METHOD OF MEASURING HISTAMINE RELEASE FROM MAST CELLS

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Related U.S. Application Data

Continuation-in-part of Ser. No. 156,076, June 23, 1971, abandoned.

U.S. Cl. ...................... 424/8; 195/1.7; 424/3; 424/12; 424/91

Int. Cl. .................. G01n 21/38; G01n 31/22; G01n 33/16

Field of Search .......... 424/3, 8, 12, 91; 195/1.7

References Cited


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ABSTRACT

The histamine released by mast cells on contact with an allergen can be determined by measuring the uptake by the mast cells of a fluorescent acridone, which uptake is proportional to the amount of histamine released by action of the antigen. Fluorescence of the acridone absorbed by the mast cells is measured in conventional manner and the amount of histamine released is determined thereby.

7 Claims, No Drawings
METHOD OF MEASURING HISTAMINE RELEASE FROM MAST CELLS

This application is a continuation-in-part of copending application Ser. No. 156,076 filed June 23, 1971, now abandoned.

BACKGROUND OF THE INVENTION

The usual method of determining the sensitivity of humans to specific allergens is a long, tedious and expensive process which involves intradermal injection of various suspected allergens and, after a waiting period, observing the response to the injection. The extent of the wheal and flare which follows the injection of an allergen to which the patient is sensitive is then evaluated by visual inspection by an allergist. Because of the very many different allergens to which the patient may be sensitive, it is often necessary for him to make additional visits to the allergist's office for further tests for a complete diagnosis. While the procedure is usually painful and obnoxious to the patient, it is, on rare occasions, extremely dangerous in that anaphylactic shock and death have followed such skin testing procedures in the case of highly allergic individuals.

It has been established that hypersensitivity to specific allergens resides in a special class of immunoglobulins, called IgE, which have the characteristic of fixing avidly to mast cells which are concentrated in the skin, lung and nasal mucosa. The tissue fixed antibody, also referred to as reagin, in combination with the specific allergen to which the patient may be sensitive, causes the release of histamine and other allergy mediators, which is responsible for the condition which the patient experiences when being subjected to an allergic reaction. It has also been established that human reaginic sera which contains IgE circulating antibodies will activate mast cells from rats in vitro and when these sensitized cells are contacted with suitable antigens, histamine is released.

The quantitative determination of the amount of histamine in human serum is a long and involved process. Samples of the serum must be diluted, centrifuged, and the serum assayed by the technique of Shore et al., J. Pharmacol. and Exp. Therap., 1959, 127, 182, or a suitable modification thereof. This procedure involves a deproteinization with perchloric acid, repeated extractions with suitable solvents, and conjugation of the histamine with o-phthalaldehyde to form a fluorophore. The histamine determination is then made by determining the degree of fluorescence of the preparation.

THE INVENTION

The present invention is based upon my discovery that fluorescent aridones are strongly absorbed by intact mast cells from which histamine has been released under the influence of an antigen. The amount of an aridone that is absorbed is directly proportional to the amount of histamine that has been released by the mast cells. Thus, by measuring the amount of the fluorescent aridone that is absorbed by the mast cells, it is possible to quantitatively determine the amount of histamine that has been released by the same cells as a result of their contact with an allergen.

The process of determining the sensitivity of an individual to specific allergens in accordance with the present invention can be carried out by a general practitioner, a nurse or a laboratory technician. It does not require the services of a highly trained allergist. It is not necessary that the patient remain in the doctor's office while a reaction to the allergen is taking place and observations made of the reaction. It is simply necessary that the patient present himself at a convenient time for withdrawal of a small amount of blood on which the various tests may be made. The determination of allergy by the process of the present invention requires less than one hour and can be carried out in a doctor's office or hospital prior to administering drugs which might cause a severe allergic reaction in the patient, such as, for example, penicillin. Many tests for different allergens can be made simultaneously with the small amount of blood that has been donated by the patient.

The cost of the procedure is relatively low, as is the equipment for conducting the tests. The results may be observed on a qualitative or quantitative basis, whereby particularly dangerous allergens to the individual patient may be quickly determined.

SUMMARY OF THE INVENTION

In brief, the invention comprises the steps of obtaining a sample of whole blood from the patient, permitting the blood to clot, and removing the serum, which may be stored in a refrigerator until ready for use. Meanwhile, a preparation of intact mast cells in a buffered saline solution is prepared. These mast cells may be from various sources, the most convenient being obtained from the abdominal cavity of mice, or rats. Intact mast cells frozen in glycerol can be used as a source of supply in practicing the invention. A 1-ml aliquot of the mast cell suspension containing from 100,000 to 1,000,000 mast cells is pipetted into a test tube and a definite amount, conveniently one drop, that is, about 0.05 ml of the patient's serum is added to each tube of the suspended mast cells and the preparation is allowed to incubate, preferably at 37°C, for about 3 minutes. A drop or about 0.05 ml of an allergen solution, many of which are commercially available, is added to the test tube containing the mast cells and the patient's serum and the tube is incubated for an additional 3 minutes. 1 ml of a dilute solution of an aridone is then added to the tube, thoroughly mixed and incubated for 10 to 20 minutes. The tube is then centrifuged at 200 g's for 5 minutes. The fluorescence of the liquid in the tube is then measured with suitable apparatus and compared with that of the control. The degree of fluorescence due to the aridone absorbed by the mast cells indicates the degree of sensitivity of the patient to the particular antigen used in the test. Obviously, many different allergens can be tested at the same time in individual tubes containing the mast cells, the patient's serum, the allergen and the fluorescent aridone.

DESCRIPTION OF A PREFERRED EMBODIMENT

Mast cells were obtained as follows: Male rats (200-400 grams) of the Sprague-Dawley strain were anesthetized with ether and exsanguinated following decapitation. Mast cells were obtained by lavage of the peritoneal and pleural cavities with 8-10 ml of buffered salt solution (pH 6.5-7.0). The standard medium employed consisted of: 150 mM NaCl, 2.7 Mm KCl, 0.9 mM CaCl₂, 3 mM NaH₂PO₄, 3.5 mM KH₂PO₄, 5.6 mM dextrose and 0.1% human serum albumin (fraction V, Nutritional Biochemicals).

The mast cells were isolated from other serous cavity cells by centrifugation into 35% Ficoll solutions by a

The number of mast cells in each preparation was estimated by the method of Bray and Van Arsdale, Proc. Soc. Expt. Biol. Med. 106: 255, 1951, i.e., aliquots of cell suspension were diluted with toluidine blue (0.1%) in 0.9% NaCl in a white blood cell-counting pipette and the stained cells were counted in a hemocytometer.

Most cells suitable for practicing the process of the present invention may be obtained from other sources. A more plentiful supply of the cells may be obtained from the peritoneal cavity of mice which have been infected with a mastocytoma, as described by Dunn et al. J. Natl. Cancer Inst. 18, 587, 1957. The large quantities of mast cells which are grown intraperitoneally may be harvested and stored indefinitely by freezing in glycerol. When required, these cells can be thawed and reconstituted to the appropriate cell concentration for practicing the invention. Mast cells from still other sources, including the hamster, may also be used. As noted above, the mast cells are used at a concentration of about 100,000 to 1,000,000 mast cells per test. The larger number of mast cells used per test will give more definitive results. These cells are usually suspended in about 1 ml. of a buffered isotonic salt solution containing calcium, sodium and other cations at a pH of 6.8 to 7.2. A suitable buffered isotonic salt solution is the well-known Hanks solution.

Only a small amount of the human serum is sufficient to activate the mast cells. For convenience, one drop of serum is used. The amount of serum may range from about 0.05 to 0.5 ml. To eliminate factors which might have a small adverse effect on the quantitative readings, it is preferred that the serum be filtered before being used to activate the mast cells. In order for the IgE antibodies of the serum to activate the mast cells completely, the preparation should be incubated for 1 to 5 minutes at temperatures between 30°C. and 45°C. Preferred conditions are incubation for 3 minutes at 37°C.

After the mast cells have been activated with the antibodies of the serum, a small amount of the desired allergen is added and the solution is incubated between 30°C. and 40°C. for a few minutes. These allergens, which are water-soluble extracts of allergenic substances such as the pollen of ragweed, timothy and trees, cat dander, house dust, and various foods and drugs, are commercially available, and only a small amount, usually the amount recommended by the manufacturer of the extract for a single intradermal test, is added to the activated mast cell suspension. Generally about 0.05 ml. of an allergen solution is employed.

The release of histamine by the mast cells is proportional to concentrations of the antigen within which they have been treated up to a certain limit. For example, 0.05 micrograms of Compound 48/80, a basic condensation product of p-methoxyphenylmethylamine available from Burroughs-Wellcome, Tuckahoe, New York, which mimics antigen action on mast cells under certain conditions, will release 10 nanomoles of histamine from 1,000,000 mast cells, and these cells will absorb about 0.2 nanomoles of 2,10-dimethylaminopropyl acridone. On the other hand, 1.0 micrograms of the same compound will release approximately 80 nanomoles of histamine from 1,000,000 mast cells, which in turn will absorb about 3.0 nanomoles of acridone. Although the ratio of histamine released to acridone uptake remains constant for any concentration of antigen at an acridone concentration of any particular valve, higher concentrations of acridone solution give lower histamine release-acridone uptake ratios.

Any water-soluble absorbable acridone which fluoresces in the present invention. The acridone employed must be capable of penetrating the mast cell. Acridone itself and 2,10-dimethylaminopropyl acridone have been found to be particularly useful. These acridones fluoresce in 1N hydrochloric acid at 4700 angstroms wavelength when activated with light at 4000 angstroms. The fluorescence was found to be linearly related to the concentration over a range of about 0.3 to 150 μg/ml. (millimicrograms per millilitre).

Although the uptake of allergen by the mast cells and release of histamine is relatively rapid and only a short incubation period is required, the complete uptake of the acridone by the activated mast cells is somewhat slower. Some acridone is taken up almost immediately, even by unactivated mast cells. On the other hand, complete equilbrium uptake of acridone requires about 20 minutes. The temperature at which this occurs is not critical. Room temperature is satisfactory.

As indicated above, the uptake of acridone is in direct proportion to the amount of histamine released by the allergen up to certain maximum limits. Acridone itself will not release histamine from mast cells, but a relatively small amount of acridone is absorbed by the unactivated cells. For example, 1,000,000 mast cells will, in 20 minutes, absorb about 5 nanomoles of 2,10-dimethylaminopropyl acridone from a solution containing 50 micrograms of the compound and about 9 nanomoles from a solution containing 100 micrograms of the same acridone. As will be seen, the exact amount of acridone taken up is dependent on the concentration of acridone present in the solution. For this reason a control tube to which no antigen has been added is necessary. However, when histamine is released from the activated mast cells by action of an allergen, larger amounts of acridone are taken up by the mast cells and this augmented take-up is directly proportional to the amount of histamine released. For example, when 1,000,000 mast cells have been subject to the action of Compound 48/80, about 17 nanomoles of acridone is absorbed in 20 minutes from a solution containing 50 micrograms of 2,10-dimethylaminopropyl acridone and when 1,000,000 mast cells are activated with the same amount of antigen and allowed to come to equilibrium with a solution containing 100 micrograms of 2,10-dimethylaminopropyl acridone, approximately 37 nanomoles of the acridone are taken up by the mast cells. The uptake of acridone is linearly related to the concentration of acridone present up to a concentration of about 500 μM or 0.015% by weight. It is preferred that the acridone solution have a concentration of 50 to 100 μM. Concentration are not necessary. However, the solution should have a concentration greater than 5 μM when 1,000,000 mast cells are used per test so that the cells alone will not absorb all of the acridone.

It is essential that the mast cells remain intact during their treatment with the patient's serum, allergen and acridone, and accordingly conditions which tend to break up the cells are to be avoided. The solutions
should be isotonic. Heat or cold should be avoided. The test procedures should take place between 30°-45°C. Reagents which tend to lyse the cells or physical forces which might break up the cells are also to be avoided. The hydrogen ion concentration should be close to pH 7.0.

After the treatment cells have been in contact with the acridone solution for 10 to 20 minutes, the suspension is centrifuged at relatively low gravity, for example, about 200 g’s for 5 minutes, the supernatant is removed, the tubes inverted and drained, and the mast cells, which have been thrown out of the solution, are lysed for 1 to 5 minutes with 1 ml of 1 N hydrochloric acid. As a matter of fact, however, the mast cells may be lysed by other means, even distilled water. Hydrochloric acid is preferred as its use enhances the fluorescence of the acridone.

The fluorescence of the liquid in the tube is then measured and compared with that of the control. If the patient’s serum has contained IgE antibody to a specific allergen tested, histamine will have been released from the mast cells and the acridone absorbed, much more than would be expected if no histamine had been released. The fluorescence of the solution due to the acridone which has been removed from the mast cells by the lysing procedure is measured in any convenient manner. It is preferred, however, that light from a suitable source having an activating band of 4000 angstroms is first passed through a filter which blocks out light having a wave length above 4100 angstroms. This light is caused to pass through the solution which fluoresces to a degree depending upon the amount of acridone in it. The fluorescent light may then be measured with a phototube which is sensitive at about 4700 angstrom. Preferably, the light is passed through a filter, which allows light above 4000 angstroms to pass. Although it is preferred that a phototube sensitive to light at a wave length of 4700 angstroms such as a 1-P-28 phototube be used, it will be apparent that for qualitative determinations the fluorescence can be observed with the naked eye.

The following data summarized in Table 1 demonstrate the uptake of acridone by sensitized mast cells in response to histamine release as a result of incubation of the sensitized mast cell with various allergens. Tests 1 through 4 are carried out with serum from subjects known to be sensitive to certain allergens. The known allergen sensitivities are indicated in column 2. Test number 5 is carried out on serum from a subject of no known allergen sensitivities and serves as a control. In each test 1.0 ml of a mast cell solution containing approximately 100,000 mast cells in Hank’s solution is incubated with approximately 0.05 ml of serum for 5 minutes at 37°C after which approximately 0.05 ml of the antigen is added and mixed. Followed by the addition of 1.0 ml of a 5 x 10−4 M concentration of 2,10-dimethylaminopropyl acridone. Incubation is continued for 20 minutes. Each test solution is then centrifuged, and the sedimented cells are lysed and the fluorescence measured in the manner described hereinbefore. The values contained in columns 3 through 8 of Table 1 represent the uptake of acridone in response to histamine release as measured by the fluorescence of each test solution and are expressed in nanomoles of the acridone uptake per 100,000 mast cells. The allergens employed in each test are commercially available and in each test 0.05 ml of the allergen solution prepared in the manner prescribed by the commercial source is used. These data indicate that in each instance of known allergen sensitivity a significant amount of the acridone was absorbed by the mast cell.

Although the process of the present invention will probably find its greatest usage in the determination of allergies and drug sensitivities in humans, it is noted that many warm-blooded animals also suffer from allergies and the procedure described herein is applicable to the determination of such allergies in these susceptible animals. Many different tests on samples of the subject’s serum for different antigens may be conducted at the same time. 10 ml of the subject’s blood should be enough for the testing of about 50 different allergens. It will also be obvious that the procedure may be easily automated, whereby the fluorescence is recorded on paper to provide a permanent record which may be filed along with the subject’s medical history.

1. A method of determining the susceptibility of a subject to allergens which comprises the steps of incubating mast cells with serum of the subject at temperatures of 30° to 45°C., contacting the incubated mast cells with an allergen and thereafter adding a dilute solution of an absorbable fluorescent acridone to the allergen treated cells and measuring the acridone absorbed by the mast cells.

2. The method of claim 1 in which 100,000 to 1,000,000 mast cells are incubated for 1 to 5 minutes with 0.05 to 0.5 ml of blood serum of a subject.

3. The method of claim 2 in which the mast cells are suspended in isotonic saline at a pH within the range 6.8 to 7.2 while in contact with the blood serum, allergen and acridone.

4. The method of claim 1 in which the acridone absorbed by the mast cells is recovered therefrom by lysis.

TABLE 1

<table>
<thead>
<tr>
<th>Test no.</th>
<th>Known Allergen Sensitivity</th>
<th>Egg Albumin</th>
<th>Mixed Ragweed</th>
<th>Mixed Trees</th>
<th>Mixed Molds</th>
<th>Cat Epithelial</th>
<th>Mixed House Dust</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ragweed Trees</td>
<td>0.54</td>
<td>2.89*</td>
<td>3.17*</td>
<td>0.64</td>
<td>0.50</td>
<td>0.48</td>
</tr>
<tr>
<td>2</td>
<td>Ragweed Molds</td>
<td>0.54</td>
<td>2.34*</td>
<td>0.29</td>
<td>3.29*</td>
<td>0.47</td>
<td>0.42</td>
</tr>
<tr>
<td>3</td>
<td>Animal dander and hair</td>
<td>0.51</td>
<td>0.54</td>
<td>0.27</td>
<td>0.60</td>
<td>2.73*</td>
<td>0.45</td>
</tr>
<tr>
<td>4</td>
<td>Ragweed House dust Trees</td>
<td>0.59</td>
<td>3.04*</td>
<td>2.31*</td>
<td>0.59</td>
<td>0.51</td>
<td>2.81*</td>
</tr>
<tr>
<td>5</td>
<td>None (control)</td>
<td>0.56</td>
<td>0.50</td>
<td>0.33</td>
<td>0.58</td>
<td>0.49</td>
<td>0.41</td>
</tr>
</tbody>
</table>

* = Established difference from control.
and the amount of the acridone absorbed by the mast cells and recovered therefrom is determined by observing the fluorescence of the acridone.

5. A method of measuring the amount of histamine released from activated mast cells in response to their reaction to an allergen while suspended in isotonic saline at pH 6.8 to 7.2 which comprises the steps of allowing the mast cells to absorb a fluorescent acridone from a dilute aqueous isotonic solution thereof, recovering the mast cells from the liquid in which they are suspended, lysing the mast cells to release the fluorescent acridone and measuring the fluorescence of said absorbed acridone.

6. The method of claim 5 in which the fluorescent acridone is 2,10-dimethylaminopropyl acridone.

7. The method of claim 5 in which the fluorescent acridone is acridone.

* * * * *
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 3,900,558
DATED : August 19, 1975
INVENTOR(S) : C. Richard Kinsolving

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 2, line 16 "... on a qualitative or qualitative basis..." should read "... on a qualitative or quantitative basis...".

Signed and Sealed this
Twentieth Day of July 1976

[SEAL]

Attest:

RUTH C. MASON
Attesting Officer

C. MARSHALL DANN
Commissioner of Patents and Trademarks