STUDIES ON THE MECHANISMS OF HYPERSENSITIVITY PHENOMENA

IX. Histamine Release from Human Leukocytes by Ragweed Pollen Antigen

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This report summarizes a series of experiments directed towards the development of an *in vitro* reaction system for the study of hypersensitivity phenomena of the immediate type. The investigation was initiated with a cell suspension, rather than with intact tissues or tissue fragments, in the thought that the use of dispersed cells might offer several advantages. These would ensue provided certain criteria were fulfilled; *i.e.*, (*a*) the cells bound the immunoglobulins which mediate anaphylaxis, (*b*) the cells served as reservoirs for those compounds whose action characterizes the anaphylactic process, and (*c*) the immunologic release of these pharmacologically active compounds could be instigated under physiologically compatible conditions.

The human leukocyte appeared as a promising candidate for study in view of information already available indicating that the addition of antigen to whole human blood is followed by the release of histamine. The initial demonstration of this phenomenon by Katz and Cohen (1) has been confirmed and extended in the reports of Noah, Middleton, VanArsdel, and Sherman (2–10). The use of human leukocytes, rather than cells from another animal, was considered important in view of the known species variations with respect to the site of storage, nature, quantity, and tissue reactivity to those vasoactive compounds whose release by antigen-antibody interaction marks the anaphylactic process.

With few exceptions, the previous work with human material has utilized

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whole blood wherein the contributions of the cellular and fluid elements are not readily discernible. It was therefore decided to design a reaction system based upon the use of isolated and washed human leukocytes suspended in a relatively simple, serum-free buffer. It was anticipated that the availability of a standardized and well defined model, amenable to experimental manipulation, would facilitate the investigation of the manner in which antigen-antibody union induces the anaphylactic response, particularly as exemplified in reaginic allergy of man. The data which follow characterize several parameters of the reaction and demonstrate that the release of histamine from human leukocytes by specific antigen is a multistep phenomenon.[1]

Materials and Methods

Buffer.—The diluent used in these experiments contains: tris[2], 0.025 M; NaCl, 0.120 M; KCl, 0.005 M; human serum albumin (HSA)[3], 0.3 mg per ml; as well as calcium and magnesium as indicated below. The tris may be used as the base, in which case, the pH is adjusted to 7.6 at 25°C with N/1 HCl. Alternatively, "preset" tris has been used at pH 7.60, 25°C. This diluent, designated "tris-A," lacks added divalent cations and is used for washing the leukocytes. Calcium and magnesium are added to final concentrations of 6×10^{-4} M and 1×10^{-3} M, respectively, from stock solutions standardized by conductance measurements. The pH of the buffer containing these metals (tris-ACM) should be 7.35 ± 0.05 at 37°C, or 7.6 ± 0.05 at 25°C. The osmolarity of the buffer is identical with that of 0.150 M NaCl as measured by the freeze point depression technique.

Glassware.—All glassware which comes into contact with the cells is siliconized in a 5 per cent solution of silicone[4] in *n*-heptane. More recently, transparent polycarbonate tubes[5] have been used successfully. These do not require silicone treatment.

Separation of Leukocytes.—The technique of Lapin *et al.* (13) is used with the following modifications: 100 ml of blood are drawn with a sterile needle attached to teflon tubing into a siliconized centrifuge bottle (250-ml) containing either 10 ml of 0.1 M ethylenediaminetetraacetic acid (EDTA) at pH 7.70, or 5.0 mg of heparin, as anticoagulant, as well as 25 ml of 6 per cent clinical dextran in isotonic saline[6], and 750 mg of glucose. The mixture of blood and additives is kept at ambient temperatures (24–26°C) for 60 to 90 minutes, the time required for the formation of a sharp interface between the erythrocytes and the platelet-, leukocyte-rich supernate. This supernate is aspirated with a siliconized pipette and centrifuged in an International PR-2 centrifuge at 900 RPM (110 *g*), at 4°C for 8 minutes. Most of the platelets remain in the plasma supernate which is discarded. The button of cells is gently resuspended with a siliconized Pasteur pipette, washed twice in tris-A (total volume, 100 ml) and brought to 25.0 ml in tris-ACM buffer. At this stage the ratio of erythrocytes to leukocytes is approximately 2:1.

Ragweed Antigen.—A preparation of purified antigen derived from ragweed was supplied

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[1] Preliminary reports outlining some of these studies have been presented in references 11 and 12.

[2] Trizma 7.60, tris(hydroxymethyl)aminomethane, Sigma Chemical Co., St. Louis, Lot No. 100 B-708-2.

[3] Behringwerke Ag., Marburg-Lahn, Germany.

[4] General Electric Co., Schenectady, New York, SC-87 dri-film.

[5] International Equipment Co., Boston, Cat. No. 1648.

[6] Cutter Laboratories, Berkeley, California.

by Dr. King and Dr. Norman as a lyophilized powder, prepared as in reference 14. The antigen (IV_2) is considered to be a protein with a molecular weight of about 37,800 (14 *a*). The relationship of this antigen to others in the initial ragweed extract will be dealt with in a subsequent report. This reagent is stored in tris-ACM buffer at −70°C, at concentrations of 100 μg and 2.0 μg per ml. The activity of these frozen preparations has remained constant during the 2 year period of this study.

Reaction Mixture.—The reaction is carried out in siliconized or polycarbonate tubes (100 x 16 mm) in a volume of 4.0 ml which includes 1.0 ml of cells, 1.0 ml of the appropriate dilution of antigen, and 2.0 ml of buffer or other reagents as required.

Histamine Assay.—The spectrophotofluorometric technique of Shore *et al.* (15), as modified by Kremzner and Wilson (16), was used with slight alterations. The reaction mixture supernates were deproteinized with perchloric acid, extracted into alkalinized butanol, and then reextracted from heptane-butanol into 0.1 N HCl. The acid phase was removed and the histamine conjugated with *o*-phthaldialdehyde[7] to form a fluorophore. Two modifications were introduced. In view of the instability of histamine in alkaline solutions, the extraction of the test sample into butanol was modified so as to add the sodium hydroxide as the last reagent in this step. The tubes were then shaken immediately. In this manner, significant and unpredictable losses of histamine were avoided. Formation of the fluorophore was carried out for 40 minutes at 4°C rather than for 4 minutes at room temperature. This led to greater specific fluorescence and a longer period of fluorophore stability. Under these conditions the recovery of histamine exceeded 90 per cent.[8]

Reference Histamine Solutions.—Histamine dihydrochloride[9] is diluted in 0.1 M HCl to a concentration of 10 μg per ml and stored at −20°C. A calibration curve was included in each experiment. All histamine values are reported as micrograms of the dihydrochloride.

Cell Viability Studies.—Eosin Y has been used to estimate cell viability as in references 17 and 18. Cells are diluted either 10- or 20-fold in eosin Y buffer (0.05 per cent), mixed in a white cell pipette, and counted in a hemocytometer. When ragweed-treated cells from allergic or non-allergic donors are incubated for 60 minutes at 37°C, nuclear staining has never exceeded the value of approximately 2 per cent.

Cell Number and Histamine Content.—The number of leukocytes used in these experiments is generally $1.5 \pm 0.3 \times 10^7$ in a reaction volume of 4.0 ml. The histamine content of the cells from any single donor has remained fairly constant over the course of these experiments in agreement with Rorsman's observation (19) that the basophil counts of individuals tend to remain constant. The histamine content does, however, vary greatly from one individual to another. The cellular histamine content of most donors ranges between 0.15 and 0.30 μg per 1.5×10^7 cells, although values of less than 0.1 μg have occasionally been encountered. At the other extreme, the cells of one individual contained 0.8 μg of histamine.

The studies of Graham *et al.* (20) reveal the virtual absence of histamine in human plasma, platelets, erythrocytes, or lymphocytes. Approximately half of the total amount is found in the basophils, the remainder being distributed among the eosinophils and neutrophils. Since 90 to 100 per cent of the total cellular histamine is released in many of the experiments reported below, the inference is drawn that a major proportion of the leukocytes (the basophils, eosinophils, and neutrophils) participates in the reaction under study.

Calculations.—Due to the variation in the histamine content of a constant number of cells derived from different donors, many of the results obtained in this study are plotted in terms of per cent histamine released. However, the absolute quantity of histamine released in each

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[7] CAL BIO CHEM, c-grade.

[8] A detailed description of the technique used in this laboratory is available upon request.

[9] Fisher Scientific Co., Lot No. 700829.

tube may be readily calculated from the value given for the total histamine content of the cell preparation in use.

An example of this calculation is taken from a typical protocol detailed in Table I which also summarizes the basic experimental design. The values obtained in the first 5 tubes indi-

TABLE I

The Release of Histamine by Increasing Quantities of Ragweed Pollen Antigen (IV_2) from Leukocytes of a Ragweed-Sensitive Donor (R.Da.—012263)

Tube No.	Human leukocytes*	Ragweed extract (IV_2), 1.0 ml	Tris-ACM buffer	Relative fluorescent intensity (RFI)‡	Specific RFI (less cell blank§)	Histamine released‖	Histamine released¶
	ml	*μg protein*	*ml*			*per cent*	*μg*
1	1.0	2×10^{-4}	2.0	0.048	0.034	18	0.07
2	1.0	6×10^{-4}	2.0	0.120	0.106	55	0.21
3	1.0	2×10^{-3}	2.0	0.185	0.171	89	0.35
4	1.0	2×10^{-2}	2.0	0.180	0.166	86	0.34
5	1.0	2×10^{-1}	2.0	0.140	0.126	65	0.25
6	1.0	None	3.0	0.014	—		—
7	1.0	None	3.0	0.207**	0.193		0.39
8	None	None	4.0	0.012	—		—

After incubation for 60 minutes at 37°C all tubes, save the "complete" (tube 7), are centrifuged and the cell-free supernate is extracted and analyzed for histamine.

* Washed and resuspended in tris-ACM, 1.5×10^7 leukocytes per ml.

‡ Readings taken in an aminco-bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Maryland), quartz cuvettes, 1.0 cm light path, activation 350 mμ; fluorescence 450 mμ.

§ Fluorescence contributed by the reagent blank (tube 8) plus the spontaneous release from cells incubated for 60 minutes at 37°C. The sum of these two values is given by the reading in tube 6.

‖ $\dfrac{\text{RFI of (sample less cell blank)}}{\text{RFI of ("complete" less cell blank)}} \times 100$

¶ The volume relationships during extraction lead to a dilution (2.23) which, when multiplied by the volume of the sample in the cuvette (2.7 ml), gives a correction factor of 6.02. The calculation of histamine in each tube is based on a standard sample of histamine extracted the same day, as follows:

$$\frac{\text{RFI of sample less cell blank} \times 6.02}{\text{RFI of standard solution per } \mu\text{g of histamine } (HCl)_2}$$

** This value (the complete) represents the total cellular histamine extractable by perchloric acid.

cate the extent of histamine release resulting from incubation of washed leukocytes with the several recorded levels of ragweed extract. In tube 6, the cells are incubated without ragweed and the release observed constitutes the cell blank which also includes the fluorescence contributed by the reagents, as seen in tube 8. The value for the total extractable histamine in the cells (the "complete") is supplied by the readings obtained from tube 7, usually assayed in duplicate. To obtain this value, an aliquot of the cell suspension in tube 7 is removed *prior* to centrifugation, and added directly to perchloric acid. In every other tube the histamine

is extracted from the supernate obtained *after* centrifugation in order to estimate the release of histamine from the cells into the fluid phase. The spontaneous (non-specific) release of histamine by the cells is reflected by the difference in readings between the cell blank (tube 6) and the reagent blank (tube 8). In this case it is 1 per cent of the total histamine. The calculations in the following text refer to the specific release as follows:

The per cent histamine release $= \frac{E - CB}{C - CB} \times 100$ where E is the histamine released in a given tube under the experimental condition in question, C is the complete (tube 7), and CB is the cell blank (tube 6). The complete in the protocol under discussion contained 0.39 μg of histamine $(HCl)_2$ as derived from the reference calibration curve. The quantity of histamine $(HCl)_2$ released in any tube is therefore obtained from the product (per cent release × 0.39 μg).

EXPERIMENTAL RESULTS

Parameters of the Immune Histamine Release Process.—

Divalent cation requirements: The data in Fig. 1 describe the effect of calcium and of magnesium in the release of histamine from human leukocytes by ragweed pollen antigen. Histamine is not detected in the fluid phase of reaction mixtures until added calcium levels exceed 1×10^{-4} M (open triangles, Fig. 1). The release process is augmented with further increments of this cation, attaining maximal values at calcium levels of 6 to 9×10^{-4} M. With still higher concentrations of calcium, histamine release is diminished so that at 6×10^{-3} M only 30 per cent of the maximal values are found. Calcium may be replaced by strontium although levels 2- to 4-fold higher are required for optimal activity. With strontium, also, inhibition occurs with supraoptimal levels. Barium is completely ineffectual in mediating this response, while in the

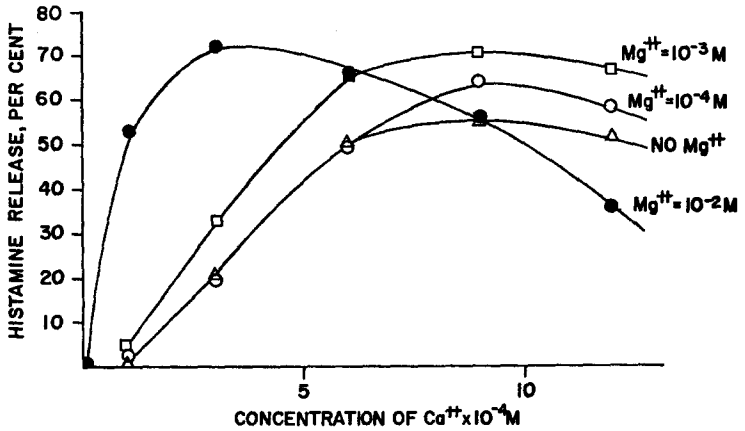

FIG. 1. Histamine release as a function of calcium and magnesium concentrations. Histamine available, 0.15 μg per 1.3×10^7 w.b.c.

presence of manganese or magnesium the response is limited to about 5 and 15 per cent, respectively.

Although magnesium alone shows but slight activity, it does enhance the maximal release mediated by calcium. Fig. 1 illustrates the synergistic relationship between the two metals. Referring to the histamine release obtained with each cation alone, it is seen that neither calcium at 1×10^{-4} M nor magnesium at 1×10^{-2} M is effective. However, when both metals are present, at the above concentrations, the response to antigen exceeds 50 per cent. With calcium as the sole cation, the amount of histamine detected in the cell-free supernate is rarely greater than 50 per cent. With both calcium and magnesium the response

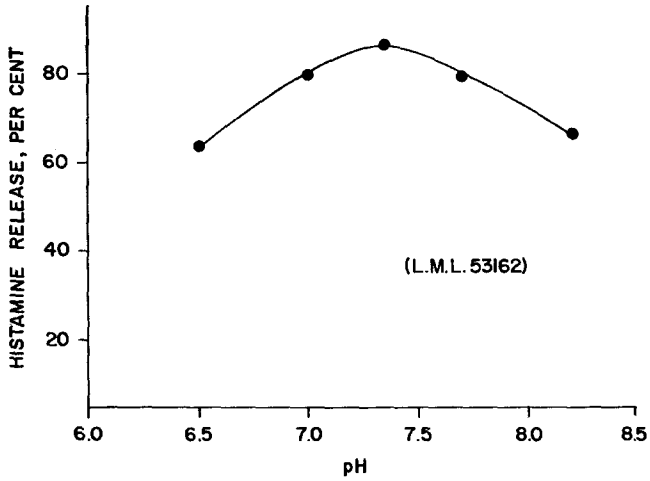

FIG. 2. Effect of variation in pH on histamine release from human leukocytes by ragweed pollen extract. Ragweed pollen extract (IV_2), 1.5×10^{-4} μg/ml; No. w.b.c., 1.3×10^7; histamine available, 0.430 μg.

usually exceeds 80 per cent, often attaining values of 100 per cent. Within the range of concentrations specified in Fig. 1, calcium and magnesium are mutually substitutive. The requirement for calcium as an essential element in the anaphylactic reaction has long been recognized, but the role of magnesium has not heretofore been clearly demonstrated (21–23). As a result of these findings, the buffer used for the present studies contains 6×10^{-4} M calcium and 1×10^{-3} M magnesium, unless otherwise specified.

pH: The release of histamine is readily influenced by changes in pH of the reaction medium. The data in Fig. 2 summarize an experiment in which the pH of the reaction medium was varied by the addition of HCl or NaOH. The ionic strength was kept constant through compensatory changes in NaCl concentrations. Optimal pH values for histamine release fall between 7.3 and 7.4 with a significant diminution on either side of these values.

Ionic strength: The sensitivity of the histamine release process to changes in ionic strength is shown in Fig. 3. Optimal values are found in the range μ = 0.14 to 0.16. Studies of the influence of this variable on histamine release, using other *in vitro* systems, have yielded conflicting data (23).

Temperature: The immune release of histamine is temperature-dependent, as shown by the time course studies depicted in Fig. 4. The response is optimal at about 37°C and almost inapparent below 22°C. No response occurs at 20°C or below. The values for the individual points which describe these time course experiments were obtained by stopping the reactions through chilling, the addition of EDTA, and centrifugation as described at length below. The major

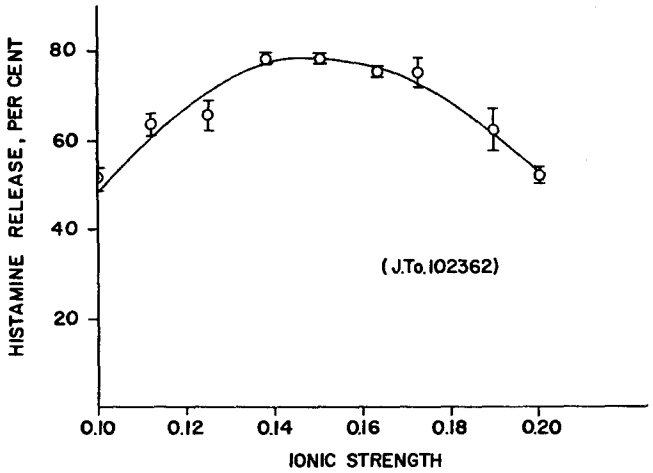

FIG. 3. Effect of ionic strength on histamine release from human leukocytes. Ragweed pollen extract (IV_2), 5×10^{-4} μg/ml; No. w.b.c., 2.4×10^7; histamine available, 0.438 μg.

difference between the kinetics of the reaction at 30°C and at 36.4°C may be found in a considerable extension of the lag period at the lower temperature. This indicates a high temperature coefficient for some reaction step during the lag phase.

The diminished response at 39.8°C (closed circles, Fig. 4) is attributable to thermal alteration of the cells (*cf.* reference 24), as demonstrated in the following experiment. Cells were prewarmed at 40°C for 20 minutes without antigen, equilibrated at 37°C, and then admixed with antigen for an additional 60 minutes at 37°C. This treatment led to a release process similar in time to that shown in Fig. 4 at 39.8°C, the rate and extent of the reaction being diminished as compared to cells maintained at 36.4°C.

For the experimental conditions described at 36.4°C (Fig. 4), the reaction is essentially complete in 40 minutes, a finding confirmed by similar experiments

with the cells of numerous donors. Not infrequently, however, the response continues beyond the 40 minute period, albeit at a much slower rate. Unless otherwise noted, the experiments have been terminated at 60 minutes.

Non-specific protein concentration: As indicated above, purified HSA is a constituent of the buffer at a concentration of 0.03 per cent. The choice of this protein and its concentration stems from the following considerations. The presence of HSA or other macromolecules in the reaction medium is required to support the viability of the cells during the extended time period including several cycles of centrifugation and incubation at 37°C. Concentrations of HSA in excess of 0.1 per cent have been found to inhibit histamine release.

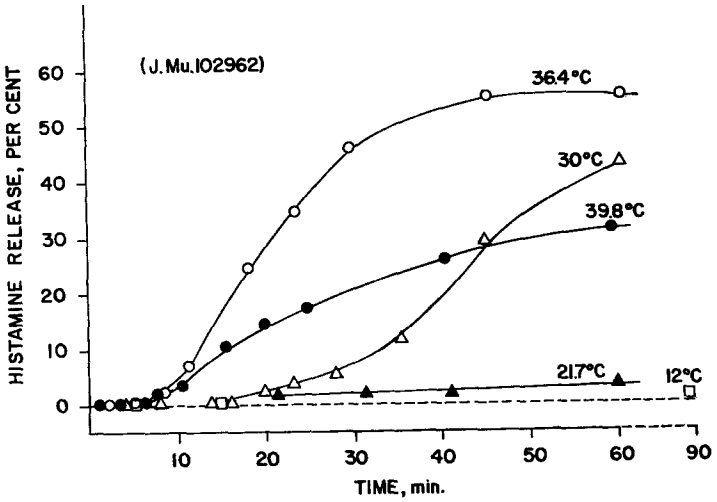

FIG. 4. Time course of histamine release at different temperatures. Ragweed pollen extract (IV_2), 5×10^{-5} μg/ml; No. w.b.c., 1.4×10^7; histamine available, 0.213 μg.

Thus, at 0.5 per cent of HSA, the release process is suppressed about 50 per cent. Other purified macromolecules are also inhibitory, although the quantitative relationships vary. In contrast to HSA, serum, even when undiluted, exerts no inhibitory effect. Highly reproducible results and good maintenance of cell viability can be achieved with the buffer described above. Furthermore, the use of this diluent is not attended by some of the complications observed when serum is added to the reaction mixture, a matter which will be discussed in a forthcoming report.

The Influence of Cell and Antigen Concentration.—The effects of antigen concentration and cell number were explored in the following experiments in a reaction system which incorporated the optimal conditions described above.

The effect of ragweed concentration: The data in Fig. 5 detail three dose-response curves, each with the cells of a different allergic donor. The cells of

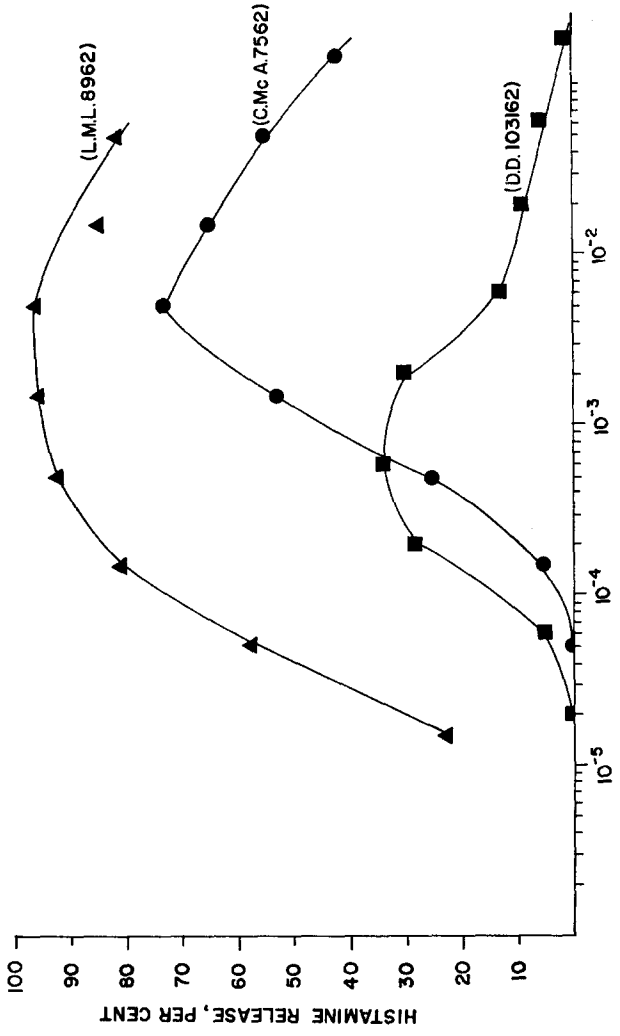

FIG. 5. Histamine release from ragweed-sensitive leukocytes obtained from three donors. L.M.L., 1.8×10^7 w.b.c.; histamine available, 0.38 μg. C.McA., 2.2×10^7 w.b.c.; histamine available, 0.42 μg. D.D., 2.3×10^7 w.b.c.; histamine available, 0.21 μg.

most donors studied thus far yield dose-response relationships similar to that shown for L.M.L. in Fig. 5, releasing 80 to 100 per cent of the available histamine under optimal conditions. In a minority of cases, as with the cells of D.D., the response does not exceed 30 to 50 per cent. Since it is not yet clear whether this diminished response is attributable to a deficiency of cell-bound antibody or to other factors, cells yielding this limited response to antigen have not been used in the experiments reported below.

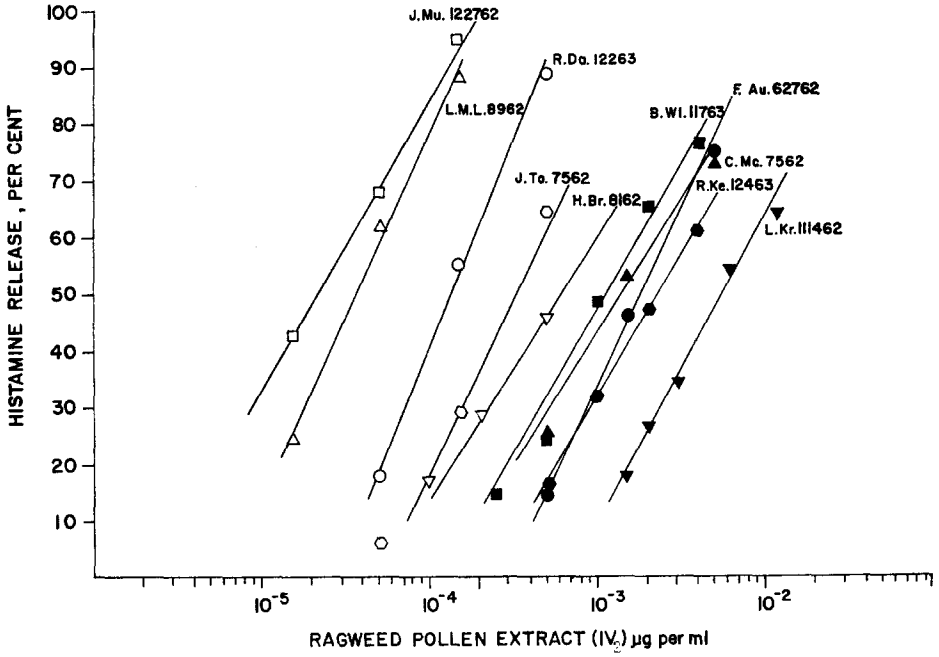

FIG. 6. Dose-response relationships for histamine release as a function of antigen concentration (cells from 10 ragweed-sensitive donors).

Fig. 6 consists of selected portions taken from the dose-response curves of 10 sensitive individuals. The graph has been constructed in this fashion to demonstrate that the log dose-response relationship is linear over a range of about 20 to 80 per cent. The slopes of these linear portions, though laterally displaced, tend to be similar. Inhibition occurs as the ragweed concentration is increased above that required for maximal release, a phenomenon observed in other anaphylactic-type reactions (6, 25–28).

The sensitivity of cell populations from different individuals to ragweed is seen to vary, in Fig. 6, over more than a 100-fold range in terms of the quantity of antigen required to release 50 per cent of the available histamine. At this writing, studies have been conducted with cell suspensions from about

200 individuals. When histamine release was observed, the amount of antigen required for a 50 per cent response generally fell between 10^{-3} and 10^{-4} μg per ml, although the range extends from 10^{-2} to 5×10^{-6} μg per ml. In contrast, cells from the remaining individuals showed no perceptible histamine release at ragweed levels up to 0.5 to 10 μg per ml, the highest levels that have been used. The present data do not bear on the diagnostic usefulness of the reaction, since only those subjects have been selected for study who claimed complete freedom from ragweed allergy or who manifested the type of seasonal symptomatology associated with ragweed sensitivity.

In contrast to the great range of individual sensitivity noted above, the cells from a single individual yield uniform results when tested on repeated occasions. Data of the type shown in Fig. 6 permit the following simple calculations. With some cell populations, perceptible histamine release is noted with 10^{-6} μg of antigen per ml. This quantity of antigen (mol. wt., 37,800) is equivalent to 3×10^{-17} moles, or about 2×10^{7} molecules. Since the number of leukocytes in the reaction mixture is of the same order of magnitude, it might be concluded that histamine release is mediated by only a few molecules of antigen per cell, assuming that all the leukocytes participate at the same level of efficiency in this process. This question has been further explored in the following experiments.

The effect of cell number: The quantity of histamine released by a constant but limiting amount of antigen (sufficient to release 20 to 30 per cent of the histamine in 1.5×10^{7} leukocytes) is not altered when the cell number is varied over a 3- to 4-fold range, as shown by the data summarized in Fig. 7, line *A*. When the level of antigen, though still limiting, is raised to yield 50 to 60 per cent release with 1.5×10^{7} cells, the effect of increasing the cell concentration differs from that described above (*cf.* Fig. 7, line *B*). Under these conditions the absolute response increases as the cell number rises, but at a decreasing rate. Finally, when the amount of antigen in the reaction mixture suffices for a maximal response (Fig. 7, line *C*), the release is directly proportional to the cell number. Extension of this experimental design to higher cell populations is impractical because the leukocytes tend to become agglutinated.

It should be noted that in the experiments described by the data in Fig. 7, line *A*, antigen, though limited in terms of the cell response, is still available in the fluid phase of the reaction mixtures. Numerous attempts have been made to demonstrate the physical uptake of antigen by the leukocytes. In every instance, supernatant fluids obtained after incubation of ragweed antigen with allergic or non-allergic donor cells, showed no loss of antigenic activity when assayed with fresh aliquots of cells. However, in view of the log dose-response relationships, differences in release due to 10 to 20 per cent uptake of antigen might not be detected. A meaningful interpretation of these data cannot be offered until more precise information is available with respect to the

following: (*a*) the avidity of the antigen-antibody union, (*b*) the possibility that a single antigen molecule may act upon more than one cell, and (*c*) the contribution of each cell type to the total response.

Kinetic Studies.—The following experiments were designed to characterize the kinetic aspects of the reaction.

Effect of EDTA: In view of the divalent cation requirements for the immune histamine release process, it was anticipated that chelating agents, such as EDTA, could be useful in kinetic experiments as a means of terminating the reaction at will. The data in Fig. 8 demonstrate the capacity of EDTA to

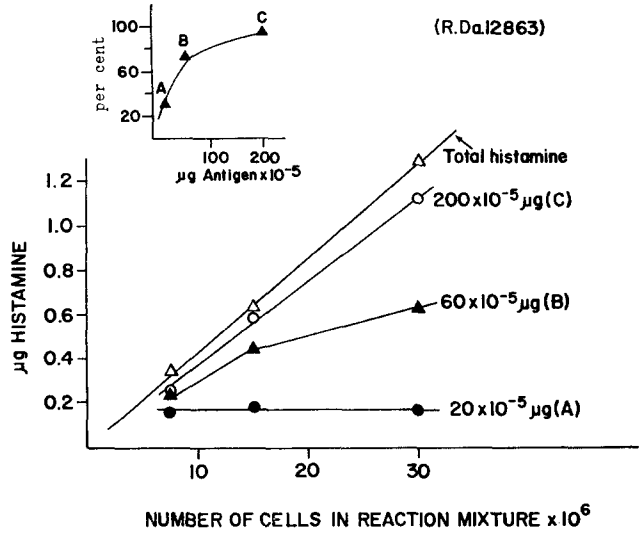

FIG. 7. Histamine release as a function of cell number at three different antigen levels (see inset). Histamine available, 0.63 μg/1.5 $\times$ 10^7 w.b.c.

prevent the release of histamine if added during the lag phase, as was also noted in reference 8. Further, the release process can also be stopped by EDTA at any time throughout its course. For this experiment a suspension of leukocytes (in a volume of 275 ml) containing 0.4 $\times$ 10^7 cells per ml was brought to 37°C. The reaction was initiated in this flask (parent flask) by the addition of ragweed antigen to a final concentration of 2.5 $\times$ 10^{-3} μg per ml. At various intervals, aliquots of 4.0 ml were removed from the parent flask and added to tubes containing 1.0 ml of cold 0.1 M EDTA. The contents of each tube were immediately mixed and centrifuged in the cold. Analyses of the supernates yielded the points which describe the reference time course of histamine release under these conditions (circles, Fig. 8). At designated intervals following the antigen addition (shown by the arrows in Fig. 8 at 2.5, 7, 14, and 20 minutes),

50.0 ml aliquots were removed from the parent reaction flask into subsidiary flasks containing 12.5 ml of 0.1 M EDTA. The incubation of these flasks was continued at 37°C, and aliquots of 5.0 ml, in duplicate, were removed at various intervals during the next hour, chilled, and immediately centrifuged. The assay of these supernates provided the points plotted as squares in Fig. 8. It can be seen that the release process was abruptly terminated in each subsidiary flask immediately upon chelation of the divalent cations, and that no further release of histamine occurred within the following 60 minutes of incubation at 37°C. That EDTA does not otherwise interfere with the reaction has been

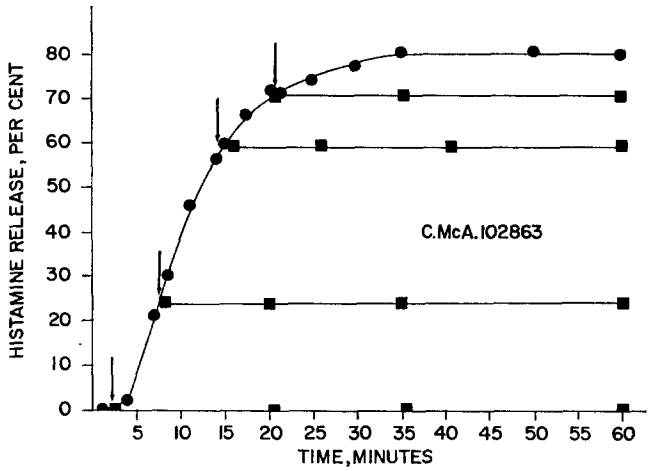

FIG. 8. Effect of EDTA addition on the release of histamine from human leukocytes by ragweed pollen extract (IV_2, 2.5×10^{-3} μg/ml). EDTA (0.02 M final concentration) added at times indicated by arrows. See text for full explanation. No. w.b.c., 1.6×10^7; histamine available, 0.24 μg.

demonstrated by the addition of an amount of EDTA equimolar to that of the calcium in the buffer. Histamine release ceased until calcium was restored to the reaction medium, at which point there was a resumption of the release process, *cf.* also reference 25.

Experiments were carried out to determine whether the reaction could be supported only by magnesium, once it had been initiated in the presence of both calcium and magnesium. For this purpose, a flask was prepared with cells and antigen in a buffer containing 1.0×10^{-4} M calcium and 1.0×10^{-2} M magnesium, (*cf.* Fig. 1). At various intervals after the addition of antigen, aliquots were removed from this flask to subsidiary flasks containing sufficient EDTA to yield a final concentration of 2.0×10^{-4} M. Since EDTA binds divalent cations stoichiometrically, and has a much higher affinity for calcium

than for magnesium, this treatment could be expected to bind essentially all the calcium, leaving the magnesium virtually free and presumably available to the reactants. The reaction ceased immediately upon addition of the EDTA, indicating that magnesium alone is incapable of mediating histamine release even when it has been initiated in the presence of both metals.

Effect of chilling: The data in Fig. 9 summarize an experiment designed to show that sudden chilling stops the release of histamine as effectively as does EDTA, both of which were used for comparative purposes. In this instance, aliquots were removed at designated time intervals from a parent flask prepared as described in the previous section and incubated at 37°C. These aliquots were

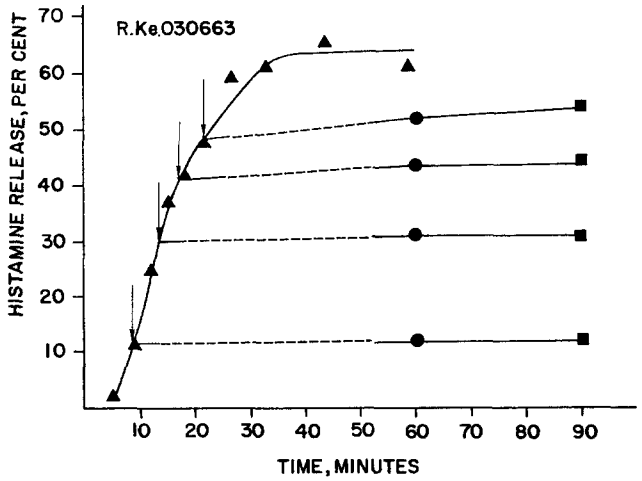

FIG. 9. Histamine release from ragweed-sensitive cells at 4°C after prior incubation at 37°C for varying periods of time. ▲, cells chilled in EDTA and centrifuged; ●, cells chilled at times indicated by arrows; incubated for 60 minutes at 4°C; then EDTA added and centrifuged. ■, same as above except incubated for 90 minutes at 4°C.

treated in one of three ways: (*a*) Samples of 4.0 ml were withdrawn at intervals, added to 1.0 ml of chilled 0.1 M EDTA, and centrifuged in the cold. The assay results of these supernates are plotted as the solid triangles in Fig. 9 and describe the reference time course of the release process. (*b*) Samples of 4.0 ml (in duplicate) were withdrawn at the points in time shown by the arrows in Fig. 9, and placed immediately in a 4°C bath. The tubes were kept at this temperature for 60 minutes after which 1.0 ml of 0.1 M EDTA was added (to equalize extraction conditions and volumes) and the tubes centrifuged. The assays of these supernates yielded the points plotted as closed circles. (*c*) Samples of 4.0 ml were removed at the same time as in (*b*) above and were handled identically except that they remained at 4°C for 90 minutes (closed squares). It is clear that the release of histamine was abruptly terminated after removal to the cold bath and showed no increase during the 90 minutes of continued cold incubation.

This finding suggests that the release of histamine from an intracellular store is a continuous, energy-requiring process. Were the release process to follow a pattern of simple diffusion due to membrane alteration and concomitant permeability increase, it might be expected that chilling from 37° to 4°C would diminish the rate of histamine appearance in the supernate by a factor of $\frac{33}{310}$, the absolute temperature differential. This is manifestly not the case, since the increment of histamine release following equilibration at 4°C was only 2 to 3 per cent, an increase within the experimental error of the assay and which would be expected to occur during the 2 to 3 minutes required for temperature equilibration.

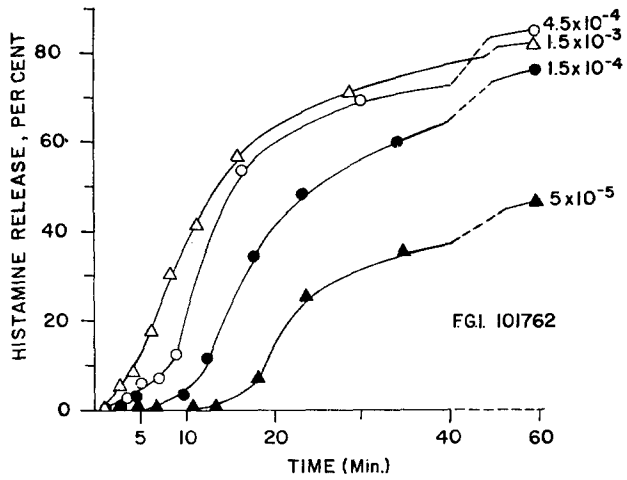

FIG. 10. Effect of antigen concentration on time course of histamine release by ragweed pollen extract (IV_2). w.b.c., 1.2×10^7; histamine available, 0.27 μg.

The effects of EDTA and of chilling are consistent with the thought that there is no period during the course of histamine release when the reaction has reached an irreversible stage such that it will progress in the absence of cations or at low temperature. This process may therefore be contrasted with the cytotoxic type of reaction which involves an initial cell lesion, osmotic imbalance, and the continued leakage of cell contents despite lowered reaction temperatures or the presence of chelating agents (29). The logical inference may therefore be drawn that histamine release is not necessarily a manifestation of injury as regards the cell engaged in the release process.

Effect of antigen concentration: Inspection of the data in Figs. 4, 8, and 9 indicates that an appreciable lag period is apparent before detectable levels of histamine appear in the cell-free supernates of reaction mixtures. The results obtained in a typical kinetic experiment with increasing quantities of antigen are summarized in Fig. 10. It is apparent that the reaction curves which describe the time course of histamine release show an initial lag, a period of

maximal velocity, and finally, a marked decrease in rate. As the concentration of antigen is increased, the lag period is shortened. This point is further emphasized by the experiment summarized in Fig. 11 designed to compare the lag period associated with a reaction initiated at optimal antigen concentration with that observed in marked antigen excess. Even with 140 times the optimal level, a concentration sufficient to depress the end point by 25 per cent, the lag period remains, although reduced to 0.5 minutes. The persistence of this latent period, even at inhibitory concentrations of antigen, suggests the occurrence of one or more events subsequent to antigen-antibody union but prior to histamine release.

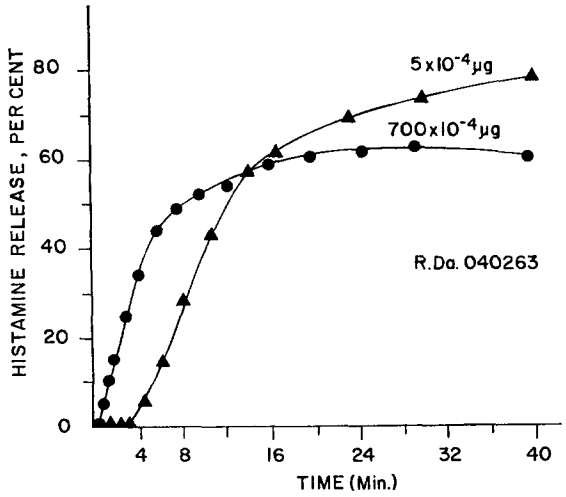

FIG. 11. Time course of histamine release at optimal and antigen excess (inhibitory) concentrations. Lag period: 5×10^{-4} µg ragweed pollen extract (IV_2) per ml, 3.75 minutes. 700×10^{-4} µg ragweed pollen extract (IV_2) per ml, 0.50 minutes. w.b.c., 1.6×10^7; histamine available, 0.30 µg.

Further Evidence for the Multistep Nature of the Histamine Release Reaction: "Desensitization".—The inability to abolish the lag phase with 100-fold excesses of antigen motivated a series of other experiments designed to establish the multistep nature of the allergic histamine release reaction. These experiments were based on the premise that if conditions for the reaction were arranged so as to be optimal in all parameters except one, the release process might be initiated but would proceed only until it reached the step requiring the missing reagent. The verification of this premise as described in the following types of experiments provides additional evidence for a multistep process.

The first experiment is based on the observation that histamine release does not occur in the absence of divalent cations, and indicates the existence of reaction(s) prior to the metal-requiring steps. Two flasks were prepared, each

containing a cell suspension with the same number of leukocytes from a single donor, in buffer lacking divalent cations. The cells were prewarmed to 37°C. To the first flask, antigen, also prewarmed at 37°C, was added in amounts which would lead to maximal histamine release were the reaction mixture optimal with respect to cation concentration. Both flasks were kept at 37°C for 40 minutes, and no release was apparent in either flask during this period. After this interval (at zero time) the first flask received a charge of calcium and magnesium at optimal levels. The second, or control flask, received, *at the same time*, both the cations and the same amount of antigen delivered to the experimental flask 40 minutes earlier. The cells preincubated with the antigen in the cation-free buffer released histamine more slowly, and to a markedly lesser extent (14 per cent in 36 minutes) than did those in the control flask (88 per cent in 36 minutes). Apparently, the cells were "desensitized"; *i.e.*, they were no longer fully responsive when all conditions were eventually adjusted to optimal levels (24). A more detailed representation of the rate and extent of inhibition is given by the data in Fig. 12, lines *A* and *C*. This experiment duplicates the one just described except that the control flask was maintained at 20°C during the 40 minute preincubation period for reasons which are discussed below. The results obtained in the two experiments are essentially similar.

A second type of experiment is based on the observation that histamine release does not occur below 20°C. Two flasks were prepared containing cells from a single donor. The cells were suspended in the usual buffer, optimal with respect to calcium and magnesium. Antigen was added to one (line *B*, Fig. 12,) and an equal volume of buffer to the other (line *A*, Fig. 12). Both antigen and buffer were at 20°C, and the flasks were maintained at this temperature for 40 minutes. No histamine release was detected in either flask during this period. At the end of this interval (at zero time), the control flask (line *A*) received the same concentration of antigen added to the other flask 40 minutes earlier, and the latter received an equal volume of buffer. Both of these additions were at 37°C, and the flasks were immediately removed to a 37°C bath. The subsequent time course of histamine release in each flask was measured. The results (Fig. 12, lines *A* and *B*) show that the cells preincubated with antigen at 20°C release significantly less histamine than the control cells. This observation indicates, again, that there must have been reactions occurring at 20°C which, though not leading to histamine release, rendered the cells less than fully reactive.

In the experiment depicted in Fig. 12 two types of desensitization were carried out, low temperature in flask B, and divalent cation deficit in flask C. After the 40 minute preincubation period equal aliquots of cells from flasks B and C were mixed, an appropriate amount of buffer added, and the histamine release from this mixed population of cells was recorded. This was done to

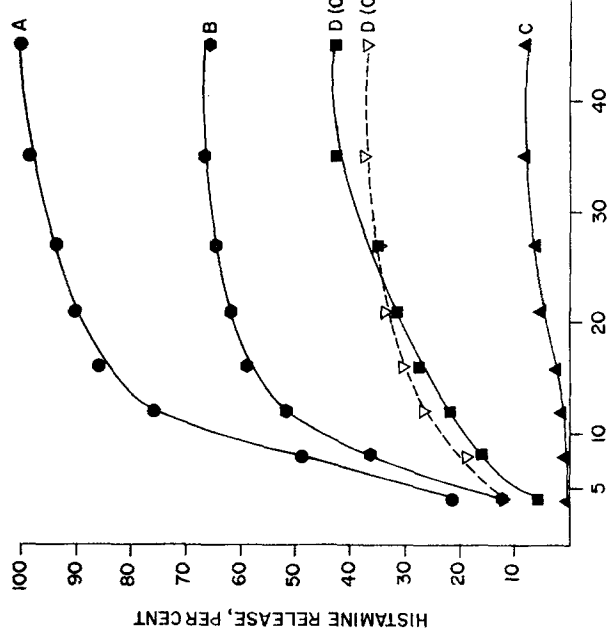

FIG. 12. Curve *A*, Cells preincubated in buffer for 40 minutes at 20°C before addition of ragweed to a final concentration of 0.01 μg per ml and incubation at 37°C. Curve *B*, Cells preincubated with ragweed (0.02 μg per ml) for 40 minutes at 20°C before adjustment of ragweed concentration to 0.01 μg per ml and incubation at 37°C. Curve *C*, Cells preincubated with ragweed (0.02 μg per ml) for 40 minutes at 37°C without added Ca^{++} and Mg^{++}, before addition of these cations at zero time, adjustment of ragweed concentration to 0.01 μg per ml and subsequent incubation at 37°C. Curve *D* (observed), Equal volumes of cells from flasks B and C were mixed, the ragweed concentration adjusted to 0.01 μg per ml and then incubated at 37°C. Curve *D* (calculated), Arithmetic means of data from curves *B* and *C*.

check the possibility that each population of cells had been affected by the pretreatment at different steps in the sequence and could complement one another, thereby yielding more release than would be expected from averaging the results obtained with each population. That this was not the case may be seen in Fig. 12, line *D*, in which the observed release is identical with the arithmetic mean of values in flasks B and C. It must be concluded therefore that either the cells were both affected at the same step or, more likely, that each cell operates independently of all others, thereby precluding complementation.

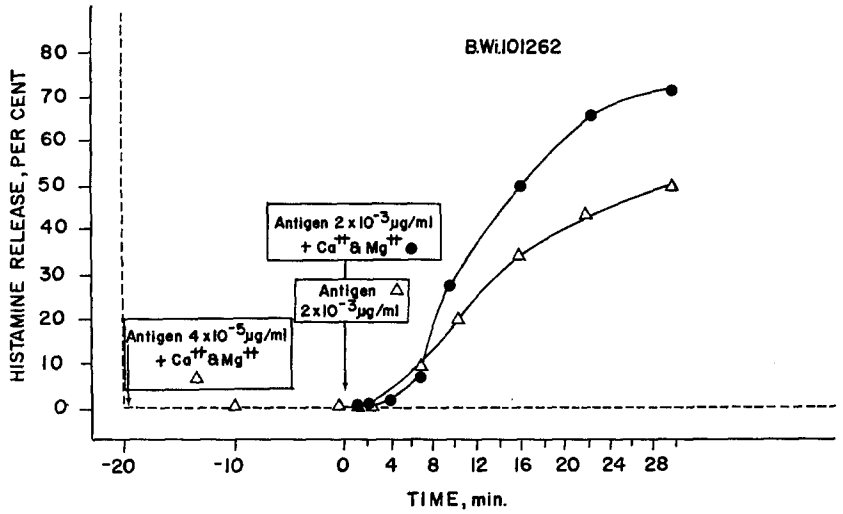

FIG. 13. Effect of preincubation of ragweed-sensitive cells with subthreshold quantities of antigen (IV_2) in the presence of divalent cations at 37°C. w.b.c., 1.2×10^7; histamine available, 0.22 µg.

In a third type of experiment, desensitization was achieved in yet another fashion (Fig. 13). In this instance, cells in one flask were preincubated at 37°C in the presence of optimal cations and a subthreshold amount of ragweed (open triangles, Fig. 13). After 20 minutes, optimal amounts of ragweed were added to this flask, as well as to a control flask containing cells incubated for the same time in buffer and cations alone (closed circles, Fig. 13). Reference to this figure shows that here, as in the previous experiments (Fig. 12), less histamine was released by the pretreated cells, the pretreatment in this case being exposure to subthreshold antigen levels.

In each of these experiments, the antigen-antibody reactions were initiated under conditions which did not lead to histamine release. Even when optimal conditions were restored, a diminished response was noted. This suggests that

the pretreatments described above altered the formation or activity of some essential intermediates in the over-all process.

DISCUSSION

The results of this study indicate the availability of an *in vitro* procedure for the study of human reaginic allergy as one example of immediate hypersensitivity phenomena. This procedure fulfills the criteria initially established for the utilization of a suspension of washed cells bearing the anaphylactic antibody as well as a store of histamine whose release can be readily effected by minute levels of specific antigen. Against the advantages inherent in the use of isolated cells and a serum-free medium must be adduced the possibility of non-physiologic alterations of the leukocytes due to repeated washing and resuspension in the tris buffer. That these alterations have not been too traumatic, however, may be judged from the data in Figs. 1 to 4 which show that the release of histamine by antigen is mediated under physiologic conditions of pH, ionic strength, and temperature.

A striking feature of this reaction system is its sensitivity. Cells of some allergic donors release detectable quantities of histamine following interaction with as little as 10^{-6} μg of antigen (3×10^{-17} moles). The maximal amount of antibody capable of combining with this quantity of antigen is about 2.5×10^{-5} μg, assuming 160,000 for the molecular weight of the antibody and a molecular composition in extreme antibody excess of Ab_5Ag. The validity of these calculations is contingent upon the demonstration that antigen is not cycling from cell to cell. If this is not the case, the minimal quantity of antibody reacting under these circumstances may be still lower in view of the observation that the major portion of antigen in the reaction mixture may be recovered from the fluid phase.

While the cells of most donors release 50 per cent of their histamine on challenge with ragweed antigen in concentrations of 5×10^{-5} to 2×10^{-3} μg per ml, the extremes extend from 5×10^{-6} to 1×10^{-2} μg per ml. There has been no attempt to correlate this variation in cell sensitivity to ragweed antigen with any clinical parameter. This relationship is now under study. However, it may be anticipated that this wide range of cell sensitivity reflects a similar range in the content or reactivity of the antibody on the cells.

There is considerable evidence to support the concept that the release of histamine is an active process resulting from stimulation of living cells by specific antigen, rather than a passive process, such as diffusion, occuring after cell injury. Thus, the process can be abruptly terminated at any point in its time course by lowering the temperature of the reaction medium (Fig. 9), or by removal of divalent cations through chelation with EDTA (Fig. 8). Further, cessation of cellular motility has not been observed following treatment of sensitive cells with ragweed, nor has the uptake of supravital dye been detected

under these conditions. Finally, the work of other investigators clearly suggests that serious alterations in cellular metabolism, as by oxygen deprivation or interference with major enzyme systems, depress the ability of the cells to respond to antigenic stimulation as judged by the release of histamine (reviewed in references 22 and 23).

The release of histamine from leukocytes differs from immune hemolysis, in that, unlike the latter, it lacks a terminal stage which proceeds at low temperatures and which cannot be halted by the addition of EDTA (29). The system under study also stands in marked contrast to that of immune hemolysis in that the release of histamine is a cellular phenomenon which proceeds in the absence of serum components in the fluid phase.

Several lines of experimentation are offered which demonstrate that antigenically induced histamine release is a multistep phenomenon. Thus, the incorporation of magnesium augments the response that can be attained with calcium as the sole additive. The latter cation alone can initiate the reaction and, in fact, yields responses of about 50 to 60 per cent. However, since calcium may displace magnesium bound in the leukocyte, it cannot be concluded that calcium participates in an earlier reaction step than does magnesium. The conclusion that each cation mediates an individual event is, however, entirely tenable.

A second line of evidence is derived from the findings summarized in Figs. 8 to 13 which show that a lag phase precedes the extracellular appearance of histamine. The duration of the latent period is influenced by the reaction temperature (Fig. 4) and antigen concentration (Figs. 10 and 11), but is demonstrable even in the presence of concentrations of antigen several orders of magnitude greater than the optimal level.

The inhibition which results from pretreatment of the cells with subthreshold levels of antigen (Fig. 13), with antigen in the absence of divalent cations, or with antigen at temperatures of 20°C or less (Fig. 12), further demonstrates the existence of reaction steps which precede the appearance of histamine in the fluid phase. These processes have not yet been characterized, but it may be inferred that some of the individual reaction steps in the sequence are enzymatically mediated, as suggested by the narrow limits of pH, temperature, ionic strength, and cation concentrations within which optimal histamine release occurs.

Furthermore, since it has been demonstrated that the histamine flux which occurs as a result of cellular interaction with antigen does not proceed by simple diffusion, the observation that 10^4 to 10^6 molecules of histamine are released for each molecule of added antigen would also seem to imply the mediation of an entity with a rapid turnover number. Indeed, one of the central questions posed in all studies of anaphylaxis relates to the identification of the enzymes brought into action by the immune event. These must be clearly differentiated

from those enzyme systems involved in the maintenance of cellular homeostasis, thereby merely providing cells which are physiologically primed for the histamine release reaction. The demonstration that the release process is multistep may provide an opportunity to clarify this problem. Isolation and study of the individual reaction steps in this sequence should allow definition of those enzymes which are immediately concerned with the translocation of histamine from the intracellular granules into the extracellular fluid.

SUMMARY

Human leukocytes, isolated from the blood of ragweed-sensitive donors, release histamine upon reaction with a purified protein antigen derived from this pollen. The release process has been studied with washed cells suspended in a defined, serum-free medium. Physiologic levels of pH, ionic strength, and temperature, as well as both calcium and magnesium, are required for optimal cellular reactivity. The level of cellular sensitivity of approximately 200 ragweed-sensitive donors has been ascertained, and the kinetics of the release process studied. The rate of histamine release is a function of antigen concentration, but even with a large excess of this reagent it is impossible to abolish a lag phase. Chelation of the divalent cations or a decrease in the reaction temperature may be utilized to stop the reaction. These measures are effective both before and after the initiation of histamine release. Diminished cellular reactivity (desensitization) has been achieved by several procedures. These have in common the addition of antigen to cells in an environment deficient in but a single respect, followed by a restoration of optimal conditions.

The significance of these data has been discussed and it has been proposed that immunologically induced histamine release is an active, enzymatically mediated process which occurs as a multistep response of viable cells to a specific antigenic stimulus.

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