

Report: Functional effects of anti-basal ganglia antibodies in patients with encephalitis lethargica and related disorders associated with streptococcal infection

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Introduction

Encephalitis lethargica (EL) affected a large number of people in the pandemic in the early 1900s (von Economo, 1930). Whilst it is now considered a rare disorder it still occurs sporadically. Its frequency, presentation, disease course, treatment response and causes are not well understood. Typically, EL presents as an acute illness in young adults with initial neuropsychiatric features, sleep disturbance and movement disorders. Histological and biochemical data suggest that autoimmune mechanisms play an important role in this disorder and recently serum anti-basal ganglia antibodies (ABGAs) have been detected in affected sporadic cases associated with evidence of recent streptococcal infection (Dale et al., 2004a). ABGA are also associated with other neuropsychiatric disorders including Sydenham's chorea, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS), Tourette syndrome (TS) and obsessive compulsive disorder (OCD). As ABGAs are strongly associated with recent streptococcal infection, these disorders represent a good model for the study of molecular mimicry and autoimmunity.

Post-streptococcal disorders are still poorly understood, largely due to the lack of suitable animal models. Therefore, in the present work, we aim to develop an animal model of these disorders by either passive transfer of EL patient IgGs or active immunization with group A streptococcus (GAS) homogenate/protein fractions, human basal ganglia tissue and recombinant proteins (putative autoantigens). In addition to the animal model we are examining the effect of immunopurified antibodies and recombinant enzymes (autoantigens) on neuronal cell function (enolase activity, ATP analysis, apoptosis etc.). Some preliminary data indicated that calcium influx via the L-type channel is significantly depressed by ABGA patient IgG, leading to neurite retraction and apoptosis. Thus, we also aim to determine the effects of purified monospecific antibodies on KCl-evoked calcium responses as well as NMDA stimulated calcium responses. Single cell calcium (Ca^{2+}) responses will be measured by fluorescence imaging as previously described (Pocock and Evans 2000).

As auto-antibody mediated diseases respond to immunomodulatory therapy, identifying and defining the pathogenesis of these disorders is important so that patients can be appropriately treated. Establishing this group of disorders as a "true" autoimmune disease of the CNS and demonstrating that ABGA have functional effects will establish a whole new paradigm.

Hypothesis and aims of the study

Group A streptococcus is the most prevalent and flexible of human pathogens. The interactions between the host and GAS are multi-dimensional and very complex. Identification of different components at the cellular and molecular level forms the first step in understanding how the

host deals with this pathogen. A combination of mouse models and molecular analysis forms the key to unveil the pathogenesis of post-streptococcal disorders.

It is hypothesised that:

1. Autoantibodies affinity-purified from patients with EL and ABGA using the recombinant proteins will have functional effects in vitro, e.g. in the case of our identified putative autoantigens ABGA will affect neuronal function and survival
2. The passive transfer of ABGA to animals will result in stereotypical movements similar to those seen in human subjects with ABGA-associated neuropsychiatric disorders, and
3. Inoculating mice with these antigens in complete Freund's adjuvant will induce the development of ABGA in mice and result in stereotypical movements similar to those seen in human subjects with ABGA-associated neuropsychiatric disorders.

The aims of the study are to use recombinant human alpha and gamma-enolase (45-46 kDa), aldolase C (40kDa) and pyruvate kinase (60kDa) to investigate the functional effects of ABGA from patients diagnosed with EL. It is also planned:

1. To use the recombinant proteins to affinity purify antigen-specific auto-antibodies to investigate the functional effects of ABGA in primary neuronal cell cultures
2. To determine if disease can be induced by passive transfer of these antibodies to animals, and
3. To attempt to induce disease in animals by inoculating animal with the individual antigens and cocktails of the relevant antigens.

Discussion of work completed to date

Group A streptococcal infections can range from a mild skin infection or a sore throat to severe, life-threatening conditions. Among the many factors involved in the virulence of this pathogen, the M protein and streptococcal superantigens (SAGs) have been the focus of interest. They have generated a lot of attention during recent years, in part because of their potential role in both autoimmunity and manifestations in acute infections. In this study we are analyzing the expression of each of the 13 superantigens in group A streptococcus samples collected from Tourette's patients together with determining their *emm* type which might help us conclude if there is any correlation between the two. Two exotoxins thought to be chromosomally encoded *speB* and *speF* were found in all 9 samples. As the number of isolates studied till now has been limited it is not possible to draw any conclusions about the prevalence of any particular *emm* type/ expressed superantigens , or about the association between the two. However, in the near future we are aiming to analyze at least a further 20 samples available to us from the same study. What can be concluded is that all GAS samples are seen expressing more than one superantigen, in addition to *speB* and *speF*. The conserved superantigen profile for different *emm* types especially in the presence or absence of chromosomally encoded SAGs (*speB* and *F*) supports the argument that surface M protein selectively influences the entry of the bacteriophages including those encoding superantigens (Mylvaganam et al., 2000, Commons et al., 2008).

The lack of a reliable animal model has made it difficult to examine the pathogenic processes of post streptococcal diseases. So one of the main aims of this study is to create an animal model of the diseases by immunizing the animals with Group A beta-hemolytic streptococcus (GABHS) homogenate/fractions, EL patient IgGs and/or the putative autoantigens (glycolytic enzymes). In a recent study, Yaddanapudi et al. (2010) found that immunizing mice with an inactivated form

of the bacteria the mice resulted in repetitive behaviours reminiscent of children with PANDAS. Injection of antibodies from the immunized mice into the bloodstream of non-immunized mice replicated these behaviours.

Three animal models were set up using biozzi ABH (antibody high) mice, which we have found to be susceptible to autoimmune experimental allergic encephalomyelitis (EAE), neuritis, thyroiditis, uveitis and peri-insulinitis following immunisation with CNS myelin, peripheral nerve myelin, thyroglobulin and pancreas homogenate respectively (O'Neill et al., 1992, Hankey et al., 2001). Although the animals were subjected to tests like activity monitoring and rotorod, the lack of an established rating scale and behavioural testing made it difficult to quantify any subtle physical or behavioural changes. I therefore aim to create and refine the animal models by carrying out a range of behavioural and motor tests like the grip strength assessment, gait analysis, tail immersion and balance beam at various time points during the experiment together with videotaping the animals that might highlight any understated changes. Additionally, a number of animal models will be set up using different inbred and genetically well-characterised mouse strains to better understand host susceptibility to GAS. For example, the C3H/HeN is a known susceptible strain and BALB/c mice on the other hand are resistant (Medina and Lengeling, 2005). Also, surgical implantation of osmotic pumps will be done to put IgGs purified from EL plasma directly into the mouse brain using a brain infusion kit.

There is substantial evidence for elevated antineuronal antibodies in children with Tourette's syndrome, but further work needs to be done to define the specific antibodies and determine their functional abilities. I used purified antibodies from EL plasma and various other test and control serum to analyze apoptosis and cytotoxicity in PC12 cells. The results show that addition of EL IgGs cause cytotoxicity and apoptosis in undifferentiated PC12 cells; however, similar experiments need to be carried out in PC12 differentiated into neural cells by NGF exposure. Apoptosis is characterised by distinct biochemical and morphological events triggered by signalling cascades. Two main phases in apoptotic cell death have been distinguished: *induction* and *execution*. Induction is controlled by a wide range of mechanisms that involve either surface (extrinsic) signalling and subsequent signal transduction mechanisms, or internal (intrinsic) pathways leading to the release of mitochondrial components (Fabbri et al., 2006, Fulda et al.). Both pathways converge in common events during the execution phase. The unique changes that characterise the apoptotic process provide several features that permit the recognition and quantitation of apoptotic cell death by cytometric methods. Therefore, I aim to carry out flow cytometry experiments labelling for mitochondrial dysfunction, superoxide production, changes in calcium influx, annexin V and caspase 3 in order to identify the series of events leading to cellular apoptosis after exposure to the EL, MS and animal model serum samples in comparison to controls. Ca^{2+} deregulation is a consequence of many different insults that alter Ca^{2+} homeostasis, causing and increasing damage to cells; for this reason, it may be defined as an "intrinsic stress," meaning that it is autoinduced by the cells as a consequence of an extrinsic stress of a different nature (Cerella et al.).

Immunohistochemistry experiments carried out did show that the prime target of anti-enolase antibody is neurons and not other CNS cell types. However, these experiments need further optimization. Immunohistochemistry on mouse brain and heart sections with serum samples will also be performed. These experiments would help identify the target CNS cell types and regions of localization. The H&E sections from the animals injected with GABHS appears to be showing leukocyte infiltration, but the results need to be confirmed and quantified by a trained

pathologist. Confocal microscopy also remains an option to identify the specific target of the antibodies in the cell.

Western blotting results show reactivity of serum from the animals injected with group A streptococcus to the human basal ganglia antigens together with GABHS soluble proteins. These cross-reactive antibodies do not exhibit polyspecific binding to the basal ganglia proteins, but instead bound to antigens of molecular weight of approximately 75-80 kDa, 40 and 45 kDa. Autoantigens of molecular weight 40 and 45 kDa (aldolase C and enolase) are recognized putative autoantigens in EL and Sydenham's chorea. Similar western blotting was carried out with EL IgGs using chemiluminescence, but due to a high background the results could not be analysed. I aim to carry out further Western immunoblotting using heart and brain protein samples from the animal models and various test serum samples (EL, MS, animal model) together with both human and animal control serums.

A number of ELISAs have been carried out with a variety of antigens like mouse brain tissue, human basal ganglia, GABHS homogenate and PC12 cells. All results have consistently shown a significant reactivity of EL serum to the antigens compared to controls. Discrepancy has been observed with plasma samples from the animal models compared to the control. However, antibodies from both EL and animal model serum have led to cellular cytotoxicity and apoptosis. These experiments need to be repeated and refined in the near future employing a larger number of test and control samples.

The enolase activity assay has revealed inhibition of the enzyme when the cells are treated with EL plasma, and this indicated the presence of anti-enolase antibodies in the patient sample. However, this is inconclusive as no difference in inhibition of enolase was seen between the EL and control human plasma samples. To validate this finding this assay needs to be repeated using a higher number n of samples and using a commercial anti-enolase antibody as a positive control. Also, the recombinant proteins aldolase C, α -enolase, γ -enolase and pyruvate kinase (putative autoantigens) will be expressed in an *E.coli* expression vector system. These proteins would then be used to create an animal model, and antibodies specific to these proteins would be obtained by isolating B-cells for the production of an immortal hybridoma cell line that produces antibody. These antibodies would then be employed for further *in vitro* assays.

This group of autoimmune disorders, like all other diseases, is multifactorial. It is possible that GABHS is the initial autoimmunity-iciting event, but that subsequent symptom exacerbations might be triggered by viruses, other bacteria, or noninfectious immunologic responses. In the pathogenesis of these diseases, host factors may also be of critical importance. Gender appears to be a risk factor, as three-quarters of PANDAS subjects are males, whereas Sydenham's is more prevalent amongst females. The age of the host also may influence susceptibility; it is known that rheumatic fever and SC are quite rare after puberty. It is also possible that the post pubertal decrease in incidence is related to the fact that the rate of GABHS infections falls dramatically around the age of 12 (Swedo et al., 2004). Genetic control of the immune response may contribute to differential vulnerability to PANDAS. Murine genetic models suggest that the response to infectious pathogens is strain specific. Different strains have responses that differ qualitatively and quantitatively and lead to very different clinical outcomes (Medina and Lengeling, 2005). Familial factors may also play a role in the pathogenesis of PANDAS.

The hypothesis of an autoimmune mechanism following a streptococcal infection in this group

of disorders is supported by the results of the present study. Until now, scientists have been unable to convincingly document the association between the appearance of antibodies directed against streptococcal antigens in peripheral blood and the onset of the behavioural and motor aspects of the disorder. As a result, treatment strategies were restricted to targeting symptoms rather than causes. Establishment of a suitable animal model using GABHS is essential to reach a definitive conclusion as to the mechanism. In summary, EL and other ABGA-associated disorders are still an emerging entity, with major implications for neuropsychiatry. There is an expanding evidence base associating ABGA with recent streptococcal infection and linking ABGA directly to the pathogenesis of these disorders. This work will provide insights into PANDAS pathogenesis and may lead to new strategies for identification and treatment of children at risk for autoimmune brain disorders.