# A New Method for Mast Cell Separation; and the Effect of Sera From Urticaria Patients on Purified Rat Mast Cells*

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**SUMMARY** **Biologically intact rat mast cells were purified with a modified coil planet centrifuge, since this apparatus enabled quite effective cell separation without using viscous high-density media which could impair the cell membrane or change its antigenicity. A reproducible final purity of 97 per cent was achieved with satisfactory yield. Morphological and chemical examinations indicated no change in the mast cells after separation. In order to pursue the cause of urticaria, purified rat mast cells were used as indicator cells. Antigen-specific histamine release was demonstrated. In the reactions, antigen-non-specific mast cell damage, i.e., induced by non-specific reaction with human sera, was also observed. However, the latter reaction was suppressed by either the addition of 1.5 mM EDTA to the reaction medium or heat-inactivation of human sera. It was assumed that the complement system is involved in non-specific histamine release.**

ASIAN PACIFIC J ALLERG IMMUN 1984; 2 : 77-84.

Mast cells, once referred to as unicellular grand,[1] are known to require calcium and metabolic energy for the release of histamine and other mediators of anaphylactic reaction.[2,3] To date, isolated mast cells have provided an interesting model for the study of stimulus-secretion coupling. On the other hand, mast cells are believed to be among the most fragile of cells for they easily lose their granules by cytolysis during inadequate procedures for separation.

Mast cells, readily harvested from the peritoneal cavity of rats, are usually separated by differential centrifugation through high-density solutions such as Ficoll and albumin. However, partial loss of histamine[4] or a remarkable decrease of both 5-hydroxytryptamine and 5-hydroxytryptophan decarboxylase activity[5] during centrifugation have been reported. Recently, Coutts, Nehring and Jariwala reported that IgE-specific receptors on the cell membrane of rat mast cells are either shed or inactivated by centrifugation through bovine serum albumin or Ficoll solution, and that purification through high-density media results in the loss of 50 to 80 per cent of the binding activity of the IgE-receptor.[6]

Since reception of stimuli, that initiates the specific function of a cell, occurs at certain contact point of the cell membrane, it is obviously desirable to obtain purified material with its delicate membrane properties retained after separation. In this paper, a new method for mast cell purification with a modified coil planet centrifuge is described. Preliminary communication of part of this work, especially concerning theoretical aspects of the device, have already been published.[7] This apparatus was devised for effective cell separation without the use of viscous high-density

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This work was supported in part by grants 511680137207 and 57770074 from the Ministry of Education, Japan

media which could impair the cell membrane.

Using purified mast cells, clinical studies for urticaria were carried out. Urticaria is a very common disease, but its causative factors have been rarely identified. In spite of detailed studies of disease history and various examinations *in vivo* and/or *in vitro,* there are still many cases for which no causative factors have been defined. It has been demonstrated that urticaria is related mostly to histamine released from skin mast cells.[8] Certain factors in the serum of the patient seem to play an important role in the degranulation of the mast cells. We re-examined the rat mast cell degranulation test[9-13] to detect various serum factors involved in the degranulation of the mast cells, in addition to the conventional aim of detecting specific IgE antibody in sera.

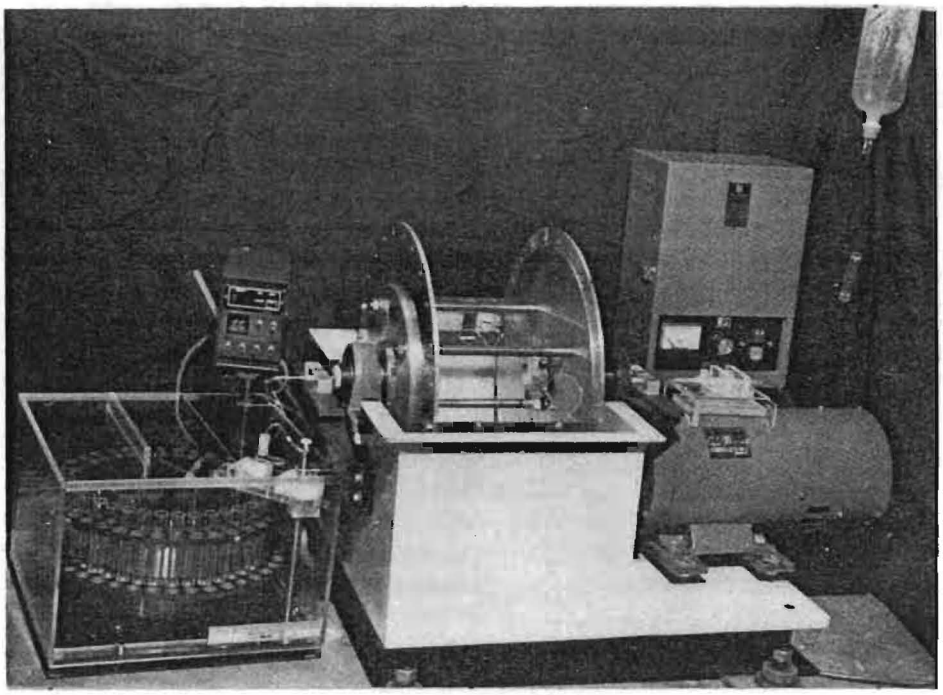

*Fig. 1* A modified coil planet centrifuge

## MATERIALS AND METHODS

### Apparatus

The modified coil planet centrifuge shown in Figures 1 and 2 was composed of the following parts: carrier fluid reservoir, joining portion, dividing joint and coiled tube. Carrier fluid was forced to flow through the coiled tubes by hydrostatic pressure via a joining portion (Fig. 3) which was a non-mobile portion connecting the fluid reservoir to the rotating coiled tube. Rotation disks, fixed with six coiled tube holders at their circumference, were equipped with a dividing joint at the centre of rotation. Carrier flow was equally divided into six flows with the dividing joint and streamed into six coiled tubes. Carrier fluid of coiled tubes was centred by another joint and collected in a fraction collector. The rotation of the disks was maintained exactly at 400 rpm by means of a controller with stroboscopic monitoring. Carrier fluid flow was controlled by adjusting the height of a fluid reservoir and

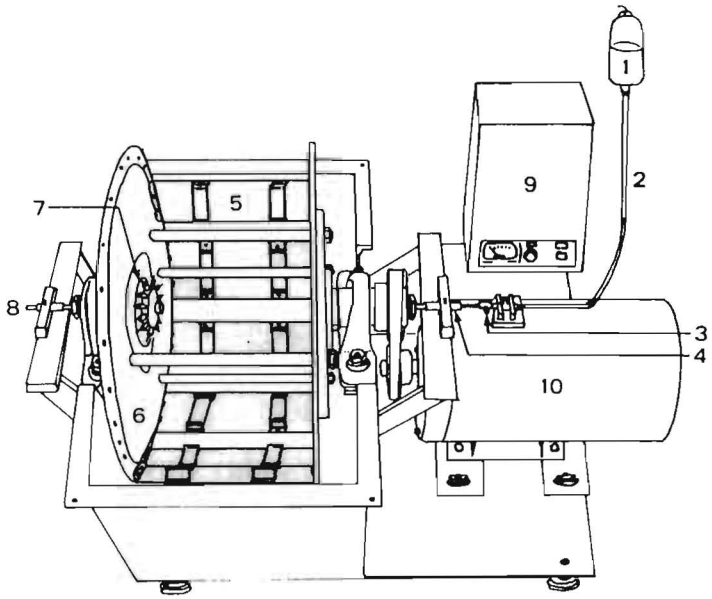


*Fig. 2* Schematic diagram of a modified coil planet centrifuge: 1. carrier fluid reservoir, 2. dripping set, 3. three-way cock for sample insertion, 4. joining portion, 5. coiled tube holder, 6. rotation disk, 7. dividing joint, 8. outlet portion, 9. rotation controller, 10. motor

checked by measuring the flow rates.

The coiled tube was prepared as follows: Teflon tubes, 10 m in length and 0.7 mm in inner diameter, were wrapped uniformly and tightly for about 100 turns around three plastic rods, 6 mm in diameter and 17 cm in length. A set of coiled tubes was fixed on a tube holder (Fig. 4). The joining portion was equipped with a three-way cock to stop the carrier flow during

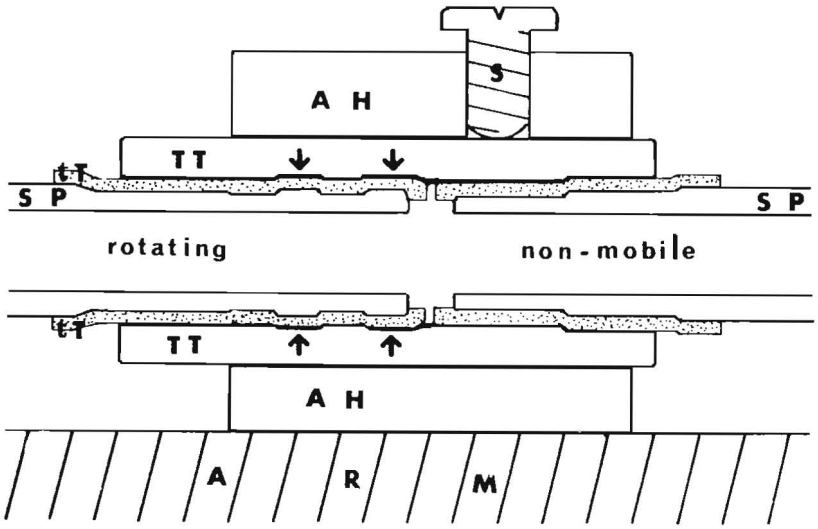


*Fig. 3* Diagrammatic illustration of the joining portion: sp, stainless pipe; tT, teflon tube; TT, thick teflon tube (3mm in inner diameter); AH, acryl holder; s, screw; ARM, arm of the centrifuge

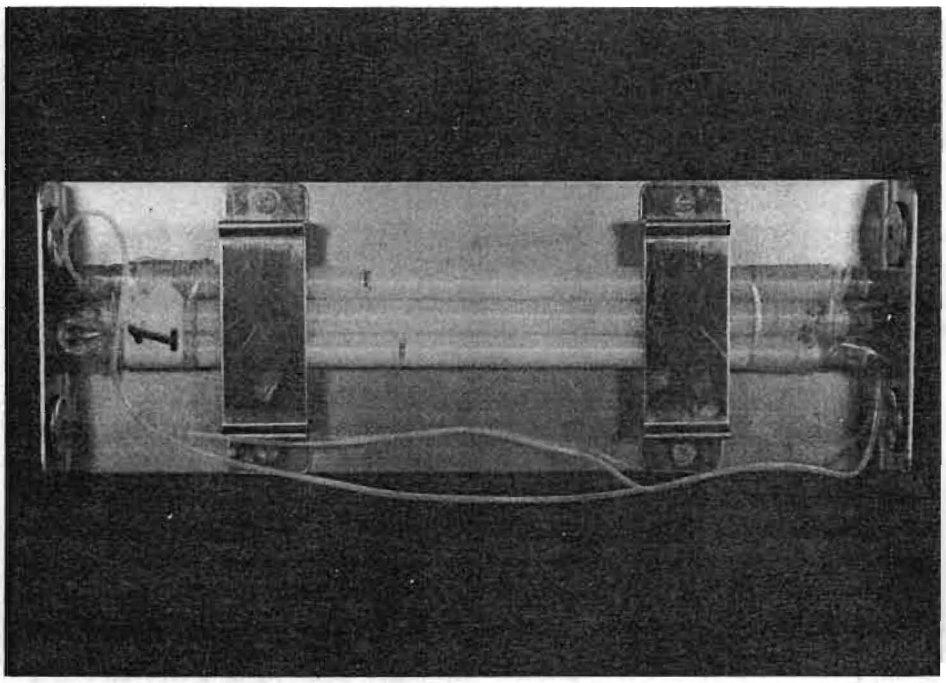

*Fig. 4* Coiled tube and tube holder

the period of loading the sample into the rotating tube system.

### Separating medium

An acid-citrate-dextrose (ACD) solution containing 15 per cent foetal calf serum was used. The ACD solution comprised 2.55 g of dihydrous trisodium citrate, 80 mg of monohydrous citric acid and 1.20 g of anhydrous dextrose in 100 ml of distilled water.

### Preparation of peritoneal and pleural cell suspension

Male albino rats of the Wistar strain weighing 150-200 g were anaesthetised with ether and exsanguinated by decapitation. The abdominal cavity was opened and flooded with 15 ml of ACD solution. After gentle massage for 30 seconds, the fluid containing the peritoneal cells was collected. Additional ACD solution was injected into the pleural cavity, and the thorax was massaged for 30 seconds, the fluid containing pleural cells was collected. Collected suspensions were pooled and centrifuged at 30xg for 10 minutes. The sedimented cells were resuspended in the separating medium at concentrations of $2\text{-}5 \times 10^7$ cells/ml.

### Separation procedure

A 5-6 ml cell suspension was loaded with a syringe into the centrifuge through a three-way cock in the joining portion. Carrier fluid flow was then started. Fractions were collected in polycarbonate tubes every seven minutes. Generally, separation was completed within one hour.

### Mast cell counting and histamine assay

Mast cells were counted before and after separation by the method of Bray and Van Arsdel.[14] Purified mast cells were washed twice and finally suspended with a modified Tyrode solution.[4] This solution was composed of 0.14 M NaCl, 3 mM KCl, 0.6 mM $CaCl_2$, 1 mM $MgCl_2$, 8 mM $Na_2HPO_4$, 3 mM $KH_2PO_4$ and 6 mM dextrose (pH 7.2 to 7.4), and supplemented with 2 per cent heat-inactivated foetal calf serum. This solution was also used as a reaction medium. To measure spontaneous histamine release, $2 \times 10^5$ mast cells in a non-separated cell mixture or the same number of separated pure mast cells were suspended in 2 ml of the medium and incubated for 30 minutes at 37°C followed by centrifugation at 100xg for 5 minutes at 4°C. Then, 1.5 ml of the supernatants were assayed for spontaneous histamine release. Total histamine content was determined on a resuspended sample. For the assay and extraction of histamine, the modified spectro-fluorometric technique of Shore *et al*,[15] (reported upon by Kremzner and Wilson[16]) was em-

ployed. The intensity of fluorescence at 450 nm excited at 360 nm was measured with a spectrofluorometer (Hitachi, Model 650-60).

**Rat mast cell degranulation test**

(a) Subjects: Chronic urticaria patients having a clinical history of one month or more were studied.

(b) Antigens and intracutaneous reactions: Housedust, *Candida* and ragweed antigens (diagnostic extract, made by Torii Pharmaceutical Company, Japan) were intracutaneously injected at the flexer aspect of the patients' forearms. Assessment was made 15 minutes later. Patients presenting erythema reactions > 20 mm or wheal reactions > 9 mm were regarded as positive.

(c) Serum: Serum was taken from the patients and immediately used for examination or kept at -80°C until use.

(d) The rat mast cell degranulation test: Purified mast cells were put into a polycarbonate tube and finally suspended in the reaction medium at a cell concentration of $1.5 \times 10^5$ cells/ml. Following the incubation of 1.5 ml of the mast cell suspension with 0.1 ml of the patient's serum for two hours at 37°C, 0.1 ml of pre-warmed antigens, which had given positive skin reactions, were added and further incubated for 30 minutes at 37°C. The final concentrations of the antigens in the reaction medium were 0.13 μg of total nitrogen/ml (housedust), 0.07 μg of protein nitrogen/ml *(Candida)* and 0.44 μg of total nitrogen/ml (ragweed). Control mast cell suspensions which contained antigen without serum (antigen control) and patient serum without antigen (serum control) were made in the same manner with every test. After centrifugation, the amount of histamine released in the supernatant and which remained in the sediment was measured separately as described under histamine assay. Per cent histamine release was calculated by the following formula:

$$\% \text{ histamine release} = \frac{\text{released histamine}}{\text{released histamine + residual histamine}} \times 100$$

All of the tests were made in duplicate and most experiments were repeated twice.

**Histamine release inhibition by EDTA**

(a) Compound 48/80-induced release: Mast cells ($1.5 \times 10^5$) were incubated with 2 μg/ml of compound 48/80 (Sigma) for 30 minutes at 37°C. The inhibition of histamine release was observed by adding various concentrations of EDTA. The dose-response curve of histamine release due to the addition of compound 48/80 is shown in Figure 5.

(b) Anti-mast cell antiserum-induced release: Anti-mast cell antiserum was obtained from rabbits immunised with purely separated rat mast cells.[17] Together with the antiserum, the serum of a guinea pig as a complement source was added to 1.0 ml of the mast cell suspension ($1.5 \times 10^5$ cells) at a final concentration of 1/90, and incubated for 30 minutes at 37°C. The antiserum was added at a final concentration of 1/30 and the inhibitory action of EDTA on per cent histamine release was examined at various concentrations of EDTA. The dose-response curve of histamine release due to the addition of anti-mast cell antiserum is shown in Figure 6.

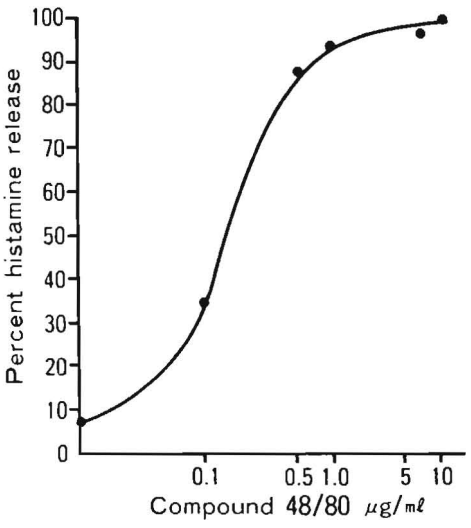


*Fig. 5* Dose-response relationship for histamine release from rat mast cells as a function of compound 48/80 concentration. The mean of the per cent histamine release is indicated for three experiments.

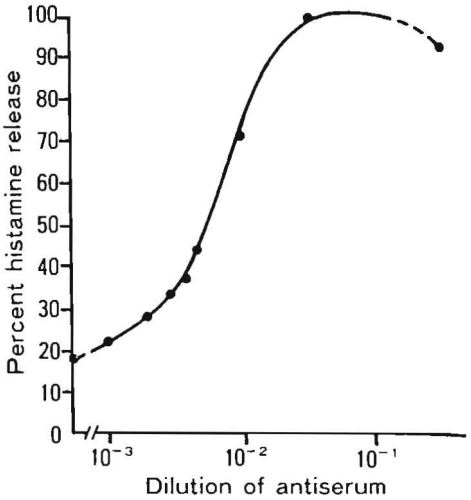


*Fig. 6* Dose-response relationship for histamine release from rat mast cells as a function of anti-mast cell antiserum dilution. The mean of the per cent histamine release is indicated for three experiments.

## RESULTS

In eight experiments, biologically intact mast cells were separated from other peritoneal and pleural cells with high purity (96.9±1.7 per cent) and good yield (40.0±2.8 per cent). The separation of mast cells during centrifugation is shown in Figure 7. The leukocytes excepting mast cells almost completely flowed out after a 47-minute centrifugation ('stop' mark in Fig. 7); mast cells flowed out later. Purely separated mast cells were collected after 'stop' mark. The purified mast cells were spherical, and the cytoplasm was tightly packed with granules (Fig. 8). Purified mast cells contained larger amounts of histamine (3.42±0.26 μg/$10^5$ mast cells) than non-separated mast cells (2.70±0.30 μg/$10^5$ mast cells),

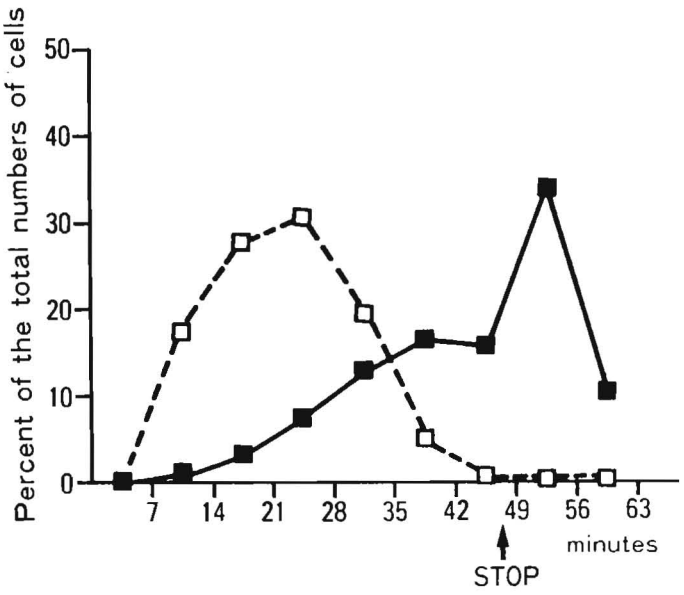


*Fig. 7* Separation of mast cells (■——■) from other leukocytes (□- - - -□). Mast cells which flowed out every seven minutes are expressed as per cent of the total number of respective cells. Flow rate in the coiled tubes was 60 cm/minutes.

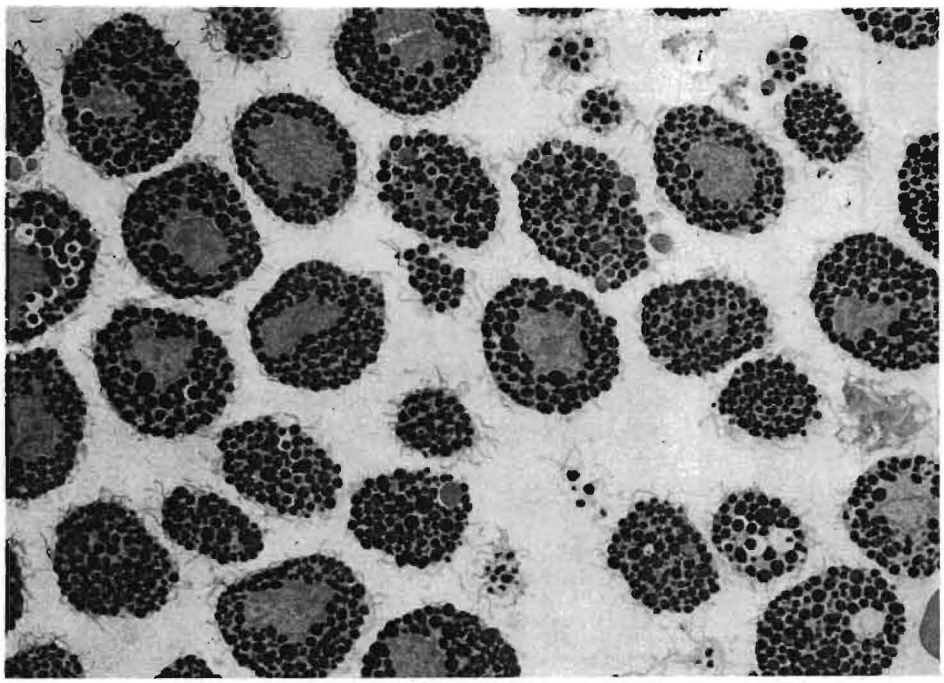

*Fig. 8* Electron microscopic appearance of purified rat mast cells

and the spontaneous release of histamine during the 30-minute incubation at 37°C was very low: 1.33±0.12 per cent. From these results, it is evident that the separation procedure did not have any unfavourable effect on mast cells.

Nineteen experiments involving the rat mast cell degranulation test were performed using purified mast cells (Table 1). In some patients sensitive to housedust, *Candida* or ragweed, a much higher percentage of histamine release was observed in the group to which both sera and antigens were added than in the group to which sera alone were added. However, the group, to which serum from the patient alone was added (serum control of Table 1, column 4), showed a considerably high percentage of histamine release, which is compatible with the results regarding histamine release from mast cells due to the addition of the sera of non-allergic persons alone (Fig. 9). There may be a tendency towards lower histamine release in the sera of non-allergic males, but no statistically significant differences were observed between the group of non-allergic persons and the patients as a whole. Such release of histamine indicates non-specific release of histamine as it shows no antigenic specificity.

The immunological release of histamine from mast cells is classified into two types of reaction.[18] The first is an active secretion, occurring when membrane-bound IgE antibodies react with a specific antigen.[19] The second is the histamine release of the cytotoxic type, in which the release of histamine occurs passively after cell membranes were damaged by immune cytolysis. Although calcium is essential for both types of reaction, there may be a difference regarding its sufficient concentration for the reaction. Compound 48/80 is a well-known histamine releaser,[20,21] and compound 48/80 was used for the model of the anaphylactic type of histamine release. For the model of the cytotoxic type, anti-mast cell antiserum and complement were used. In order to distinguish which type is related to the non-specific histamine release, the inhibitory effects of EDTA in the two types of histamine release were studied.

On the basis of the dose-response curve of compound 48/80 (Fig. 5), 2 μg/ml of the compound 48/80 was employed for the active release of histamine from separated mast cells. It was the minimal concentration for nearly 90 per cent histamine release. According to the

*Table 1* Release of histamine from rat mast cells incubated with sera of patients and challenged with antigen of housedust, *Candida* and ragweed

| Patient No. | Antigen | % histamine release | | |
|---|---|---|---|---|
| | | Antigen control | Serum control | RMCT |
| *1 | housedust | 3.2 | 20.0 | 38.7 |
| *2 | " | " | 50.9 | 69.7 |
| *3 | " | " | 31.2 | 41.2 |
| 4 | " | " | 37.9 | 41.6 |
| 5 | " | " | 70.5 | 77.4 |
| *6 | " | " | 56.4 | 78.8 |
| 7 | " | " | 67.3 | 78.8 |
| 8 | *Candida* | 4.6 | 49.6 | 49.6 |
| 9 | " | " | 62.3 | 67.9 |
| *10 | " | " | 32.8 | 41.3 |
| 11 | " | " | 38.0 | 40.9 |
| 12 | " | " | 45.2 | 46.2 |
| 13 | ragweed | 3.2 | 87.3 | 97.0 |
| 14 | " | " | 31.2 | 35.7 |
| *15 | " | " | 27.9 | 38.6 |
| 16 | " | " | 70.5 | 70.2 |
| 17 | " | " | 62.3 | 70.9 |
| *18 | " | " | 32.8 | 52.5 |
| *19 | " | " | 46.0 | 59.2 |

*In these patients, antigen-specific histamine release was observed. Experimental errors were at most 10 per cent when expressed as the coefficient of variation. RMCT: rat mast cell degranulation test.

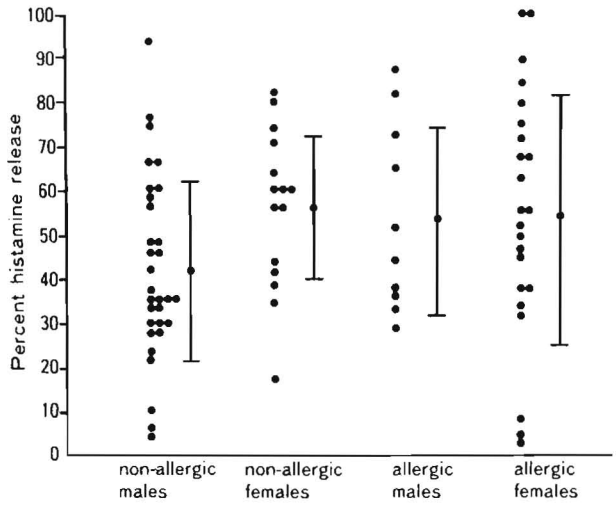


*Fig. 9* Histamine release from rat mast cells by human sera. The mean and standard deviation of the per cent histamine release are shown.

dose-response curve of anti-mast cell antiserum for histamine release (Fig. 6), a final dilution of 1/30 was employed, since this antiserum concentration induced nearly complete damage of mast cells.

The inhibitory effects of EDTA on the histamine release are shown in Figure 10. The release of histamine was inhibited at 0.5 mM of EDTA in the group to which compound 48/80 was added and at 1.5 mM in the group treated with the antiserum.

The release of histamine due to the human sera of non-allergic subjects and patients with urticaria was also inhibited at 1.5 mM of EDTA (Fig. 11). This inhibitory pattern is quite similar to that of the cytotoxic type of release, suggesting that the non-specific histamine release by the addition of human serum may be attributed to the complement system.

It was confirmed in the experiments (Table 2) that nine occurrences of human sera-induced histamine release were markedly suppressed when the complement system was inactivated by heating the serum for 30 minutes at 56°C.

## DISCUSSION

Rat mast cells, separated at 97 per cent purity by using a modified coil planet centrifuge with a common physiological buffer solution, are shown to maintain the same morphological appearance as mast cells before separation. Moreover, chemical examinations indicated that no changes occurred in the mast cells after separation.

The rat mast cell degranulation test has been proposed as a method for measuring antigen-induced histamine release from rat mast cells sensitised with human IgE antibody.[9-13] It may be seen that human IgE can bind to the IgE receptor on the membrane of rat mast cells.[22] Conversely, Kulczycki and Metzger reported that monomeric human IgE has little affinity for the IgE receptor of rat mast cells and, since the reliability of the rat mast cell degranulation test is limited, this technique has found little acceptance.[23]

However, it has been shown that human $IgG_4$ sensitises rat mast cells and causes antigen-specific histamine release (Sonak and Robenek, XI International Congress of Allergology and Clinical Immunology, No. 556, 1982). The relationship of the degranulation of rabbit baso-

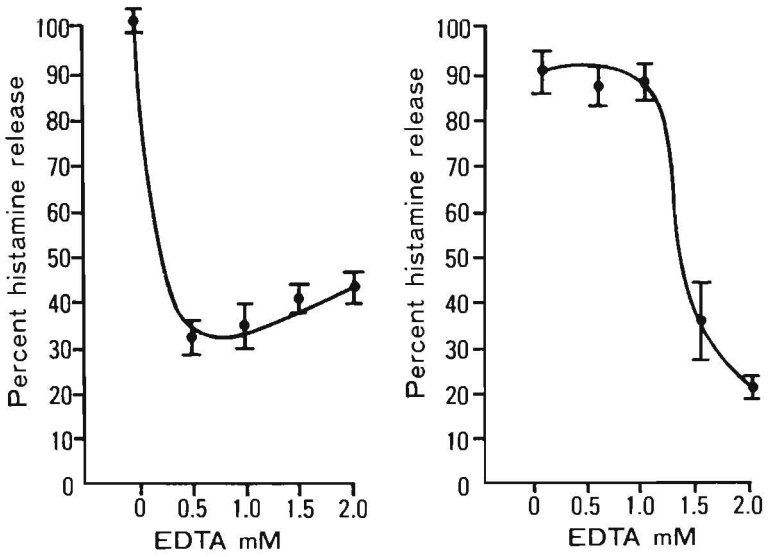


*Fig. 10* Inhibition of histamine release with EDTA (left, compound 48/80-induced release; right, anti-mast cell antiserum-induced release). EDTA was added to rat mast cells just prior to the addition of compound 48/80 or anti-mast cell antiserum. The mean and standard deviation of the per cent histamine release in three repeated experiments are shown.

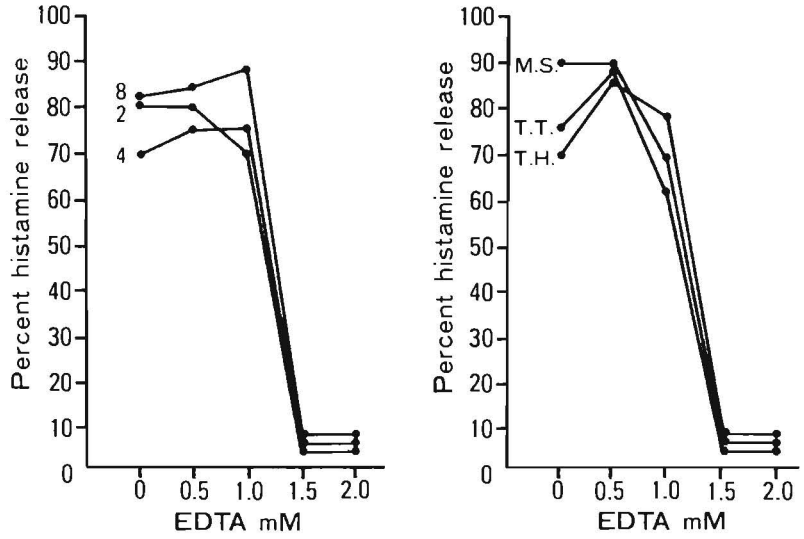


*Fig. 11* Inhibition of human sera-induced histamine release with EDTA (left, patients' sera-induced release – patients' numbers are the same as in Table 2; right, non-allergic persons' sera-induced release).

*Table 2* Non-specific action of human sera and effect of heat inactivation in histamine release

| Patient No. | % histamine release | |
|---|---|---|
| | Serum | Heat-inactivated serum |
| 1 | 88.8 | 5.9 |
| 2 | 79.8 | 4.9 |
| 3 | 30.7 | 4.9 |
| 4 | 68.8 | 5.5 |
| 5 | 35.0 | 5.9 |
| 6 | 60.5 | 6.6 |
| 7 | 31.2 | 9.4 |
| 8 | 82.7 | 11.2 |
| 9 | 83.9 | 18.5 |

% histamine release is expressed as the mean of duplicate experiments.

phil leukocytes to antibodies present in human allergic serum has also been reported.[24] Recently, IgE antibody has been measured by radioimmunoassay (RAST) or enzyme immunoassay (ELISA). IgE antibody characteristically occurs in the blood of allergic patients, but there may exist other constituents related to the pathogenesis of allergy. Thus, it should be emphasised that biological assays such as the rat mast cell degranulation test are still useful, because they can detect not only IgE antibody but also other causative factors present in allergic patients.

We consider that rat mast cells purified by a modified coil planet centrifuge may be more satisfactory for the rat mast cell degranulation test than either unpurified peritoneal cells (which tend to aggregate readily) or density-purified mast cells (which appear to lose IgE receptors[6]). As a result, histamine release with antigenic specificity after the addition of housedust, *Candida* and ragweed was observed in some patients (Table 1). It is assumed that these antigens may relate to the onset of urticaria in these patients.

Sera alone caused marked damage to rat mast cells and this phenomenon, in part, makes the rat mast cell degranulation test unreliable. However, there may be some correlation between the cause of urticaria and the damage to the mast cells induced by sera of patients; therefore, an attempt was made to clarify the non-specific action of human serum on the rat mast cells.

Urticaria appears to result from skin mast cells. Many factors have been known to act on mast cells and cause degranulation. As immunological factors, aggregated IgE antibodies and anaphylatoxins

formed as a result of cleavage of complement components have been well investigated.[25] And as non-immunological factors, physical agents such as mechanical stimuli, and chemical factors such as acetylcholine, endogenous hormone, and also exogenous histamine liberators such as drugs, parasite constituents and bacterial toxins, are known to cause degranulation of mast cells.[8,20] Most of these factors are either the constituents of the serum or are carried to the tissue by way of the blood stream.

When the effects of sera of non-allergic subjects or those of the patients on the rat mast cells were examined, the damage to mast cells caused by the sera of patients was revealed to be more severe than that caused by the sera of non-allergic subjects (Fig. 9). In order to identify the constituents of the sera which induced the non-specific damage of the mast cells, an examination was conducted in order to determine whether the reactions of histamine release are likely to be linked to the anaphylactic type or the cytotoxic type. In the anaphylactic type, the influx of calcium induced by the antigen-antibody reaction is known to trigger the activation of the enzymatic system and granules are secreted.[19] In the cytotoxic type, activation of the complement induced by the formation of the antigen-antibody complex requires calcium. Calcium is essential for both types. The experimental results on the inhibitory effect of EDTA revealed that, since calcium has a different mode of action in the two types of reactions, the required amounts of EDTA differed for both reactions. It may be presumed that the complement system is involved in the non-specific type (Figs. 10,11), since the amount of EDTA needed to suppress the release of histamine and its inhibitory patterns was very similar to the reactions of mast cells treated with anti-mast cell antibody and complement. Furthermore, this presumption is supported by the fact that the reaction was markedly decreased after serum was inactivated by heating (Table 2).

Purified mast cells were shown to be useful for the rat mast cell degranulation test in order to detect the causative factors of urticaria, although further studies may be necessary to clarify how the constitutents of the serum of patients may be involved in the damage of mast cells. The addition of a critical amount of EDTA to suppress the non-specific reaction may facilitate the detection of pathogenic factors.

## ACKNOWLEDGEMENT

The authors wish to thank Professor S. Matsuura for his kind advice throughout this experiment.

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