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ANTIBODY ASSAY FOR AMYLIN

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(57) Claim

1. A monoclonal antibody which binds within the C-terminal end of human amylin but which does not bind to unamidated human amylin.

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(54) Title: ANTIBODY ASSAY FOR AMYLIN

(57) Abstract

A monoclonal antibody which binds at the C-terminal end of human amylin, and assays using such monoclonals for the detection of amylin and amylin analogs.

ANTIBODY ASSAY FOR AMYLIN

Related Application

This application is a continuation-in-part of Phelps, et al. U.S. Serial No. 07/883,754 filed May 15, 1992, entitled "Antibody Assay for Amylin", the contents of which is hereby incorporated by reference in its entirety.

The inventions may have been made with Government support under Small Business Innovative Research Contract No. N43-DK-2-2283 awarded by the National Institutes of Health. The Government may have certain rights in the inventions.

Background of the Invention

This invention relates to antibody assays for amylin.

Amylin is a protein that is expressed in pancreatic islet beta-cells and secreted into the blood in response to nutrient intake. Its structure and biological activities are described in a publication by Amylin Pharmaceuticals, Inc. (formerly, Amylin Corporation), "Science and Corporate Review", October 1991, which is hereby incorporated by reference herein.

There is a need for monitoring amylin levels in Type 1 diabetics (who have lowered or negligible levels of circulating amylin), in Type 2 diabetics and obese individuals (who have excessive levels of circulating amylin), and in other conditions in which amylin levels may

be altered and should be periodically determined or otherwise evaluated. There is also a need for an assay which is specific to amylin (because of its homology to the calcitonin gene-related peptides) and that is sensitive (because of the very small amounts of circulating amylin). There is, furthermore, a need for an assay that can be used to monitor amylin analogs (such as AC-0137) which are being evaluated for use in the treatment of Type 1 diabetics.

Summary of the Invention

Applicant has discovered that antibodies which bind specifically to the C-terminal end of human amylin are particularly advantageous in antibody based assays for detection of human amylin or analogs thereof. In particular, applicant has discovered that monoclonal antibodies to the C-terminal end of human amylin can be selected which recognize the amino acid sequence between residues 30 and 37 inclusive in amylin and, importantly, can differentiate between the amidated C-terminal end of human amylin and the non-amidated C-terminal end. (Non-amidated human amylin (also referred to as "amylin acid") is virtually, if not completely, inactive while human amylin (the 37th amino acid of which is tyrosine-amide) is fully bioactive.). Such antibodies are useful for detecting the presence or amount of an amylin analog in a test sample, and in particular, are useful in a competitive or sandwich assay for amylin.

Thus in a first aspect, the invention features a monoclonal antibody which binds or recognizes at the C-terminal end of human amylin.

By "binds or recognizes" is meant that a positive binding result is obtained using standard assay procedures as described below in Example 1.

The term "monoclonal antibody" refers to a purified antibody produced from a single clone of an antibody producing cell. Such monoclonal antibodies are distinct from polyclonal antibodies which include a plurality of antibodies produced by a plurality of antibody-producing cells in a living organism.

In preferred embodiments, the monoclonal antibody recognizes the C-terminal end of human amylin having the amidated amino acid sequence between amino acids 30 and 37 of amylin.

In a second aspect, the invention features a monoclonal antibody which binds to the amidated C-terminal end of human amylin, and not to the non-amidated C-terminal end. For example, the invention includes the monoclonal antibody ~~termed 7025-27~~ produced by the mouse hybridoma cell line 7025-27.4 deposited with the American Type Culture Collection on May 15, 1992 and assigned the number HB 11045.

In a third aspect, the invention features an assay for detecting the presence or amount of an amylin analog. The assay includes use of a monoclonal antibody which binds to the C-terminal end of human amylin. By way

of example, an assay using a monoclonal antibody for detecting the presence or amount of an amylin analog may comprise the steps of contacting said amylin analog or a sample suspected of containing said analog with said monoclonal antibody, and determining the presence or amount of said amylin analog, wherein said monoclonal antibody binds to the C-terminal end of human amylin. The assay may be a competitive assay or a sandwich assay. In sandwich format the assay includes a second monoclonal or polyclonal anti-amylin antibody, and the first or second antibody is typically detectably labelled.

By "amylin analog" is meant to include not only natural occurring forms of amylin (including human, rat, mouse, cat, and guinea pig amylin), but also proteins which have an amino acid sequence substantially similar to that of amylin but containing one or more deletions or additions of amino acids within that sequence or including substitution of one or more amino acids generally with a conserved amino acid, for example, glycine may be replaced with a valine, or a positively charged amino acid replaced with other positively charged amino acid. Such amylin analogs have similar or identical biological activity to the naturally occurring amylin most closely rated in sequence to that analog. Such analogs are useful in treatment of various diseases or disease states or conditions and the detection of such analogs is useful to determine that the appropriate level of amylin analog is

present within an organism, for example, by removing a test sample from that organism and determining the level of amylin analog within that sample. An amylin analog which may be detected or measured using the inventions described and claimed herein is ^{25,28,29}Pro-h-amylin, also referred to as AC-0137.

In preferred embodiments, the method includes use of a second monoclonal antibody to amylin or a polyclonal antibody to amylin; most preferably the monoclonal antibody which binds to the C-terminal end of human amylin is detectably labeled (e.g., with a radioisotope or a fluorescent or chemiluminescent agent, or with an enzyme); the second monoclonal antibody or polyclonal antibody binds at a location distinct from the C-terminal end of amylin; and the assay is either a competitive antibody assay or a sandwich assay.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

The general methodology and steps of antibody assays are described by Greene, U.S. Patent 4,376,110, entitled "Immunometric Assays Using Monoclonal Antibodies; Antibodies, A. Laboratory Manual, Cold Spring Harbor Laboratory, Chapter 14 (1988); Radioimmunoassay and related

methods", A.E. Bolton and W.M. Hunter, Chapter 26 of Handbook of Experimental Immunology, Volume 1, Immunochemistry, edited by D.M. Weir, Blackwell Scientific Publications, 1986; "Enzyme immunoassays: heterogenous and homogeneous systems", R.M. Nakamura, A. Voller, and D.E. Bidwell, Chapter 27 of Handbook of Experimental Immunology, Volume 1, Immunochemistry, edited by D.M. Weir, Blackwell Scientific Publications, 1986; and Current Protocols in Immunology, Chapter 2, Section I, Edited by John E. Coligan, Ada M., Druisbeek, et al., 1991 all of which are hereby incorporated by reference in their entirety.

Unless historical controls are used, in all such assays (as described below) a control or controls are typically performed, which are designed to give positive and/or negative results, for example, the test may include a known amylin, positive control and a known non-amylin negative control.

Monoclonal and polyclonal antibodies to the amino terminal end of amylin can be produced by standard techniques using either whole amylin or fragments thereof. Antibodies to other regions can similarly be produced. Standard assay formats can be used to assay for amylin analogs. Below are non-limiting examples of the invention. Equivalent procedures will be recognized by those in the art.

Kits including these antibodies are also included in this invention, and include separate containers or vials

containing separate labelled or unlabelled antibodies and reagents and controls as discussed above.

Example 1: Monoclonal Antibody Production to C Terminal End of Amylin

Monoclonal F025-27 recognizes the C-terminal portion of the amylin peptide (residues 30-37) was produced from an outbred strain of mice, ND4. Mice were immunized with a batch of synthetically produced human amylin that had formed a significant number of intermolecular disulfide cross links producing multimers of the amylin sequence (these links were formed spontaneously over time). This peptide source was administered to the mice in Complete Freund's adjuvant followed by subsequent boosts in Ribi's adjuvant at three week intervals. Each mouse received 30-50 ug of peptide per dose. One out of five mice responded to the immunization protocol with positive serum titers against human amylin, and the spleen from this mouse was fused to the myeloma fusion partner P3/X63-Ag8-6.5.3 by a modification of the method of Oi and Herzenberg, 17 Selected Methods in Cellular Immunology, ed. Barbara Mishell and Stanley Shiigi, 1980, W.H. Freeman and Co.). Positive clones were selected by radioimmunoassay using standard techniques. Hybridoma antibody in culture supernatants was immobilized on Dynatech Immulon-2 wells coated with sheep anti-mouse antisera and subsequently blocked with 1% nonfat dry milk. Positive clones were

detected by the binding of ^{125}I-human amylin labeled by chloramine T iodination. All positive clones were expanded and three clones were frozen. Two out of three clones had similar reactivity with amylin peptide fragments, but only one clone (F025-27) remained stable and was successfully subcloned. It was discovered that this antibody binds to the C-terminal end of amidated human amylin but not non-amidated amylin.

Other methods to obtain a clone expressing an antibody to the C-terminal portion of the amylin molecule is to immunize with a C-terminal fragment. The human amylin fragment glycine cysteine glycine and amino acids 28-37 of amylin (termed CGG28-37-NH_2) was conjugated to thyroglobulin using succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and used to immunize ND4, Balb/C and A/J mice. Only A/J mice (5/5) responded with positive serum titers when screened with ^{125}I-human amylin labeled by chloramine T iodination although all mice responded to the immunizing fragment. Mice with positive serum titers to intact amylin can be fused and screened as outlined above to yield clones binding to the C-terminal portion of the molecule (residues 28-37).

Example 2: Sandwich Assay Using Radiolabelled F025-27

Either monoclonal antibodies F002-2.18 or F024-4.4 (which recognize epitopes on amylin distinct from the C-terminal end, and were produced using standard techniques

such as described above using human amylin cross-linked to thyroglobulin with glutaraldehyde), or other monoclonals or polyclonal antisera can be used to generate a sandwich assay (IRMA) that quantitates amylin. Dynatech Immulon-2 wells are coated with F002-2.18 or F024-4.4 by overnight incubation of antibody at 20 μg/ml in carbonate buffer, pH9. Wells are subsequently blocked with 1% nonfat dry milk for one hour at room temperature (about 20°C). Sample or standard (50 μl) containing amylin (0-750 pM) is added to the well and incubated for three hours at room temperature. Wells are washed with phosphate buffered saline (PBS), 0.1% Tween-20 detergent, and ^{125}I-F025-27 (200,000 cpm/50 μl) is added to each well and incubated an additional three hours at room temperature. Wells are washed with PBS, 0.1% Tween-20 detergent and counted in a Pharmacia LKB gamma counter.

Example 3: Sandwich Assay Using Enzyme-Labelled F025-27

As in Example 2, monoclonal antibodies F002-2.18 or F024-4.4 or other monoclonal antibodies or polyclonal antisera can be used to generate a sandwich assay (IEMA) that quantitates amylin. Dynatech Immulon-2 wells are coated with F002-2.18 or F024-4.4 by overnight incubation of antibody and 20 μg/ml in carbonate buffer, pH9. Wells are subsequently blocked with 1% nonfat dry milk for one hour at room temperature (about 20°C). Sample or standard (50 μl) containing amylin (0-750 pM) is added to the well

and incubated for three hours at room temperature. Cells are washed with phosphate buffered saline (PBS), 0.1% Tween-20 detergent, and F025-27 conjugated to alkaline phosphatase is added to each well and incubated an additional three hours at room temperature. Wells are washed with PBS, 0.1% Tween-20 detergent and enzyme activity is detected using the fluorescent substrate, 4-methyl feryl phosphate and signal is read in a fluorescent plate reader (IDEXX or Dynatech).

Example 4: Comparison of IRMA and IEMA for Amylin

The Example 2 IRMA and the Example 3 IEMA for human amylin were validated and compared to one another.

The average Minimum Detectable Concentration (MDC) for the amylin IRMA was 2.07 pM. The mean intra-assay variability was 6.7%. The inter-assay variability of 8 clinical samples was 17.8%. Quantitation of amylin controls in the IRMA is shown below:

Control	Mean
Low	5.4 pM
Mid	32.4 pM
High	110.4 pM

The average MDC for the amylin IEMA was 1.4 pM. The mean intra-assay variability was 6.3%. The inter-assay variability for the same 8 clinical samples referenced above was 16.2%. Quantitation of amylin controls in the IEMA is shown below:

Control	Mean
Low	5.8 pM
Mid	28.6 pM
High	107.7 pM

Example 5: IRMA and IEMA Sandwich Assays for AC-0137

Assays for AC-0137 were prepared and carried out as described in Example 2 (IRMA) and Example 3 (IEMA), and compared to one another in an assay validation study.

The average MDC for the AC-0137 IRMA was 9.3 pM. The mean intra-assay variability was 7.5%. For clinical samples tested, the inter-assay variability was 16.6%. Quantitation of AC-0137 controls in the IRMA is shown below:

Control	Mean
Low	14.4 pM
Mid	55.0 pM
High	147.6 pM

The average MDC for the AC-0137 IEMA was 1.1 pM. The mean intra-assay variability was 3.5% and the inter-assay variability (for clinical samples) was 16.7%. Quantitation of AC-0137 controls in the IEMA is shown below:

Control	Mean
Low	13.2 pM
Mid	58.8 pM
High	239.3 pM

Deposit

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whichever is the longer, and its responsibility to notify the depository of the issuance of such a patent, at which time the deposit will be made irrevocably available to the public. Until that time the deposit will be made available to the Commissioner of Patents under the terms of 37 C.F.R. Section 1.14 and 35 U.S.C. Section 122.

Other embodiments are within the following claims.

The claims defining the invention are as follows:

1. A monoclonal antibody which binds within the C-terminal end of human amylin but which does not bind to unamidated human amylin.

2. A monoclonal antibody according to claim 1 which binds within the C-terminal end of human amylin, wherein said C-terminal end comprises the amino acid sequence between amino acids 28 and 37.

3. A monoclonal antibody according to claim 2 wherein said C-terminal end comprises the amino acid sequence between amino acids 30 and 37.

4. A monoclonal antibody according to claim 1 wherein said monoclonal antibody binds within said C-terminal end of said human amylin, but does not bind within the remainder of the sequence of said human amylin.

5. A monoclonal antibody according to claim 2 wherein said monoclonal antibody binds within said C-terminal end of said human amylin, but does not bind within the remainder of the sequence of human amylin.

6. A monoclonal antibody according to claim 3 wherein said monoclonal antibody binds within said C-terminal end of said human amylin, but does not bind within the remainder of the sequence of human amylin.

7. The monoclonal antibody produced by the hybridoma number HB 11045 deposited at the American Type Culture Collection.

8. An assay for determining the presence or amount of amylin or an amylin analog, said amylin analog having a C-terminal amide, in a sample suspected of containing said amylin or amylin analog, comprising the steps of:

(a) contacting said sample suspected of containing amylin or amylin analog with a monoclonal antibody according to any of claims 1 to 7;

(b) contacting positive and/or negative control samples with a monoclonal antibody according to any one of claims 1 to 7; and

(c) determining the presence or amount of said amylin or amylin analog.

9. The assay according to claim 8 wherein said assay is a competitive assay.

10. The assay according to claim 8 wherein said assay is a sandwich assay.

11. The assay according to claim 8 wherein said monoclonal antibody is detectably labelled and wherein said labelled monoclonal antibody binds to said amylin or to said amylin analog and is used to determine the presence or amount of said amylin or amylin analog.

12. An assay for determining the presence or amount of amylin or an amylin analog, said amylin analog having a C-terminal amide, in fluid sample suspected of containing said amylin or amylin analog, comprising the steps of;

(a) contacting said sample with a measured amount of a first antibody directed to amylin to form a soluble complex of said first antibody and said amylin or amylin analog present in said sample, said first antibody being labelled;

(b) contacting said soluble complex with a second monoclonal antibody directed to amylin, said second monoclonal antibody being bound to a solid carrier, to form a ternary complex of said first antibody, said amylin or amylin analog and said second antibody bound to said solid carrier;

(c) separating said solid carrier from said sample and unreacted labelled first antibody;

(d) measuring either the amount of labelled first antibody associated with said solid carrier or the amount of unreacted labelled first antibody; and

(e) relating the amount of labelled antibody with the amount of labelled antibody measured for a control sample prepared in accordance with steps (a) to (d), said control sample being known to be free of said amylin or amylin analog, to determine the presence of said amylin or amylin analog in said fluid sample, or relating the amount of labelled antibody measured with the amount of labelled antibody measured for samples containing known amounts of said amylin or amylin analog prepared in accordance with steps (a) to (d) to determine the amount of said amylin or amylin analog in said fluid sample, said first or second antibody being according to any one of claims 1 to 7.

13. An assay for determining the presence or amount of amylin or an amylin analog, said amylin analog having a C-terminal amide, in a fluid sample suspected of containing said amylin or amylin analog, comprising the steps of:

(a) contacting said sample with a first antibody directed to said amylin or amylin analog, wherein said first antibody is bound to a solid carrier, to form a complex of said amylin or amylin analog present in said sample and said first antibody;

(b) separating unreacted sample from said complex;

(c) contacting said complex with a measured amount of a second antibody directed to said amylin or amylin analog, wherein said second antibody is labelled;

(d) measuring either the amount of labelled second antibody associated with said complex or the amount of unreacted labelled second antibody; and

(e) relating the amount of labelled antibody with the amount of labelled antibody measured for a control sample prepared in accordance with steps (a)-(d), said control sample being known to be free of said amylin or amylin analog, to determine the presence of said amylin or amylin analog in said fluid sample, or relating the amount of labelled antibody measured with the amount of labelled antibody measured for samples containing known amounts of said amylin or amylin analog prepared in accordance with steps (a)-(d) to determine the amount of said amylin or amylin analog in said fluid sample, said first or second antibody being according to any one of claims 1 to 7.

14. An immunometric assay to determine the presence or amount of amylin or an amylin analog, said amylin analog having a C-terminal amide, in a sample suspected of containing said amylin or amylin analog, wherein a monoclonal antibody according to any one of claims 1 to 7 is employed.

15. An assay for determining the presence or amount of amylin or an amylin analog, said amylin analog having a C-terminal amide, in a sample suspected of containing said amylin or amylin analog, comprising the steps of:

(a) contacting said sample with a known quantity of added labelled amylin or amylin analog;

(b) contacting said sample with a monoclonal antibody according to any one of claims 1 to 7; and

(c) determining the amount of labelled amylin or amylin analog bound to said monoclonal antibody or the amount of labelled amylin or amylin analog which is not bound to said monoclonal antibody.

16. The assay of claim 8 wherein said amylin analog is 25, 28, 29Pro-human amylin.

17. The assay of claim 11 wherein said labelled monoclonal antibody is radioactively labelled.

18. The assay of claim 11 wherein said labelled monoclonal antibody is enzyme labelled.

19. A kit comprising a monoclonal antibody which binds to human amylin but which does not bind to unamidated human amylin.

20. A kit comprising a monoclonal antibody which binds to human amylin but which does not bind to unamidated human amylin and which binds within the C-terminal end of human amylin, wherein said C-terminal end comprises the amino acid sequence between amino acids 28 and 37; and suitable control samples.

21. A kit comprising a monoclonal antibody which binds to human amylin but which does not bind to unamidated human amylin and which binds within the C-terminal end of human amylin, wherein said C-terminal end comprises the amino acid sequence between amino acids 30 and 37; and suitable control samples.

22. A kit according to claim 20 or 21 wherein said monoclonal antibody binds within said C-terminal end of said human amylin, but does not bind within the remainder of the sequence of said human amylin.

23. The assay of claim 12 wherein said amylin analog is 25, 28, 29Pro-human amylin.

24. The assay of claim 13 wherein said amylin analog is 25, 28, 29Pro-human amylin.

25. The assay of claim 14 wherein said amylin analog is 25, 28, 29Pro-human amylin

26. The assay of claim 15 wherein said amylin analog is 25, 28, 29Pro-human amylin.

27. The assay of claim 12 wherein said labelled monoclonal antibody is radioactively labelled.

28. The assay of claim 13 wherein said labelled monoclonal antibody is radioactively labelled.

29. The assay of claim 12 wherein said labelled monoclonal antibody is enzyme labelled.

30. The assay of claim 13 wherein said labelled monoclonal antibody is enzyme labelled.

31. The assay of claim 8 wherein said amylin is human amylin.

32. The assay of claim 12 wherein said amylin is human amylin.

33. The assay of claim 13 wherein said amylin is human amylin.

34. The assay of claim 14 wherein said amylin is human amylin.

35. The assay of claim 15 wherein said amylin is human amylin.

36. A monoclonal antibody which binds to human amylin but which does not bind to unamidated human amylin, substantially as hereinbefore described with reference to the Examples.

Dated 25 March, 1997
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