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Immunologic Aspects of Human Reaginic Allergy: An in vitro Method and some Applications*. By Abraham G. Osler, Lawrence M. Lichtenstein**, and David A. Levy**. With 11 Figures in the Text

Studies of human reaginic allergy have long been hampered by the lack of suitable *in vitro* methods. Initial efforts to fill this gap were undertaken by Katz and Cohen[9] and their findings relative to the release of histamine from human blood by specific antigen have been amply confirmed during the past fifteen years+. More recently, a systematic exploration of this *in vitro* phenomenon has led to the design of a standardized reaction system appropriate for mechanistic studies[12]. This report will summarize experiments based on this procedure and describe

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some of the factors which govern the release of histamine from human leukocytes by ragweed pollen antigen.

A slight digression may be appropriate, however, in order to distinguish between two types of immune events which are associated with cell and tissue injury, the immediate allergic reaction and cytotoxic phenomena. Delayed type reactions involving only interaction between cells will not be considered.

The cytotoxic process is best exemplified by immune hemolysis, a reaction in which antibody reacts with antigens that are part of the erythrocyte membrane. In the presence of the system of serum components called "complement", there ensues the activation of an esterolytic enzyme and the subsequent unleashing of a reaction sequence terminating in cell death[3]. The integrity of the cell membrane is violated, the cell leaks potassium, osmotic relationships with the environment are disrupted, and finally, the macromolecular contents are extruded[7].

For the erythrocyte, this is manifested usually by the release of hemoglobin. Gram negative bacteria, spirochaetes, and mammalian cells, normal or malignant, are also subject to this destructive process in the presence of specific antibody and complement. The cell involved in the process is destroyed, and the resulting tissue injury is not necessarily due to the release of toxic cell constituents.

A variant of this cytotoxic reaction is that of passive hemolysis. Here, an antigen unrelated to the erythrocyte becomes fixed to the cell surface. The interaction of antibody with the cell-fixed antigen in the presence of complement also leads to hemolysis. The cell that is destroyed is an innocent victim of the ongoing immune reaction at its surface, but the consequences are the same as in immune hemolysis.

The second type of reaction is that of immediate hypersensitivity. In this case the union of antigen with antibody may occur *at* or *near* a cell surface* and results in the release of vasoactive compounds like histamine or 5-hydroxytryptamine. As in passive immune hemolysis, the cell is unrelated in an immunologic sense to the specific reactants. The tissue damage which follows is attributable to the release of pharmacologically active compounds from appropriate cell or tissue reservoirs, and not necessarily to injury of the cell containing the vasoactive agents. The participation of the complement system in this process still remains to be established, particulary since the process can proceed in the absence of added serum factors.

* The immune event at a cell surface characterizes the reaction sequence with leukocytes, mast cells, or tissue minces. The release of histamine from rabbit platelets[5] does not require cell-fixed antibody and the formation of anaphylatoxin proceeds entirely in the fluid phase of reaction mixtures.

The experiments now to be described concern the release of histamine from human leukocytes[12]. For these studies, leukocytes are separated from the blood of ragweed sensitive individuals by gravity sedimentation in the presence of dextran and an anticoagulant like EDTA. The cells are washed, resuspended to a convenient volume and mixed with ragweed pollen antigen, a highly purified protein isolated by KING and NORMAN (mol. wt. = ca. 38,000)[10]. The reaction system contains leukocytes suspended in a NaCl-KCl-tris buffer with 0.03 per cent human albumin, appropriate metals as discussed below, and antigen. After incubation at 37°C, the cells are sedimented and the supernatant fluid is assayed for histamine by the spectrophotofluorometric procedure described by SHORE et al.[24].

Factors Influencing the Release of Histamine by Antigen

Nonimmune parameters. The overall reaction proceeds most efficiently at physiologic conditions of pH (optimum = 7.4) and ionic strength (optimum, μ = 0.15). Studies of metal requirements have led to the

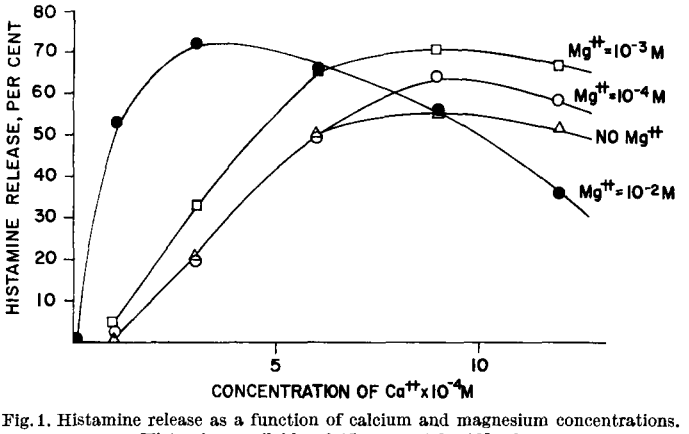

Fig. 1. Histamine release as a function of calcium and magnesium concentrations. Histamine available—0.15 μg per 1.3×10^7 w.b.c.

conclusion that both calcium and magnesium participate in this allergic response. As shown by the data in Fig. 1, histamine release is not initiated until the concentration of calcium exceeds 10^{-4} M, optimal levels for this cation having been localized at about 6 to 9×10^{-4} M. With calcium as the sole divalent cation additive, histamine release rarely exceeds 60 per cent of the cellular reservoir. However, the incorporation of magnesium potentiates the response so that 90 to 100 per cent of the cellular histamine may be recovered in the extracellular fluid. Since magnesium alone barely supports more than 15 per cent of the histamine release, it may be concluded that calcium and magnesium perform independent roles in the transfer of histamine from its intracellular location to the fluid phase of

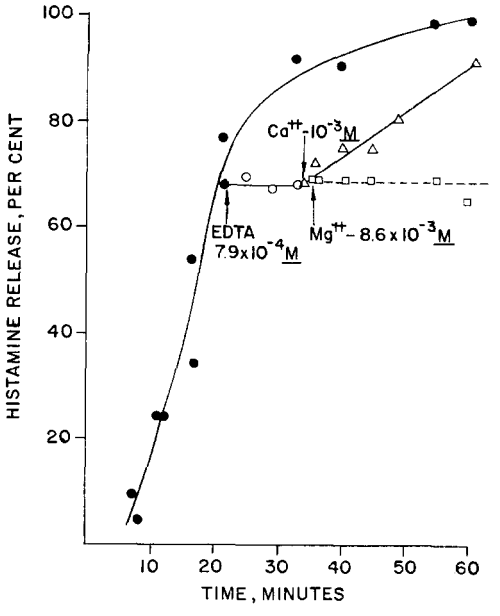

Fig. 2. Inhibition of histamine release from human leukocytes by EDTA: Reversal by calcium but not by magnesium. Ca^{++} in buffer = 6×10^{-4} M Exp. 100763; Cells: BeWi 0.16 μg histamine per 10^7 WBC; IV_2: 0.002 μg per ml

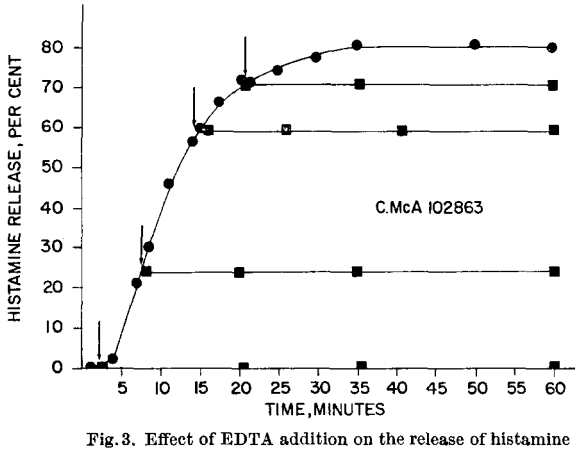

Fig. 3. Effect of EDTA addition on the release of histamine from human leukocytes by ragweed pollen extract (IV_2, 2.5×10^{-3} μg/ml). EDTA (0.02 M final concentration) added at times indicated by arrows. See text for full explanation. No. w.b.c., 1.6×10^7; Histamine available, 0.24 μg

reaction mixtures. The isolation of reaction step intermediates requiring only calcium or magnesium is necessary to provide definitive evidence in this regard.

The individual activities of calcium and magnesium also emerge from experiments of the type depicted in Fig. 2. In this experiment, the reaction was terminated with EDTA but could be restored only with calcium, showing that the inhibitor had not damaged the cells. This finding and the observation that magnesium potentiates the response beyond that achieved with calcium alone, support the inference that the two metals act upon distinct reaction steps in the sequence.

These experiments also confirmed many of the earlier studies which showed that the release of histamine can be terminated by EDTA. It was also observed that EDTA can halt the histamine release process at any time during its course (Fig. 3), thus providing a point of departure between the histamine release reaction and immune hemolysis. In the latter process, EDTA is effective only during the early reaction steps of a limited antibody system and involves those complement components whose activity is divalent cation-dependent, e.g., C'1 and C'2. The later steps which require the intervention of the C'3 components are not metaldependent. Subsequent to the utilization of the known complement components, an irreversible

lesion is produced which leads to the loss of hemoglobin irrespective of the presence of divalent cations[8]. No such terminal phase has yet been detected in the allergic reaction.

As has been noted by others[2,17], histamine release is most efficient at physiologic temperatures (Fig. 4). As the temperature approaches 20°C, the reaction ceases. This finding, coupled with the action of EDTA, provides an efficient and immediate means of arresting the release process at will, thereby facilitating kinetic studies. The data in Fig. 4

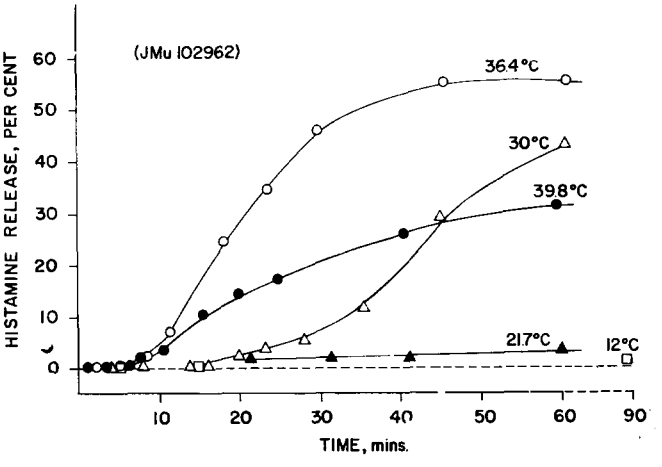

Fig. 4. Time course of histamine release at different temperatures. Ragweed pollen extract (IV_a) = 5×10^{-5} µg per ml. No. w.b.c. = 1.4×10^7. Histamine available = 0.213 µg

also show that the rate and extent of histamine release is curtailed at 39.8°C. To elucidate the meaning of this finding, leukocytes were maintained at 40°C for 30 min and then equilibrated at 37°C before the addition of antigen and further incubation at the latter temperature. Cells treated in this manner were less responsive than another aliquot of the same cells which were not subjected to the elevated temperature. The inference is clear that incubation of the leukocytes at the higher temperature may have damaged the cells with respect to their subsequent capacity to release histamine.

The results of this experiment suggest that the release of histamine via the allergic process requires a physiologically competent cell. Leukocytes examined at the end of the histamine release reaction still exclude vital dyes and retain their motility. These observations, together with the need for divalent cations and a physiologic temperature throughout the reaction course are consistent with the concept that the release of histamine, even in its terminal stages, is not one of simple diffusion through a ruptured membrane. The process seems to require a meta-

bolically active cell which is not necessarily injured after release of its histamine content.

Recent experiments on the effect of two inhibitors of the allergic reaction also support these contentions, The data in Tables 1 and 2

Table 1. *Inhibition of Immune Histamine Release from Human Leukocytes by DFP*

DFP concentration molar $\times 10^{-4}$	Histamine release per cent	Inhibition per cent
None	91	0
0.62	91	0
1.8	74	19
5.5	38	58
18.0	0	100
50.0	0	100

Table 2. *Inhibition of Immune Histamine Release from Human Leukocytes Previously Incubated with DFP*

DFP concentration in preincubation medium molar $\times 10^{-4}$	Histamine release per cent
None	45
2.0	22
10.0	4
50.0	1

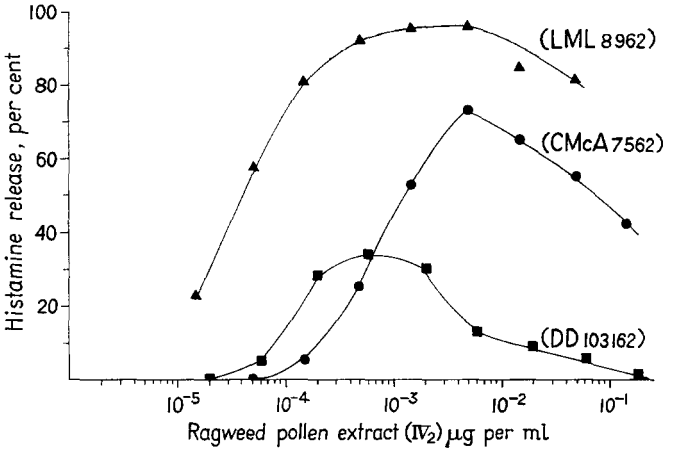

Fig. 5. Histamine release from ragweed sensitive leukocytes obtained from three donors. *LML* 1.8×10^7 w.b.c., histamine available—0.38 µg; *CMcA* 2.2×10^7 w.b.c., histamine available—0.42 µg; *DD* 2.3×10^7 w.b.c., histamine available—0.21 µg

describe experiments relating to the suppressive action of di-isopropyl-fluorophosphate (DFP), an inhibitor of esterase activity. The results leave little doubt that DFP does indeed stop the release of histamine, but its action may be directed towards the cell, rather than towards a subsequent step initiated by antigen[2,17]. Similar findings were obtained with salicylaldoxime, an inhibitor of complement activity[16].

The effect of antigen concentration. The three dose-response curves shown in Fig. 5 illustrate some of the differences encountered with cells from individual donors. The curve describing the release process for cells, LML, is characteristic of most donors in that virtually all the histamine

can be recovered in the cell-free supernatant fluid after reaction with antigen. The cells of some donors (e.g., DD) never release more than a fraction of their available histamine, for reasons which are not entirely clear. The threshold quantities of antigen required to initiate the release process, and the quantity of antigen needed for a maximal response are seen to vary with different cell suspensions. Excess antigen suppresses the reaction. A more extensive quantitative comparison of cellular reactivity may be drawn from the data in Fig. 6 which indicate that cells from different

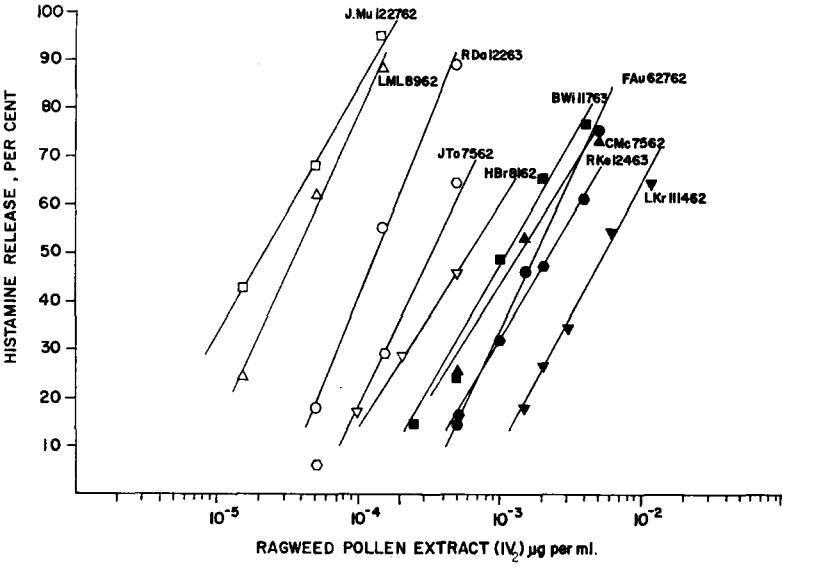

Fig. 6. Dose-response relationships for histamine release as a function of antigen concentration (cells from 10 ragweed sensitive donors)

individuals may vary over more than a 100-fold range in their responsiveness to antigen. This difference may be associated with an increased amount and/or reactivity of the antibody presumed to be on the cells. With some cells, reactions have been noted with as little as 10^{-6} μg of antigenic protein, or slightly more than 10^7 molecules, whose distribution with respect to the equivalent number of leukocytes in the reaction mixture poses an interesting and unsolved problem. It has been reported that about half of the cellular histamine in human blood cells is contained in the basophils which constitute less than one per cent of all leukocytes. The remainder is found in the eosinophilic and neutrophilic polymorphonuclear leukocytes[6]. Since the cells of many donors studied in this laboratory release almost all of their cellular histamine into the fluid phase on contact with antigen, it may be concluded that this allergic event involves cells other than the basophils

Time course studies such as described in Figs. 4 and 7 reveal the existence of a temperature- and antigen-dependent lag phase which precedes the appearance of histamine in the fluid phase. Attempts to reduce this lag phase below detectable levels, even with 100-fold excesses of antigen, have failed. These results, as well as the findings with respect to the divalent cation requirements, permit the deduction that the release of histamine from human leukocytes is a multi-step phenomenon. Additional evidence for this conclusion has been drawn from "desensitization" experiments, described in reference[12].

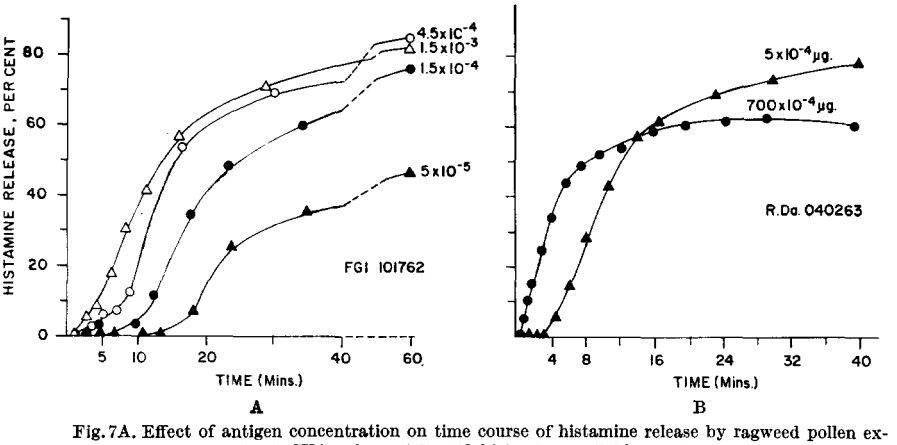

Fig. 7A. Effect of antigen concentration on time course of histamine release by ragweed pollen extract (IV_3). w.b.c.—1.2×10^7, histamine available—0.27 µg

Fig. 7B. Time course of histamine release at optimal and antigen excess (inhibitory) concentrations. Lag period—5×10^{-4} µg ragweed pollen extract (IV_3) per ml, 3.75 min; 700×10^{-4} µg ragweed pollen extract (IV_3) per ml, 0.50 min; w.b.c.—1.6×10^7; Histamine available—0.30 µg

Evidence for the presence of cell-bound antibodies. The design of experiments discussed thus far implies that leukocytes from ragweed sensitive individuals bear the so-called reaginic antibodies. Evidence to validate this assumption has been obtained by several independent procedures.

The first concerns the detection of γ-globulin on the surface of human leukocytes in complement fixation experiments with rabbit antibody to human γ-globulin. Gamma globulin has been identified on cells from allergic donors as well as from cells of individuals who are not sensitive to ragweed pollen. Neither the specificities of these immunoglobulins, nor their electrophoretic behavior have yet been ascertained, but it is entirely reasonable to assume that they will prove to be diversified[20,25].

Preliminary autoradiographic studies suggest that leukocytes from allergic donors bind radio-iodinated ragweed pollen. The binding of the isotopically labeled antigen to these cells can be greatly reduced by the prior addition of an excess of noniodinated antigen[23].

The results of the complement fixation experiments mentioned above simply demonstrate that one or more classes of immunoglobulins are present on the cell surface, without commitment as to their specificity characteristics with respect to the ragweed pollen antigen. The autoradiographic experiments are also limited in failing to indicate whether the cells which bind antigen actually participate in the histamine

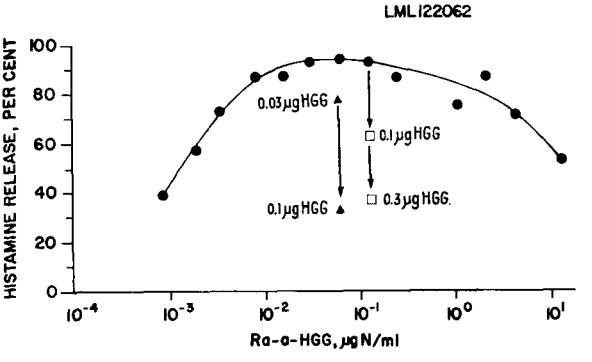

Fig. 8. Histamine release from human w.b.c. by rabbit antihuman gamma globulin; inhibition by human gamma globulin. Histamine available—0.23 μg

Table 3. *Effect of Preincubation of Leukocytes with Ra-a-Hu-γ-Gl on their Subsequent Release of Histamine by Ragweed Extract (IV_2)*

	Rabbit anti-Hu-γ-Gl added—μg N per ml				
	0	0.01	0.1	1.0	10
Ragweed (IV_2) μg per ml × 10^{-5}	Histamine release, per cent				
2	26	14	0	0	0
5	38	21	1	0	0
100	61	46	24	6	1

Preincubation with Ra-a-Hu-γ-Gl—15 min at 4° C. Cfg. at 1200 rpm—12 min at 4° C. Resuspended in buffer with ragweed—60 min at 37° C.

release response. It is also conceivable that a cell can react with but one or a few molecules of antigen and release its store of histamine, yet escape detection by the autoradiographic procedure.

An experiment which diminishes some of these uncertainties is described by the data in Fig. 8 which show that the addition of rabbit antihuman globulin to human leukocytes results in the release of histamine by virtue of its interaction with human γ-globulin on the cell surface. Moreover, the reaction of antibody to human γ-globulin inhibits the release of histamine from these cells by ragweed pollen antigen (Table 3). The latter finding may be explicable in terms of the steric hindrance imposed to the ragweed antigen by the prior immune event[11].

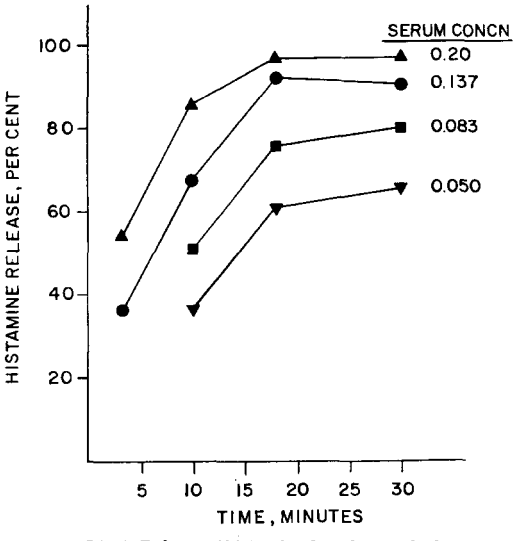

Fig. 9. Release of histamine from human leukocytes sensitized passively *in vitro*: Effect of serum dilution at optimal antigen levels. Exp. 081864; Cells: FrAt; Histamine content: 0.31 μg per 1.2×10^7 w.b.c.; Serum: DiGu 042964 with EDTA 0.004 M; Sensitization at 37° C; Antigen at maximum of 0.045 μg per ml

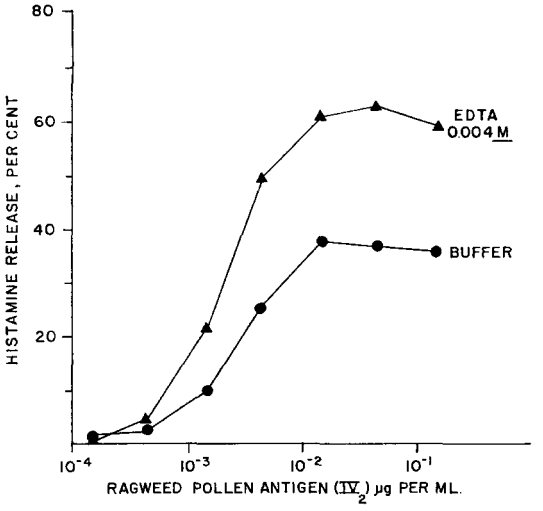

Fig. 10. Release of histamine from human leukocytes sensitized passively *in vitro*: Effect of EDTA during sensitization. Exp 082864; Cells: FrAt; Histamine content: 0.20 μg per 9×10^6 w.b.c.; Serum: FrGl 072464, 1/5 dilution; Sensitization: 60 min at 37° C

Alternatively, the cell may have been "desensitized" as a consequence of the initial reaction between the cell-bound human γ-globulin and its specific rabbit antibody. As a result, the capacity of the leukocyte to release histamine in the subsequent reaction with pollen antigen may have been reduced. However, desensitization due to the previous immune event need not follow inevitably, as was noted in studies of immediate hypersensitivity reactions in the guinea pig small intestine[21]. Notwithstanding these considerations, the date in Fig. 8 confirm the results of the complement fixation studies in the demonstration that human leukocytes carry γ-globulin, even after extensive washing with a serum-free buffer.

Studies of passive sensitization in vitro. Human leukocytes from nonallergic donors may be sensitized passively by the incorporation of reaginic serum to the cell suspension[13,22,29]. This *in vitro* model of the Prausnitz-Küstner reaction appeared suitable for studies of antibody fixation to tissues, a crucial and early event in the mediation of certain types of allergic tissue damage (cf. however[5]). The procedure involves the incubation of leukocytes isolated from the blood of nonallergic individuals with reagin-containing serum

under physiological conditions of pH and ionic strength. After thorough removal of serum constituents from the fluid phase, the addition of pollen antigen leads to the release of histamine. Studies of the cell-antibody interaction have shown that human leukocytes resemble other tissues in that the fixation process is much more efficient at 37°C than at lower temperatures. The rate and extent of this reaction varies directly with serum dilution, thus jus-

Table 4. *Histamine Release from Human Leukocytes Sensitized Passively In Vitro: Effect of Calcium and Magnesium during Sensitization*

Reagents added	Histamine release by ragweed pollen antigen, μg per ml $\times 10^{-3}$	
	4.8	36.0
	per cent	
None	25	41
Sodium EDTA (2×10^{-3} M)	42	58
Sodium EDTA (2×10^{-3} M) + Ca^{2+} (2.5×10^{-3} M)	25	45
Sodium EDTA (2×10^{-3} M) + Mg^{2+} (2.6×10^{-3} M)	40	58

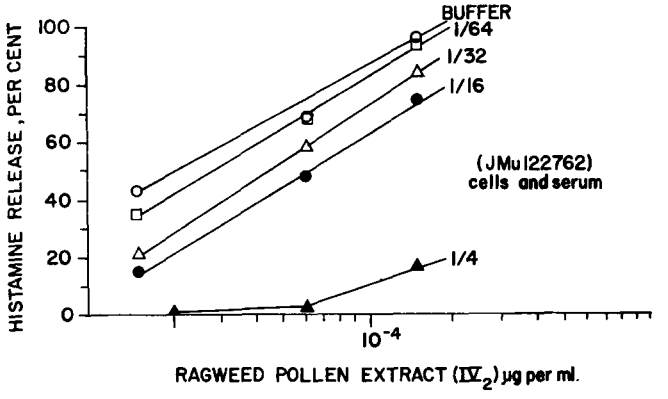

Fig. 11. Inhibition of histamine release by unheated autologous serum. w.b.c.—1.4×10^7, histamine available—0.21 μg

tifying the use of histamine release as an index of the amount of antibody passively bound to the cells (Fig. 9). It was of considerable interest to observe that EDTA enhances the fixation of antibody (Fig. 10), a potentiation which is nullified by calcium, but not by magnesium (Table 4). The level of EDTA necessary for optimal enhancement is approximately that required to bind the serum calcium. The potentiation of fixation by EDTA and the relatively high temperature coefficient of this process suggest that sensitization of the leukocytes cannot be adequately explained in terms of adsorption[4]. Calcium ions seem to play a dual role in the human allergic reaction, impeding antibody fixation to the cell but enhancing the release of histamine by antigen.

The passive sensitization studies have also demonstrated a relatively unique serum-cell relationship in that cells from different individuals vary in their capacity to bind the histamine-releasing antibody. Further studies of this observation may prove fruitful in characterizing the nature of the cell receptor and the mechanism of its interaction with reaginic antibody.

Inhibition of histamine release by reaginic serum antibody. The presence of antibodies in sera of ragweed-sensitive patients, demonstrable by the passive transfer experiments, was also ascertained by studies of inhibition of histamine release. Admixture of reactive cells to serum-antigen mixtures led to a suppression of histamine release. A typical experiment is summarized by the data in Fig. 11. Of interest to a better understanding of the mechanism of the wheal and flare reaction in humans is the finding that the serum of untreated ragweed-sensitive individuals also inhibits the immune release of histamine, a property usually ascribed only to the sera of patients undergoing parenteral immunization.

Summary

Suspensions of human leukocytes have been used for *in vitro* studies of reaginic allergy. These cells when isolated from ragweed sensitive patients, release histamine on reaction with antigen at threshold levels of 10^{-6} μg. The release process is characterized by a lag phase and a requirement for both calcium and magnesium. The divalent metal requirement has been observed throughout the reaction course. Physiologically competent cells are required for the release process, since cells subjected to 40° C, or treated with di-isopropylfluorophosphate show an impaired response. On these grounds, it has been concluded that the release process is a multi-step phenomenon and that the passage of histamine to the extracellular environment cannot be accounted for in terms of diffusion following membrane injury. Human leukocytes carry γ-globulins which can react with specific antibody in complement fixation, or in histamine release experiments.

Leukocytes from nonallergic individuals can be sensitized passively *in vitro*. The fixation of antibody to this cell surface is characterized by a high temperature coefficient and is enhanced by ethylenediaminetetraacetate.

Serum from untreated allergic donors as well as from donors who have received specific pollen therapy, binds antigen in the fluid phase of reaction mixtures, thereby inhibiting the release of histamine from the leukocytes.

Figs. 1—7, as well as Tables 1—3 have been taken from a dissertation to be submitted by Dr. Lawrence M. Lichtenstein in partial fulfillment of the requirements for the Ph. D. degree.

Figs. 1, and 3—7 — Reprinted by permission of The Rockefeller Institute Press, from The Journal of Experimental Medicine, October, 1964, volume 120, No. 4.

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Mechanisms of Passive Sensitization. By J. L. MONGAR. With 8 Figures in the Text

Passive sensitization can be considered to be a fixation of preformed antibody γ-globulin on to histamine-containing mast cells. Sensitization is measured by challenging with the specific antigen. When antigen combines with antibody various enzymes are activated (MONGAR and SCHILD 1962) and, by some mechanism that is not yet understood, the mast cells lose their granules and the granules lose their histamine (and other pharmacologically active agents).

The amount of passive sensitization can therefore be measured either by:

1. Counting the number of mast cells that have lost their granules (HUMPHREY and MOTA 1959).

2. Measuring the amount of histamine that has been released (MONGAR and SCHILD 1960; BROCKLEHURST, HUMPHREY and PERRY 1961).

3. Measuring some effect of the released histamine such as smooth muscle contraction as in the Schultz-Dale reaction (HALPERN et al. 1959; NIELSON et al. 1959), or increase in capillary permeability as in the studies on passive cutaneous anaphylaxis where the amount of dye that escapes from the damaged capillaries is measured (OVARY 1958; BIOZZI et al. 1959).

Although a cellular theory was clearly stated by FRIEDBERGER and HARTOCH as early as 1909 it was Dale's work in 1913 which formed the