Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2437675 A1 2005/02/20

(21) 2 437 675

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(22) Date de dépôt/Filing Date: 2003/08/20

(41) Mise à la disp. pub./Open to Public Insp.: 2005/02/20

(51) Cl.Int.^{7}/Int.Cl.^{7} G01N 33/68, G01N 33/569, G01N 33/543, G01N 33/53

(71) Demandeurs/Applicants:
CASHMAN, NEIL, CA;
LEHTO, MARTY, CA

(72) Inventeurs/Inventors:
CASHMAN, NEIL, CA;
LEHTO, MARTY, CA

(74) Agent: BERESKIN & PARR

(54) Titre : METHODES DE DETECTION DE PROTEINE PRION
(54) Title: METHODS OF DETECTING PRION PROTEIN

(57) Abrégé/Abstract:
The invention relates to an epitope protection assay for use in diagnosis, prognosis and therapeutic intervention in diseases involving polypeptide aggregation such as prion infections. The methods of the invention first block accessible polypeptide target epitope with a protecting agent. After denaturation of the polyeptide, a detecting agent is used to detect protein with target epitope that was inaccessible during contact with the protecting agent.

Abstract

The invention relates to an epitope protection assay for use in diagnosis, prognosis and therapeutic intervention in diseases involving polypeptide aggregation such as prion infections. The methods of the invention first block accessible polypeptide target epitope with a protecting agent. After denaturation of the polyeptide, a detecting agent is used to detect protein with target epitope that was inaccessible during contact with the protecting agent.

TITLE: Methods of Detecting Prion Protein

FIELD OF INVENTION

The invention relates to an epitope protection assay for use in diagnosis, prognosis and therapeutic intervention in diseases involving polypeptide aggregation such as prion infections.

BACKGROUND OF THE INVENTION

Prion diseases have become a major health concern since the outbreak of BSE or "Mad Cow Disease" (reviewed above, refs 1,2). BSE was first discovered in the United Kingdom but has now spread to many other countries in Europe and Japan. In the UK alone there has been close to 180,000 cases of BSE, which resulted in the destruction of cattle and possible infection of close to 2 million head. The total cost estimated to the UK was in excess of $2.5 billion. BSE is believed to be transmitted among cattle through feed that contains polypeptides rendered from infected cattle, and it is thought to be transmitted to humans through eating beef or other cattle products from infected animals.

Emerging Prion Diseases.

The prion diseases are a group of rapidly progressive and untreatable neurodegenerative syndromes, neuropathologically characterized by spongiform change, neuronal cell loss, gliosis, and brain accumulation of abnormal amyloid polypeptide. Human prion diseases include classical Creutzfeldt-Jakob disease (CJD), which has sporadic, iatrogenic, and familial forms. Since 1996, a "new variant" of CJD (vCJD) has been identified in the United Kingdom, France, the Republic of Ireland, Hong Kong, Italy, the United States, and Canada [1,2]. Variant CJD is capable of killing individuals as young as age 14 with unknown incubation period. There is little doubt that vCJD is a human form of bovine spongiform encephalopathy (BSE)[3]. The primary epidemic from consumption of contaminated cattle tissue has affected about 120 individuals at present [4], with the number of new cases increasing by 30% per year [2].

The spectre of vCJD "secondary epidemics" through blood, blood products, surgery, dentistry, vaccines, and cosmetics is of great concern [2,4]. Detection of blood prion infectivity in experimental BSE/vCJD infections of mice and sheep [5-7] suggests a special risk exists for the transmission of vCJD through blood and blood products. Canada and the United States have recently expanded vCJD blood donor deferrals to all countries in Western Europe.

Although sheep scrapie has been known for centuries, the most important animal prion disease at present is BSE. More than 173,000 cattle, primarily from Britain, have developed symptomatic BSE, and as many as a million have entered the food supply undetected [8]. BSE is now being increasingly reported in cattle which were "born after the ban" in 1996 of food supplementation with meat and bone meal, suggesting that alternate routes may exist to keep the epidemic from being readily extinguished. Another troubling issue is the possible transmission of BSE to sheep, which may expose additional human populations to the BSE/vCJD prion strain [5,9]. A recent report showed that prions can replicate in certain muscle groups [10], indicating a potential risk in tissues previously considered safe for human consumption.

Chronic wasting disease (CWD) of captive and wild cervids (deer and elk) represents another newly emergent animal prion disease in North America [11,12], whose impact on human health is yet unknown. It is apparent that newly-recognized prion diseases pose a threat to the safety of foods, blood products, and medical-surgical treatments.

Prions: Atypical Pathogens.

Newly emergent prion diseases, and the polypeptide-only nature of prions, have created serious medical, veterinary, and economic challenges worldwide. To date, the only commercialised tests for prion infection have been based on post-mortem brain samples. No biochemical test exists to detect prions in the blood of infected animals, despite detection by experimental transmission studies. The development of sensitive and specific diagnostic tests for prion infection is a challenging task, in part due to the unusual nature of the prion infectious agent. The infectious agents that

transmit the prion diseases differ from other pathogens in that no nucleic acid component has been detected in infectious materials [13]. According to the prion theory developed by Nobel Laureate Dr. Stanley Prusiner, infectivity resides in PrP^{Sc}, a misfolded conformational isoform of the near-ubiquitous normal cellular prion polypeptide PrP^{C} [14]. PrP^{Sc} is indeed the most prominent (or perhaps sole) macromolecule in preparations of prion infectivity, and minimally appears to be a reliable surrogate for prion infection. PrP^{Sc} is partially resistant to protease digestion, poorly soluble, and exists in an aggregated state, in contrast to the protease sensitive, soluble, monomeric isoform PrP^{C} [15-17].

PrP^{Sc} is derived from its normal cellular isoform (PrP^{C}), which is rich in α-helical structure, by a posttranslational process involving a conformational transition. While the primary structure of PrP^{C} is identical to that of PrP^{Sc}, secondary and tertiary structural changes are responsible for the distinct physicochemical properties of the two isoforms.

One of the difficulties in assessing the safety of food or blood products from potentially infected humans with prions is the lack of an accurate diagnostic test for blood or other accessible biosamples. Currently, there are no diagnostic tests that can be applied for screening live animals, humans, blood or blood products at an early stage. This also provides a further problem in organ transplantation, adding unknown risk to organ recipients. Therefore, as a preventative measure, countries such as the UK no longer source plasma from its inhabitants. The risk of spreading prion diseases has affected other countries as well. For example, the United States and Canada do not accept blood donations from individuals who have resided in the UK or France for more than 6 months.

Currently, the diagnosis of vCJD can only be confirmed following pathological examination of the brain at autopsy or biopsy. Some complimentary strategies in early CJD detection include electroencephalograms (EEG), magnetic resonance imaging (MRI) scans, and cerebrospinal fluid (CSF) tests, which may be useful "surrogate" or "proxy" markers. The absence of a

"direct test" for prion infection stands in stark contrast to conventional infectious agents, such as viruses and bacteria.

Some tests that are in the process of being commercialized are based on surrogate markers of infection which are "once removed" from actual infectious prions.

PrP protease resistance, the basis of most commercially available diagnostic tests for prion disease. In the current methodologies, a sample of brain is removed and digested with proteases, enzymes that can digest PrP^{C}, but leave a protease-resistant core of PrP^{Sc}. The protease-resistant fragment of PrP^{Sc} is then detected by immunoblotting (as in the Prionics test) or by capture ELISA (as in the BioRad and Enfer tests, and in a new test from Prionics). However, digestion with proteases is cumbersome and variable, leading to false negatives and positives. Moreover, there are some prion strains which are reported to contain PrP^{Sc} which is infectious and aggregated, but which is not protease resistant. Protease-sensitive PrP^{Sc} also predominates early in infection and in cross-species transmission of disease.

Detection of protease-resistant PrP fragments is also the basis of a urine diagnostic test pioneered by Dr. Ruth Gabizon of Hadadassa Hospital in Jerusalem, and confirmed by Dr. Yusei Shiga of Tohoku University in Sendai, Japan, which is being commercially developed by Prionics. However, detection of protease-resistant PrP in urine is subject to the same limitations as the post-mortem brain test, and has the additional disadvantage of requiring precipitation from large volumes of urine, and poor sensitivity (for example, only detecting PrP^{Sc} in late stages of the disease, not pre-symptomatically).

SUMMARY OF THE INVENTION

The inventors have recently discovered the "epitope protection assay" (EPA), a novel method that promises to yield sensitive and specific antemortem detection of prions in blood and other accessible tissues and fluids. In prion

diseases, the normal cellular monomeric prion polypeptide PrP^{C} undergoes refolding to an abnormal, aggregated isoform, generically designated PrP^{Sc}.

According to the invention, the methods are useful where a target epitope is accessible in either one of a disease protein or a wild type protein and inaccessible in the other.

Our prion detection method consists of:

1) reacting a sample with a chemical modifying agent;
2) disaggregating and denaturing the sample; and
3) probing with antibodies against prion polypeptide epitopes.

PrP^{C} is rendered "invisible" in the assay, because epitopes on the monomeric molecules are blocked to antibody recognition by the chemical modifying agent, whereas molecules of PrP^{Sc} are "protected" from chemical modification by virtue of being sequestered within aggregates.

The method of the invention has many advantages over existing technology. In one embodiment, the invention is referred to as "EPA", which is a simple, efficient method for detecting aggregated PrP^{Sc}, the pathogenic molecule which constitutes the infectious particle.

EPA lends itself to high-throughput robotic-capable platforms. For example, EPA is not dependent on PrP protease resistance, the basis of most commercially available diagnostic tests for prion disease. Epitope protection technology does not require a protease digestion step, which may make it more sensitive to early infection. Certainly, the absence of a protease digestion step permits EPA to be more amenable to high-throughput robotic platforms.

According to the invention, the methods are useful where a target epitope is accessible in either one of a disease protein or a wild type protein and inaccessible in the other.

The invention includes a method of detecting whether a candidate polypeptide including a target epitope is in a wildtype conformation or a non-wildtype conformation, comprising:

contacting the polypeptide with a protecting agent that selectively blocks accessible target epitope, wherein in one of the non-wildtype conformation or the wildtype conformation, the target epitope is accessible and reacts with the protecting agent, and wherein in the other conformation, the target epitope is inaccessible and does not react with the protecting agent;

removing unreacted protecting agent from contact with the polypeptide;

next modifying the candidate polypeptide to convert any inaccessible target epitope to accessible target epitope;

next contacting the polypeptide with a detection agent that binds selectively to target epitope that was converted from inaccessible target epitope to accessible target epitope, wherein binding between detection agent and converted target epitope indicates that prior to conversion the candidate polypeptide was in a conformation in which the target epitope was inaccessible and wherein lack of binding between the detection agent and the target epitope indicates that the polypeptide was in a conformation in which the target epitope was inaccessible, thereby indicating whether the polypeptide was in a wildtype conformation or a non-wildtype conformation.

The invention also includes a method of detecting whether a candidate polypeptide including a target epitope is in a wildtype conformation or a non-wildtype conformation, comprising:

contacting the polypeptide with a protecting agent that selectively blocks accessible target epitope, wherein in the wildtype conformation, the target epitope is accessible and reacts with the protecting agent, and wherein in the non-wildtype conformation, the target epitope is inaccessible and does not react with the protecting agent;

removing unreacted protecting agent from contact with the polypeptide;

next modifying the candidate polypeptide to convert any inaccessible target epitope to accessible target epitope;

next contacting the polypeptide with a detection agent that binds selectively to target epitope that was converted from inaccessible target epitope to accessible target epitope, wherein binding between detection agent and converted target epitope indicates that the candidate polypeptide was in a non-wildtype conformation and wherein lack of binding between the detection agent and the target epitope indicates that the polypeptide was in a wildtype conformation.

The invention also includes a method of detecting whether a candidate polypeptide including a target epitope is in a wildtype conformation or a non-wildtype conformation, comprising:

contacting the polypeptide with a protecting agent that selectively blocks accessible target epitope, wherein in the non-wildtype conformation, the target epitope is accessible and reacts with the protecting agent, and wherein in the wildtype conformation, the target epitope is inaccessible and does not react with the protecting agent;

removing unreacted protecting agent from contact with the polypeptide;

next modifying the candidate polypeptide to convert any inaccessible target epitope to accessible target epitope;

next contacting the polypeptide with a detection agent that binds selectively to target epitope that was converted from inaccessible target epitope to accessible target epitope, wherein binding between detection agent and converted target epitope indicates that the candidate polypeptide was in a wildtype conformation and wherein lack of binding between the detection

agent and the target epitope indicates that the polypeptide was in a non-wildtype conformation.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the invention are described in relation to the drawings in which:

Fig. 1. PrP in acid treated brain homogenate is protected from modification by peroxynitrite

Mock or acid treated human brain homogenate was treated with increasing concentrations of peroxynitrite (ONOO) and then subjected to immunoblotting with 3F4(panel A) or 6H4 (panel B). Effect of peroxynitrite on the 3F4 (C) and 6H4 (D) epitope in mock (□) and acid treated (●) brain homogenate. Immunoblot films were scanned and band intensities determined by Unscanit software. The results are the combined relative intensities of 3 separate experiments.

Fig. 2. PrP in scrapie infected hamster brain is protected from modification by peroxynitrite

(**A**) Effect of peroxynitrite treatment on the 6H4 epitope in scrapie infected hamster brain. (**B**) The blot in (**A**) was scanned and relative band intensities determined using Unscanit software. (●) Scrapie infected hamster brain. (□) Normal hamster brain.

Fig. 3 Protection from peroxynitrite induced modification is due to aggregation in acid treated brain

(**A**) Effect of peroxynitrite on the immunoprecipitation (IP) of PrP in mock and acid treated brain homogenate. Brain homogenate was treated with 10 mM peroxynitrite followed by incubation for 2 h at RT with (+) or without (-) 2.5 M guanidine hydrochloride (Gu). The resulting samples were immunoprecipitated with 6H4 or 3F4. More PrP is precipitated in the acid treated sample following treatment with peroxynitrite + Gu whereas in the mock sample, Gu has no effect. This suggests that Gu is able to break up aggregated PrP in the acid sample that is protected from destruction by peroxynitrite. (**B**) Effect of peroxynitrite on PrP in mock and acid treated brain homogenate as measured by ELISA. Brain homogenate was treated with increasing concentrations of peroxynitrite followed by 2.5 M Gu. Following a 10-fold dilution, the samples were analyzed by sandwich ELISA with 6H4 as the capture Ab and 3F4 as the detection Ab. Similar to the immunoblot and IP data, the results suggest that misfolded PrP is protected from destruction by peroxynitrite treatment, possibly due to aggregation.

DETAILED DESCRIPTION OF THE INVENTION

The current invention provides a useful method for the detection of a disease related polypeptide counterpart of a normal cellular polypeptide which forms aggregates or otherwise leads to the obscuration of one or more epitopes that are not obscured in the normal polypeptide. In one embodiment, the method of the invention is applied to the detection of PrP^{Sc} in plasma, serum, urine or other biological sample.

"**epitope**" refers to a portion of an antigen which is recognized by and bound by an antibody. Preferably, the epitope is a linear epitope on a polypeptide typically includes 6 to 10 contiguous amino acids that are recognized and bound by an antibody. A conformational epitope includes non-contiguous amino acids. Sometimes confomational epitopes can r-establish themselves by partial refolding on the immunoblot membrane.The antibody recognizes only the 3-dimensional structure. When a protein molecule is folded into a

three dimensional structure the amino acids forming the epitope are positioned in a manner that permits the antibody to recognize and bind to the amino acids. In an unfolded (denatured), protein only the linear epitope is recognized and bound by the antibody. Since the protein is unfolded prior to contact with the detection agent, the inaccessible epitope will typically be a linear epitope.

"**protecting agent" or "blocking agent"** refers to an agent that covalently modifies and destroys epitope reactivity, for example on an amino acid side group within a linear epitope, so that the epitope is prevented from binding to detection agent (usually but not always an antibody). An example of a blocking agent is peroxynitrite. Other examples would include hydrogen peroxide, diethyl pyrocarbonate, 4-hydroxynonenal (4HNE) and diazerine, Chemical modifying agents that completely saturate accessible amino acids critical for epitope recognition in native conditions are most useful in the applications of epitope protection technology.

"**inaccessible epitope**" means that target epitope modification by the chemical blocking agent is prevented or reduced either by differential misfolding relative to the wild type polypeptide or by aggregation of misfolded polypeptide;

"**detection agent**" refers to an agent that binds to epitope and which may be detected, such an antibody specific for prion polypeptide epitopes that can be used to probe the sample containing the polypeptide. The detection agent is used after the polypeptide is unfolded such that the detection agent only has the opportunity to bind to unblocked epitopes.

"wildtype folded conformation" refers to the wild type, folded conformation of protein in a non-disease or non-disorder state.

"misfolded conformation" refers to the folded conformation of polypeptide in a disease or disorder state where the conformation differs from the wild type

conformation. The difference in conformation is as a result of differential folding. The differential folding may cause protein aggregation.

"**wildtype conformation**" refers to the wild type conformation of protein in a non-disease or non-disorder state.

"**non-wildtype conformation**" refers to the conformation of polypeptide in a disease or disorder state where the conformation differs from the wild type conformation. The difference in conformation may be as a result of differential folding, polypeptide aggregation or differential post-translational modification compared to the wild type polypeptide. In the case of polypeptide aggregation, the aggregation may prevent accessibility of the epitope rather than the changed conformation.

The present inventors have found that treatment of recombinant mouse prion polypeptide (rmPrP) at low pH in the presence of low concentrations of denaturants causes the polypeptide to acquire increased beta-sheet content, reminiscent of the misfolded disease-associated prion polypeptide isoform, PrP^{Sc}. This conversion of rmPrP is associated with increased solvent accessibility of tyrosine side chains. The inventors have found that treatment of normal brain homogenate with acid and denaturants causes PrP to become detergent insoluble. In order to probe the surface accessibility of tyrosines and other residues in normal and misfolded PrP^{C}, normal and acid-misfolded human brain tissue was treated with the chemical nitrating compound peroxynitrite. Peroxynitrite treatment of brain tissue caused a reduction in the binding of the anti-PrP antibodies 3F4 and 6H4 as measured by immunoblotting, immunoprecipitation and ELISA. Peroxynitrite-induced epitope blocking was more pronounced on normal brain PrP than on misfolded PrP, suggesting a protective effect of aggregation. Similar findings were observed in normal and scrapie-infected hamster brain, in which 3F4 and 6H4 epitopes of scrapie brain PrP were partially protected from peroxynitrite-induced modification. Immunoprecipitation of peroxynitrite-treated brain with anti-nitrotyrosine antibodies suggests that either PrP is

nitrated on tyrosine residues or another polypeptide in proximity to PrP is nitrated and coimmunoprecipitates PrP.

The invention includes a method of determining polypeptide aggregation, including but not limited to PrP^{Sc}, comprising:

reacting said sample with a chemical modifying agent where such agent could be, but not limited to, peroxynitrite

disaggregating and denaturing the chemically modified sample with heat or detergent

probing with antibodies specific for prion polypeptide epitopes.

Identifying prion conversion inhibitors

Since the invention is useful for detecting differences between polypeptides, the invention further includes an assay for evaluating whether a candidate compound is capable of inhibiting or stabilizing prion conversion. The invention also includes compounds for inhibiting or stabilizing prion conversion identified by the methods described in the application. Decreased protein conversion to an intermediate prion protein substrate or PrP^{Sc} indicates that the candidate compound is useful for treating prion disease.

The assays of the invention may be used to screen candidate compounds to determine if they inhibit PrP^{Sc} formation. Protein may be contacted with a candidate compound *in vivo* or *in vitro* and then used in the methods of the invention to determine if wild type protein has been converted to PrP^{Sc} or if PrP^{Sc} has been converted to wild type protein.

Therefore, the invention also provides methods for identifying substances that inhibit conversion to PrP^{Sc} (eg. prion protein conversion from wild type protein or intermediate to PrP^{Sc}) comprising the steps of:

(a) reacting a protein and a candidate substance, and

(b) determining whether the protein has been converted to PrP^{Sc} using the methods of the invention.

Similar methods may also be performed to identify compounds which stabilize the wildtype prion state, or bind to PrP^{Sc} and block conversion of recruitable PrP isoforms.

Another aspect of the invention provides a method of identifying substances which reverse PrP^{Sc} formation comprising the steps of:

(a) reacting a protein and a candidate substance, and

(b) determining whether the PrP^{Sc} has been converted to wild type protein using the methods of the invention.

Biological samples and commercially available libraries may be tested for substances such as proteins or small organic molecules that bind to a protein. Inhibitors are preferably directed towards specific domains of prion protein. To achieve specificity, inhibitors should target the unique sequences and or conformational features of prion protein.

Prion protein conversion may be periodically monitored in a subject over time (eg. at a first time and a second time at least a week or at least a month after the first time) to identify, for example, increased or decreased levels of PrP^{C} or increased or decreased levels of in the subject. The methods of the invention may also be used to measure a subject's level of PrP^{C} or PrP^{Sc} to determine the subject's response to drug therapy. Decreasing levels of prion protein in the subject over time indicate a positive response to drug therapy.

Since many neurological diseases are associated with aggregated proteins, similar diagnostic methods are useful for these diseases and their aggregated proteins: amyotrophic lateral sclerosis (superoxide dismutase 1), Alzheimer's disease (amyloid beta), Parkinson's disease (alpha synuclein), Huntington's disease (Huntingtin), and others diseases involving abnormal protein folding, aggregation or post-translational modification. Such a test would work in the spinal fluid in addition to peripheral blood. In Alzherimer' disease two inaccessible epitopes 6E10 and 4G8 are present and these may be detected using the methods described in this application with an anti-6E10 or anti-4G8 antibody (detection agent) known in the art.

All such assays could be adapted and optimised to a simple high-throughput platform.

EXAMPLES

Example 1

Peroxynitrite reacts differently with PrP in normal and acid treated or scrapie brain homogenate

When brain homogenate is incubated at pH 3.5 in the presence of guanidine, PrP becomes detergent insoluble and is more suspectible to misfolding to a PK-resistant isoform in the presence of PrP^{Sc} (5). This acid treated PrP is a 'model prion' which is partially misfolded and/or aggregated resembling characteristics of PrP^{Sc}. When mock or acid treated brain homogenate is incubated with increasing concentrations of peroxynitrate and then subjected to immunoblotting, there is less PrP recognized by both 3F4 (Figure 1A and C) and 6H4 (Figure 1B and D) in mock treated brain homogenate than in acid treated brain homogenate. The PrP in the acid treated brain homogenate is protected from modification by peroxynitrate.

Example 2

Detection of genuine prion proteins

The epitope protection phenomenon for 'model prions' was also observed for genuine prions in scrapie infected hamster brain (Figure 2A and B). As with model prions, the 3F4 and 6H4 epitopes of PrP in Ha^{Sc} brain homogenate are protected from modification by peroxynitrite. It is clear that 'model prions' and $haPrP^{Sc}$ share characteristics that provide protection from chemical modification by peroxynitrite, such as differential misfolding or aggregation.

Example 3

Is aggregation responsible for the reduction in peroxynitrite-induced epitope modification of misfolded PrP?

To show that epitope protection of acid treated and scrapie brain was due to aggregation, samples were treated with peroxynitrite and then incubated with or without guanidine before immunoprecipitation. Treatment of the samples with guanidine breaks up any aggregates of PrP (6-8) that protect the polypeptide from modification by peroxynitrite. Incubation of mock treated brain with 2.5 M guanidine after peroxynitrite treatment did not show an

increase in 3F4 and 6H4 epitopes as revealed by immunoprecipitation (Figure 3A lanes 1-4). However, when peroxynitrite-treated acid brain homogenate was incubated with guanidine, there was an increase in PrP that could be detected by 3F4 and 6H4 immunobeads (Figure 3A lanes 5-8). This shows that guanidine is able to break up aggregates of acid treated brain homogenate and release PrP that is protected from modification by peroxynitrite. Other means of solubilizing PrP aggregates were used and boiling samples in SDS loading buffer resulted in the greatest solubilization.

Example 4

Optimization of EPA parameters

Titration experiments with peroxynitrite, hydrogen peroxide and methylene (based on UV light photolysis of the precursor diazirine) or other modying agents, are conducted to identify the optimal conditions for epitope protection in :

1. Normal hamster and human brain “model prions”, using immunoblotting and conventional fluorescence ELISA, conducted at BSL1 (low level containment);
2. Infectious prions from hamster and human brain, using immunoblotting analysis and time-resolved fluorescence, conducted in BSL3 (high-level containment).

In each case, brain homogenates are prepared and mixed with increasing concentrations of the modifying agent and processed as described (immunoblotting, and time resolved fluorescence). This will define the type and concentration of chemical agent allowing the maximal distinction between monomeric and aggregated prion proteins. Additional informative control experiments include using recombinant hamster PrP^{C} in buffer and in PrP $^{-/-}$ knockout mouse brain, and by mouse normal and scrapie-infected brain (murine PrP is 6H4+ and 3F4-). Methylene will be a useful modifying agent for epitope protection of prions, due to its more uniform and complete chemical modification of many epitopes.

Infectious prions may have different properties for chemical modification than do "model prions," and that brain prions may display different chemical modification properties than do endogenous prions circulating in blood [26], or PrP^{Sc} detectable in urine of infected animals [27]. If these phenomena are observed, then the optimal conditions for authentic endogenous prions will be established for EPA as a prelude to licensing.

Example 5

EPA adapted to a fluorescent ELISA system

The epitope protection assay for aggregated PrP was adapted to a fluorescent sandwich ELISA system using 6H4 as the capture antibody and 3F4 as the detection antibody (Figure 3B). The sandwich ELISA assay system is able to identify aggregated PrP in acid treated brain homogenate but only if the samples are boiled in SDS loading buffer after peroxynitrite treatment (Figure 3B – compare open circles and closed circles). At peroxynitrite concentrations greater than 8 mM, there is 2.5-3x as much PrP detected in the acid treated sample as compared to the mock treated sample.

Example 6

Detection of a single brain prion protein

Detection of single brain prions (estimated to comprise 10^5-10^6 molecules of PrP^{Sc} [16,28]) has been (controversially) published by two groups [29,30]. Currently, detection of 10^8-10^9 molecules of recombinant PrP using conventional fluorescence ELISA has been accomplished, suggesting that an assay ~1000-fold more sensitive will permit single-prion detection. The necessary sensitivity will be provided by the Dissociation enhanced lanthanide fluoroimmunoassay (Delfia) assay. The Delfia assay uses a chelated lanthanide-labeled tracer, such as europium (Eu) and time-resolved fluorescence (TRF) to measure output signal. The benefit of lanthanide chelates is that their fluorescence lasts up to 200,000 times longer than conventional fluorophors, allowing signal capture after non-specific interfering fluorescence has faded (particularly critical for biological samples, which may possess considerable non-specific fluorescence). Delfia-based systems can measure as little as 100 fmol/well of Eu which is >1000 times more sensitive

than conventional ELISA assays, which will be able to detect single prions by EPA. The optimal TRF 96-well plate reader for the Delfia system is manufactured by Wallac-Victor (Perkin-Elmer), and will be used to automate sample analysis.

Using an optimal chemical modifier and optimal conditions a sensitive capture 96-well plate assay for detection of hamster and human prions, using the Delfia TRF system is provided. This is used to:

1. Characterize, optimize and quantify detection of recombinant prion protein by TRF;
2. Determine the sensitivity of the Delphia-TRF for hamster and human brain prions.

Example 7

Detection of Prion Proteins in biological fluids

The EPA achieves commercial utility by detecting PrP^{Sc} in biological tissues and fluids for which no present technology exists. Blood prions are in very low abundance (10-100 prions/mL by bioassay [26], and protease-resistant PrP in urine is only intermittently/sporadically detectable by precipitation of large fluid volumes [27]. Also, any prospective blood test must contend with high concentrations of PrP^{C} (10^6-fold more than PrP^{Sc} [31]) and "blocking" by heterologous plasma proteins [32,33]. Using the optimized chemical modification regimen and the Delfia-TRF system, the sensitivity thresholds for EPA in blood and urine is determined using:

1. Hamster and human plasma and urine "spiked" with a titration of 263K hamster prions;
2. Plasma and urine from Syrian hamsters "endogenously" infected with 263K prion disease

Biological fluids clinically accessible by non-invasive routes will provide a substrate for a practical antemortem test for diagnosis and screening of prion infection in humans and animals. If EPA with "prion spike" titration in normal

blood and urine reveals similar Delfia-TRF signals to the same prion titration in buffer, the EPA is not affected by "blocking factors" in these biological fluids. Interestingly, preferential "blocking" of PrP^{Sc} by heterologous proteins may actually *enhance* epitope protection to chemical modifying agents. If decreased detection of prions in blood or urine is observed, pre-clearing strategies will be employed to enhance PrP^{Sc} detection with detergents, precipitating agents, and adsorbents typically used in commercial ELISA assays which are known to one skilled in the art.

Human and bovine plasma and urine are tested using optimized EPA conditions and compared to samples from human variant CJD and BSE, respectively. Although the monoclonal antibody 6H4 recognizes PrP from all species, other antibodies (commercially available) are used for the Delfia TRF system for cattle, sheep, and cervids, which lack the 3F4 epitope.

Materials and Methods

Materials

Recombinant hamster PrP (rhaPrP) and 6H4 was from Prionics. Recombinant human PrP (rhuPrP) was from Roboscreen. Biotin-3F4 and 3F4 were from Signet. 3F4 reacts against MKHV and 6H4 reacts against DYEDRYYRE.

Preparation of Acid-misfolded PrP

Acid misfolded PrP was used as "model prions" in this study and was prepared as in (5). Briefly, 100 µl of 10% brain homogenate was mixed with an equal volume of 3.0 M GdnHCl (final concentration 1.5 M) in PBS at pH 7.4 or pH 3.5 adjusted with 1 N HCl, followed by rotation at room temperature. After 5 h incubation, samples were methanol precipitated with 5 volumes of ice-cold methanol and pellets were resuspended in 100 µl of lysis buffer. The samples treated at pH 7.4 were designated as mock-treated samples.

Peroxynitrite treatment of Brain Homogenates

An aliquot (18µl) of normal or misfolded/diseased brain homogenate was vortexed while 2 µl of peroxynitrite in 100mM NaOH/60 mM H_2O_2 was added to give a final peroxynitrite concentration of 0-15 mM. After vortexing for a

further 15 s, the samples were subjected to Western blotting, immunoprecipitation or sandwich ELISA.

Western Blotting

Samples were boiled in SDS loading buffer (62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% beta-mercaptoethanol and 0.01% bromphenol blue) for 5 min. and separated on 12% Tris-Glycine polyacrylamide gels followed by transfer to Hybond-P. PrP was detected using 3F4 (1:50000) or 6H4 (1:10000) as the primary antibody and HRP-conjugated goat anti-mouse (1:10000) as the secondary antibody followed by exposure to ECL-Plus and visualization by exposure to Kodak X-OMAT film. Band intensities were quantitated using UnScan-IT software.

Immunoprecipitation

Samples were incubated with 50 µl of Ab-conjugated (100µg/ml) Dynal M-280 magnetic beads in a final volume of 1 ml binding buffer (3 % NP-40; 3% Tween-20) for 3 h at room temperature with rotation. Beads were washed in wash buffer (2% NP-40; 2% Tween-20) x3 and boiled in 30 µl SDS loading buffer without beta-mercaptoethanol for 5 min. Supernatants were analyzed by Western blotting as described above.

Sandwich ELISA

The capture antibody (6H4; 1:5000 in 50 mM bicarbonate binding buffer, pH 9.6) was bound to an opaque 96-well plate (Nunc Maxisorp) by overnight incubation at 4 °C. After blocking with 1% BSA in 0.05% TBST for 2 h, plates were washed 3x in TBST and incubated overnight at 4°C with standard concentrations of rhuPrP or rHaPrP along with unknown brain homogenates. Plates were washed 3x and incubated with the detecting antibody biotin-3F4 (1:5000) at RT for 1h. After washing 3x, avidin-HRP (1:5000) was added and incubated for 30 min. at RT. Following a final wash step (x3) the plate was developed with Quantablu fluorescent substrate for 10-90 min at RT and fluorescent intensities determined with an excitation of 325nm and emission of 420 nm.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the

invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

WE CLAIM

1. A method of detecting whether a candidate polypeptide including a target epitope is in a wildtype folded conformation or a misfolded conformation, comprising:

contacting the polypeptide with a protecting agent that selectively blocks accessible target epitope, wherein in the wildtype folded conformation, the target epitope is accessible and reacts with the protecting agent, and wherein in the misfolded conformation, the target epitope is inaccessible and does not react with the protecting agent;

removing unreacted protecting agent from contact with the polypeptide;

next modifying the candidate polypeptide to convert any inaccessible target epitope to accessible target epitope;

next contacting the polypeptide with a detection agent that binds selectively to target epitope that was converted from inaccessible target epitope to accessible target epitope, wherein binding between detection agent and converted target epitope indicates that the candidate polypeptide was in a misfolded conformation and wherein lack of binding between the detection agent and the target epitope indicates that the polypeptide was in a wild type folded conformation.

2. The method of claim 1, wherein the candidate polypeptide comprises prion protein, the wild type folded conformation comprises the conformation of wild type folded prion protein and the misfolded conformation comprises the conformation of PrP^{Sc}.

3. The method of claim 1 or 2, wherein the blocking agent is selected from the group consisting of peroxynitrite.

4. The method of claims 1-3, wherein the polypeptide is modified by denaturing the polypeptide.

5. The method of claims 1-4 , wherein the polypeptide is denatured by heat and/or detergent.

6. The method of claims 1-5, wherein the polypeptide is modified by treatment with a disaggregation agent to disaggregate the polypeptide from other polyeptides.

7. The method of claims 1-6, wherein the disaggregation agent is selected from at least one of the group consisting of guanidine, detergent and heat.

8. The method of claims 1-7, wherein the detection agent comprises an antibody directed against a prion polypeptide epitope.

9.The method of claims 1-8, wherein the antibody comprises 6H4 or 3F4 .

10. The method of claims 1-9, wherein the misfolded conformation is indicative of a disease caused by protein misfolding.

11. The method of claims 1-10, wherein the disease comprises BSE or CJD.

12. The method of any one of claims 1 to 11, wherein the epitope is inaccessible in the misfolded conformation because i) the differential misfolding of the polypeptide compared to the wild type folded polypeptide prevents or reduces reaction between the blocking agent and the epitope or ii) the polypeptide in the misfolded conformation aggregates with other polypeptides in the misfolded conformation to prevents or reduces reaction between the blocking agent and the epitope.

Application number : numéro de demande: 2437675

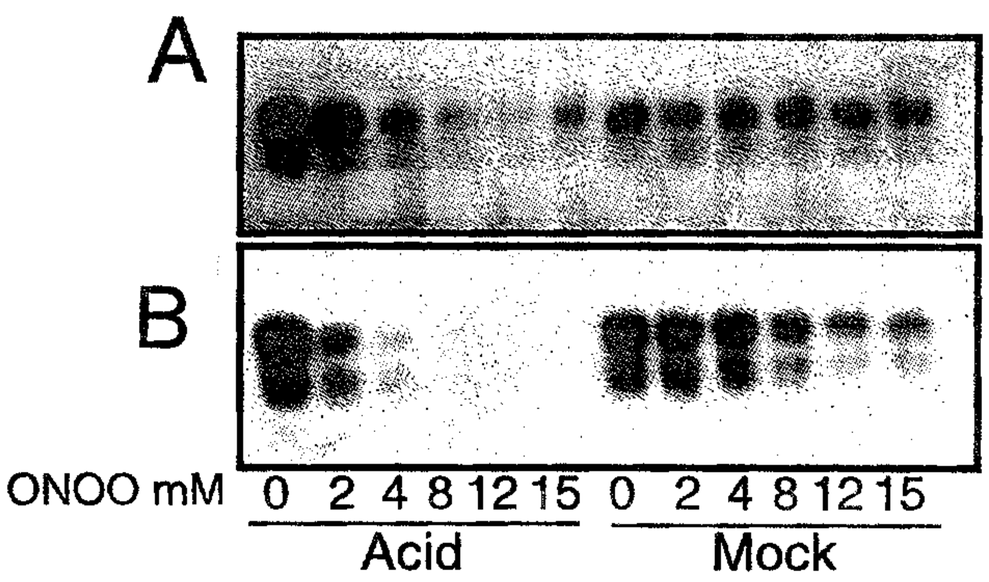
Figures: 1a, b, 2a, 3a.

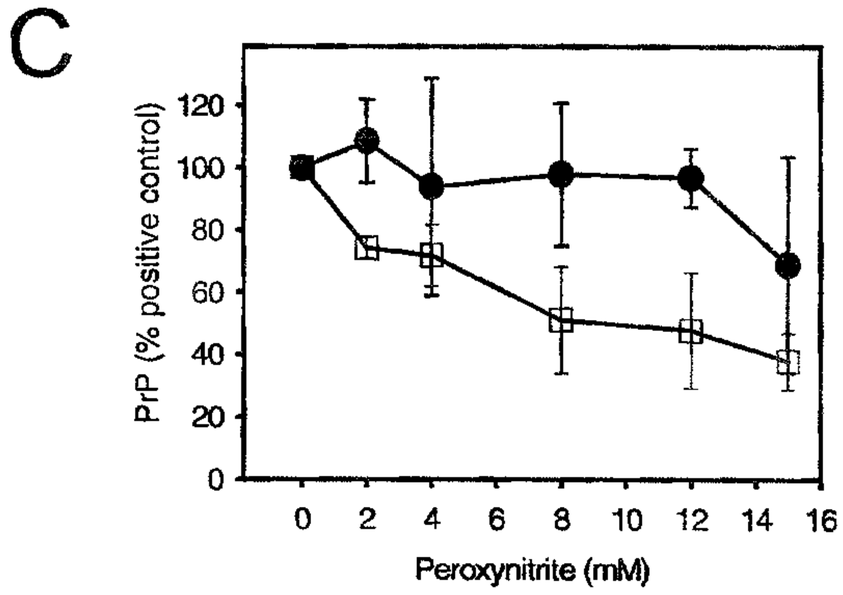
Pages:

Unscannable items
received with this application
(Request original documents in File Prep. Section on the 10th floor)

Documents reçu avec cette demande ne pouvant être balayés
(Commander les documents originaux dans la section de préparation des dossiers au 10ème étage)

Fig 1


A
B
ONOO mM 0 2 4 8 12 15 0 2 4 8 12 15
Acid
Mock


C
PrP (% positive control)
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Peroxynitrite (mM)

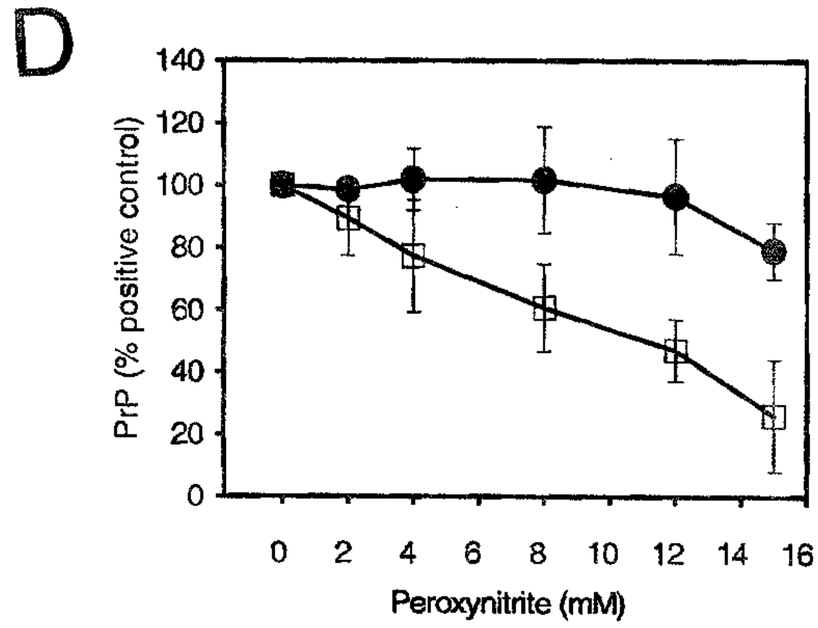

D
PrP (% positive control)
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Peroxynitrite (mM)

Fig 2

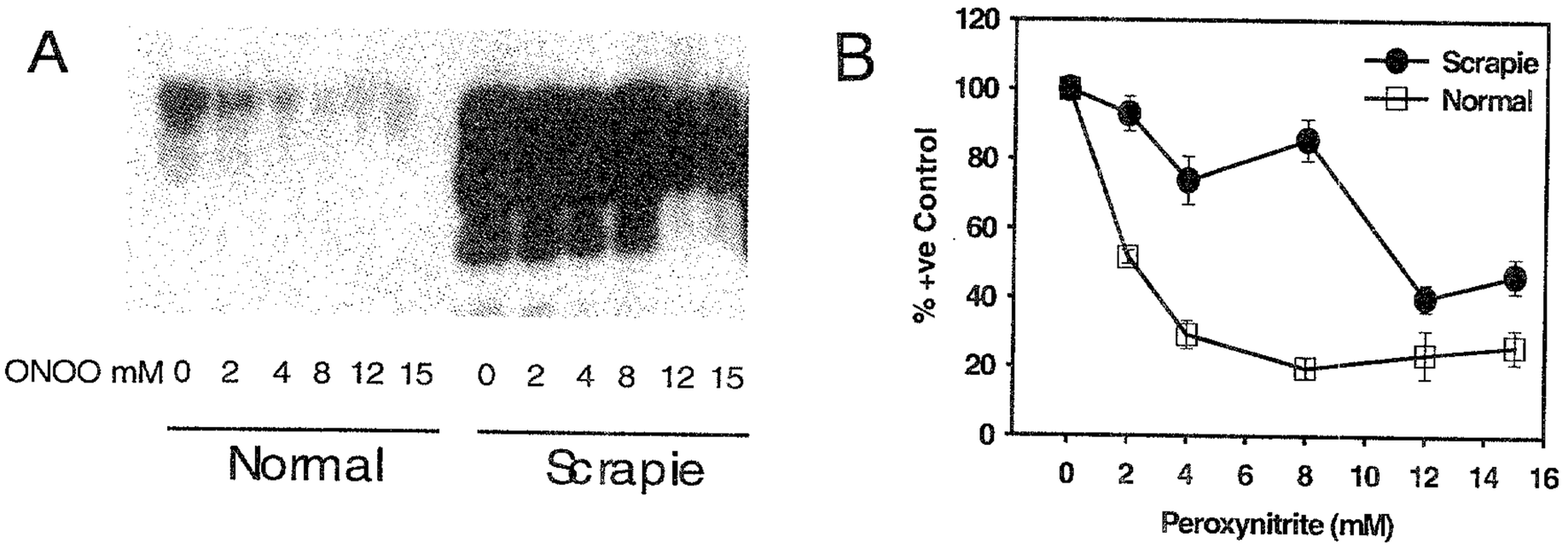
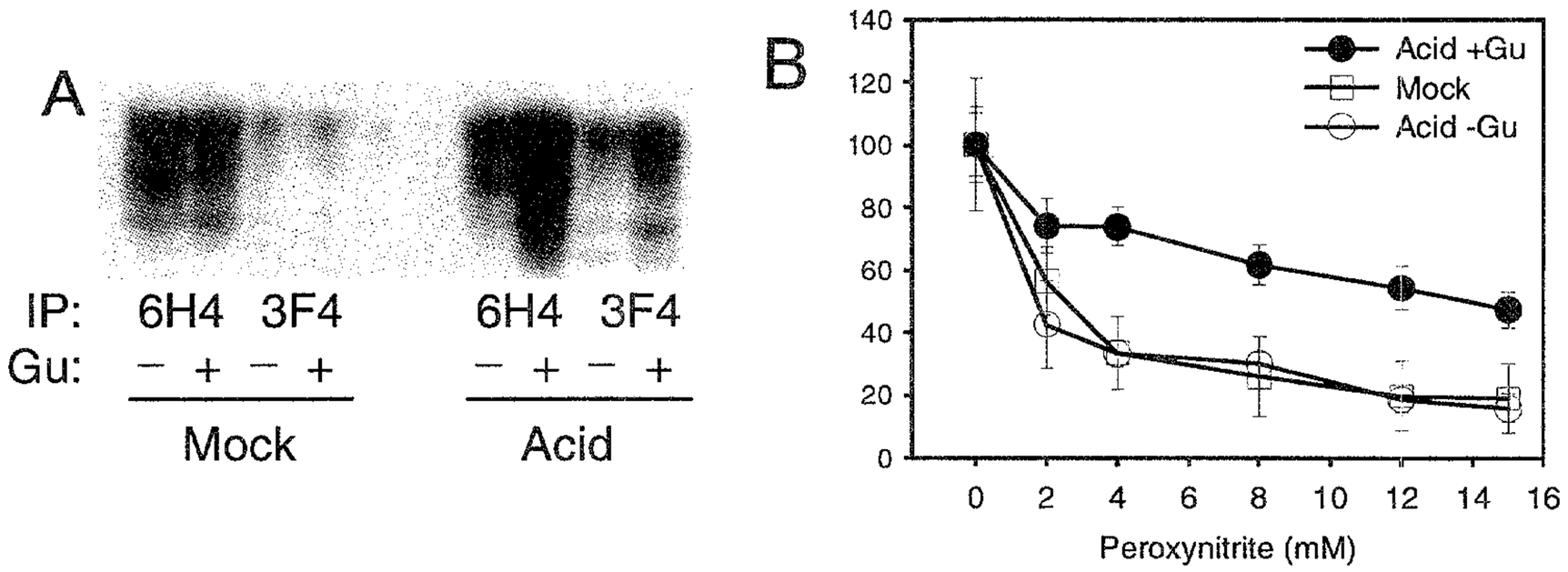

A
ONOO mM 0 2 4 8 12 15 0 2 4 8 12 15
Nomal
Scrapie
B
% +ve Control
120
100
80
60
40
20
0
Scrapie
Normal
0 2 4 6 8 10 12 14 16
Peroxynitrite (mM)

Fig 3


A
IP: 6H4 3F4 6H4 3F4
Gu: − + − + − + − +
Mock
Acid
B
Acid +Gu
Mock
Acid -Gu
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Peroxynitrite (mM)