CLONING AND SEQUENCING OF A MITOCHONDRIAL AUTOANTIGEN WITH IMMUNOGLOBULIN G FROM PATIENTS WITH MULTIPLE SCLEROSIS

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Abstract

Multiple Sclerosis (MS) is a chronic neurological disease of the central nervous system (CNS), characterised by a cellular immune response in early stages and demyelination of the CNS later. Although the cause of MS is unknown, there is much evidence that points to MS as an autoimmune disease. To test the hypotheses that an Autoantigen is involved in MS, we screened a λgt11 human foetal spinal cord cDNA library using IgG from patients with MS. From screening 2×10^6 recombinant phage, 6 positive clones were identified. Properties expressed by these lambda phage on the bacterial lysate plate appeared to react specially with pooled MS IgG but not with pooled IgG purified from normal human sera. The positive clones were amplified by the polymerase chain reaction (PCR) and sequenced. After searching in GenBank analysis of the sequences showed a high percent similarity between three clones called M64, M63 and M62 (0.6, 0.9 and 1.8 Kb length, respectively) and the mitochondrial gene encoding the human NADH:ubiquinone reductase (complex I). This is the first time a mitochondrially encoded protein has been shown to be an autoantigen. This discovery adds to the growing list of intracellular enzymes which are considered as involved autoantigens in autoimmune diseases.

Introduction

There is general agreement that abnormal auto-immune responses are involved in the pathogenesis of Multiple Sclerosis (MS). Despite the intensive work over the last 30 years, there is no consensus as to the importance of the various antigens which have been shown to react with autoantibodies or T cells from patients with MS [1]. In other autoimmune disease there has been considerable success in cloning new antigens through the use of antibodies from patients to probe cDNA libraries. This hassled to the isolation of some very interesting new antigens which are useful in diagnosis and understanding the pathogenesis of disease. For example, probing of a human λgt11 fibroblast cDNA library with sera from patients with

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connective tissue disease led to the isolation of a "U1 small nuclear ribonucleoprotein C polypeptide" autoantigen [2]. A "P70(KU) lupus" autoantigen was isolated by screening of a human hepatoma λgt11 cDNA library against sera from patients with systemic lupus erythematosus [3]. A human thyroid λgt11 cDNA library was also screened against sera from patients with Hashimoto's thyroiditis and a "64-kDa" autoantigen was isolated [4]. Probing of a human cerebellar λgt11 cDNA library degeneration led to the isolation of a zinc-finger protein as an autoantigen [5]. Screening of a human fetal adrenal λgt11 cDNA library against sera from patients with Addison's disease led to the isolation of a steroid (17α-hydroxylase) autoantigen [6]. MHC class I-restricted autoantigen in type 1 diabetes [7], formiminotransferase cyclodeaminase in hepatitis [8], follistatin-related protein in rheumatoid arthritis [9] and canine bullous pemphigoid (CBP) antigens in CBP patients [10] are also some other examples, which have recently been identified as novel autoantigens using the screening of organ-specific cDNA libraries. In some cases the discovery has even proceeded to the development of useful immunoassays which can be used in the early diagnosis of the disease [11]. Islet cell autoantigen 69 in insulin dependent diabetes mellitus [12] and zinc-finger like protein in paraneoplastic cerebellar degeneration [13] are two examples of this category. One of the big surprises in autoimmunity in recent years has been the frequent occurrence of enzymes that are clearly autoantigens [14]. Some of these antigens, for example thyroid peroxidase in thyroid disease, make some sort of sense in relation to the pathogenesis; others such as pyruvate dehydrogenase in primary biliary cirrhosis [15] make little or no sense with one of the main problems being their general occurrence in the body, yet the autoimmune attack is localized to a particular organ. It is interesting that several of the autoantibodies which have been identified, actually bind to the active site and inhibit their target enzyme [14].

To test the hypothesis that an autoimmune mechanism is involved in MS, we have screened a λgt11 foetal spinal cord (HFSC) cDNA library with pooled IgG purified from 5 MS patients sera. A totally unexpected new autoantigen was identified which could indicate, that mitochondria are involved in MS.

Materials and Methods

Materials

Luria-B [16] media was prepared using 'Difco'-certified yeast extract, tryptone and bactoagar (FSE, Australia). Supported nitrocellulose filters (Hybond C-extra), secondary antibody (sheep anti-human IgG F(Ab´)2 HRP-linked) and the chemiluminescence detection reagents (ECL for Western bolt) were obtained from Amersham, Australia. RX (blue-sensitive) autoradiography film (Fujii) was from Hanimex, Perth. Normal and MS sera were obtained from collections used by Dr. R. D. Cook, Murdoch University. IgG was purified using protein A-Sepharose (Pharmacia, Australia). A λgt11 library was obtained from Dr. C. Campagnoni, Mental Retardation Research Center, University of California, Los Angeles. It was made from poly A+RNA from human spinal cord at 14-16 weeks gestation. The cDNA were cloned into λgt11 using EcoR1 linkers. The base titre was 1.5×10^9 pfu.

Obtaining the Disease Specific Clones

The positive clones were isolated from cDNA library by probing with a pool of IgG prepared from the individual sera. A new method has been used for screening cDNA library [17]. About 2×10^6 phage particles from the cDNA library were plated out on 150 mm Petri dishes (40000 in each). Expressed proteins were transferred to nitrocellulose filters and probed with pooled IgG purified from 5 MS patients. Positive plaques, which produced signals in duplicate filters, were picked after alignment with the original plate by stabbing a sterile Pasteur pipette through both the top overlay and underlying agar. The plaques were transferred to a 1.5 ml microcentrifuge tube containing 300 μl of SM buffer, vortexed for 30 seconds after adding one drop of chloroform and incubated at 4°C overnight. In order to obtain clones which only react with antibodies associated with the autoimmune disease, it is necessary to remove any clones which also react with IgG present in normal sera. After stripping the nitrocellulose filters according to the recommended method by the manufacture of the ECL kit they were reacted with pooled normal human IgG. The method for testing the stripped filters against the pooled normal IgG was the same as above. Any clone, which also reacted with normal IgG, was then discarded.

Positive clones were rescreened twice on small plates (90 mm, about 100 plaques each) until all plaques produced positive signals in duplicate filters. A second screening with normal IgG was performed. Finally, a total of 6 clones which reacted with the pooled MS IgG, but did not react with pooled normal IgG, survived after tertiary screening. Five μl of each phage solution (containing approximately 100 recombination phage) was added on the top of an agar plate containing a Y1090 bacterial lawn in duplicate places. Protein lifts were carried out after 2-3 h incubation at 37°C. The filter was then cut into two pieces, each carrying the same protein for incubation with MS and normal IgG, and processed for screening with the same method as above. With this method, it is possible to check cross

reactivity of more than 20 clones against two kinds of IgG using just one 137 mm nitrocellulose filter.

Amplification and Sequencing of the Positive Clones

The polymerase chain reaction (PCR) technique was employed to amplify the inserted DNA from all isolated positive clones using the following materials purchased from Biotech International, Perth, Western Australia. Lambda gt11 forward (5′-ggtggcgacgactcctggagcccg-3′) and reverse (5′-ttgacaccagaccaactggtaatg-3′) primers (20 ng each) were mixed with dNTP (1 ml of 2 mM), $MgCl_2$ (1 ml of 25 mM), 0.7 unit of Tth plus DNA polymerase and 10×PCR reaction buffer in a 25 µl total volume. As DNA template, 40 ng of phage DNA, 0.5 µl of phage solution or a touch fresh plaque by the tip of a pipette and then was left in PCR solution for 1 min was used. To prevent the evaporation of the solution in high temperature, 1 drop of paraffin oil was added to the top of the solution and spun in a microcentrifuge for 5 sec. The amplification proceeded for 25 cycles in a DNA thermal cycler (Perkin Elemer Cetus, Norwalk, USA). Each cycle involved a 94°C denaturation step for 1 min followed by 1 min annealing step at 62°C and then 2 min polymerization step at 72°C. Denaturing time for the forst cycle was 5 min to destroy the phage protein coat and DNA super-coil. Polymerization time for the last cycle was 10 min to get full length of amplified products. Each PCR products (5 µl) was run on 1% agarose gel for identification of the amplified DAN. The PCR products were run on low melting agarose gel and sequenced after direct purification from gel. Sequencing reactions were performed by the dideoxynucleotide chain termination method using a PRISM Ready Reaction DyeDeoxy Termination cycle sequencing kit. The samples were applied on 6% (w/v) acrylamide gel and the sequences were analyzed using the Applied Biosystem sequencing system, Model 373A.

Results and Discussion

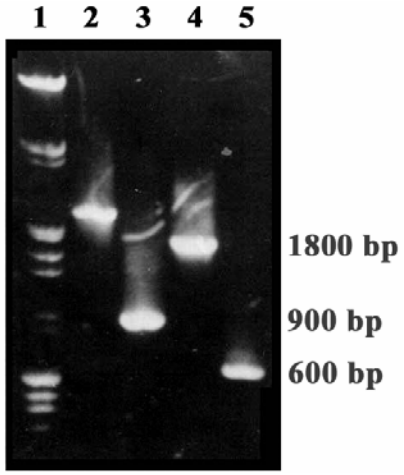
Clones have been successfully isolated from a cDNA expression library by application of the parallel lift method. By eliminating all clones which also react with normal IgG and MS IgG, antigen-antibody reactions specific for MS were detected. Six positive clones survived the tertiary screening. Purified DNA from the positive clones were amplified by the PCR method. Figure 1 shows the electrophoresis of the PCR product from 4 of these clones, run in 1% agarose gel. These clones had inserts ranging from approximately 600 to 2000 bp. Direct sequencing of the inserts of the positive clones is a fast way to identify the sequence of the antigen. This method is only applicable when amplification of the insert, using the external primers for the bacteriophage vector DNA, produces a clear sharp band. A good quality template for sequencing was produced by purification of the DNA from the agarose gel of clones M62, M63 and M64. The PCR product from clones M62, M63 and M64 was purified from low melting agarose gel and fully sequenced. The full sequence of clone M64 is shown in Figure 2. A consensus sequence was determined from that in M62, M63 and M64 (Figure 3). Three of the clones M62, M63 and M64 had a very high degree of similarity over the first 300 nucleotides. The sequence shows the presence of a double stop codon at position 45 and 46 which meant that the epitope recognized by MS IgG had to be within the first 44 amino acids expressed in λgt11 clones.

Sequence analyses were conducted on GenBank (release 87, National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA) to determine if this nucleotide sequence shared identity with any known or characterized nucleotide sequences. The analysis identified a greater than 99% identity between the above nucleotide sequence and a portion of a mitochondrial gene (Figure 4) called ND4, which encodes NADH:ubiquinone reductase, a part of complex I in the mitochondrial electron transport chain. The region of ND4 that was cloned contains the quinone-binding site for Complex I [18].

In summary, we have isolated yet another auto-antigen which is an enzyme. This enzyme adds to the mystery of the pathogenic role of enzyme autoantigens. It is the first mitochondrially-encoded autoantigen, which has been discovered. While other mitochondrial autoantigens have been reported (e.g., pyruvate dehydrogenase [15]), these come from nuclear-encoded molecules.

At first sight, it is difficult to see how the antibody to Complex I could be involved in the pathogenesis of MS, as it would have to get inside the oligodendrocyte and then inside the mitochondria. At this stage, we have proposed a new hypothesis which involves damage to mitochondria and a plasma membrane localization for the autoantigen in plasma membrane oxidoreductase. If correct, apart from the implications with regard to a better understanding of the pathogenesis of MS, there are potential new therapies and new diagnostic tests. However, this hypothesis will need to be examined by additional experiments.

Affinity-purified ND4 antibody could also be a very useful tool for investigating the structure and function of NADH:ubiquinone reductase enzyme in normal people and in diseases associated with abnormalities in Complex I.



PCR amplification of the lambda gt11 inserts. Lane 1 is MW marker, 2, 3, 4 and 5 are positive clones from screening of a human fetal cDNA library with purified IgG from MS sera.

Figure 1. Presents the electrophoretic pattern of products of amplification by PCR of the DNA clones isolated with MS IgG. Lambda gt11 primers were used for the amplification. The marker DNA in lane 1 was a mixture of the digested pUC19 DNA/MpA II and λ DNA Eco RI/Hind III. Lane 2, 3, 4 and 5 represents the products from clones M61, M62, M63 and M64, respectively.

λgt11 ⟶ | EcoRI site |
```
TCAGTATCGGCGGAATTCCGGTCATTCTCATAATCGCCCACGGGCTTACA
1        10        20        30        40        50
TCCTCATTACTATTCTGCCTAGCAAACTCAAACTACGAACGCACTCACAG
51       60        70        80        90        100
TCGCATCATAATCCTCTCTCAAGGACTTCAAACTCTACTCCCACTAATAG
101      110       120       130       140       150
TTTTTGGGGTGACTTCTAGCAAGCCTCGCTAACCTCGCCTTACCCCCCAC
151      160       170       180       190       200
TATTAACCTACTGGGAGAACTCTCTGTGCTAGTAACCACGTTCTCCTGAT
201      210       220       230       240       250
CAAATATCACTCTCCTACTTACAGGACTCAACATACTAGTCACAGGCCTA
251      260       270       280       290       300
TACTCCCTCTACATATTTACCACAACACAATGGGGGTCACTCACCCACCA
301      310       320       330       340       350
CATTAACAACATAAAACCCTCATTCACACGAGAAAACAACCTCATGTTCA
351      360       370       380       390       400
TACAACTATCCCCCATTCTCCTCCTATCCCTCAACCCCGACATCATTACC
401      410       420       430       440       450
GGGTTTTCCTCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
451      460       470       480       490       500
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| EcoRI site | ⟵ λgt11
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CGGAATTCCAGCTGAGCGCCGTCGCTAC
501      510       520     528
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Figure 2. Shows the full sequence of clone M64.

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                                10        20        30        40
m62/63/64                 NNTATCGGCGGAATCCCCGGTAATTCTCATAATCGCCCACGGGCT
                                        : : :: X:::::::::::::::::::::
HUMMTC    TCTCATCCAAACCCCCTGAAGCTTCACCGGCGCAGTCATTCTCATAATCGCCCACGGGCT
              11670     11680     11690     11700     11710     11720

             50        60        70        80        90       100
m62/63/64 TACATCCTCATTACTATTCTGCCTAGCAAACTCAAACTACGAACGCACTCACAGTCGCAT
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
HUMMTC    TACATCCTCATTACTATTCTGCCTAGCAAACTCAAACTACGAACGCACTCACAGTCGCAT
              11730     11740     11750     11760     11770     11780

            110       120       130       140       150       160
m62/63/64 CATAATCCTCTCTCAAGGACTTCAAACTCTACTCCCACTAATAGCTTTTTGGATGACTTC
          ::::::::::::::::::::::::::::::::::::::::::::::::: ::::::::::
HUMMTC    CATAATCCTCTCTCAAGGACTTCAAACTCTACTCCCACTAATAGCTTTTT-GATGACTTC
              11790     11800     11810     11820     11830      11840

            170       180       190       200       210       220
m62/63/64 TAGCAAGCCTCGCTAACCTCGCCTTACCCCCCACTATTAACCTACTGGGAGAACTCTCTG
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
HUMMTC    TAGCAAGCCTCGCTAACCTCGCCTTACCCCCCACTATTAACCTACTGGGAGAACTCTCTG
               11850     11860     11870     11880     11890     11900

            230       240       250        260      270        280
m62/63/64 TGCTAGTAACCACGTTCTCCTGATCAAATATCACTCTCCTACTTACAGGACTCAACATA
          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
HUMMTC    TGCTAGTAACCACGTTCTCCTGATCAAATATCACTCTCCTACTTACAGGACTCAACATA
               11910     11920     11930     11940     11950
```

Figure 3. Shows the consensus sequence from analysis of nucleotides in clones m62, m63 and m64 which were found to express peptides and polypeptides specific for the multiple sclerosis.

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m62con, 261 nt vs GenBank - %AW library
using DNA matrix
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	initl	initl	opt
H. sapiens mitochondrial genome	536	536	1032
H. sapiens mitochondrial genome	556	536	1032
Human mitochondrion, complete genome	536	536	1032
Human mitochondrial tRNAs and partial protein	529	529	1025
H. sapiens partial cDNA sequence; clone HEEF	522	522	1018
Human mitochondrial DNA, complete sequence	549	529	1018
H. sapiens cDNA clone A338	504	440	930
Pygmy chimpanzee mitochondrial DNA	844	453	878
Chimpanzee (*P. troglodytes*) mitochondrial genes	849	451	871
Chimpanzee mitochondrial DNA, complete sequence	835	444	857
Gorilla mitochondrial genome fragment	792	407	832
Gorilla mitochondrial tRNAs and partial protein	785	401	825
Gorilla mitochondrial DNA, complete sequence	785	401	825
Human keratinocyte cDNA, clone 515	404	360	809
Orangutan mitochondrial DNA, complete sequence	728	372	775
Orangutan (*P. pygmaeus*) mitochondrial genes	716	372	768
Gibbon mitochondrial genome fragment	652	357	685
Mitochondrion *M. fuscata* NADH-dehydrogenase	523	304	626
Mitochondrion *M. sylvanus* NADH-dehydrogenase	523	290	612
Mitochondrion *M. mulatta* NADH-dehydrogenase	523	290	605

Figure 4. Shows that the consensus nucleotide sequence in m62/m63/m64 is over 99% identical to six depositions of human mitochondrial DNA in GenBank (release number 87). It is also homologous to 10 depositions of mitochondrial DNA from other primates.

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