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ANTIBODY TO MITOCHONDRIAL COMPLEX-I IN SOME PATIENTS WITH MULTIPLE SCLEROSIS

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ABSTRACT

When pooled immunoglobulin G (IgG) from patients with multiple sclerosis (MS) was used to probe a human fetal spinal cord λgt11 cDNA library, the IgG was found to bind to a predicted epitope of human mitochondrial ND4 sequence. To investigate the involvement of the ND4 as an autoantigen in MS, we determined the presence of specific antibody in the serum of MS patients and serum samples of some other autoimmune disease as controls.

A peptide, which is part of the ND4 protein in human mitochondrial complex I, CysLeuAlaAsnSerAsnTyrGluArgThrHisSerArg, was conjugated with a maleimido-thiol bond to diphtheria toxoid and used as an autoantigen. To remove any IgG which bound to diphtheria toxoid and the bovine serum albumin (BSA) blocking agent in the ELISA, the sera were preadsorbed before being incubated with the conjugate. About 20% of patients with multiple sclerosis (MS) had antibody to the peptide and when present, the level was found to fluctuate. In preliminary experiments autoantibody to ND4 was found to be not specifically associated with MS. The prevalence and involvement of the autoantibody in multiple sclerosis remains to be determined.

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INTRODUCTION

While there is general agreement that genetic, autoimmune and environmental factors are involved in the pathogenesis of MS, there is still no consensus as to the respective roles of susceptibility genes, antigens and microorganisms in the pathogenesis of MS.[1] Evidence for autoimmune responses to myelin and non-myelin proteins has been frequently presented and the list was recently extended by the addition of αB-crystallin.[2] In several autoimmune diseases patients have been found to produce antibodies to an interesting variety of enzymes[3] and some of these have been useful in differential diagnosis. Several of these autoantibodies to enzymes have been discovered by probing λgtl1 cDNA libraries.[4]

To locate new autoantigens in MS, a control pool of 5 samples of IgG from patients with MS was thoroughly adsorbed with the *E. coli* host (Y1090) which was used for expression of proteins in a λg11 cDNA library from human fetal spinal cord.[5] When this MS IgG was used to probe the cDNA, 6 clones were obtained and 3 of them contained part of the human ND4 sequence.[6] ND4 is one of the 7 mitochondrially encoded proteins which together with over 34 nuclearly encoded proteins comprise NADH: ubiquinone reductase (Complex I).[7] This sequence is particularly interesting as it contains the quinone binding site for Complex I,[8] the arginine which is mutated to histidine in Leber's Hereditary Optic Neuropathy (MTND4*LHON11778A)[9] and a major epitope for the induction of antibodies in rabbits.[10] The epitope

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reacting with the MS IgG is part of a consensus sequence which Degli Esposti et al.[8] have shown is involved in the quinone binding site of Complex I. They deduced this from a study of the activity of Complex I with rotenone and quinones, in patients with MTND4*LHON11778A where arginine-340 is replaced by a histidine. In its early stages MTND4*LHON11778A can be mistaken for MS1 1 and a few carriers of this mutation have been found to have MS rather than LHON.[12] In this report we present data on the occurrence of antibody to a peptide within the cloned region of ND4.

MATERIAL AND METHODS

Epitope prediction analysis and peptide synthesis

The location of antigenic determinants was predicted using a protein toolbox program. Application of the MacVector 3.5 antigenic index program (IBI, New Haven, CT, USA) showed a likely epitope involving amino acids 331 to 336 in ND4 (Figure 1). Because of differences in codon usage between *E. coli* and mitochondrial protein synthesis systems, the codons for the two tryptophans at 358 and 359 in ND4 would have been recognized as stope codons in the *E. coli* expression system.[13] Therefore, the predicted amino acid sequence for the protein expressed in the λgt1 1 cDNA library and recognized by IgG, from people with MS would comprise only 44 amino acids (Figure 2).

A peptide, Cys LeuAlaAsnSer AsnTyrGluAgrThr HisSer Arg, was synthesized and conjugated to diphtheria toxoid with a maleimido-thiol bond involving the N-terminal cysteine[14] (Chiron Mimotopes, Melbourne). The diphtheria toxoid-ND4-peptide conjugate was used to immunize a rabbit and to coat wells of microtiter plates for ELISA assays. The conjugate produced a good immune reponse in the rabbit. The rabbit antibody was used to check the evenness of coating of wells in a microtiter plate with the 13-amino acid peptide.

ELISA assay with ND4 peptide

For the assay of IgG binding to the ND4 peptide the sera were pretreated to remove any nonspecific binding to plastic, BSA blocker and the diphtheria toxoid carrier. Wells 1 to 5 of a microtiter plate were coated with diphtheria toxoid (5.8 μg) and well 6 with the ND4 peptide conjugated to diphtheria toxoid (6.3 μg/well of conjugate containing 0.5 μg peptide). Triplicate samples 100 μL of 1 in 100 dilution of sera in phosphate buffered saline (PBS) were incubated in well 1 for 1 hour before being transferred sequentially to wells 2 to 5, with 1 hour incubation in each to remove any binding of IgG to the diphtheria toxoid, BSA used as a blocking agent (5% in PBS) or to the plastic, before transfer into well 6. After 1 hour incubation, wells were washed with PBS-Tween and the bound IgG measured using an alkaline phosphatase conjugate (A3150, Sigma, Sydney) and the colour determined at 405 nm. Figures 3 and 4 show the

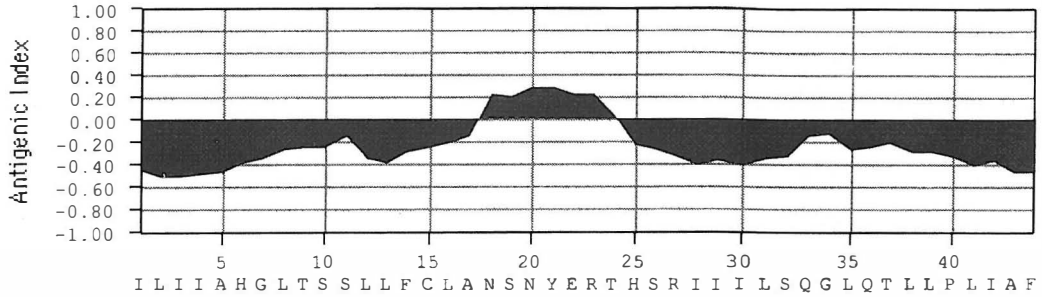


Fig. 1. Epitope prediction in cloned ND4. Predicted amino acid sequences are in the equatorial, versus the antigenic index determined by the computer analysis in vertical position.

315 **325**
a. IleLeuMetIleAlaHisGlyLeuThrSerSerLeuLeuPheCys
335 **His**
LeuAlaAsnSerAsnTyrGluArgThrHisSerArgIleMetIle
345 **355**
LeuSerGlnGlyLeuGlnThrLeuLeuProLeuMetAlaPheTrp
b.
IleLeuIleIleAlaHisGlyLeuThrSerSerLeuLeuPheCys
LeuAlaAsnSerAsnTyrGluArgThrHisSerArgIleIleIle
LeuSerGlnGlyLeugInThrLeuLeuProLeuIleAlaPhe

Fig. 2. a, Predicted amino acid sequence of human mitochondrial ND4 (Anderson et al., 1981). The mutation in Leber's Hereditary Optic Neuropathy (MTND4*LHON11778A) results in a histidine replacing an arginine at amino acid 340 (Wallace et al., 1988). **b**, Predicted amino acid sequence, using *E. coli* codons, in clones isolated from a λgt1 1 human fetal spinal cord cDNA library which was probed by pooled IgG from people with MS.

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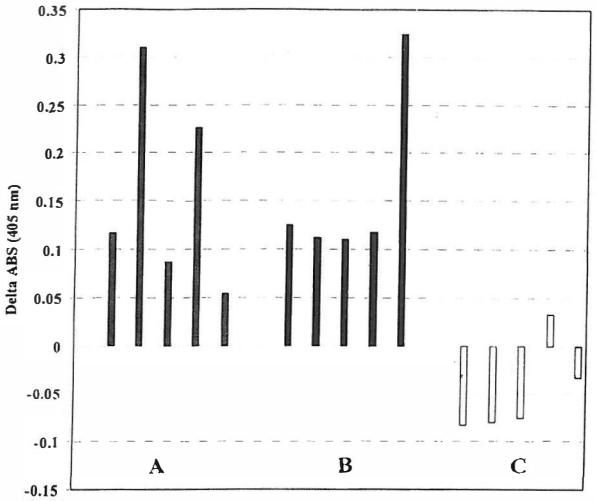


Fig. 3. Binding of IgG to diphtheria toxoid conjugate of the ND4 peptide CLANSNYERTHSR in sera from 3 patients (A, B and C) with multiple sclerosis as determined by ELISA. The numbers indicate the interval in weeks between investigations.

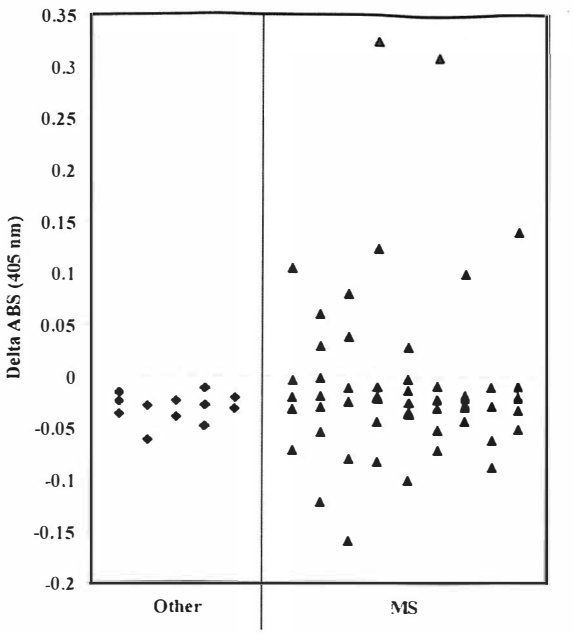


Fig. 4. Binding of IgG to the ND4 peptide conjugated to diphtheria toxoid in serum from patients with MS and in other human sera.

difference between the optical density in wells 6 and 5.

Source of sera

Sequential samples (Figure 3) were obtained from 3 patients with typical MS from Professor W.W. Tourtellotte, Los Angeles. The MS sera (Figure 4) were randomly selected from samples supplied by professor D.A.S. Compston, Cambridge; professor C.C.A. Bernard, Melbourne; Dr W.M. Carroll, Perth; and Dr R.H. Edis, Perth. The other sera included samples from people with no disease (9), breast cancer (1) and Guillain-Barre Syndrome (2).

RESULTS

Autoantibody to ND4

While the wells were shown to be evenly coated using the rabbit antibody to the 13-amino acid peptide, the results with selected human sera were not satisfactory as there was a highly variable level of binding of human IgG to bovine serum albumin (BSA) and/or plastic, especially with the MS sera. Similar problems are frequently encountered in peptide ELISA assays used with human IgG.[15] To ensure efficient presentation of the peptide and to eliminate all non-specific binding of the IgG to the diphtheria toxoid carrier, BSA and plastic, each serum was exposed sequentially in 5 wells to these compounds before being presented to the ND4-peptide conjugate to diphtheria toxoid in well 6. The specific binding of the IgG to the peptide was assessed as the difference in optical density between wells 5 and 6.

The assay was applied to serial samples from 3 patients with MS (Figure 3). Antibody to the ND4 peptide conjugate was found to fluctuate in 2 of the patients while the third (patient C) showed no antibody except for a very low level in one sample. Patient C tended to have a lower level of total IgG in his cerebrospinal fluid on each of the sampling dates when compared to patient A and B.

The same assay was applied to 54 sera from patients with MS and a selection of sera from normal people and those with other diseases. Approximately 20% of the sera from patients with MS contained antibody to the ND4 peptide (Figure 4). In 8 of the samples from Professor Compston which had antibody to the ND4 peptide conjugate, there was no obvious association between the presence of the antibody and disease course, year of diagnosis, presenting syndrome, nor current disease status. Studies are in progress to determine if there is an association between the presence of antibody to ND4 and the presence of HLA-DR2 as the ND4 peptide contains the motif which is required for peptides to bind to DR2 (Brusic V, personal communication).

In preliminary experiments with sera from patients who attended a clinic for assessment of autoimmune disease, moderately high levels of antibody to the ND4 peptide were present in association with some other autoantibodies. For example, it was found in sets of sera where there were antibodies to DNA (3/5), islet cell (3/5), nuclear antigen (2/5) and acetylcholine receptor (2/5) but

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not in association with autoantibodies to smooth muscle nor thyroid microsomal antigens nor in 10 sera where no autoantibodies had been detected (Carnegie PR, Sanati MH and Hollingsworth PN, in preparation).

DISCUSSION

Mutations in Complex I, autoimmunity and MS

Baum[16] has suggested that mutations could cause abnormal assembly of the proteins in Complex I, which could result in disruption of the finely coordinated formation of the enzyme and the degradation of the excess components. It is known from work on minor transplantation antigens in mice that a peptide from the ND1 component of Complex I can be presented on cell surfaces, in association with class I-like MHC molecule, and act as a transplantation antigen.[17] Therefore it is quite conceivable that this known antigenic region[10] of ND4 could also be presented on the surface of cells which had damaged Complex I.

Since the initial report by Harding et al.[12] on the 5 female carriers for the 11778A mutation who had typical symptoms of MS rather than LHON, there have been several similar cases reported.[18] A survey of 307 MS patients in the UK for the 11778A mutation failed to identify this as a mutation associated with MS;[19] however, there are now 2 independent studies[20,21] which claim that there is a significantly higher frequency of the 4216C mutation in both adults and children with MS. This mutation involves the ND1 component of Complex I. Since Complex I has a major role in energy production and in temperature regulation,[9] it is possible that the well known and puzzling abnormalities of fatigue and temperature regulation[22] in MS patients are linked to subtle deficiencies in the function of Complex I in MS. It is clear from studies on possible genetic factors involved in MS that MS is not a typical maternally inherited diseases;[1,19] however, one of the genes involved in susceptibility to MS could be a component of Complex I encoded in the nucleus. The damage to Complex I in MS could be a result of a mutation in nuclear DNA, somatic mutation in the mitochondrial DNA, toxins or other agents which lead to the misassembly of Complex I. Autoimmune disease would not occur unless the immune system were stimulated directly by the ND4 epitope or by an epitope which mimicked its structure in a virus or bacteria.

Possible role of antibody to Complex I in MS

Firstly, the autoantibody could be involved in a primary autoimmune attack. While there is increasing evidence that some antibodies can be taken up by cells,[23] it is highly unlikely that the antibody could come in contact with ND4 as it is inside of the mitochondria.[7,8] However, as there are reports of another NADH: quinone reductase in the plasma membrane,[24] it is quite likely that the quinone binding site in this enzyme could be similar in structure to that of ND4 in Complex I and thus provide an alternative target for the antibody to ND4. This plasmamembrane enzyme is upregulated when mitochondria are damaged.[24] An immune response to a pathogenic or even a non-pathogenic microorganism, if it contained a similar antigenic determinant would start an autoimmune attack on the plasma membrane enzyme.

Secondly, the autoantibody could be one component in a chain of disturbances from which autoimmune disease would result. The primary defect would have to be an internal abnormality in the mitochondria which leads to an exposure of the ND4 epitope on the cell surface[19] in association with a MHC molecule. Damage would only occur when the patient was infected with a microorganism which expressed a similar epitope to ND4 on its surface, and triggered an immune response to this epitope via a class I MHC molecule. However in MS, as discussed above, the mitochondrial genes involved in Complex I appear to be normal, with the possible exception of ND1 in some patients.[20,21] It is conceivable that one of the other 34 nuclear genes which encode the Complex I proteins[7] may be abnormal or alternatively, damage to mitochondria could be caused by toxins or even a virus which could lead to the presentation of the ND4 epitope on the cell surface.

This discovery of an antibody to Complex I, when taken together with the reports of mutations to Complex I in some patients[20,21] and the report on abnormal numbers of mitochondria in epithelial cells in the blood brain barrier of MS patients,[25] points to a need for further study of mitochondria in MS.

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