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(57) Abstract

Adrenal autoantigen is isolated and characterised as a protein obtainable from the microsome fraction of the adrenal gland, having a molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody. The protein innative, recombinant or modified form as well as fragments thereof comprising an epitope for adrenal autoantibody are used in a method and kit for detecting adrenal autoantibodies. The protein is also useful as a medicament for treating Addisson's disease. Absorption studies show that the autoantigen is steroid 21-hydroxylase.

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Assay for Adrenal Autoantigen

The present invention relates to adrenal autoantigen. More particularly, it relates to immunoassays for adrenal autoantibodies involving adrenal autoantigen. The invention further relates to methods of using adrenal autoantigen in the diagnosis of autoimmune Addison's disease.

Addison's disease is a disorder characterised by failure of the adrenal gland and is often an autoimmune disorder involving destruction of the adrenal cortex and the presence of adrenal autoantibodies in the patient's serum. The adrenal cortex is responsible for producing several steroid hormones including cortisol, aldosterone and testosterone. In autoimmune Addison's disease and other forms of the disease, levels of these hormones are reduced. This reduction in hormone levels is responsible for the clinical symptoms of the disease which include low blood pressure, muscle weakness, increased skin pigmentation and electrolyte imbalance.

Autoantibodies to the adrenal cortex were first described in 1957 using the technique of complement fixation[1]. Subsequently other laboratories confirmed the presence of these autoantibodies using complement fixation or immunofluorescence[2-4]. Radioimmunoassay and ELISA techniques have also been described using crude adrenal membrane preparations[5-6].

The preparation of purified rabbit adrenal autoantigen was reported in 1973[7] but the isolation of human adrenal autoantigen remained an elusive goal. The human adrenal autoantigen(s) has/have been localised to the microsomal fraction of adrenal tissue homogenates and in 1988 it was shown that the antigen is a membrane protein with a molecular weight of about 55,000[11]. In 1990 there was described an assay to detect binding of adrenal autoantibodies to subcellular fractions of the human adrenal gland[8]. The assay indicated that the autoantibody has a plasma membrane and/or microsomal source and was described as providing

a more rational approach to "eventual" antigen isolation and purification.

Measurement of adrenal autoantibodies is important in the diagnosis of Addison's disease and currently immunofluorescence is used routinely.

The present inventor has for the first time succeeded in isolating and characterising an adrenal antigen. The isolation and characterisation of the antigen enables the production of the antigen, for example, by recombinant techniques, the generation of antibodies to the antigen, purification of adrenal autoantibody and assays for the antigen. Such assays are useful for the diagnosis of latent or actual autoimmune Addison's disease.

The present invention relates to assays for adrenal autoantibodies. Preferred assays of the invention involve the use of a protein obtainable from the microsome fraction of human or animal adrenal glands. The protein has an observed molecular weight of approximately 50,000 to 60,000 and is antigenic towards adrenal autoantibody. Preferably the protein is of human origin.

The invention further relates to assays involving the use of protein comprising an epitope for adrenal autoantibody, having an observed molecular weight of from about 50,000 to about 60,000 and obtainable by:

homogenising adrenal glands,

subjecting the homogenate to differential centrifugation to obtain a microsome fraction,

suspending the microsome fraction in a phosphate buffer,

centrifuging the suspension in the presence of sodium cholate to form a supernatant,

adding polyethylene glycol and further sodium cholate to the supernatant and mixing the supernatant,

centrifuging the thus mixed supernatant to form a pellet,

resuspending the pellet in aqueous sodium cholate to form a suspension,

dialysing the suspension against aqueous sodium cholate to form a solubilised microsome preparation, and

purifying the solubilised microsome preparation by column chromatography to obtain a column fraction containing the protein.

The protein is preferably obtainable from human or mammalian adrenal glands.

Experiments indicate that the protein obtainable by the above techniques is steroid 21-hydroxylase (p450c21). Steroid 21-hydroxylase is an enzyme prominent in the zona glomerulosa of the adrenal cortex and which catalyses one step of the biological pathway from cholesterol to hydrocortisone. cDNA encoding 21-hydroxylase has been cloned and sequenced and an amino acid sequence derived for the protein[10].

The adrenal protein obtainable as described above may be modified by one or more amino acid modifications (deletions, additions or substitutions) which do not cause loss of adrenal autoantibody binding properties.

The invention includes the use of a fragment of the protein which comprises an epitope for adrenal autoantibody. Suitable fragments include synthetic peptides.

The protein may be in crude or purified form. Preferably, it is substantially pure. The protein may be native protein derived from a human or animal source or it may be of recombinant origin.

The adrenal proteins, especially human adrenal protein, form one aspect of the invention. The proteins of the invention may be in the form of a modified protein or a protein fragment as described above. The proteins of the invention (including modified proteins and fragments) may be labelled. The proteins are preferably mammalian and more preferably human.

In yet other aspects the invention resides in kits for detecting adrenal autoantibodies in a sample. In one aspect the kit comprises the adrenal protein. The invention includes the use in an assay for adrenal autoantibodies of a protein or a protein fragment of the invention or of an antibody to such a protein or protein fragment.

The invention opens the way to the therapy of adrenal autoimmune disorders as described more fully hereafter, by the disruption of the lymphocytes involved in the disorder. In particular, the invention provides pharmaceutical formulations comprising a protein or protein fragment, especially a peptide, of the invention for pharmaceutical use. In another aspect there is provided a pharmaceutical formulation comprising a protein or protein fragment of the invention formulated for pharmaceutical use; the formulation may be a composition which comprises a pharmaceutically acceptable diluent, excipient or carrier as well as the active protein or protein fragment.

Figure 1 is an elution profile of proteins eluted from a column chromatograph;

Figure 2 shows a Western blot of eluted column fractions contacted with (A) serum containing adrenal autoantibodies and (B) normal serum;

Figure 3 shows the protein staining pattern of eluted column fractions after gel electrophoresis;

Figure 4 is a dot blot assay of eluted column fractions contacted with (A) serum containing adrenal autoantibodies and (B) normal serum;

Figure 5 shows a dot blot assay of eluted column fraction VI contacted with ten different serum samples.

Figure 6 is a Western blot analysis of eluted column fraction VI; and

Figure 7 is a Western blot analysis of the protein of column fraction VI after SDS-PAGE analysis and electroelution.

The invention relates to assays for adrenal autoantibodies. The assays involve the use of antigenic protein described in more detail below.

The Antigenic Protein

The protein useful in the invention is a protein obtainable from the microsome fraction of human or animal adrenal glands. The protein is an antigen for adrenal autoantibodies. The protein is further characterised by an observed molecular weight of from about 50,000 to 60,000 and preferably from about 52,000 to 58,000 (eg approximately 55,000). The molecular weight is that observed against the following standards in analysis by gel electrophoresis using a 9% polyacrylamide gel : myosin (205,000), beta-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000).

The adrenal protein preferably binds to human adrenal autoantibodies but less preferably may bind exclusively to animal adrenal autoantibodies.

The protein may be native protein extracted from human adrenal glands or, less preferably, adrenal glands from another mammal. The protein may be obtained by the following illustrative procedure. Adrenal glands are homogenised and the homogenate subjected to differential centrifugation to obtain a microsome fraction. The microsome fraction is suspended, eg in phosphate buffer containing glycerol.

The suspension is then centrifuged, typically in the presence of sodium cholate and normally also EDTA and dithiothreitol (DTT). After centrifugation polyethylene

glycol (PEG) and further sodium cholate and normally glycerol, EDTA and DTT are added to the supernatant and the preparation mixed before further centrifuging. The pellet resulting from centrifuging is resuspended in aqueous sodium cholate normally containing glycerol, EDTA and DTT and dialysed against aqueous sodium cholate, again normally containing glycerol, EDTA and DTT.

After dialysis, the solubilised microsomes are preferably centrifuged, to remove any precipitated material. The solubilised microsome preparation may be stored at -70°C.

Throughout the procedure, the liquids are buffered at pH7, for example using phosphate buffer.

The solubilised microsome preparation is further purified by chromatography, for example using octyl-Sepharose CL-4B and, as the eluant, phosphate buffer (which may contain glycerol, EDTA and DTT), followed by increasing concentrations of one or more detergents, for example that sold under the trade mark EMULGEN.

Column fractions are typically monitored by optical absorbance.

The column fraction containing the adrenal autoantigen may be determined by Western blotting. The column fractions are separated by gel electrophoresis followed by blotting onto nitrocellulose. The nitrocellulose is reacted with serum from a patient with a high level of adrenal autoantibodies and incubated with for example anti-human immunoglobulin-horseradish peroxidase conjugate and, reacted with a chemiluminescent substance. Chemiluminescence is detected with photographic film.

The above procedures are described to illustrate the invention by way of example only, and alternative protocols may be followed to obtain crude, purified or substantially pure antigen.

As described in more detail in the Examples, the adrenal autoantigen isolated by the described procedures was found to have a molecular weight of 55,000 ± 5,000. The protein was found to have the biochemical characteristics of steroid 21-hydroxylase and to react on Western blots with rabbit antibodies to recombinant 21-hydroxylase. Absorption of the native human adrenal protein with human adrenal autoantibodies prevented the subsequent reaction of the native human protein with rabbit antibodies to 21-hydroxylase in Western blot analysis. In addition, human adrenal autoantibodies reacted with recombinant 21-hydroxylase expressed in yeast. These experiments indicate that the protein is 21-hydroxylase.

Whilst the adrenal protein is preferably of human origin, the invention embraces antigens derived from animals. The antigen may be obtained by purification of animal or, preferably, human adrenal microsome fractions or by expression of recombinant protein.

As the antigen there may also be used synthetic peptides containing an epitope against adrenal autoantibody. Synthetic peptides may be prepared by any known synthetic procedure, for example. Such synthetic peptides represent a fragment of the native or modified protein.

Steroid 21-hydroxylase

As described elsewhere herein, the adrenal protein has immunological properties which equate it with steroid 21-hydroxylase.

Steroid 21-hydroxylase is an enzyme which has been studied because deficiency in 21-hydroxylase activity results in inefficient synthesis of cortisol and elevated levels of corticotropin. Such 21-hydroxylase deficiency is one of several syndromes termed congenital adrenal hyperplasia and is inherited as a monogenic autosomal recessive trait. A plasmid designated pC21a comprising a portion of the coding sequence of bovine 21-hydroxylase was prepared for use in the diagnosis

of congenital adrenal hyperplasia[9]. Plasmid pC21a was subsequently used to prepare a plasmid pC21/3c (pCD//pC21/3c) containing an insert encoding the complete cDNA for 21-hydroxylase except for about 30 base pairs at the 5' end[10].

Plasmid pC21/3c has been sequenced[10] and deposited at the American Type Culture Collection (ATCC) under ATCC Nos 57420 (freeze dried E. Coli containing the plasmid and 57421 (purified plasmid DNA). Both deposits are freely available to the public.

Saccharomyces cerevisiae capable of producing steroid 21-hydroxylase may be made by a method comprising ligating into a yeast expression vector the steroid 21-hydroxylase gene sequence obtainable from plasmid pCD//pC21/3c (ATCC 57421) modified by replacement of the bases coding for the first 13 amino acids with the bases coding for a yeast leader sequence, and transforming saccharomyces cerevisiae with the resultant vector.

Preferably, the steroid 21-hydroxylase gene sequence obtainable from said plasmid is subcloned as a BamHI fragment into vector pTZ18, and the resultant vector containing a 21-hydroxylase gene sequence is digested to obtain a fragment comprising a coding region for 21-hydroxylase and ligated into yeast expression vector pYES, as cut with BamHI and SphI, using a BamHI - NarI linker comprising 11 base pairs of non-coding and 42 base pairs of coding STE2 gene sequence.

Included in the invention is a method of producing a yeast expression vector comprising a DNA sequence encoding steroid 21-hydroxylase, comprising ligating into a yeast expression vector the steroid 21-hydroxylase gene sequence obtainable from plasmid pCD//pC21/3c (ATCC 57421) modified by replacement of the bases coding for the first 13 amino acids with the bases coding for a yeast leader sequence and a method of expressing steroid 21-hydroxylase, comprising culturing Saccharomyces cerevisiae transformants prepared by a method of the invention.

Steroid 21-hydroxylase may therefore be used in assays and kits for detecting adrenal autoantibody, especially in the diagnosis of Addison's disease. Steroid 21-hydroxylase may also be used in the therapy of adrenal autoimmune disorders.

The expression of bovine 21-hydroxylase as part of a process for the preparation of steroids is described in EP 0340878 (Gist-Brocades).

The 21-hydroxylase is preferably human 21-hydroxylase , but may alternatively be an animal (e.g. bovine) 21-hydroxylase. The 21-hydroxylase may be purified from human or animal sources but is preferably recombinant 21-hydroxylase. The 21-hydroxylase may have a native sequence or may be modified by one or more amino acid alterations which do not result in loss of antigenicity towards adrenal autoantibodies.

Also useful in the invention are fragments of 21-hydroxylase which retain the desired antigenic property.

The protein or protein fragment may be in crude, purified or pure form.

Cloning Antigenic Protein

Recombinant protein may be prepared by isolating polyadenylated adrenal mRNA and reverse transcribing it into cDNA to prepare a cDNA library. A host organism is transformed with the cDNA using an appropriate expression vector and the transformants screened for organisms expressing the adrenal autoantigen, which organisms may then be cloned. Hosts may be prokaryotic or eukaryotic, for example E. Coli, yeast or CHO cells. Of course, when a host is transformed with a vector, appropriate promoter-operator sequences are also introduced in order for the protein to be expressed. The protein obtained may be glycosylated or unglycosylated.

An illustrative procedure for preparing recombinant adrenal protein will now be described in more detail by way of example only. Polyadenylated adrenal RNA is isolated and the cDNA sequence based on the RNA sequence is generated with the use of reverse transcriptase. The cDNA is then cloned into a bacteriophage vector (eg lambda gt10, lambda gt11, lambda Zap) and an adrenal cDNA library is created. The library is then screened using 32-P labelled oligonucleotide probes synthesised on the basis of known parts of the amino acid sequence of adrenal autoantigen. The library (when subcloned into the expression vector, eg lambda gt11) can alternatively be screened with autoantibodies to adrenal antigen or with monoclonal antibodies. In this case the positive plaques are identified in a reaction with radiolabelled Protein A or enzyme-labelled anti-IgG.

Positive plaques (ie containing the cDNA sequence complementary to the probes or expressing the antibody binding protein) are purified to homogeneity and ligated to create a full coding sequence. The full-length cDNA is then sequenced by the Sanger method and subcloned into a plasmid vector, eg pBR322, pTZ18 or Bluescript.

Alternatively, the gene coding for adrenal autoantigen can be synthesised in a polymerase chain reaction (PCR). In this method, the first strand of cDNA is synthesised from RNA using reverse transcriptase and then used as a template for DNA amplification with Taq DNA polymerase. The primers for initiation of each round of amplification are synthesised based on the known amino acid sequence of the N terminus of the adrenal protein. In this case a modification of cDNA amplification using one-sided (anchored) PCR is used. The amplified gene is purified on an agarose gel and cloned into plasmid vectors as above.

For expression of recombinant protein, the full length of the isolated gene is cloned into a mammalian expression vector (eg pRC/CMV). CHO cells, for example, are then transfected (using the $CaPO_4$ precipitation method) with vectors containing the adrenal autoantigen gene. Cells are collected, concentrated by

centrifugation and a microsomal preparation prepared. The presence of recombinant proteins in the microsomal fraction is detected by Western blotting using adrenal autoantibodies and the material purified using Octyl Sepharose chromatography as described above in relation to purification of native adrenal autoantigen.

Glycosylation of the recombinant protein may be studied using lectins and compared to the native protein.

Detailed methodology of cloning techniques may be found in J Fambrook; E F Fritch and T Maniatis, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, Second Edition 1989.

A specific method of expressing human steroid 21-hydroxylase will now be described.

Human 21-hydroxylase may be expressed using the 21-hydroxylase gene in plasmid pCD//pC21/3c[10]. The purified plasmid is publically available from ATCC deposit No. 57421. Alternatively, a suitable plasmid may be prepared following the procedure of White et al[10].

In an exemplary technique, the 21-hydroxylase gene in pCD//pC21/3c was modified by replacing the first 13 amino acids with the first 14 amino acids of STE2 gene and placed under the control of GAL1 promoter in pYES 2.0 (Invitrogen).

In this technique the 21-hydroxylase gene sequence was subcloned from pCD//pC21/3c as a BamHI fragment into the vector pTZ18 (Pharmacia). Digestion with SphI (Northumbria Biologicals Ltd, Cramlington NE23 9HL, UK) followed by partial digestion with NarI (Northumbria Biologicals Ltd, Cramlington NE23 9HL, UK) yielded a large fragment comprising the entire coding region of

21-hydroxylase apart from 41 base pairs at the 5' end. This was then ligated into pYES cut with BamHI (Northumbria Biologicals Ltd, Cramlington NE23 9HL, UK) and SphI using a BamHI-NarI linker comprising 11 base pairs of non-coding and 42 base pairs of STE2 coding sequence[58]. Transformants of Saccharomyces cerevisiae (C13 ABYS 8B - Yeast Genetic Stock Center, Berkeley, California) were grown in selective media and used to inoculate expression cultures in YEP-glucose (2%) or YEP-galactose (2%). [YND is yeast nitrogen base from Difco Laboratories + 2% dextrose and YEP is yeast extract + 2% peptone (both from Oxoid)]. After 48 hours at 30°C, the cells were harvested, broken by vortexing with glass beads in 1% sodium deoxycholate and analysed on SDS-PAGE followed by Western blotting.

Bovine 21-hydroxylase may be expressed as described in EP 0340878.

The protein may be a variant of the native form containing amino acid alterations which do not result in loss of antigenicity to adrenal autoantibodies.

Such modified proteins may be prepared, for example, by the use of oligonucleotide directed mutagenesis with a synthetic oligonucleotide (typically 7 to 24 bases long) that is complementary to the wild-type sequence but which contains one or more base changes compared to the wild-type sequence.

The size of the oligonucleotide primer is determined by the requirement for stable hybridization of the primer to the region of the gene in which the mutation is to be induced, and by the limitations of the available methods for synthesizing oligonucleotides. In general the overall length of the oligonucleotide will be such as to optimize stable, unique hybridization at the mutation site with the 5' and 3' extensions from the mutation site being of sufficient size to avoid editing of the mutation by the exonuclease activity of the DNA polymerase. Oligonucleotides used for mutagenesis usually contain from about 7 and more usually about 12 to about 24 bases, preferably from about 14 to about 20 bases and still more

preferably from about 15 to about 18 bases. They will usually contain at least about three bases at the centre or positions immediately 3' of the centre of the altered or missing codon.

The primer is hybridized to single-stranded phage such as M13 into which a strand of DNA of the invention has been cloned. It will be appreciated that the phage may carry either the sense strand or antisense strand of the DNA. When the phage carries the antisense strand the primer is identical to the region of the sense strand that contains the codon(s) to be mutated except for a mismatch with said codon(s) that defines a deletion of the codon or a triplet that codes for another amino acid.

When the phage carries the sense strand the primer is complementary to the region of the sense strand that contains the codon(s) to be mutated except for an appropriate mismatch in the triplet that is paired with the or each codon to be mutated. The hybridization is carried out at a temperature which will usually range between about 0°C and 70°C, more usually about 10°C to 50°C.

Melting temperature (Tm) of synthesised oligonucleotides is calculated and used as a guidance to hybridisation temperature conditions. Usually the template and the oligonucleotides are heated briefly to 20°C above the estimated Tm. Hybrids form as the temperature of the reaction mixture drops below Tm. The resulting mixture of double-stranded heteroduplex DNA is then transfected directly into a bacterial host and mutants can be identified by screening plaques by hybridisation, using the radiolabelled mutagenic oligonucleotide as a probe. Hybridisation is usually carried out under conditions that allow the labelled oligonucleotide to form hybrids with both mutant and wild-type DNA. By progressively increasing the stringency of the subsequent washes it is possible to establish conditions under which hybrids between mutagenic oligonucleotide and wild-type DNA will dissociate and perfect hybrids formed by the oligonucleotide and desired mutant will not dissociate. Identified in this way mutants are then purified and the presence of the desired mutation confirmed by DNA sequencing of the

mutagenised and adjacent regions or of the full insert sequence carried in the vector.

After the hybridization, the primer is extended on the phage DNA by reaction with DNA polymerase I, T_4 DNA polymerase, reverse transcriptase or other suitable DNA polymerase. The resulting dsDNA is converted to closed circular dsDNA by treatment with a DNA ligase such as T_4 DNA ligase. DNA molecules containing single-stranded regions may be destroyed by S1 endonuclease treatment.

The resulting mutational heteroduplex is then used to transform a competent host organism or cell. Replication of the heteroduplex by the host provides progeny from both strands. Following replication the mutant gene may be isolated from progeny of the mutant strand, inserted into an appropriate expression vector, and the vector used to transform a suitable host organism or cell. Exemplary vectors and hosts are described above.

Directed mutagenesis techniques are well known and are described in the literature[24-28]:

The invention accordingly includes not only DNA sequences encoding protein of the invention but also DNA sequences which hybridise to such sequences and encode an antigenic determinant to adrenal autoantibody. Such hybridisable sequences may be sequences encoding a modified protein or a protein fragment, whether of modified or unmodified protein. Hybridisation conditions are described, for example, by Smith M and Gillam S[25] and by Fambrook et al. (supra). Modified DNA is normally capable of hybridising to DNA sequences encoding protein of the invention under conventional hybridising conditions and preferably under stringent hybridising conditions.

Antibodies to the Antigenic Protein

In some assays, antibodies to the adrenal protein are useful. Such antibodies may be prepared by any known method, for example.

Polyclonal antibodies may be prepared by administering the adrenal autoantigen or cells expressing the antigen to an animal in order to induce the production of serum containing polyclonal antibodies.

Monoclonal antibodies can be prepared using the hybridoma technique of Kohler and Milstein[29]. Further exemplary literature references are listed in the attached bibliography[30-34]. According to the hybridoma method, a human or other animal or lymphocytes therefrom is/are immunised with a protein or protein fragment of the invention. Lymphocytes from the immunised individual or culture are fused with myelomas into single cells. The resulting continuous cell lines are screened for antibody producing hybrids. Such hybrids may be cultured and the antibody collected.

The antibodies useful in the invention include not only intact molecules, including human monoclonal antibodies, but also fragments thereof which are capable of binding to the antigenic protein. Also useful are recombinant antibodies.

Antibodies to 21-hydroxylase may be made by such techniques as described above.

Labelling

The assays of the invention normally involve antigen or antibody labelled so as to be detectable. Numerous methods of labelling are known and any such known technique may be used, for example. Examples of the extensive literature describing labelling techniques are to be found in the attached bibliography[35].

Exemplary labels are radioactive labels, enzymes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds and metal chelates. The

binding of such labels to the antigen or antibody may be performed using any conventional technique, for example.

Assays

The assays of the invention include those assays known to the skilled person for the quantitative or non-quantitative detection of antibodies and all involve contacting antigenic protein (the adrenal protein obtainable as previously described or fragments thereof) with a sample. The assays may be competitive or non-competitive and examples are radioimmunoassays (RIA), enzyme immunoassays (EIA), and enzyme-linked immunosorbent assays (ELISA). The reader is referred to the literature for details of suitable assays[35].

The competitive assays of the invention include assays which involve contacting an antigenic protein (which expression embraces protein fragments) with a biological sample (typically serum or urine). Normally the sample is diluted, for example, 1 part sample in from 10 to 30 parts diluent such as phosphate buffered saline or another buffer. Either simultaneously or subsequently there is added labelled antibody against the antigenic protein. The labelled antibody and any antibody in the sample compete to bind to the antigen and the bound labelled antibody after competition is measured. The antigenic protein in such techniques is preferably immobilised on a substrate by known techniques such as covalent bonding via an amide or ester linkage or adsorption, for example.

The invention also includes sandwich assays (eg RIA, EIA, ELISA) in which the antigenic protein immobilised on a substrate is contacted with a biological sample suspected of containing adrenal autoantibodies. After washing, there is added labelled antigenic protein or labelled anti-adrenal autoantibody antibody, either of which selectively binds to any adrenal autoantibody which has bound to the immobilised antigen. The appropriate detection technique for the label is then employed, eg quantitative or non-quantitative detection of radioactive emissions or

colorimetric techniques. Sandwich assays can also be employed using, as the labelled ligand for the antibody, any agent capable of recognising an antibody, for example labelled anti-immunoglobulins, protein A or protein G.

Other assays comprise contacting a sample with labelled antigenic protein, separating bound and unbound labelled antigen (eg using a column having adsorbed thereon antibody to the antigen) and detecting the labelling by a quantitative or non-quantitative technique.

Assays may also be performed by contacting with a sample immobilised anti-antigenic protein antibody having bound thereto labelled antigen and detecting labelled antigen bound to immobilised antibody or labelled antigen bound to antibody in the sample.

Two illustrative assay techniques for adrenal autoantibodies

1. Assay based on a radioactive label

Purified adrenal autoantigen is labelled with a radioactive label such as ^{125}I using one of many well-known techniques. The labelled material will then be incubated (1h at room temperature) with a suitably diluted (eg 1 in 20 in phosphate buffered saline) serum sample. Adrenal autoantibodies present in the test sample will bind to the ^{125}I-labelled adrenal autoantigen and the resulting complex is precipitated by addition of antibodies to human immunoglobulins or addition of a similar reagent (eg solid phase Protein A). The amount of ^{125}I-labelled antigen in the precipitate is then determined. The amount of adrenal autoantibody in the test serum sample will be a function of the amount of radioactivity precipitated. The amount of adrenal autoantibody can be expressed as the amount of radioactivity in the pellet or more usually by including dilution of an adrenal autoantibody positive reference serum in the assay.

2. Assay based on an enzyme label

Purified adrenal autoantigen is coated onto plastic wells of ELISA plates either directly onto plain wells or indirectly. The indirect method could involve coating the wells first with a monoclonal or polyclonal antibody to adrenal autoantigen (the antibody would be selected so as not to bind to the same site as adrenal autoantibodies) followed by addition of adrenal autoantigen. Several other indirect coating methods are well known.

After coating with autoantigen, suitably diluted (eg 1 in 20 in phosphate buffered saline) test sera are added to the wells and incubated (1h at room temperature) to allow binding of adrenal autoantibody to the antigen coated onto the wells.

The wells are then washed and a reagent such as anti-human IgG conjugated to horseradish peroxidase is added. After further incubation (eg 1h at room temperature) and washing, an enzyme substrate such as orthophenylene diamine is added and the colour generated measured by light absorbance. The amount of adrenal autoantibody in the test sample will be a function of the final colour intensity generated. Results can be expressed as light absorbance or more usually by including dilution of an adrenal autoantibody positive reference serum in the assay.

The assays of the invention involving the interaction of antigenic protein and a sample may be performed using a kit comprising antigenic protein. The sandwich assays of the invention may conveniently be performed using a kit comprising an antigenic protein, normally immobilised on a substrate, and labelled anti-adrenal autoantibody antibody or labelled antigenic protein.

The invention also includes kits for use in competitive assays comprising antigenic protein and labelled antibody to the antigenic protein. The antigenic protein is preferably immobilised on a substrate.

Further included in the invention are kits comprising labelled antigenic protein for use in simple assays involving detection of antigen/antibody complex. Other kits of the invention comprise anti-antigenic protein antibody which is immobilised on a substrate and which has bound thereto labelled antigenic protein.

In those kits comprising immobilised protein, the protein is preferably immobilised on the internal surface of a plastics tube.

The invention includes assays and kits as described above which use 21-hydroxylase as the antigen.

In a variant of the invention, the antigenic protein is replaced by antibody to the autoantibody. This variant is not suitable for sandwich assays using ligands which bind to any antibody.

The invention also includes the use of antibody against the adrenal protein of the invention to detect steroid 21-hydroxylase. Methods and kits as described above may be used for such assays. The antibody may be human monoclonal antibody but alternatively is human polyclonal antibody or animal polyclonal or monoclonal antibody. Optionally, the antibody used in assays of the invention may comprise more than one antibody.

Therapy of Adrenal Autoimmune Disease

It is thought that autoimmune diseases are caused at least in part by autoantigens persistently activating T cells[36]. The autoantigen in the case of adrenal autoimmune disease can be any epitope of the antigenic molecule which is recognised by a T cell receptor capable of helping a B cell make antibody to the antigenic molecule, or a T cell otherwise involved in the autoimmune disorder.

Adrenal autoimmune disorders should therefore be treatable by disrupting the

action of T cells involved in the disorder. T cells such as, for example, those infiltrating the adrenal gland, in lymphoid organs or in the circulation can act as T helper (Th) cells in responding to adrenal autoantigen epitopes. Such Th cells help B cells make specific anti-autoantigen antibodies. T cells can also act in mediating cell-mediated immune responses and act on adrenal cortex cells by the release of cytokines or directly. The destruction of adrenal cortex cells can result, when cytotoxic T cells specific for the adrenal autoantigen are activated, or by an inflammatory response mediated by a different T cell class.

If such T cells were interfered with, the production of anti-autoantigen antibodies and of T cells destructive of adrenal cortex cells would be suppressed. The T cells could be disrupted by a protein or protein fragment (eg peptide) capable of binding to the T cell receptor (TCR) of an adrenal autoantigen specific T cell, ie containing a T cell eptiope of the autoantigen.

Such a protein or peptide acts as a competitive antagonist for the autoantigen in the adrenal cortex and thereby inhibits antigen specific cellular interactions which result in the production of adrenal autoantibody or adrenal autoantigen specific cell-mediated immunity. Exemplary references describing the use of peptides to treat autoimmune disease are included in the attached bibliography[36-42].

Another therapeutic approach is to suppress the autoimmune response to adrenal autoantigen by administering adrenal autoantigen specific T cells[43-49] or a protein or protein fragment (eg peptide) mimicking the TCR of such cells[48.49]. Administration of such a "vaccine" induces "counter-autoimmunity" which is thought to be mediated by T cells specific to the TCR of the autoimmune T cells[43,48,50,51].

The autoimmune response may also be suppressed by T suppressor (Ts) cells capable of specifically recognising an anti-adrenal autoantigen B cell or T cell[52,53].

Such therapy may be effected by administering to the patient a protein or protein fragment comprising an adrenal autoantigen epitope capable of inducing Ts cells or by administering adrenal autoantigen - specific Ts cells capable of suppressing an anti-adrenal autoantigen response.

The T cell epitope or epitopes of adrenal autoantigen may be determined by, for example, standard techniques in the art.

The autoimmune TCR specific to the adrenal autoantigen may be characterised using methods described in the literature[54]. Disruption of interaction of the autoimmune T cells with the adrenal autoantigen may alleviate or prevent adrenal autoimmune disease. T cells which will disrupt such interaction and which are specific for the autoimmune TCR for adrenal autoantigen may be prepared[55].

The present invention provides pharmaceutical (including veterinary pharmaceutical) formulations comprising the adrenal protein obtainable from human or animal adrenal glands as defined above, which protein may optionally be modified by one or more amino acid alterations. The invention also includes formulations comprising a fragment of such protein, eg a peptide containing an antigenic determinant to adrenal autoantibody.

In another aspect, the invention provides pharmaceutical formulations comprising 21-hydroxylase or a fragment thereof, eg a peptide containing an antigenic determinant to adrenal autoantibody.

The invention also includes pharmaceutical formulations comprising a ligand, eg a peptide, capable of binding to the TCR of an adrenal autoantigen specific T cell or B cell.

In other aspects, the invention provides T cells specific for adrenal autoantigen and proteins or protein fragments (eg peptides) which mimick the receptor of such T

cells. Included in the invention are counter-autoimmune T cells, ie T cells induced by such adrenal autoantibody specific T cell or TCR mimicking protein or protein fragment. Pharmaceutical compositions including a pharmaceutically acceptable diluent, excipient or carrier as well as any of the aforesaid agents are further provided by the invention.

Also provided by the invention are Ts cells capable of interacting specifically with an anti-adrenal autoantigen B cell or T cell and pharmaceutical formulations comprising such cells. Similarly included is the use of the adrenal protein defined above or of an 21-hydroxylase or a fragment of either (eg a peptide comprising a suitable epitope) to generate such anti-autoimmune T cells.

Other Uses of the Antigenic Proteins

Antigenic protein may be used to purify adrenal autoantibody, for example by adsorbing the adrenal autoantigen or 21-hydroxylase on a substrate, passing a sample containing the antibody over the substrate and then washing the substrate to release the antibody.

The adrenal autoantibody may be used to raise antibody to the adrenal autoantibody for use in assays or, for example, for screening transformed host cells to detect expression of recombinant adrenal autoantibody, which itself could be labelled and used in a competitive assay, for example.

Example 1

Preparation and solubilisation of adrenal microsomes.

Human adrenal glands were recovered from the redundant tissue surrounding kidneys removed from cadaveric donors and then stored at -70°C in small (100mg) pieces. A microsome fraction was isolated from adrenal tissue homogenates by

differential centrifugation[11-13] and suspended in 100mM phosphate buffer pH 7.0 containing 20% glycerol (1ml of buffer for each g of starting adrenal tissue). The protein concentration was then estimated[14] and the mixture adjusted to a final concentration of 25-30mg of adrenal protein per ml.

Sodium cholate (solid) was then added to a final concentration of 3mg of cholate per 1mg of adrenal proteins and the mixture was adjusted to a final concentration of 0.1mM EDTA and 0.1mM dithiothreitol (DTT). Sodium cholate was allowed to dissolve by mixing on a magnetic stirrer at 4°C for 1h and then the mixture was centrifuged at 100,000 x g for 1h at 4°C. After centrifugation, an equal volume of 50% PEG 6000 in 100mM phosphate buffer pH 7.0, 20% glycerol, 0.1mM DTT, 0.1mM EDTA was added to the supernatant and the preparation mixed on a magnetic stirrer for 30min at 4°C. This was followed by centrifugation at 12,000 x g for 1h at 4°C. The pellet was then resuspended in 3% sodium cholate in 100mM phosphate buffer pH 7.0 containing 20% glycerol, 0.1mM EDTA. 0.1mM DTT (1.3ml per 1g equivalent of starting adrenal tissue) and dialysed overnight at 4°C against 0.3% sodium cholate in 50mM phosphate buffer pH7.0, 20% glycerol, 0.1mM EDTA, 0.1mM DTT (basal buffer). After dialysis the solubilised microsomes were spun at 12,000 x g for 15 min at 4°C to remove any precipitated material.

The protein concentration of the solubilised microsome preparation was then estimated[10] and the preparation stored in aliquots at -70°C.

Example 2

Purification of adrenal autoantigen.

The methods described by Kominami et al[15] and Bumpus and Dus[16] were used. In particular, Octyl-Sepharose CL-4B (Pharmacia LKB) was extensively washed in 50mM phosphate buffer pH 7.0, 20% glycerol, 0.1mM EDTA, 0.1mM DTT

according to the manufacturer's instructions, poured into 1 x 10cm column and equilibrated with basal buffer.

6ml aliquots of solublished microsomes were applied to the column at 25ml/h and eluted initially with basal buffer. 3.3ml fractions were collected and monitored by light absorbance at 280nm and 418nm. Washing of the column was continued until the OD 280nm was below 0.01 and OD 418nm was zero. Further elution from the column was then carried out using increasing concentrations of Emulgen 913 (Kayo-Atlas, Tokyo) in the basal buffer (step-wise increases; 0.025%, 0.05%, 0.1%, 0.15% and 0.2%; 25ml each step).

The column fractions were monitored at 418nm (monitoring at 280nm is not possible in the presence of Emulgen) and aliquots (0.5ml) taken from each fraction. The elution profile of proteins from the column is shown in Figure 1. Aliquots from groups of fractions were then pooled, concentrated (Centricon 10 microconcentrators) and analysed by gel electrophoresis. In particular, samples from the Octyl-Sepharose column were analysed on polyacrylamide gels (9%) under reducing conditions[17]. A mixture of molecular weight markers containing myosin (205,000), beta-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) was run on each gel.. After electrophoresis, gels were either stained with coomassie blue, fixed and dried or blotted onto nitrocellulose membranes.

In some experiments, protein bands of interest were cut out from the stained gel and the protein electroeluted (40V and 70mA for 20 hours) into 0.1M sodium bicarbonate pH 7.8 containing 0.1% SDS. The eluted material was then dialysed extensively against distilled water, dried under vacuum and stored at -40°C.

After proteins were blotted onto nitrocellulose membranes, the membranes were cut into strips and incubated in phosphate buffered saline (PBS) at 37°C overnight with gentle shaking. The membranes were then reacted with 10% newborn calf

serum (NCS) in PBS containing 0.5% Tween 20 for 2h at 37°C with gentle shaking. After rinsing and washing with PBS containing 0.5% Tween 20 (3 x 5ml) followed by reaction with serum from a patient with high levels of adrenal autoantibodies or with normal serum diluted 1:400 in 10% NCS, 10% glycerol, 1M glucose in PBS with 0.5% Tween 20 for 2h at 37°C with gentle shaking, the membranes were washed extensively with PBS containing 0.5% Tween 20. They were then incubated with dilute anti-human immunoglobulin-horseradish peroxidase conjugate in PBS containing 0.05% Tween 20 for 1h at room temperature with gentle shaking. This was followed by washing with PBS containing 0.5% Tween 20 and reaction with a chemiluminescence generating substrate according to the substrate manufacturer's protocol (ECL System from Amersham). Nitrocellulose membrane strips and photographic film were set for 10-60 seconds and then developed.

Experiments were also performed in which rabbit antibody to recombinant steroid 21-hydroxylase[56] and rabbit antibody to microsomal epoxide hydrolase[57] were used in combination with anti-rabbit IgG horseradish peroxidase conjugate.

Example 3

Analysis of Electrophoresis Gel

As can be seen in Fig. 2, the adrenal autoantibodies only reacted with protein bands with an observed molecular weight of 55,000 $\pm$ 5,000. In further studies, the 55,000 mol. wt. band of fraction VI was cut out of the gel, electroeluted and re-run in the gel electrophoresis system. This showed that the protein migrated as a single band with mol. wt. 55,000. In addition, when the electroeluted material was blotted into nitrocellulose, it reacted strongly with adrenal autoantibodies. These studies indicated that 55,000 molecular weight protein is the major adrenal autoantigen involved in autoimmune Addison's disease.

Protein staining of the gel obtained by electrophoresis of the column fractions showed that fraction VI contained a protein with a molecular weight of 55,000 (Fig. 3). Some of the fractions surrounding fraction VI also contained smaller amounts of the 55,000 mol. wt protein.

A further Western blot analysis of fraction VI is shown in Figure 6, in which Lane 1 shows reaction with normal pool serum, lane 2 reaction with adrenal autoantibody positive pool serum, lane 3 reaction with rabbit 21-hydroxylase antibody, lane 4 reaction with normal rabbit serum and lane 5 reaction with rabbit epoxide hydrolase antibody. The rabbit antibody to 21-hydroxylase, is shown to react with the 55,000 mol. wt. protein whereas the antibody to epoxide hydrolase reacted with a protein of molecular weight 49,000.

In some experiments, the 55k band from SDS-PAGE analysis of fraction VI was cut out of the gel, electroeluted, run on PAGE to confirm homogeneity (data not shown) and analysed by Western blot. The eluted band reacted strongly with adrenal autoantibody positive serum and with rabbit antibody to 21-hydroxylase but not with epoxide hydrolase antibody nor with normal pool human serum, nor with normal rabbit serum.

These observations indicate that the autoantigen is steroid 21-hydroxylase and that a previous suggestion that it is epoxide hydrolase (UK patent application No 9205040.0) resulted from sequencing the wrong band on a gel.

Example 4

Absorption of Adrenal Antigen with Adrenal Antibodies.

IgG was purified from normal pool sera and individual patient sera using chromatography on protein A-glass bead columns (Bioprocessing Consett, UK). Aliquots of purified adrenal protein were incubated with the IgGs (2-5mgml^{-1}) for 1h at 37°C. A protein A-glass bead suspension in PBS was then added to bind

free IgG and IgG complexed to adrenal antigen(s). After 1h at room temperature, the mixture was centrifuged (12,000xg, 10min, 4°C) and the supernatants analysed by SDS-PAGE and Western blotting using Addison sera and rabbit antibodies to recombinant 21-hydroxylase.

Analysis of the Western blots indicated that rabbit antibody to recombinant 21-hydroxylase did not react with native adrenal 55k protein which had been pre-absorbed with IgG from human adrenal autoantibody positive Addison sera (data not shown). Pre-absorption with normal pool serum IgG had no effect on reactivity with 21-hydroxylase antibody.

Example 5

Assay for adrenal autoantibodies using adrenal autoantigen.

Multiple small (1ul) aliquots of fractions I-VIII from the experiment shown in Fig. 1 were blotted into nitrocellulose membranes. The membranes were cut into strips and washed and reacted with serum containing adrenal autoantibodies or normal pool serum.

As can be seen in Fig. 4B, dot blots of fractions I-III reacted strongly with normal pool serum and there was a weak interaction between fraction IV and normal pool serum. The Western blotting studies shown in Fig. 2 indicated that this effect of normal human serum was not due to an interaction with adrenal autoantigen. However, fractions I-IV were clearly unsuitable for use in analysis of serum adrenal autoantibodies. Fraction VI however did not show any interaction with normal pool serum in the dot blot system, suggesting that it could be used in a specific assay system for adrenal autoantibodies. Typical results using fraction VI in a dot-blot assay are shown in Fig. 5. The dot-blot assay whose results are shown in Fig. 5 was conducted using ten serum samples from different patients, as follows: samples 1 & 2: Addison's disease; sample 3: normal pool; samples 4

& 5: systemic lupus erythematoses; sample 6: Hashimoto's disease; samples 7 & 8: primary biliary cirrhosis; samples 9 & 10: Graves' disease.

Table 1 summarises data obtained with a group of Addison patients. Out of the 36 Addison sera analysed by dot blot assay, 26 (72%) were positive. Analysis of the sera by one or more of three different methods (immunofluorescence, immunoprecipitation or Western blot) confirmed the presence of adrenal autoantibodies in all 26 dot blot positive sera (Table 1). The 10 Addison sera negative by dot blot assay were also negative in the three other systems used to detect adrenal autoantibodies (Table 1). The 26 adrenal autoantibody positive patients had a high proportion of thyroid autoantibodies. In particular, TPO (thyroid peroxidase) autoantibodies in 22/26 (85%), Tg (thyroglobulin) autoantibodies in 17/26 (65%) and TRAb (thyroid stimulating hormone receptor antibodies) in 4/26 (15%). At least two of the TRAb positive patients (nos. 23 and 26) had well-documentated histories of thyrotoxicosis. In contrast, the 10 Addison patients shown in Table 1 who were adrenal autoantibody negative had a similar proportion of thyroid autoantibodies as the normal population[18].

In dot blot assays of sera from 10 patients with Hashimoto's disease, 10 patients with Graves' disease, 5 patients with rheumatoid arthritis, 5 patients with systemic lupus erythematoses, 4 patients with primary biliary cirrhosis and 10 normal individuals, only one serum (from a patient with Graves' disease) was found to be positive. This positive Graves' serum was confirmed to have antibodies reactive with the 55,000 band on Western blot.

Our observations with the dot blot assay are in good agreement with previous studies of large numbers of sera using immunofluorescence[2,19-23]. For example, Nerup[20] and Blizzard[2] found that respectively 74% and 53% of patients with idiopathic Addison's disease had serum adrenal autoantibodies by immunofluorescene. Healthy normal donors rarely have adrenal autoantibodies detectable by immunofluorescence but most studies report adrenal autoantibodies

in a few patients with non-adrenal autoimmune disease, including Graves' disease[20-23]. This is consistent with the detection of adrenal autoantibodies by dot blot in one Graves' serum.

Table 1

Adrenal Autoantibody Assays

Addison Patient No.	Dot Blot	Western Blot	Immuno-fluorescence	Immuno-precip.
1	+	++++	nt	nt
2	+	++++	nt	nt
3	+	+++	nt	nt
4	+	+++	nt	nt
5	+	++	nt	nt
6	+	+	+	+
7	+	++	nt	nt
8	+	+	nt	nt
9	+	+	nt	nt
10	+	+	nt	nt
11	+	+++	nt	nt
12	+	++	+	+nt
13	+	++	nt	nt
14	+	+	nt	+
15	+	nt	+	+
16	+	nt	+	+
17	+	nt	++	+
18	+	+	+	+
19	+	+	+	+
20	+	+	+	+
21	+	+	+	+
22	+	+	+	+
23	+	+	+	+
24	+	+	+	+
25	+	+	+	nt
26	+	+	+	nt
27	neg	nt	neg	neg
28	neg	nt	neg	neg
29	neg	nt	neg	neg
30	neg	nt	neg	neg
31	neg	nt	neg	neg
32	neg	nt	neg	neg
33	neg	nt	neg	neg
34	neg	neg	nt	nt
35	neg	neg	nt	nt
36	neg	neg	neg	neg

Thyroid Autoantibodies

Addison Patient No.	TgAb U/ml	TPOAb U/ml	TRAb	Other Auto-antibodies
1	0.6	30	neg	
2	16	30	neg	
3	14.5	30	45.5	
4	6	30	neg	
5	neg	2.7	neg	
6	30	30	91.9	a,b,c
7	1.2	21	neg	
8	neg	2.0	neg	
9	neg	26	neg	
10	12.5	17	neg	
11	7.5	1.9	neg	
12	20	8.9	neg	a,c,d
13	neg	neg	neg	
14	0.5	21	neg	
15	neg	neg	neg	c,e
16	neg	neg	neg	
17	2.2	30	neg	b,c,g
18	neg	neg	neg	c
19	1.7	21	neg	c
20	neg	1.7	neg	c
21	neg	25	neg	c
22	16	34	18.3	
23	30	30	neg	d,e,f
24	8.4	12	6.3	
25	30	30	47.6	c
26	8.2	30	neg	c
27	neg	neg	neg	
28	neg	neg	neg	
29	neg	neg	neg	
30	0.4	8.6	neg	d
31	neg	neg	neg	c,d
32	neg	5.0	neg	
33	neg	neg	neg	
34	neg	neg	neg	
35	neg	neg	neg	
36	neg	neg	neg	

Key: a = antitubulin antibodies
b = anti-islet cell antibodies
c = anti-parietal cell antibodies
d = anti-nuclear antibodies
e = anti-mitochondrial antibodies
f = anti-desmin antibodies
g = anti-steroid producing cell antibodies
nt = not tested

Example 6

Expression of 21-hydroxylase in Saccharomyces cerevisiae

For expression in the yeast Saccharomyces cerevisiae, the plasmid yeast vector pYES 2.0 (obtained from Invitrogen) was used. The steroid 21-hydroxylase gene sequence in the plasmid vector pCD//pC21/3c[10] was obtained from the American Type Culture Collection, Rockville, USA (Deposit No 57421). The 21-hydroxylase gene sequence was subcloned as BamHI fragments (BamHI obtained from Northumbria Biologicals Ltd, Cramlington NE23 9HL, UK) into the cloning vector pTZ18 (obtained from Pharmacia).

After subcloning, further digestion was carried out using the enzyme SphI (Northumbria Biologicals Ltd, Cramlington NE23 9HL, UK) followed by NarI (Northumbria Biologicals Ltd, Cramlington NE23 9HL, UK) to obtain a large fragment comprising the entire coding region of the 21-hydroxylase gene apart from 41 base pairs at the 5' end.

The 21-hydroxylase gene so prepared was then ligated into the plasmid yeast vector pYES (cut with the enzymes BamHI and SphI) using a linker molecule consisting of parts of the STE2 gene (11 base pairs of non-coding sequence and 42 base pairs of coding sequence)[58]. The linker molecule was synthesised using an oligonucleotide synthesiser.

The 21-hydroxylase gene incorporated into the plasmid yeast vector in this way was then incorporated into yeast cells (Saccharomyces cerevisiae C13 ABYS 8B, Yeast Genetic Stock Center, Berkeley, California) by electroporation (equipment obtained from Invitrogen) and the cells grown at 30°C for 48h on selective media (YND-glucose supplemented with amino acids except for uracil). The yeast cultures grown on selective media were used to inoculate larger expression cultures in YEP-glucose (2%) or YEP galaclose (2%).

Yeast cells from 100ml 48h expression cultures were harvested at late-log phase, washed in 50mM Tris-HC1 pH 8.0 with addition of 1mM phenyl-methyl-sulphonyl-fluoride (PMSF) and resuspended in 1ml of 1% sodium deoxycholate in 50mM Tris-HC1, pH 8.0 containing 1mM PMSF. Acid treated beads (Sigma 450-500um) were added and the cells broken by vortexing 10 times for 30sec with 30sec incubation on ice between such vortex period. After the removal of glass beads, the homogenates were centrifuged at 12,000 x g for 2min and the supernatants analysed on SDS-PAGE followed by Western blotting.

The subject matter contained in this application includes the subject matter of UK patent applications Nos 9205040.0 and 9216306.2 (published as GB 2256046), from which priority is claimed and copies of which are filed herewith.

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CLAIMS

1. A method for detecting adrenal autoantibody in a sample, comprising contacting with the sample

(i) a protein obtainable from the microsome fraction of human or mammal adrenal glands and having an observed molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody;

(ii) the adrenal protein (i) modified by one or more amino acid modifications and comprising an epitope for the adrenal autoantibody; or

(iii) a fragment of modified or unmodified adrenal protein (i) comprising an epitope for the adrenal autoantibody.

2. A method as claimed in claim 1, wherein the protein has a molecular weight of about 55,000.

3. A method as claimed in claim 1 or claim 2, wherein the protein or protein fragment is of recombinant origin.

4. A method as claimed in claim 1 or claim 2, wherein there is contacted with the sample a said fragment which is a synthetic peptide.

5. A method as claimed in any of claims 1 to 4, wherein the protein or protein fragment is labelled and the method further comprises detecting for labelled protein (fragment)/autoantibody complex or unbound labelled protein (fragment).

6. A method as claimed in any of claims 1 to 4 and which further comprises contacting the protein or protein fragment and the sample with labelled antibody to the protein or protein fragment and then detecting labelled antibody bound to

the protein or protein fragment.

7. A method as claimed in claim 6, wherein the protein or protein fragment is immobilised on a substrate.

8. A method as claimed in any of claims 1 to 4, wherein the protein or protein fragment is immobilised on a substrate and the method further comprises, after contacting the protein or protein fragment with the sample, contacting the protein or protein fragment with labelled ligand for the adrenal autoantibody and detecting bound labelled ligand.

9. A method as claimed in claim 8, wherein the ligand is a protein or protein fragment as defined in claim 1, an antibody to the adrenal autoantibody, an anti-immunoglobulin, protein A or protein G.

10. A method as claimed in any one of claims 1 to 9, wherein the protein or protein fragment is human.

11. A method for detecting adrenal autoantibody in a sample, comprising contacting with the sample:

a) protein comprising an epitope for adrenal autoantibody, having an observed molecular weight of from about 50,000 to about 60,000 and obtainable by:

homogenising mammalian adrenal glands,

subjecting the homogenate to differential centrifugation to obtain a microsome fraction,

suspending the microsome fraction in a phosphate buffer,

centrifuging the suspension in the presence of sodium cholate to form a supernatant,

adding polyethylene glycol and further sodium cholate to the supernatant and mixing the supernatant,

defined in any of claims 1 to 3, 10 or 11 or a protein fragment as defined in any of claims 1 to 4, 10 or 11 and a pharmaceutically acceptable diluent, excipient or carrier.

24. A pharmaceutical composition as claimed in claim 23, wherein the active ingredient is a protein fragment which is a peptide.

25. A pharmaceutical composition comprising a ligand capable of binding to the receptor of an adrenal autoantigen specific T cell and a pharmaceutically acceptable diluent, excipient or carrier.

26. A pharmaceutical composition as claimed in claim 25 wherein the ligand is peptide.

27. An isolated T cell specific for a protein obtainable from the microsome fraction of human or mammal adrenal glands and having an observed molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody.

28. A protein or protein fragment which mimicks the receptor of a T cell specific for a protein obtainable from the microsome fraction of human or mammal adrenal glands and having an observed molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody.

29. An isolated T cell induced by a T cell as claimed in claim 27 or by a protein or protein fragment as claimed in claim 28.

30. An isolated Ts cell specific for a B cell or T cell specific for a protein obtainable from the microsome fraction of human or mammal adrenal glands and having an observed molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody.

31. A pharmaceutical composition comprising T cells specific for a protein obtainable from the microsome fraction of human or mammal adrenal glands and having an observed molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody, T cells induced by T cells specific for a protein obtainable from the microsome fraction of human or mammal adrenal glands and having an observed molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody, Ts cells specific for B cells or T cells specific in either case for a protein obtainable from the microsome fraction of human or mammal adrenal glands and having an observed molecular weight of about 50,000 to 60,000 and antigenic towards adrenal autoantibody, or a protein or protein fragment which mimicks the receptor of such a T cell and a pharmaceutically acceptable diluent, excipient or carrier.

32. A method of producing a transformant of Saccharomyces cerevisiae capable of producing steroid 21-hydroxylase, comprising ligating into a yeast expression vector the steroid 21-hydroxylase gene sequence obtainable from plasmid pCD//pC21/3c (ATCC 57421) modified by replacement of the bases coding for the first 13 amino acids with the bases coding for a yeast leader sequence, and transforming Saccharomyces cerevisiae with the resultant vector.

33. A method as claimed in claim 32, wherein the steroid 21-hydroxylase gene sequence obtainable from said plasmid is subcloned as a BamHI fragment into vector pTZ18, and the resultant vector containing a 21-hydroxylase gene sequence is digested to obtain a fragment comprising a coding region for 21-hydroxylase and ligated into yeast expression vector pYES, as cut with BamHI and SphI, using a BamHI - NarI linker comprising 11 base pairs of non-coding and 42 base pairs of coding STE2 gene sequence.

34. A method of expressing steroid 21-hydroxylase, comprising culturing Saccharomyces cerevisiae transformants prepared by a method as claimed in claim 32.

35. A method of producing a yeast expression vector comprising a DNA sequence encoding steroid 21-hydroxylase, comprising ligating into a yeast expression vector the steroid 21-hydroxylase gene sequence obtainable from plasmid pCD//pC21/3c (ATCC 57421) modified by replacement of the bases coding for the first 13 amino acids with the bases coding for a yeast leader sequence.

36. Transformed cells of Saccharomyces cerevisiae capable of expressing steroid 21-hydroxylase and obtainable by a method as claimed in claim 32.

37. A yeast expression vector capable of transforming Saccharomyces cerevisiae to express steroid 21-hydroxylase and obtainable by a method as claimed in claim 35.

Fig. 1

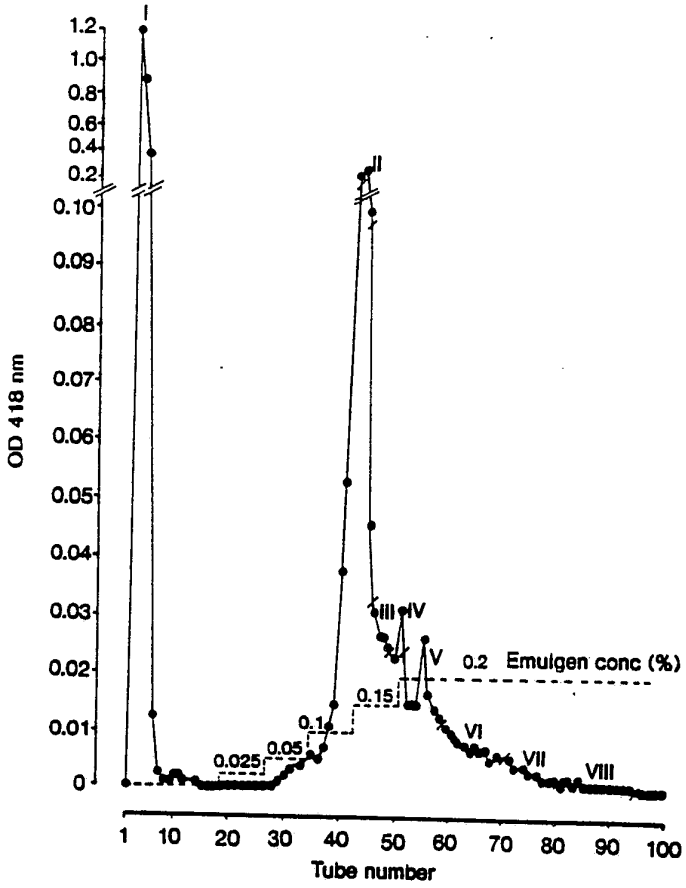

OD 418 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
I
II
III
IV
V
VI
VII
VIII
0.025
0.05
0.1
0.15
0.2 Emulgen conc (%)
1 10 20 30 40 50 60 70 80 90 100
Tube number

Fig. 2

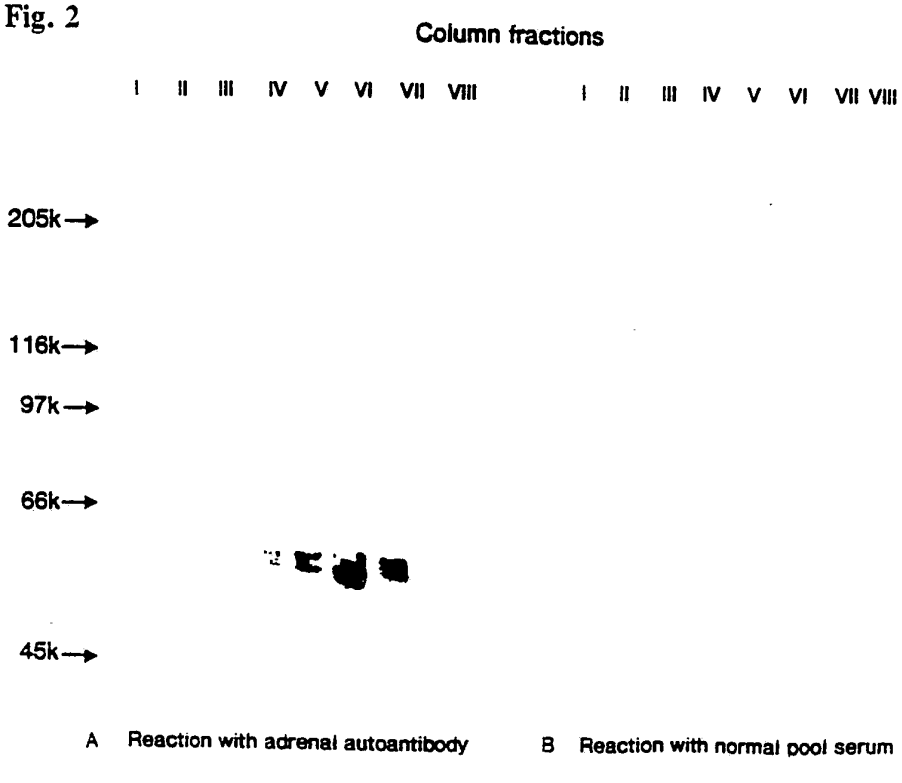

Column fractions
I II III IV V VI VII VIII I II III IV V VI VII VIII
205k
116k
97k
66k
45k
A Reaction with adrenal autoantibody
B Reaction with normal pool serum

Fig. 3

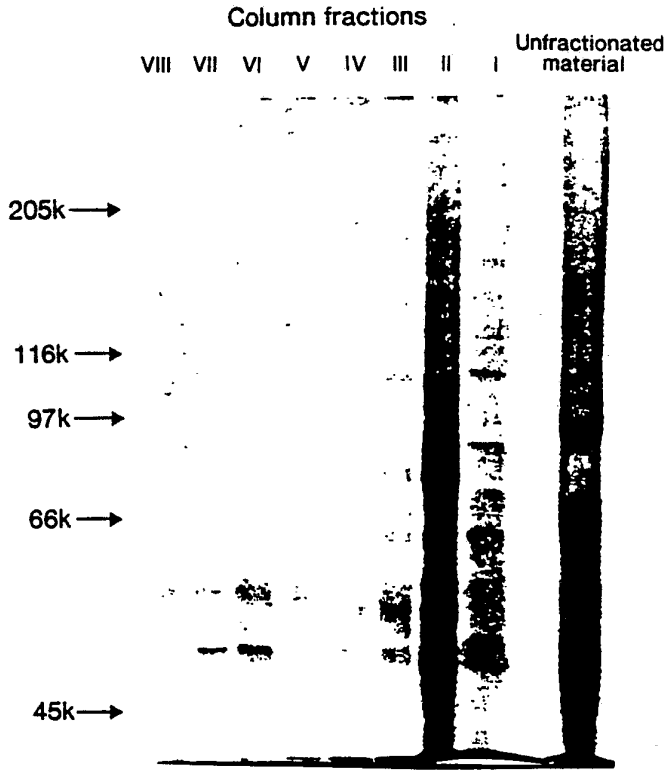

Column fractions
VIII VII VI V IV III II I
Unfractionated material
205k
116k
97k
66k
45k

Fig. 4

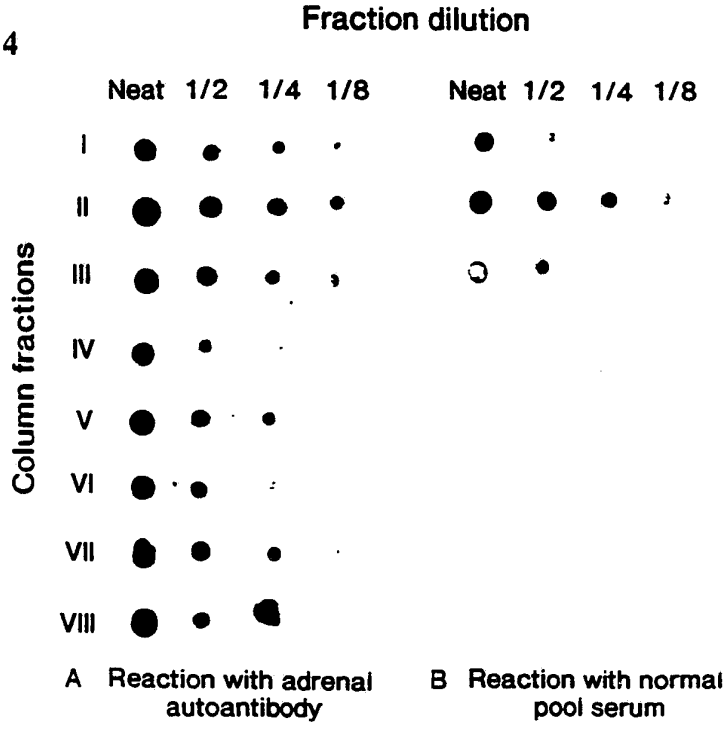
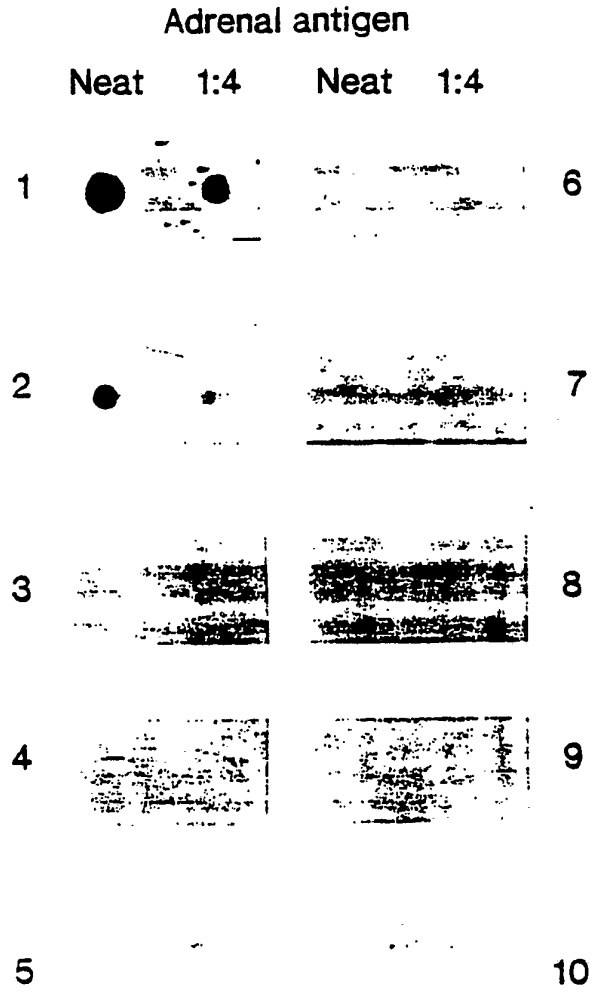

Fraction dilution
Neat 1/2 1/4 1/8
Neat 1/2 1/4 1/8
Column fractions
I
II
III
IV
V
VI
VII
VIII
A Reaction with adrenal autoantibody
B Reaction with normal pool serum

Fig. 5



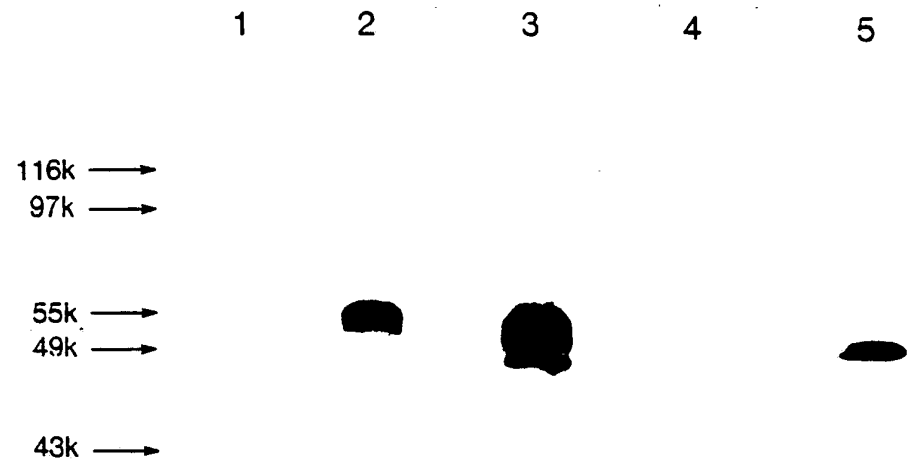


Fig. 6

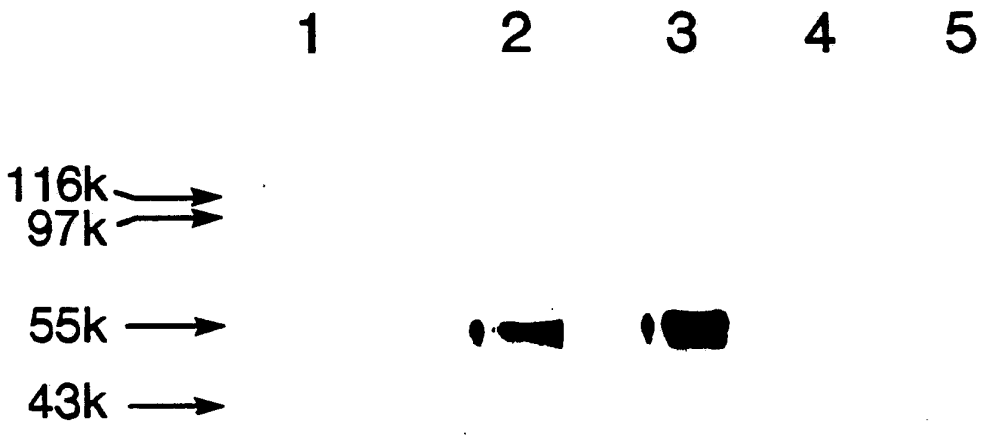


Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00443

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/564; G01N33/68; G01N33/58; G01N33/543
A61K37/52; C12N5/08; C12N9/02; C12N15/81

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N ; A61K ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	FEBS LETTERS vol. 231, no. 1, April 1988, AMSTERDAM NL pages 25 - 28 J. FURMANIAK; D. TALBOT; D. REINWEIN; G. BENKER; F. M. CREAGH AND B. REES SMITH 'Immunoprecipitation of human adrenal microsomal antigen' cited in the application see the whole document ---	1-24
P,X	THE LANCET vol. 339, 28 March 1992, LONDON GB pages 770 - 773 KAI KROHN ET AL. 'Identification by molecular cloning of an autoantigen associated with Addison's didease as steroid 17alpha-hydroxylase' cited in the application see the whole document --- -/--	1-24

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14 JUNE 1993	28. 06. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	DöPFER K.P.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	MOLECULAR AND CELLULAR ENDOCRINOLOGY vol. 68, no. 1, 2 January 1990, SHANNON, IE pages 29 - 34 M. L. CHOUINARD AND H. R. FEVOLD 'ACTH-induced increases in rabbit adrenal immunoreactive P-450-17alpha and P-450-21' cited in the application see the whole document	1-24
A	THE JOURNAL OF CLINICAL ENDOCRINOLOGY & METABOLISM vol. 70, no. 1, January 1990, PHILADELPHIA, PA, US pages 95 - 99 GEORGE M. BRIGHT AND INDERJIT SINGH 'Adrenal Autoantibodies Bind to Adrenal Subcellular Fractons Enriched in Cytochrome-c Reductase and 5'-Nucleotidase' cited in the application see the whole document	1-24
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 83, no. 14, July 1986, WASHINGTON US pages 5111 - 5115 PERRIN C. WHITE, MARIA I. NEW, AND BO DUPONT 'Structure of human steroid 21-hydroxylase genes' cited in the application see the whole document	32-37
A	EP,A,0 340 878 (GIST-BROCADES N. V.) 8 November 1989 cited in the application see the whole document	32-37

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9300443 SA 70723

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 14/06/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0340878	08-11-89	AU-B- 635494	25-03-93
		AU-A- 3575989	29-11-89
		JP-T- 4500303	23-01-92
		WO-A- 8910963	16-11-89
		AU-A- 4428289	18-04-90
		CN-A- 1042567	30-05-90
		EP-A- 0360361	28-03-90
		JP-T- 3501445	04-04-91
		WO-A- 9003428	05-04-90