

Compounds used to inhibit mediators of PPA were given i.v. in 0.3 ml of physiological saline 5 min before i.v. challenge with OA.

RESULTS

Production of homocytotropic antibodies in different strains of rats

Repeated attempts to sensitize male Fullinsdorf rats to OA by injection with OA and *B. pertussis* met with little success. Homocytotropic antibodies, as detected by PCA reactions in rats, were frequently absent in injected animals or present in low titre (range 2–8). Similar results were obtained with the few male and female August rats studied. Oxford Hooded and Albany rats proved better

in that PCA titres of anti-OA sera raised in these animals were frequently in the range 4–32 and, with the former strain, titres of 64–256 were sometimes obtained. BN rats were chosen, however, for PPA studies since this strain consistently produced high titre anti-OA sera (PCA titres 128–8192). A striking and significant difference was observed in the PCA titres obtained with sera from male and female BN rats, the mean titres of the latter being twice that found in male rat sera. Table 1 lists the mean PCA titres found in the five different rat strains studied.

Effect of serum dilution on paw thickness in PPA experiments

Preliminary studies using dilutions of BN rat anti-OA serum together with a 48-h sensitization period before i.v. challenge with OA, were carried out using Fullinsdorf and Oxford Hooded rats. Using dilutions of an anti-OA serum (PCA titre 1600), greater swelling was observed in the paws of Oxford Hooded rats than in paws of the Fullinsdorf strain. To obtain paw thickness increases of about 3 mm or more, dilutions of no more than about 1–20 of the antiserum were used (Table 2).

To investigate the response in passively sensitized paws after shorter sensitization periods, Oxford Hooded rats were challenged

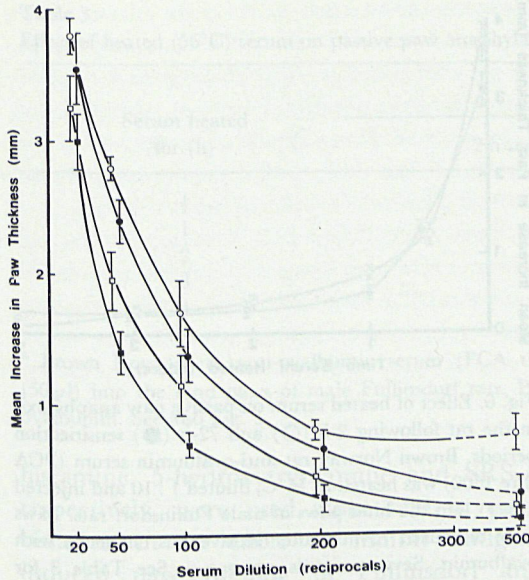


Fig. 2. Effect of antiserum dilution and short sensitization periods on paw responses in passively sensitized rats. Dilutions of anti-ovalbumin serum (PCA titre 1600) prepared in Brown Norway rats were injected ($50\mu\text{l}$) into the hind paws of male Oxford Hooded rats. Recipients were challenged i.v. with ovalbumin 1 h ($\circ$), 2 h ($\bullet$), 4 h ($\square$) or 6 h ($\blacksquare$) after paw injections and the paw sizes were measured 15 min later. Eight rats per group.

with OA 1, 2, 4 and 6 h after the injection of dilutions of rat anti-OA serum. For all dilutions, paw size was maximum at 1 h and decreased steadily over the 6-h period (Fig. 2). Of the dilutions examined, only the most concentrated solutions (1:20 and 1:50), produced size changes that were sufficiently large to be used successfully in studies on passive anaphylaxis. Paw sizes in Hooded rats challenged with antigen after 6 h were approximately the same as with rats challenged after 24 and 48 h.

Determination of optimum time for measuring paw thickness following antigen challenge

Following injection into the hind paws of rat anti-OA serum and a 2-h sensitization period, groups of rats were challenged i.v. with OA and paw thicknesses were assessed at various time intervals up to a maximum of 24 h. Fig. 3 shows that maximum swelling was apparent 15

min after antigen challenge. To check that this time interval for measuring paw thickness following antigen challenge was also suitable for sensitization periods in excess of 2 h, groups of rats were injected in the hind paws and then challenged with OA after 2, 4, 6, 24, 48, 72 and 96 h. Paw thicknesses in these animals were monitored after 15 min and 30 min and, as shown in Fig. 4, large increases in paw sizes could be detected over the entire time range studied. As with the studies after a 2-h sensitization period (Fig. 3), the interval of 15 min after antigen challenge revealed greater paw swelling. This time interval was therefore used in all subsequent experiments reported in this communication.

Sensitization period: Persistence of homocytotropic antibodies in the rat paw

Rat anti-OA serum that gave a PCA titre in rats of 4096 was diluted 1:10 and injected into the hind paws of groups of rats. After 2 h and 3, 6, 10, 15, 20, 22, 24, 30 and 35 days, groups of animals were injected i.v. with OA and the paws read 15 min later. Paw thicknesses remained elevated for 10 days before dropping in a linear fashion (Fig. 5). Marked swelling was apparent 20 days after injection of

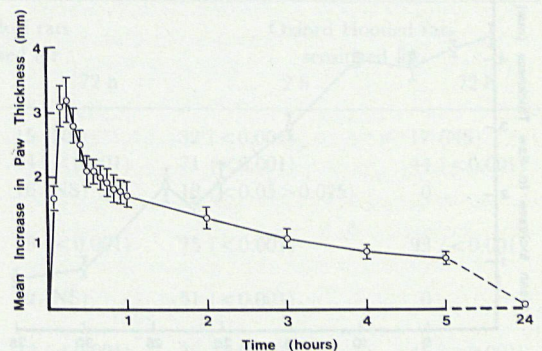


Fig. 3. Changes in paw thickness with time following antigenic challenge of sensitized rats. Both hind paws of male Oxford Hooded rats were sensitized by injection of $50\mu\text{l}$ of a 1:20 dilution of Brown Norway rat anti-ovalbumin serum (PCA titre 1600). Two hours later animals were challenged i.v. with ovalbumin and paw sizes were measured at 5-min intervals up to 1 h and then at 2, 3, 4, 5 and 24 h. Ten animals in test and control groups.

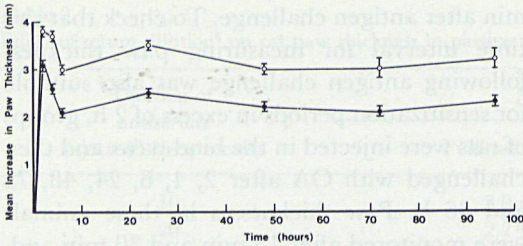


Fig. 4. Changes in paw thickness following passive paw anaphylaxis in rats 15 min (○) and 30 min (●) after i.v. challenge with sensitizing antigen. Hind paws of groups of male Oxford Hooded rats were sensitized with 50μl of a 1 : 20 dilution of Brown Norway anti-ovalbumin serum (PCA titre 1600) for periods of 2, 4, 6, 24, 48, 72 and 96 h. Eight animals per group.

antiserum and, even at day 35 when the experiment was concluded, the paws were still significantly swollen.

Effect of heated serum on PPA

Unheated rat anti-OA serum and antiserum heated in a water bath at 56°C for periods of 30 min, 1 h, 2 h, 3 h and 4 h, were diluted and injected into the hind paws of groups of Fullinsdorf and Oxford Hooded rats. After sensitization periods of 2 h and 72 h, animals were challenged i.v. with OA and the paws

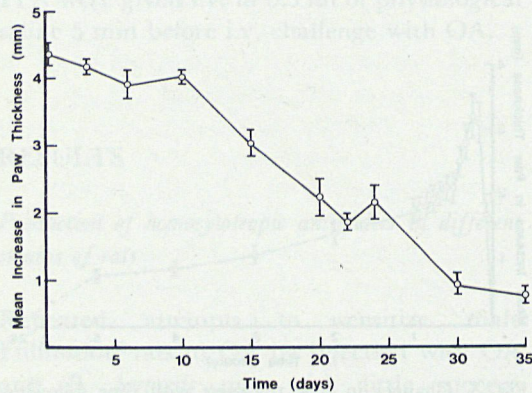


Fig. 5. Persistence of homocytotropic antibody in the paw in passive paw anaphylaxis in male Oxford Hooded rats. Paws were sensitized with 50μl of a 1 : 10 dilution of Brown Norway rat anti-ovalbumin serum (PCA titre 4096) for periods of 2 h or 3, 6, 10, 15, 20, 22, 24, 30 or 35 days before i.v. challenge with ovalbumin and measuring paw thickness 15 min later. Seven animals per group.

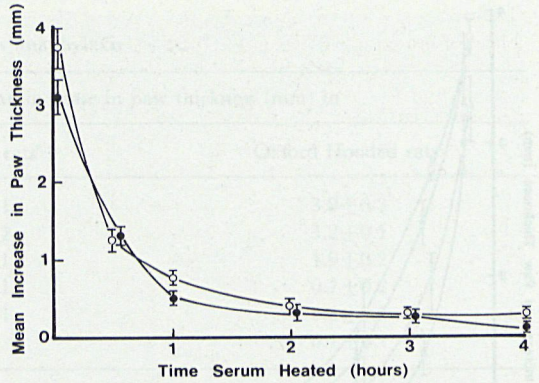


Fig. 6. Effect of heated serum on passive paw anaphylaxis in the rat following 2-h (○) and 72-h (●) sensitization periods. Brown Norway rat anti-ovalbumin serum (PCA titre 1600) was heated at 56°C, diluted 1 : 10 and injected (50μl) into the hind paws of male Fullinsdorf rats. Paws were measured 15 min after intravenous challenge with ovalbumin. Seven animals per group. See Table 3 for percentage inhibitions of paw swellings observed.

measured 15 min later. Fig. 6 and Table 3 show the results obtained in one experiment with Fullinsdorf rats. Animals sensitized with serum heated for 30 min showed reductions in paw sizes after 2 h and 72 h of 66% and 57% respectively when compared with rats sensitized with unheated serum. Following the 2-h latent period, maximum reduction in paw size was obtained after serum had been heated for 2 h. Heating beyond this time did not significantly reduce the swelling and residual activity (approximately 10%) remained in the samples heated for from 2–4 h. An 84% reduction in paw thickness was obtained when rats were passively sensitized for 72 h with serum heated at 56°C for 1 h. Heating serum for 2–4 h virtually abolished all tissue sensitizing activity after a 72-h latent period (Fig. 6, Table 3). Results obtained with Oxford Hooded rats were similar except that paw thicknesses obtained after injection of unheated serum were greater.

Mediators of rat PPA. Inhibition of PPA using antagonists of known mediators of anaphylaxis

Mepyramine, methysergide and diethylcarbamazine, selected as known antagonists for

Table 3.
Effect of heated (56°C) serum on passive paw anaphylaxis in the rat^a

Serum heated for (h)	Percentage inhibition of paw swelling after	
	2 h sensitization	72 h sensitization
$\frac{1}{2}$	66	59
1	79	84
2	89	89
3	92	92
4	91	96

^a Brown Norway rat anti-ovalbumin serum (PCA titre 1600) was heated at 56°C, diluted 1:10 and injected (50 µl) into the hind paws of male Fullinsdorf rats. Paws were measured 15 min after intravenous challenge with ovalbumin. See also Fig. 5.

histamine, 5-hydroxytryptamine and SRS-A respectively, were used alone and in combination in an effort to inhibit antigen-induced paw swelling in Fullinsdorf and Oxford Hooded rats following 2-h and 72-h sensitization periods (Table 4). Diethylcarbamazine showed little or no inhibitory capacity and this was also true of mepyramine when used in rats sensitized for 72 h. After a 2-h sensitization period, mepyramine produced approximately 30% inhibition of paw swelling

in both rat strains examined. Methysergide was the best of the inhibitors studied although at 72 h in Oxford Hooded rats only 44% inhibition was obtained. The most striking result, however, was the finding that a mixture of mepyramine and methysergide significantly enhanced the inhibition of paw swelling in both strains at both 2 h and 72 h. This was most clearly seen in hooded rats sensitized for 72 h before injection of mepyramine, methysergide or a mixture of these two. Injection of

Table 4.
Inhibition of passive paw anaphylaxis^a in the rat using antagonists of known mediators of anaphylaxis

Compounds	Dose (mg/kg)	Percentage inhibition in paws from			
		Fullinsdorf rats sensitized for		Oxford Hooded rats sensitized for	
		2 h	72 h	2 h	72 h
Mepyramine	2.5	31 (<0.001) ^b	15 (NS)	32 (<0.001)	17 (NS)
Methysergide	2.5	72 (<0.001)	74 (<0.001)	71 (<0.001)	44 (<0.001)
Diethylcarbamazine	20	15 (<0.02>0.01)	16 (NS)	18 (<0.05>0.025)	0
Mepyramine and methysergide	2.5	81 (<0.001)	89 (<0.001)	75 (<0.001)	93 (<0.001)
Mepyramine and diethylcarbamazine	2.5	35 (<0.001)	12 (NS)	51 (<0.001)	0
Methysergide and diethylcarbamazine	2.5	76 (<0.001)	72 (<0.001)	72 (<0.001)	45 (<0.001)
Mepyramine, Methysergide and diethylcarbamazine	2.5	80 (<0.001)	95 (<0.001)	81 (<0.001)	89 (<0.001)
Indomethacin	3	NT	0	NT	0

^a Hind paws sensitized with 50 µl of a 1:10 dilution of Brown Norway rat anti-ovalbumin serum (PCA titre 4096). Antagonists given intravenously 5 min before ovalbumin. Paws measured 15 min after antigen injection. ^b Numbers in parenthesis show statistical significance (Student's t-test). NS, not significant. NT, not tested.

all three inhibitors, mepyramine, methysergide and diethylcarbamazine in the same admixture, did not produce significantly greater inhibition than was seen with mixed mepyramine and methysergide.

Indomethacin, an inhibitor of prostaglandin synthetase, was inactive in both strains following sensitization for 72 h (Table 4).

DISCUSSION

Results in this study have confirmed and extended the finding of Martel & Klicius (8) that the rat paw can be passively sensitized with homocytotropic antibody and used, like the PCA reaction, to study immediate hypersensitivity reactions *in vivo*. We attempted to define optimum conditions for PPA experiments in rats and to obtain information on the mediators released following the interaction of antigen with its complementary tissue-bound antibody. Generation of such antibodies in a number of different rat strains was investigated and these experiments revealed that BN females were excellent for producing high titre antisera after only a single injection of antigen and adjuvant. Like Martel & Klicius (8) we found that a higher antibody concentration was needed for PPA than for PCA. In general, PCA titres of 1000 or better must be diluted to only approximately 1 : 10 or 1 : 20 to produce experimentally useful increases in paw size. It should be recognized, however, that the dilution of antiserum necessary to provoke large paw responses is dependent on the strain of rats used. Experiments designed to reveal the optimum time for measuring paw thickness after antigen challenge clearly showed that maximum oedema occurred 15 min after i.v. injection of antigen. Our findings on heat lability of the paw-sensitizing antibodies together with persistence of the antibodies in the tissues for long periods (> 35 days), indicated that the rat homocytotropic antibodies studied here probably all belong to the IgE class (23). Residual paw swelling observed in rats sensitized for 2 h with heated (56°C, 2–4 h) serum may indicate, however, that small

amounts of antibody of the IgG_a class (2, 10) were present in the BN rat antiserum. Experiments designed to investigate the tissue-sensitizing antibody classes in the BN rat antisera are planned, but, as yet, we have obtained no data on the physicochemical nature of the tissue-sensitizing antibodies.

Rowley & Benditt (22) showed that paw oedema resulting from mast cell damage in the rat is mediated largely by 5-hydroxytryptamine. The property of antagonists of 5-hydroxytryptamine to inhibit cutaneous anaphylaxis in rats following a 48-h or 72-h sensitization period is well established (5, 9, 11). Assuming that mepyramine and methysergide are fairly specific antagonists of histamine and 5-hydroxytryptamine respectively, our inhibition results with these compounds indicate that 5-hydroxytryptamine is the most important mediator of PPA at both 2 h and 72 h after injection of antiserum into the rat paw. Mepyramine showed some inhibitory effect after the 2-h latent period but this compound produced little inhibition of the paw reaction when given just before antigen challenge 72 h after sensitization of the rat paws. Evidence that histamine is not an important mediator of PCA reactions in rats was provided by Brocklehurst et al. (3) and the poor inhibition by mepyramine of PCA reactions observed by Mota (11) supports this conclusion. A third mediator of anaphylaxis in the rat has been studied by Austen's group who showed that both IgG_a and IgE antibodies may promote antigen-induced release of slow reacting substance of anaphylaxis (SRS-A) in this species (13, 15). The release of SRS-A in the rat was shown to be inhibited by diethylcarbamazine citrate (14, 16) but, we found that this compound showed little or no significant inhibition of OA-induced PCA 2 h and 72 h after sensitization of the paw with rat anti-OA serum. Our inhibition results with mepyramine, methysergide and diethylcarbamazine therefore indicate that 5-hydroxytryptamine is the most important mediator involved in PPA in the rat after both a short (2 h) and a long (72 h)

sensitization period. The enhanced inhibitory capacity observed following injection of both mepyramine and methysergide also suggested that together these two may be preventing the liberation of a third mediator. This possibility suggests itself especially in the results obtained with Oxford Hooded rats following a 72-h sensitization period. If such a mediator is involved, then it does not appear to be a prostaglandin since indomethacin was without activity in inhibiting the 72-h PPA response.

PCA is an invaluable immunological technique for studying immediate allergic reactions, detecting antigens and antibodies *in vivo*, studying immunological cross-reactivity and screening potential anti-allergic drugs. Unfortunately, the method does not easily lend itself to quantitation and although the size of a skin reaction is roughly proportional to the antibody content (24), use of the diameter of a reaction for quantitation requires a high degree of skill and many animals. The use of radiolabelled antigen (21) and extraction of extravasated dye and measurement of its blue colour intensity (see (6) for references) have been used for quantitation but, at best, the PCA reaction must be considered to be difficult and time-consuming to quantitate. Although PPA requires the use of more concentrated antibody dilutions than PCA, the former method is at least as easy to perform, especially if suitable instrumentation is available for paw measurement. Accurate measurement of paw thickness in the rat is not difficult and this permits ready detection of inhibitors of PPA reactions. PPA in the rat is therefore a viable alternative to PCA and offers advantages over the latter method especially in the search for, and assessment of, anti-allergic drugs.

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