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**METHOD FOR DETERMINING THE PRESENCE OF REAGIN-IMMUNOGLOBULINS (REAGIN-Ig) DIRECTED AGAINST CERTAIN ALLERGENS, IN AQUEOUS SAMPLES**

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No Drawing. Filed Sept. 6, 1968, Ser. No. 758,131

Int. Cl. A61k 27/04

U.S. Cl. 424-1

26 Claims

**ABSTRACT OF THE DISCLOSURE**

An in vitro method for analyzing a body fluid for reagin-immunoglobulins therein that are reactive with allergens which comprises contacting a sample of body fluid with a water-insoluble polymer to which is attached at least one test allergen for a long enough time to allow reaction therebetween and then contacting with either labelled antibodies that are reactive with the reagin immunoglobulins or with antibodies against reagin-Ig and reagin-Ig labelled with a radiation emitting atom or group, separating into a liquid phase and a solid phase, and measuring the radiation emitted from at least one of the two separated phases.

It is known that a number of immunogenic substances, so-called allergens, of which many are of a protein, peptide or carbohydrate nature, can give rise to allergic reactions in the form of, for instance, asthma and hay fever. (Such allergens are very widespread and may be derived from, for example, animal dandruffs, plant pollens, microorganisms, some parasites, etc.) In this connection a special type of antibodies, so-called reagins which are immunoglobulins—which are directed specifically against the allergen or allergens—appears as a rule in very low concentrations, in the patient's blood, for instance, and can be shown in blood serum. These special immunoglobulins will hereinafter be referred to as reagin-immunoglobulin or eagin-Ig.

The present invention is concerned with a method for determining the presence of reagin-Ig directed against certain allergens in aqueous samples. It is particularly important to determine the presence of reagin-Ig in conjunction with diagnosing the hypersensitivity of animals and people.

Prior testing procedures have included skin tests, wherein a test allergen has been applied to the skin of the patient with for instance a hypodermic syringe and needle; if the patient is hypersensitive (allergic) a reaction takes place between the allergen and the antibodies directed against the allergen in question, in vivo; which results in certain symptoms such as reddening or swelling of the skin at the place where the injection was made, which can be observed and assessed with respect to the degree of reaction.

In vivo tests are also the so-called provocation tests, wherein the patient inhales an allergen in the form of an aerosol. If the patient is hypersensitive to the allergen a reaction is obtained, which may show asthma symptoms. Both of the aforementioned tests are time consuming and, in certain instances, are uncomfortable or dangerous to the patient. In a number of cases no tests can be made. Further, a multiplicity of in vivo tests are required to diagnose whether, or not, the patient is hypersensitive to a certain allergen or allergens.

Previously tested in vitro-methods are of no practical significance.

The object of the invention is to provide a simple and reliable in vitro method, which can be effected without

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unpleasantness or risk to the patient, and thus in which the above-mentioned disadvantages are eliminated.

The invention is mainly characterized in that the test sample, e.g. a body fluid such as blood serum or blood plasma is contacted, in vitro, with a water insoluble polymer to which has been bound a test allergen, a reaction between the test allergen on the polymer and the reagin-Ig directed thereagainst taking place, so that said reagin is bound to the test allergen on the insoluble polymer, and the polymer with the attached test allergen and reagin-Ig is contacted with either antibodies against reagin-Ig which have been labelled with a radiation emitting atom or group, or with antibodies against reagin-Ig and reagin-Ig labelled with a radiation emitting atom or group, whereafter the insoluble polymer with substances attached thereto is separated from the fluid, whereupon the radiation emitted from the insoluble polymer with the substances attached thereto or the radiation emitted from the separated fluid is measured, or the effect thereof examined.

Thus, if reagin-Ig directed against the allergen is present in the sample, labelled reagent will be bound to the insoluble phase which will then emit radiation. The latter increases with increasing concentration of said reagin-Ig in the test sample. The radiation of the liquid phase will instead decrease with increasing concentration of said reagin-Ig, as more labelled reagent is bound to the insoluble phase. The measured radiation values obtained in this procedure for the test sample can be compared with values for control samples.

Water insoluble polymers are used as a carrier for the test allergen. They can, for example, exist in the form of particles of varying form and size. Polymers, for example, in the form of flat objects such as discs or strips or also, for example, in the form of the wall of a tube such as the internal side of a test tube can also be used. The bond between the polymer and the test allergen should be such that in normal washing procedures the test allergen cannot be released from the polymer. To this effect it may, for instance, be of chemical or optionally physical nature. A suitable form of chemical binding is to provide bridges of covalent character between the polymer and the test allergen. For this purpose the polymer is selected so that it contains or can be provided with suitable reactive groups, for instance amino groups, hydroxyl groups and carboxyl groups, to enable the test allergen to be bound easily to the polymer. For this purpose bridges between the polymer and allergen with chemical bonds of a covalent character are preferably selected.

It is particularly suitable to select polymers consisting of a three-dimensional network, held together by bonds of a covalent character. Such polymers, even though they are swellable in water or aqueous media, are completely insoluble and thus cannot release any of the polymeric material or the substances bound thereto, for instance during washing procedures. Examples of such polymers are the copolymerisates obtained by cross linking substances containing a plurality of hydroxyl groups such as carbohydrates and sugar alcohols, e.g. dextran, starch, dextrans and other polysaccharides, and polyvinyl alcohol, with a bifunctional substance, e.g. bifunctional substances of the type X—R—Z, wherein, for instance, X and Z are halogen or epoxy groups and R is the residue of the bifunctional substance, e.g. an aliphatic radical containing 3 to 10 carbon atoms.

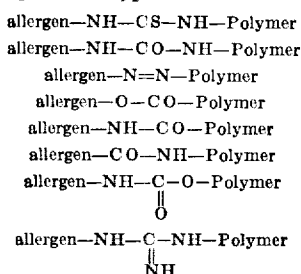
For example, for this purpose the commercially available product Sephadex can be used; this product consists of dextran cross-linked with glycerin ether-bridges, obtained by treating dextran with epichlorohydrin. The product can be used, for example, in the form of small grains. Sephadex and products obtained in a similar man-

ner are water swellable but insoluble gels and can be made in particle form. They contain hydroxyl groups and can therefore be readily substituted with other groups, e.g. groups containing amino groups or carboxyl groups, which are well suited for forming bridges with bonds of covalent character to the test allergen.

The polymer is preferably used in such a form that a large contact area is obtained, for example, in the form of particles.

The test allergen is bound to the carrier polymer under mild conditions, in order not to appreciably reduce the immunochemical reactivity of the allergen.

Used in chemically binding the allergen to the polymer are such reactive groups as amino groups, hydroxyl groups, mercapto groups, amido groups and carboxyl groups, a bridge being formed having chemical bonds, preferably of covalent character, from the test allergen to the polymer, e.g. of the type:



The bridge between the allergen and the polymer need not be known as to its structure and can be selected from a number of widely varying types, as the only purpose of the bridge is to prevent the allergen from being washed away from the insoluble polymer.

The new method is based upon the observation that reagin-Ig can be bound both to the allergen which they are specifically directed against and to antibodies generally directed against reagin-Ig. Such antibodies are at least bivalent, i.e. they can be bound to at least two molecules of reagin-Ig. Antibodies directed to reagin-Ig and also reagin-Ig itself can be labelled with a radiation producing atom or group, e.g. with radioactive isotopes or a fluorescent group.

When compared with the known methods to perform skin tests and provocation tests the present method offers the advantage that the presence and also the quantity of reagin-Ig capable of reacting with a specific type of allergen and a specific group of allergens such as animal allergens or plant allergens, can be determined in a blood sample from the patient in vitro by a very sensitive and simple method completely safe to the patient. It is essential for the method that the test allergen against which the reagin-Ig to be determined is directed, is very firmly bound to a water-insoluble carrier. The reagin-Ig which has reacted with the allergen can thus easily be separated from non-allergen-bound immunoglobulins. Furthermore, during further stages in the method the antibodies and the reagin-Ig which have not been bound to the polymer can be very easily separated from those which have been bound to the polymer.

The polymer with substance attached thereto can be readily separated from the fluid. The separation is insensitive to variations in salt and protein concentration of the fluid within physiological limits. The complete determining process including the separation of free, labelled antibodies and reagin-Ig and polymer bound labelled antibodies and reagin-Ig, respectively, can for instance, be carried out in the same test tubes without the addition of precipitating agents or the like.

The aforementioned method presumes access to isolated reagin-Ig and antibodies directed thereagainst.

Reagin-Ig can be recovered from serum taken from allergic patients, and in rare instances from serum taken from patients having tumors in the tissues which form

reagin-Ig. Reagin-Ig can be purified by different methods for separating proteins, such as gel filtration, ion exchange chromatography and electrophoresis. Similarly to other immunoglobulins the reagin-Ig can be split chemically or enzymatically into an antigen binding fragment and a fragment which is the carrier of the class-specific determinants.

Antibodies against reagin-Ig can be prepared in accordance with known methods for immunizing test animals, for instance, by repeated sub-cutaneous injections of small quantities of antigens in mixture with a suitable adjuvant, e.g. Freund's mineral oil suspension. The antibodies formed in the animal, e.g. a rabbit, can be recovered from the blood serum of the animal.

Specific antibodies, i.e. the antibodies directed against the class-specific determinants of reagin-Ig, can be obtained by immunizing with split fragments of the reagin-Ig molecules containing these determinants, or also by isolating the specific antibodies from a mixture of antibodies obtained by immunizing with the native reagin-Ig.

Specific antibodies directed against reagin-Ig can, if desired, be isolated from the antiserum by conventional immunisorbent techniques; reagin-Ig being coupled to, for instance, bromoacetyl cellulose and the antibodies which have been bound to the coupled reagin-Ig, are released by lowering the pH.

It is sometimes an advantage to use specific antibodies against the class-specific determinants in reagin-Ig, to obtain a very high assurance and accuracy during the tests.

Labelling with radioactive isotopes of reagin-Ig and antibodies against reagin-Ig can be effected in a conventional manner, a suitable isotope for the purpose being selected, e.g.  $^{125}\text{I}$  (method according to Hunter and Greenwood). Similarly, labelling with a fluorescent group can be effected in a conventional manner, e.g. with a fluorescein-derivative such as fluorescein-isothiocyanate.

Allergen-containing extracts, e.g. from dog or horse epithelium, timothy or birch pollen, are commercially available.

The radioactivity determining processes can be effected by conventional methods, i.e. with the use of scintillation detectors. Similarly the fluorescence measuring processes can also be effected by conventional methods.

The quantity of polymers having allergen bound thereto is selected inter alia, with thought to the level of sensitivity required during the test. The quantity of antibodies against reagin-Ig added to the reaction is selected, for instance, so that there is an excess in relation to the number of combining sites for these antibodies on the attached substances of the polymers after the allergens have been completely saturated with reagin-Ig. Similarly, the quantity of labelled antibodies and labelled reagin-Ig is suitably selected so that there is an excess in relation to the maximum number of combining sites on the substances attached to the polymer.

According to the invention the test allergen may be one single allergen or a mixture of two or more allergens. In the latter case the method affords the advantage whereby a quick "yes" or "no" can be obtained during the examination to the question of whether hypersensitivity against one or more allergens in a large group of allergens is manifest. If the answer is yes further determinations must be made to establish against which of the allergens the hypersensitivity of the patient exists within the large group.

According to the invention the method can also be applied for the quantitative determination of reagin-Ig. For this purpose a serial dilution of test serum is analysed, and the result is expressed after a comparison with the effect of a standard solution analysed in an analogous manner.

The invention also encompasses a kit for the determination of reagin immunoglobulins (reagin-Ig) directed against certain allergens in aqueous test samples, adapted to be used for the method as specified above, comprising a first reagent comprising a water insoluble polymer to which has been bound a test allergen, a second reagent

comprising antibodies against reagin-Ig and optionally a third reagent comprising reagin-Ig, said antibodies against reagin-Ig having been rendered capable, by labelling, of emitting radiation, if the third reagent is not present, and reagin-Ig of the third reagent having been rendered capable, by labelling, of emitting radiation, said first reagent being intended to contact, in vitro, with an aqueous test sample obtaining reagin-Ig to bind said reagin-Ig to the test allergen on the insoluble polymer, and said second reagent, optionally together with said third reagent, being intended to contact with the insoluble polymer with the attached test allergen and reagin-Ig to provide a mixture comprising radiating insoluble material comprising insoluble polymer and substances attached thereto and radiating liquid phase, the radiation of said insoluble material and said liquid phase being each a function of the concentration of reagin-Ig directed against the test allergen in the test sample.

According to a preferred embodiment of the invention, the insoluble polymer is in particle form.

The kit may, according to the invention, be included in a test pack, adapted to be used for testing samples taken from allergic patients.

According to the invention the kit may include an ampoule or the like containing antibodies against reagin-Ig preferably in dried, e.g. lyophilized form, and possibly also an ampoule containing labelled reagin-Ig, preferably in dried, e.g. lyophilized form; whereby in the case where the last mentioned ampoule is not used the antibodies against reagin-Ig are labelled.

The invention will be illustrated in the following by examples, which illustrate in more detail the manner in which the method according to the invention is applied.

#### EXAMPLE 1

##### Determining reagin-Ig directed against an animal allergen (dog epithelium)

###### (A) Preparation of particles with chemically bound allergen

Fine particles of a copolymer of dextran and epichlorohydrin were used as starting material, the dextran being crosslinked with glycerine ether-bridges (Sephadex, particle size 1-10 microns). The copolymer is insoluble but swellable in water, the substance absorbing about 2.5 grams of water per gram of dry substance. The starting material was first activated with cyanogen bromide in an alkaline solution, whereupon the particles of the activated copolymer were washed. A commercially available allergen extract of dog epithelium was added to an aqueous suspension of the particles, the extract containing 0.2 mg. per ml., in the ratio of 1 ml. of allergen extract to 100 mg. of activated polymer. The allergen was then bound chemically to the activated copolymer. Subsequent to the reaction the particles of the copolymer were centrifuged with the allergen bound thereto, and washed twice with 0.5 M NaHCO<sub>3</sub>; 0.1 M acetate buffer; 0.1 M trisbuffer with 1% bovine serum albumin. The particles were homogenized and suspended in 0.1 M trisbuffer with 1% bovine serum albumin.

###### (B) Preparation of reagin-Ig

Reagin-Ig was obtained from serum taken from a myeloma patient, having a high content of reagin-Ig in the blood. Reagin-Ig was isolated from the serum by salt precipitation, gel filtration and ion exchange-chromatography.

Samples of thawed or freshly collected plasma from a patient with myelomatosis were diluted with 0.15 M sodium chloride to contain about 15 mg./ml. in respect to the M-component (reagin-Ig). Precipitation with anhydrous sodium sulphate (18 g./100 ml.) was made at 25° C. After one hour the precipitate was collected by centrifugation (10,000×g, 20 minutes, 25° C.). The sediment was collected and washed with sodium sulphate solution (18 g./100 ml.) and dissolved in 0.8 volume of 0.1 M sodium phosphate buffer, pH 7.5. The material was reprecipitated once as described. Following the last pre-

cipitation, the sediment was dissolved in 0.1 M tris-HCl buffer pH 8.0 (20° C.) and applied to a column (3.2 x 30 cm.) of DEAE-Sephadex® A-50 equilibrated with the same buffer. Elution of the material was performed by a continuous gradient from 0.1 M to 1 M tris-HCl at a constant pH of 8.0 at 20° C. Fractions containing reagin-Ig were collected and concentrated and applied to a column (3.2 x 95 cm.) of Sephadex® G 150 equilibrated with 0.1 M tris-HCl-0.2 M NaCl-0.002 M EDTANa<sub>2</sub> pH 7.7 and containing 0.02% sodium azide. The material was allowed to pass through the column three times (recycling chromatography technique). Fractions containing reagin-Ig were collected and concentrated by ultrafiltration (Visking tubing ½") at 4° C.

Reagin-Ig prepared as described was contaminated with less than 0.1 percent of total protein of other immunoglobulins (IgA, IgD, IgG and IgM). The purified reagin-Ig gave a single peak in electrophoresis pH 8.6 I=0.05 migrating in the β-γ region. Ultracentrifugation studies revealed a single boundary and the sedimentation constant, S<sub>20,w</sub>, was calculated to 8.20S. The molecular weight of reagin-Ig was calculated to 200,000±5,000 using a partial specific volume of 0.173 cm.<sup>3</sup>/g. as determined on basis of the amino acid and carbohydrate compositions of reagin-Ig. The diffusion constant, D<sub>20,w</sub>, was calculated to be 3.71×10<sup>-7</sup> cm.<sup>2</sup>/sec.

Reduction experiments revealed that reagin-Ig consists of two kinds of polypeptide chains, thus resembling other serum immunoglobulins. Evidence has been obtained for the presence of 2 light polypeptide chains of 22,600 molecular weight in reagin-Ig. The molecular weight of the heavy polypeptide chains, including its carbohydrate prosthetic group(s) was calculated to be about 77,400 assuming a molar ratio of 1:1 for heavy and light polypeptide chains in the native reagin-Ig. The amino acid and carbohydrate composition is shown in Table 1.

TABLE 1

| Amino acid and carbohydrate composition of reagin-Ig |                                   |
|--|-----------------------------------|
| Amino acid:  | Amino acid residue <sup>1</sup>   |
| Tryptophan   | 3.67                              |
| Lysine   | 4.20                              |
| Histidine  | 2.16                              |
| Amide N  | 1.34                              |
| Arginine   | 5.63                              |
| Aspartic acid  | 6.73                              |
| Threonine  | 9.01                              |
| Serine   | 8.52                              |
| Glutamic acid  | 8.93                              |
| Proline  | 5.24                              |
| Glycine  | 3.13                              |
| Alanine  | 3.92                              |
| Half-cystin  | 2.15                              |
| Valine   | 5.59                              |
| Methionine   | 1.17                              |
| Isoleucine   | 2.29                              |
| Leucine  | 6.08                              |
| Tyrosine   | 4.63                              |
| Phenylalanine  | 3.91                              |
|  | 88.29                             |
| Carbohydrate:  | Carbohydrate residue <sup>1</sup> |
| N-Acetyl-glucosamine                                 | 4.60                              |
| Hexose (galactose+mannose)                           | 5.34                              |
| L-fucose   | 0.51                              |
| N-acetyl-neuraminic acid                             | 1.26                              |
|  | 11.71                             |

<sup>1</sup> Grams per 100 grams of protein.

###### (C) Preparation of antibodies against reagin-Ig

Rabbits were injected intramuscularly with a mixture of 0.5 mg. of reagin-Ig in 0.2 ml. of 0.15 M sodium chloride solution and 0.8 ml. of complete Freund's adjuvant. The immunisation was repeated after 14 days and then each

week for three weeks. After a further ten days had passed blood was removed from the rabbits and antiserum recovered from the blood by allowing it to coagulate and removing the clot.

The antiserum was made specific by adsorption during 1 hour at +37° C. and 16 hours at +4° C. After centrifuging the specificity of the antiserum was tested by immunoelectrophoresis against normal human serum and with Ochterlony-gel diffusion analysis against pure immunoglobulin A, G and M, so-called light polypeptide chains from normal immunoglobulin G and Bence-Jones proteins, whereupon it proved to be specific against reagin-Ig. The antiserum was used as antibody material for the determining process.

(D) Preparation of labelled reagin-IG

Reagin-Ig was labelled with <sup>125</sup>I in accordance with a method described by Hunter and Greenwood, Nature 144 (1962), page 495.

(E) Determining process

The analyses were made in glass or plastic tubes of 50 x 10 mm. in dimension.

- (1) 0.5 ml. of suspension of particles with chemically bound allergen obtained according to A above, the suspension containing 1 mg. of particles per ml., was introduced into each of two test tubes.
- (2) 0.05 ml. of the patient serum to be tested was introduced to one of the tubes.
- (3) 0.05 ml. of control serum from a non-allergic source was introduced into the second tube.
- (4) The content of these tubes was incubated for 20 hours at room temperature, the tubes being rotated slowly in the vertical plane.
- (5) The particles were centrifuged down at 3000 r.p.m. for 1 minute, whereupon the supernatant was removed.
- (6) The particles were washed three times with the 0.1 M tris-buffer (pH 7.4) with 1 percent, by weight, of bovine serum albumin, the suspension being centrifuged and the supernatant being removed by suction after each washing operation. It was ensured during each suction operation that solid particles were not entrained.
- (7) 0.1 mole of the antiserum obtained according to C above was introduced to each tube.
- (8) The content of the tubes was incubated in accordance with step 4 above.
- (9) The particles were centrifuged down in accordance with step 5 above.
- (10) The particles were washed in accordance with step 6 above.
- (11) 0.1 ml. of a solution having <sup>125</sup>I-reagins according to D above were introduced to the tubes.
- (12) The content of the tubes was incubated according to step 4.
- (13) The particles were centrifuged down according to step 5.
- (14) The particles were washed according to step 6.
- (15) The tubes were placed in scintillation detectors for measuring the gamma radiation.

For the purpose of evaluation, the intensity of radiation from the one tube was compared with the intensity of radiation from the other tube, which contained control serum taken from non-allergic patients.

Alternatively, subsequent to centrifuging according to step 13 above, a certain volume of supernatant can be transferred to count tubes, whereafter the gamma radiation from <sup>125</sup>I-reagin free in the solution can be determined.

For quantitative determination 0.05 ml. of test serum in varying degrees of dilution can be added in step 2 above to each of a number of test tubes. A number of tubes were provided in the same manner with varying dilutions of a standard serum. The activity can then be expressed in relation to that of a standard serum.

EXAMPLE 2

Determination of reagin-Ig directed against a plant allergen (birch pollen)

(A) Preparation of particles with chemically bound allergen

Fine particles of dextran cross-linked with glycerine ether bridges (Sephadex G 25, superfine) and which had been substituted by isothiocyanatophenoxyhydroxypropyl groups (according to the method by R. Axén and J. Porath, Acta Chem. Scand. 18 (1964), p. 2193) were used as starting material. To a suspension of 100 mg. of the particles in an aqueous solution of sodium hydrocarbonate was added 1 ml. of allergen solution (commercially available allergen extracts) having approximately the same concentration as the allergen solution in Example 1. The mixture was incubated for three days at room temperature whilst being constantly rotated slowly in the vertical plane. The particles were then centrifuged and washed twice with each of 0.5 M NaHCO<sub>3</sub>, 0.1 M acetate buffer, 0.1 M tris buffer having 1% bovine serum albumin. The particles were homogenized and suspended in 0.1 M tris buffer with 1% bovine serum albumin.

(B) Isolation of fragments of reagin-Ig containing class-specific determinants (so-called Fc-fragments)

100 mg. of reagin-Ig produced according to Example 1B in 0.1 M sodium phosphate-buffer (pH 7.0) with 0.1 M cystein was incubated at 37° C. for 4 hours with 1 mg. of papain, whereupon the reaction was interrupted by the addition of iodoacetamide solution to a final concentration of 0.05 M. The reaction mixture was fractionated by gel filtration (Sephadex G 150), a copolymer of dextran and epichlorohydrin being used, with a water regain of 15 g. per g. of solid substance and elution of the gel bed was effected with 0.1 M tris-HCl, 0.2 M NaCl buffer (pH 7.7). Fractions containing Fc-fragment were collected for immunization purposes.

(C) Preparation of antibodies against the Fc-fragment of reagin-Ig

Antiserum against the Fc-fragment of reagin-Ig obtained according to B above was obtained in a manner similar to that described in Example 1C for antiserum against reagin-Ig. Antibodies directed against the Fc-fragment were isolated from this antiserum, which had been adsorbed in a manner corresponding to that described in the Example 1C, in the following manner: 2 grams of a complex between bromoacetyl cellulose and reagin-Ig were mixed with 34 ml. of a solution containing approximately 95 mg. immunoglobulin isolated from antiserum by ion-change chromatography. The obtained suspension was activated at pH 7 and +4° C. for 2 hours, whereafter it was centrifuged with 20.000×g for 20 minutes at +10° C. The supernatant was removed, the cellulose washed with 0.15 M NaCl-8 M urea, pH 6-7, centrifuged and the supernatant removed. This washing procedure was repeated until the supernatant was free from protein. The cellulose protein complex was added to 9 ml. of 0.1 M glycine HCl-buffer (pH 3.1) and incubated at +37° C. for 40 minutes whilst being stirred. The whole solution was centrifuged and the supernatant was dialyzed against 1200 ml. of 0.01 M tris-HCl 0.1 M NaCl (pH 7.1) at +4° C. for 48 hours. This solution contained antibodies in a quantity of approximately 1 mg. per ml., which are specific for the Fc-fragment of reagin-Ig, and thus also for the whole reagin-Ig. This antibody material is sufficient to complete some 10 million determinations.

(D) Labelling of antibodies specific for the Fc-fragment of reagin-Ig

The labelling process was effected in a manner similar to that described above in Example 1 for reagin-Ig.

## (E) Determining process

- (1) 0.5 ml. of a suspension of particles having chemically bound plant allergens obtained according to A above was introduced into each of two test tubes; the suspension containing 1 mg. particles per ml.
- (2) 0.05 ml. of the patient serum to be tested was added to one of the tubes.
- (3) 0.05 ml. of a control serum taken from a non-allergic source was introduced to the other tube.
- (4) The content of the tubes was incubated for 5 hours at room temperature, the tubes being rotated slowly in the vertical plane.
- (5) The particles were centrifuged down at 3000 r.p.m. for 1 minute, whereupon the supernatant was removed.
- (6) The particles were washed three times with 0.1 M tris-buffer pH 7.4 with 1% bovine serum albumin. The supernatant was removed by suction after each centrifuging operation. It was ensured when removing the supernatant that solid particles did not follow therewith.
- (7) 0.1 mol of the solution obtained according to C above with antibodies against the Fc-fraction of reagin-Ig and labelled with  $^{125}\text{I}$  was added to all tubes.
- (8) The particles were centrifuged to 3000 r.p.m. for 1 minute whereupon the supernatant was removed.
- (9) The contents of the tube was incubated for 15 hours at room temperature, the tubes being rotated slowly in the vertical plane.
- (10) The particles were washed three times with 0.1 M tris-buffer (pH 7.4) with 1 percent, by weight, of bovine serum albumin with centrifuging and removal by suction of the supernatant after each washing operation. It was ensured during the removal of the supernatant that solid particles were not entrained therewith.
- (11) The tubes were placed in scintillation detectors for measuring gamma radiation.

For estimating purposes the intensity of radiation from the first tube was compared with the intensity of radiation from the second tube, which contained a control serum from a non-allergic source.

Alternatively, after the centrifuging operation according to stage 8 a certain volume of the supernatant can be transferred to count tubes, whereafter the gamma radiation from labelled antibodies against the Fc-portion free in the solution can be measured.

For quantitative determination purposes 0.05 ml. test serum in various solutions can be added in step 2 above to each of a number of test tubes. A number of test tubes was provided in the same manner with various dilutions of a standard serum. The activity can be expressed in relation to the activity of the standard serum.

What we claim is:

1. An in vitro method for analyzing a body fluid so as to obtain an indication as to the amount of reagin-immunoglobulins therein that are reactive with one or more allergens capable of producing allergic reactions in humans and animals which method comprises:
- (a) contacting an aqueous sample derived from body fluid with a water-insoluble polymer to which is attached at least one test allergen by bonds capable of withstanding normal washing procedures,
- (b) continuing the contacting set forth in step (a) for a long enough time to allow reaction between said polymer attached test allergen and any reagin-immunoglobulins in the aqueous sample of body fluid that are reactive therewith,
- (c) contacting the product of step (b) with antibodies that have atoms or groups capable of emitting radiation for labelling purposes and which antibodies are reactive with the reagin-immunoglobulins that would be reactive with the test allergen,
- (d) separating the product of step (c) into a liquid phase and a solid phase, and

(e) measuring the radiation emitted from at least one of the phases separated in step (d).

2. The method of claim 1 wherein said polymer is a cross-linked copolymerizate of a material selected from the group consisting of carbohydrates, sugar alcohols and polyvinyl alcohol, with a bifunctional substance of the general formula  $\text{X}-\text{R}-\text{Z}$  wherein X and Z individually are selected from the group consisting of halogen and epoxy groups and R is the residue of the bifunctional substance.

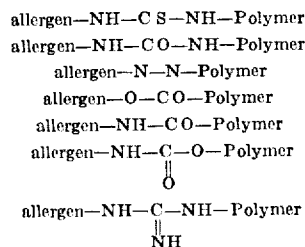
3. The method of claim 2 wherein R is an aliphatic radical containing 3 to 10 carbon atoms.

4. The method of claim 2 wherein said carbohydrate is selected from the group consisting of dextran, starch and dextrans.

5. The method of claim 1 wherein said polymer is dextran cross-linked with glycerin ether-bridges obtained by treating dextran with epichlorohydrin.

6. The method of claim 1 wherein said water-insoluble polymer is provided with suitable reactive groups to enable the test allergen to be bound easily to the polymer.

7. The method of claim 6 wherein the reactive groups will form a bridge between the allergen and the polymer which is selected from the following group:



8. The method of claim 1 wherein the polymer is in the form of fine particles having a large contact area.

9. The method of claim 1 wherein the antibodies have been labelled with a radioactive isotope.

10. The method of claim 9 wherein the radioactive isotope is iodine.

11. The method of claim 1 wherein the antibodies against reagin-Ig have been labelled with a group capable of emitting fluorescent light.

12. The method of claim 1 wherein at least two allergens capable of producing allergic reactions in humans and animals are attached to the water insoluble polymer for step (a).

13. An in vitro method for analyzing a body fluid so as to obtain an indication as to the amount of reagin-immunoglobulins therein that are reactive with one or more allergens capable of producing allergic reactions in humans and animals which method comprises:

- (a) contacting an aqueous sample derived from body fluid with a water-insoluble polymer to which is attached at least one test allergen by bonds capable of withstanding normal washing procedures,
- (b) continuing the contacting set forth in step (a) for a long enough time to allow reaction between said polymer attached test allergen and any reagin-immunoglobulins in the aqueous sample of body fluid that are reactive therewith,
- (c) contacting the product of step (b) with (1) antibodies that are reactive with the reagin-immunoglobulins that would be reactive with the test allergen and with (2) additional reagin-immunoglobulins labelled with a radiation emitting atom or group,
- (d) separating the product of step (c) into a liquid phase and a solid phase, and
- (e) measuring the radiation emitted from at least one of the phases separated in step (d).

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14. The method of claim 13 wherein said polymer is a cross-linked copolymerizate of a material selected from the group consisting of carbohydrates, sugar alcohols and polyvinyl alcohol, with a bifunctional substance of the general formula X—R—Z wherein X and Z individually are selected from the group consisting of halogen and epoxy groups and R is the residue of the bifunctional substance.

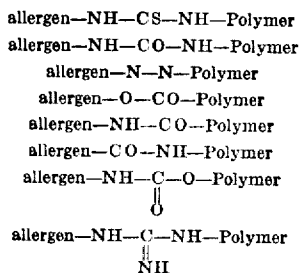
15. The method of claim 14 wherein R is an aliphatic radical containing 3 to 10 carbon atoms.

16. The method of claim 14 wherein said carbohydrate is selected from the group consisting of dextran, starch and dextrans.

17. The method of claim 13 wherein said polymer is dextran cross-linked with glycerin ether-bridges obtained by treating dextran with epichlorohydrin.

18. The method of claim 13 wherein said water-insoluble polymer is provided with suitable reactive groups to enable the test allergen to be bound easily to the polymer.

19. The method of claim 13 wherein the reactive groups will form a bridge between the allergen and the polymer which is selected from the following group:



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20. The method of claim 13 wherein the polymer is in the form of fine particles having a large contact area

21. The method of claim 13 wherein the additional reagin-Ig has been labelled with a radioactive isotope.

22. The method of claim 21 wherein the radioactive isotope is iodine.

23. The method of claim 13 wherein the additional reagin-Ig has been labelled with a group of emitting fluorescent light.

24. The method of claim 13 wherein at least two allergens capable of producing allergic reactions in humans and animals are attached to the water-insoluble polymer of step (a).

25. Antibodies against reagin-Ig which have been labelled with a radioactive isotope of iodine.

26. Reagin-Ig which has been labelled with a radioactive isotope of iodine.

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BENJAMIN R. PADGETT, Primary Examiner

U.S. Cl. X.R.

23—230 B; 250—106 T; 424—8, 12

UNITED STATES PATENT OFFICE  
CERTIFICATE OF CORRECTION

Patent No. 3,720,760 Dated March 13, 1973

Inventor(s) HANS H:son BENNICH et al

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

In column 1, line 8, insert:

"Claims priority, application Sweden,  
September 6, 1967, No. 12297/1967"

Signed and sealed this 19th day of November 1974.

(SEAL)  
Attest:

McCoy M. GIBSON JR.  
Attesting Officer

C. MARSHALL DANN  
Commissioner of Patents

# REEXAMINATION CERTIFICATE (159th)

United States Patent [19]

[11] B1 3,720,760

Bennich et al.

[45] Certificate Issued Feb. 7, 1984

[54] **METHOD FOR DETERMINING THE PRESENCE OF REAGIN-IMMUNOGLOBULINS (REAGIN-Ig) DIRECTED AGAINST CERTAIN ALLERGENS, IN AQUEOUS SAMPLES**

[75] Inventors: **Hans H. Bennich, Uppsala; Stig G. O. Johannson, Storvreta; Leif E. Wide, Uppsala, all of Sweden**

[73] Assignee: **Pharmica, Inc., Piscataway, N.J.**

**Reexamination Request:**

No. 90/000,199, May 14, 1982

**Reexamination Certificate for:**

Patent No.: **3,720,760**  
Issued: **Mar. 13, 1973**  
Appl. No.: **758,131**  
Filed: **Sep. 6, 1968**

Certificate of Correction issued Nov. 19, 1974.

- [51] Int. Cl.<sup>3</sup> ..... **G01N 33/60**  
[52] U.S. Cl. .... **436/513; 436/529;**  
                  **436/532; 436/547; 436/804; 436/811**  
[58] Field of Search ..... **424/1, 1.5; 436/547,**  
                                  **436/513, 518-534; 252/301**

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*Primary Examiner*—Benjamin R. Padgett

[57] **ABSTRACT**

An in vitro method for analyzing a body fluid for reagin-immunoglobulins therein that are reactive with allergens which comprises contacting a sample of body fluid with a water-insoluble polymer to which is attached at least one test allergen for a long enough time to allow reaction therebetween and then contacting with either labelled antibodies that are reactive with the reagin immunoglobulins or with antibodies against reagin-Ig and reagin-Ig labelled with a radiation emitting atom or group, separating into a liquid phase and a solid phase, and measuring the radiation emitted from at least one of the two separated phases.



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**REEXAMINATION CERTIFICATE  
ISSUED UNDER 35 U.S.C. 307.**

THE PATENT IS HEREBY AMENDED AS  
INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

2

AS A RESULT OF REEXAMINATION, IT HAS  
BEEN DETERMINED THAT:

The patentability of claims 1-24 is confirmed.

5 Claims 25 and 26 are determined to be patentable as amended:

10 25. [Antibodies] *Substantially pure antibodies* against reagin-Ig which have been labelled with a radioactive isotope of iodine.

26. [Reagin-Ig] *Substantially pure reagin-Ig* which has been labelled with a radioactive isotope of iodine.

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