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[54] **LABEL MODIFIED IMMUNOASSAYS**

[75] Inventors: **Robert F. Zuk**, Mountain View; **Edward T. Maggio**, Redwood City, both of Calif.

[73] Assignee: **Syva Company**, Palo Alto, Calif.

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[58] **Field of Search** ..... 195/103.5 A, 103.5 R, 195/99, 127; 424/12, 8; 23/230 B

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,996,345	12/1976	Ullman et al.	428/8 X
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Wei, et al., "Preparation of a Phospholipase C–Antihuman IgG Conjugate, and Inhibition of its Enzymatic Activity by Human IgG," *Clin. Chem.*, vol. 23, No. 8, (1977) pp. 1386–1388.

Primary Examiner—Thomas G. Wiseman
Attorney, Agent, or Firm—Bertram I. Rowland

[57] **ABSTRACT**

Methods and reagents are provided for immunoassays employing as reagents a labelled receptor, where the label is capable of providing a detectable signal, and modifying reagents, capable of modifying the signal obtained from the label. When ligand (ligand analog for monoepitopic ligands) is present in the assay medium, a complex is formed which inhibits interaction between the label modifying reagent and the label. By measuring the signal obtained in the presence of a known amount of ligand in the assay medium and comparing that signal with the signal obtained with an unknown sample suspected of containing ligand, one can qualitatively or quantitatively determine the amount of ligand in the unknown. For determinating anti(ligand) the assay is carried out in substantially the same way, except a source of ligand or ligand analog must be provided.

37 Claims, No Drawings

LABEL MODIFIED IMMUNOASSAYS

BACKGROUND OF THE INVENTION

1. Field of the Invention

There is an expanding interest in the ability to determine or monitor small amounts of organic antigenic or haptenic materials. Frequently, the concentrations of interest are micromolar or less. Various techniques have been developed which are capable of isolating and detecting a specific compound, despite the presence of myriad other compounds of similar and different structure.

One group of techniques referred to as competitive protein binding assays or immunoassays depend for their specificity on the use of a receptor, normally an antibody, which is specific for a compound of a particular spatial and polar organization. For these assays it is normally necessary to produce antibodies by injecting antigens or hapten conjugated antigens into a vertebrate to induce the formation of antibodies which may then be harvested. The bleeds which are obtained can be usually purified to separate a globulin concentrate from other proteinaceous materials. To further purify the globulin concentrate to separate the antibodies of interest from other globulins is only difficultly achieved.

While affinity chromatography will provide for some concentration of the desired antibodies, the procedure is normally slow and frequently results in substantial loss of the desired antibodies as well as reduction in the binding constant. That is, those antibodies in the composition which have the strongest binding, frequently cannot be removed from the column. Therefore, most methods have avoided labeling antibodies, since either the antibodies had to be purified to concentrate the antibodies of interest or a large amount of label was introduced associated with proteins which were not involved in the assay. This normally results in a large background signal which inevitably reduces the sensitivity of the assay, unless a physical separation of the extraneous label is incorporated into the procedure.

The alternative has been to label ligand. While labeling of ligand is feasible where the ligand is a simple hapten or the antigens are available in substantially pure form, in those situations where the antigen is only difficultly purifiable, exists in only small amounts, or is labile, labeling of antigens is not feasible for a commercial process.

An assay is therefore desirable which avoids the problems of purification and isolation of both the antibodies of interest and the ligand of interest. In addition, the assay should provide for minimizing introduction of label into the assay medium which produces a signal which interferes with or is additive with the signal which is measured.

2. Brief Description of the Prior Art

Radioimmunoassay is described in two articles by Murphy, J. Clin. Endocr. 27, 973 (1967); ibid 28, 343 (1968). U.S. Pat. No. 3,817,837 teaches a homogeneous enzyme immunoassay. U.S. Pat. Nos. 3,654,090, 3,791,932, 3,850,752 and 3,839,153 teach hetereogeneous enzyme immunoassays. In the agenda for the Ninth Annual Symposium on Advanced Analytical Concepts for the Clinical Laboratory, to be held March 17 and 18, 1977 at the Oakridge National Laboratory, a paper entitled "Phospholipase C-Labeled Antihuman IgG: Inhibition of Enzyme Activity by human IgG," to be presented by R. Wei and S. Riebe is reported. U.S. Pat. Nos. 3,935,074 and 3,998,943 disclose immunoassay techniques involving steric inhibition between two different receptors for different epitopic sites. U.S. Pat. No. 3,996,345 teaches the use of a common receptor, a portion of which is bound to a fluorescer and the remaining portion bound to quencher, whereby the presence of ligand brings the receptors together so as to allow for quenching of fluorescence. Carrico, et al, Anal. Biochem. 72 271 (1976) and Schroder, et al, ibid 72 283 (1976) teach competitive protein binding assays where a label is bonded to a hapten with the label being subject to enzymatic transformation to produce a signal. Antibody bound to the hapten inhibits the approach of enzyme to the label.

SUMMARY OF THE INVENTION

Methods and compositions are provided for use in immunoassays for the accurate determination of a member of an immunological pair i.e. ligands and ligand receptors, at concentrations down to 1 μg/ml or less. With polyepitopic ligands labeled receptors are employed, where the label is capable of modification by modifying reagents. Upon combining polyepitopic ligand, (poly(ligand analog) with haptens) labeled receptor and the modifying reagent(s), a complex is formed between the ligand and the labeled receptor which inhibits the modification of the label by the modifying reagent(s). The label outside the complex is modified, so that the observed signal is from the unmodified label in the complex and any residual signal from the modified label. By comparing the results to known standards, the concentration of ligand can be determined.

When a monoepitopic ligand is involved, a plurality of ligand analogs will be conjugated to a hub nucleus to provide a poly(ligand analog). The poly(ligand analog) will be included with the other reagents, so that a competition exists between the monoepitopic ligand and the poly(ligand analog) for the labeled receptor. The labeled receptor which binds to the poly(ligand analog) will form a complex which inhibits the modification of the label, while free labeled receptor and labeled receptor bound to the ligand will be modified. By employing known standards, the amount of monoepitopic ligand may be determined.

For receptors, the assay is carried out in substantially the same way, except that a source of ligand or ligand analog must be provided.

The compositions can be provided as kits, whereby measured amounts of the labeled antibody, and, where required, ligand or poly(ligand analog), and modifying reagent(s) are provided, particularly as dry powders or concentrated solutions which can be reconstituted as reagent solutions for use in the immunoassays.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Method and compositions are provided for sensitive immunoassays, where reagents can be prepared without cumbersome and difficult purifications and/or isolations of antigens and antibodies. The method will normally involve bringing together an analyte and a labeled receptor (with monoepitopic analytes, a poly(ligand analog) will be employed in addition), so as to form a complex which sterically inhibits the approach of macromolecules to the label. The label is capable of providing a distinctive signal by being exposed to an agent e.g. electromagnetic radiation, usually light, or

chemical reagents. After an appropriate time, a modifying agent is added which is capable of interacting with the label and modifying, preferably reducing, its distinctive signal. The agent interacts with the label and the signal from the assay medium is measured. By comparison of the determined signal to signals from assay media having known amounts of analyte, the concentration of analyte in an unknown sample may be determined.

DEFINITIONS

Analyte—the compound or composition to be measured, which may be a ligand which is mono- or polyepitopic, antigenic or haptenic, a single or plurality of compounds which share at least one common epitopic site or a receptor.

Ligand—any compound for which a receptor naturally exists or can be prepared.

Ligand analog—a modified ligand which can compete with the analogous ligand for receptor, the modification providing means to join a plurality of ligand analogs in a single molecule.

Poly(ligand analog)—a plurality of ligand analogs joined together, normally to a hub nucleus, to provide a compound having a plurality of epitopic sites capable of competing with the analogous ligand for receptor.

Label—a compound or composition capable of providing a detectable signal in conjunction with physical activation (or excitation) or chemical reagents and capable of being modified, so that the particular signal is diminished or increased.

Receptor—Any compound or composition capable of recognizing a particular spatial and polar organization of a molecule i.e. an epitopic site. Illustrative receptors include naturally occurring receptors, antibodies, enzymes or fragments thereof that contain a binding site e.g. Fab, and the like. For any specific ligand, the receptor will be referred to as anti(ligand). The receptor anti(ligand) and its reciprocal ligand form an immunological pair.

Labeled Receptor—receptor having at least one label covalently bonded to it and retaining at least one binding site.

Modifier—a macromolecule capable of physically or chemically interacting with the label to reduce the signal produced by the label.

Complex—a combination of at least one labeled receptor and one polyepitopic ligand (includes poly(ligand analog)), normally in the assay medium there being on the average at least two of one of the components in each of the complexes and frequently a total of four or more of the components bound together.

ASSAY

The subject assay is carried out in an aqueous, normally homogeneous, zone at a moderate pH, generally close to optimum label detection. The assay zone for the determination of analyte is prepared by employing an appropriately buffered aqueous solution, the unknown sample, which may have been subject to prior treatment, labeled receptor, modifier, and as appropriate poly(ligand analog) and ancillary reagents for reacting with the label to produce the detectable signal. For determination of anti(ligand) in the sample, ligand or poly(ligand analog) will normally be added. The assay zone will normally be homogeneous.

In carrying out the assay an aqueous medium will normally be employed. Other polar solvents may also be employed, usually oxygenated organic solvents of from one to six, more usually from one to four carbon atoms, including alcohols, ethers and the like. Usually, these cosolvents will be present in less than about 35 weight percent, more usually in less than about 10 weight percent.

The pH for the medium will usually be in the range from about 5 to 10, more usually in the range from about 6 to 9. Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The particular buffer employed is not critical to this invention, but in individual assays, one buffer may be preferred over another.

Moderate temperatures are normally employed for carrying out the assay and usually constant temperatures during the period of the assay will be employed. The temperatures will normally range from about 10° to 50° C., more usually from about 15° to 40° C.

The concentration of analyte which may be assayed will generally vary from about 10^{-4} to 10^{-15} M, more usually from about 10^{-6} to 10^{-13} M. Considerations such as whether the assay is qualitative, semi-quantitative or quantitative, the particular detection technique and the concentration of the analyte of interest will normally determine the concentration of the other reagents.

Receptor (antiligand) may be a mixture of labeled and unlabeled receptor, generally having from about 5 to 100% of the receptor as labeled receptor. The proportion of unlabeled receptor will depend on the nature of the label, the manner of preparation, the sensitivity of the label detection system and the like. For example, with a fluorescer label, there may be substantial self-quenching when all of the receptor is labeled, so that it is desirable to introduce a significant amount of unlabeled receptor in the assay medium.

Normally, for polyepitopic ligand analytes the concentration of total antiligand based on binding sites will be about 1–50 times either the minimum or maximum concentration of interest based on epitopic sites, usually about 1–10 times and more usually 1–3 times the maximum concentration of interest. For monoepitopic ligand analytes and receptor analytes, based on binding sites, the respective concentrations of poly(ligand analog) and labeled antiligand will have concentrations about equal to the minimum concentration of interest, normally not exceeding the maximum concentration of interest, generally not less than 10^{-4}, more usually not less than 10^{-2} of the minimum concentration of interest. Concentration ranges of interest will generally vary from about 10^{-3} to 10^{-14} g/ml. For monoepitopic analytes and receptor analytes the concentration of total antiligand other than analyte will usually be up to fifty times the concentration of polyligand analog or ligand, more usually up to 10 times, most usually up to three times.

The concentration of modifier will vary widely depending on the nature of the modifier, its effectiveness in modifying the signal, its side reactions and the like. Normally, large excesses of modifier can be used to insure that the rate of modification is rapid and the concentration of modifier is not limiting. Therefore, when the modifier is not a catalyst at least stoichiometric concentrations of modifier will be used and molar excesses of 100 or more may be employed. Where the modifier is an enzyme, concentrations of at least 10^{-2} times Km will usually be employed. For other catalysts, the catalytic concentration will generally range from

about 10^{-2} to 10^{-6} times the minimum or maximum concentration of interest.

The order of addition may vary widely. Normally, the unknown sample and labeled receptor will be combined in an appropriate medium before the introduction of the modifier. When the modifier reversibly modifies the label, the modifier and labeled receptor may be premixed. Depending on the nature of the ancillary reagents, if any, they may be added initially or with or subsequent to the addition of the modifier. After combining the unknown with the labeled receptor and, as appropriate, ligand or poly(ligand analog), the assay medium may be incubated for a sufficient time to form complexes.

The times between the various additions for the assay components and for the immunological reactions which are involved may vary widely, depending upon the particular compounds involved, the mode of addition, the concentrations involved, the binding constants of the receptors, and the like. Normally, times between additions may vary from a few seconds to many hours, usually not exceeding twelve hours, and more usually not exceeding six hours. After adding each component to the assay mixture, different incubation periods before adding the next component or taking the measurement will be involved. Since the ultimate results will be dependent upon the results obtained with standard(s) treated in substantially the same manner, and when possible in the identical manner, the particular mode and periods of time are not critical, so long as significant reproducible differentiations are obtained with varying concentrations of analyte.

Depending upon the choice of assay protocol, the equipment employed and the concentration of analyte involved, assay volumes may be as small as about 1 μl, more usually being at least 25 μl, and will usually not exceed 5 ml, more usually not exceeding about 2 ml.

In particular situations, the subject method allows for the simultaneous determination of two or more analytes, usually not more than about five analytes. By employing labels which give substantially non-interfering signals, each different label can be conjugated to a receptor for a different analyte. This embodiment is readily illustrated with fluorescers. One employs fluorescers which fluoresce by emitting light at different wavelengths. Therefore, a particular wavelength of emitted light would be associated with a particular analyte. The assay would be performed in the normal way for each analyte, except that all the reagents and sample would be included in one assay medium. The assay medium would be irradiated with light of wave lengths which correspond to the absorption bands of the different fluorescers and the amount of fluorescence from each of the fluorescers determined. By appropriate calculations the contribution to the emission spectrum of each of the fluorescers could be determined.

The same technique could be applied with other labels, but for the most part not as conveniently. With enzyme labels, the different enzymes would have to have substrates and products which were not interfering and could be independently detected. The problem of interference between labels and their associated systems will vary to lesser or greater degrees depending upon the particular label and its modifiers.

In determining anti(ligand), the procedure is the same, with the exception indicated previously, but the observed result may be an increase or decrease in the signal depending on the relative proportions of the various components. That is, the anti(ligand) may displace labeled anti(ligand) from the complex or enhance complex formation. Preferably, a protocol is employed where anti(ligand) will displace labeled anti(ligand).

In a preferred embodiment, the modifier is anti(label), that is, a receptor which specifically binds to the label, affecting the label in a variety of ways.

One effect is to inhibit the interaction of a chemical with the label. For example, with an enzyme label, antibodies to enzyme can be prepared which sterically or allosterically inhibit the enzyme. Those enzymes which become bound by anti(enzyme) will be deactivated. Another technique is to employ a label which chemically reacts with the modifier to change the chemical nature of the modifier. For example, with a redox reaction, binding of anti(label) to one of the reactants in the redox reaction will inhibit the approach of the other reactant to the label. If the label is an enzyme substrate, binding by antilabel will inhibit the enzyme catalyzed reaction. Finally as a further illustration, anti(label) may change the environment of the label, so as to change the physical characteristics of the label. With a fluorescent label, anti(fluorescer) will change the light absorption and/or emission characteristics of the fluorescer when bound to the fluorescer. Thus, by irradiating the solution with light within the absorption band of unbound fluorescer, free fluorescer will fluoresce, at a different wave length or efficiency than the bound fluorescer, allowing free and bound fluorescer to be distinguished.

The use of an anti(label) as the modifier has many advantages. It is specific for the label and will generally not be subject to interference from materials normally encountered in samples to be assayed. Furthermore, with the enzyme, the redox and the fluorescer labels, amplification can be achieved in that a single unbound label can be used to cause a plurality of measureable events. Another preferred embodiment is a fluorescent enzyme substrate which is quenched upon interaction with an enzyme.

The measurement of the detectable signal from the label will vary widely depending upon the nature of the label. The measurement will normally involve measuring electromagnetic radiation at a particular wavelength or narrow band of wavelengths in various ranges, such as radiofrequency, ultraviolet, visible, etc., although other measurements may be made e.g. electrical or microcalorimetric. For electromagnetic measurements, the absorption or emission of radiation will be involved.

Depending upon the nature of the label, various techniques may be employed. For the most part, the techniques employed will involve the absorption or emission of electromagnetic radiation. Such techniques may involve fluorescence, chemiluminescence, ultraviolet or visible light absorption, electron spin resonance, and the like.

MATERIALS

The primary components in the subject assay for analyte are: the analyte; the labelled receptor; the modifier; any ancillary reagents for the label; and, as appropriate poly(ligand analog). In addition, in the assay for anti(ligand), ligand or poly(ligand analog) will be added. The different labels allow for great variety in protocols and methods of measurement, although certain techniques will be vastly superior to other techniques. Of particular significance are those techniques

which allow for amplification, that is, where a single event results in the occurrence of a plurality of events. Within this category are redox reactions and transfer reactions, which are enzymatically mediated and result in a variety of products which may be detected in a number of different ways.

ANALYTE

The ligand analytes of this invention are characterized by being monoepitopic or polyepitopic. The polyepitopic ligand analytes will normally be poly(amino acids) i.e. polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations or assemblages include bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes, cell walls, and the like.

For the most part, the polyepitopic ligand analytes employed in the subject invention will have a molecular weight of at least about 1,000, usually 5,000, more usually at least about 10,000. In the poly(amino acid) category, the poly(amino acids) of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight; among the hormones of interest, the molecular weights will usually range from about 5,000 to 60,000 molecular weight.

The wide variety of proteins may be considered as to the family of proteins having similar structural features, proteins having particular biological functions, proteins related to specific microorganisms, particularly disease causing microorganisms, etc.

The following are classes of proteins related by structure:

protamines
histones
albumins
globulins
scleroproteins
phosphoproteins
mucoproteins
chromoproteins
lipoproteins
nucleoproteins
glycoproteins
unclassified proteins, e.g. somatotropin, prolactin, insulin, pepsin

A number of proteins found in the human plasma are important clinically and include:

Prealbumin
Albumin
α_1-Lipoprotein
α_1-Acid glycoprotein
α_1-Antitrypsin
α_1-Glycoprotein
Transcortin
4.6S-Postalbumin
Tryptophan-poor α_1-glycoprotein
α_1X-Glycoprotein
Thyroxin-binding globulin
Inter-α-trypsin-inhibitor
Gc-globulin
(Gc 1-1)
(Gc 2-1)
(Gc 2-2)
Haptoglobin
(Hp 1-1)
(Hp 2-1)
(Hp 2-2)
Ceruloplasmin
Cholinesterase
α_2-Lipoprotein(s)
α_2-Macroglobulin
α_2-HS-glycoprotein
Zn-α_2-glycoprotein
α_2-Neuramino-glycoprotein
Erythropoietin
β-lipoprotein
Transferrin
Hemopexin
Fibrinogen
Plasminogen
β_2-glycoprotein I
β_2-glycoprotein II
Immunoglobulin G (IgG) or γG-globulin
Mol. formula: $\gamma_2\kappa_2$ or $\gamma_2\gamma_2$
Immunoglobulin A (IgA) or γA-globulin
Mol. formula: $(\alpha_2\kappa_2)^n$ or $(\alpha_2\gamma_2)^n$
Immunoglobulin M (IgM) or γM-globulin
Mol. formula: $(\mu_2\kappa_2)^5$ or $(\mu_2\gamma_2)^5$
Immunoglobulin D (IgD) or γD-Globulin (γD)
Mol. formula: $\delta_2\kappa_2$) or $(\delta_2\gamma_2)$
Immunoglobulin E (IgE) or γE-Globulin (γE)
Mol. formula: $(\epsilon_2\kappa_2)$ or $(\epsilon_2\lambda_2)$
Free K and γ light chains
Complement factors:
C'1
C'1q
C'1r
C'1s
C'2
C'3
β_1A
α_2D
C'4
C'5
C'6
C'7
C'8
C'9

Important blood clotting factors include:

TABLE VII

BLOOD CLOTTING FACTORS

International designation	Name
I	Fibrinogen
II	Prothrombin
IIa	Thrombin
III	Tissue thromboplastin
V and VI	Proaccelerin, accelerator globulin
VII	Proconvertin
VIII	Antihemophilic globulin (AHG)
IX	Christmas factor, plasma thromboplastin component (PTC)
X	Stuart-Prower factor, autoprothrombin III
XI	Plasma thromboplastin

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