Identification of Rare Membrane Antigen Specific Human B Cells

Nicholas S. R. Sanderson

Department of Biomedicine, University of Basel and University Hospital Basel, and Research Center for Clinical Neuroimmunology and Neuroscience Basel (RC2NB), Hebelstrasse 20, CH-4031 Basel, Switzerland.
nicholas.sanderson@unibas.ch

ABSTRACT

The experimentally well supported model that MG pathology is caused by antibodies of the IgG class that bind to AChR at the neuromuscular junction, activate complement, and possibly cause internalization of receptors or their functional blockade has enabled the development of a range of reasonably effective treatments. A better understanding of which B cells are responsible for producing these pathogenic antibodies, and why such B cells develop would enable the development of more targeted therapies. Studies of antibodies isolated from single B cells from patients have provided some of this information that was not available from studies of polyclonal antibodies in sera, but perhaps future studies of the B cells themselves will provide deeper insight into the causes of the disease and thereby enable its prevention.

Introduction

The majority of patients (Vincent and Newsom-Davies, 1985) diagnosed with myasthenia gravis (MG) have antibodies against the muscle-type nicotinic acetylcholine receptor (AChR). The receptor is a ligand-gated ion channel built from five protein subunits, each with four transmembrane domains. In the muscles of healthy adults, the AChR is mostly found in dense clusters on the muscle membrane at the neuromuscular junction (NMJ) i.e., the point at which the terminus of the motor neuron contacts the muscle, and each receptor includes one beta subunit, one delta, one epsilon, and two alpha subunits. In fetal muscles, and in denervated muscles, the location of the epsilon subunit is taken by a similar protein encoded by a different gene, the gamma subunit (Gu and Hall, 1988). Reviewing available data in 1981, Engel et al. concluded that there was compelling evidence for a model of the disease based on IgG binding to AChR at the neuromuscular junction, followed by complement-mediated destruction of the postsynaptic membrane and depletion of the receptor. In the forty years since then, this model has been supported by numerous studies and refined in some details, but although much progress has been made in determining how best to treat the disease, our understanding of its cause has not developed extensively. The model from the nineteen seventies predicts that removal of the pathogenic antibodies, inhibition of complement activation, or measures to enhance the effect of the remaining receptors would be clinically effective, and all three predictions have been empirically supported and exploited for treatment. Is there anything more we could know, that could lead to an improvement in patients' lives? Two possibly meaningful avenues of enquiry might be a better understanding of the cells responsible for producing the pathogenic antibodies, which might enable the targeted depletion of these cells, and insight into the original cause of the disease, which might enable its prevention. Focusing narrowly on anti-AChR MG, this review will argue that for both of these goals, the isolation of rare, antigen-specific B cells from patients is a critical step. A great deal of progress has been made in this direction, but at the timepoint of the 14th MGFA conference, technical challenges still remain. Information about the monoclonal antibodies produced by single isolated B cells has already extended what had been deduced from the study of the polyclonal antibody pool in patients' sera, and an important future direction will be the study of the B cells that make these pathogenic antibodies.

Both soluble antibodies and their membrane expressed counterparts (the B cell receptor or BCR) will be discussed, and for readers from non-immunological fields, the relationships between these entities can be summarized as follows.

During its early development, a B cell links together genes encoding sections of protein, thereby generating two new compound genes that between them encode a membrane-expressed antigen receptor, the BCR, with a specificity that is (almost) unique to each B cell. A B cell that has completed this developmental stage but not yet encountered antigen is referred to as "naive". If such a B cell does encounter an antigen that is bound with high enough affinity by its BCR, it will internalize this antigen, cleave it into peptides (assuming the antigen is a protein), and re-externalize these peptide fragments in complex with proteins of the major histocompatibility complex on the extracellular side of its plasma membrane. This event of antigen capture leads to activation of the B cell, and can be followed by one of two possible outcomes. If the presented antigen-fragments are recognized by an activated T cell, the interaction between the B and T cells leads to the series of events described below (Tanaka and Baba, 2020). If no co-cognate T cell is available to provide this signal (T cell

help), then the B cell will die. If T cell help is available, the B cell survives, and undergoes phenotypic development, associated with changes in the structure of the BCR so that it is no longer expressed in the membrane, but secreted as a soluble molecule known as an antibody, with the same binding specificity as the BCR. This process of development can also include changes in the gene sequences that alter the specificity and affinity of the antibody (called somatic hypermutation), and others that do not alter the specificity, but alter other functional properties of the antibody (class switch, i.e., the change from IgM to IgG or other classes). By these developmental processes, naive B cells assume more "effector-like" phenotypes, becoming plasma cells that are specialized for antibody secretion, and memory cells that retain expression of the membrane BCR, and do not produce antibodies initially, but rather contribute to future responses against the same antigen (Suan et al., 2017) . In this review the word "immunoglobulin" will be used to refer to both the membrane-bound BCR and the secreted antibodies.

From the broad questions "Which B cells make the pathogenic antibodies?", and "Why do these cells develop?" we can extract the following narrower questions:

Which B cells?

What are the classes/subclasses, mutation status, and epitope specificity of the pathogenic antibodies?

What is the phenotype (memory, naive, plasma, long- or short-lived) of the pathogenic B cells?

Why?

What was the initial antigen encountered by the naive B cell that led to development into an antibody-secreting phenotype?

These questions have been approached so far by studying serum from patients, and also by a range of cell-oriented techniques, including the immortalization of single B cells using Epstein Barr virus, or hybridoma formation. Considerable information has also been obtained with the technique of phage display (Graus et al., 1997; Farrar et al., 1997; Fostieri et al., 2005), but since this involves the pooling of numerous B cells, rather than the investigation of single cells, this line of enquiry is outside the scope of this review.

Class/Subclass

The question of the class of AChR-binding antibodies in MG can be productively addressed by studying soluble antibodies in serum, since these are thought to contain the pathogenic agent, and subclasses of soluble antibodies can be determined accurately. Tindall (1981) compared abundance of AChR-precipitating antibodies of classes IgG, IgM, and IgA in serum from patients with MG, and reported that (compared with a cutoff at mean + 3 x standard deviations

in healthy controls of 0.39, 1.31, and 0.49 units) patients had respectively ranges of 0-1050, 0-13.34, and 0-2.43 units of IgG, IgM and IgA in their serum. Investigation of anti-AChR antibodies in patient sera has accordingly focused on IgG, although unbiased protocols to isolate AChR-specific B cells can also yield cells expressing IgM (Blair et al., 1986; Cardona et al., 1994).

Patients with MG have elevated levels of all four subclasses of IgG compared to healthy controls (Rødgaard et al., 1987; Liu et al., 2010) and although anti-AChR antibodies of all four subclasses can be found, subclass distribution within the AChR-specific fraction is also dominated by IgG1, but differs from the global pattern, with a larger than expected representation in IgG3, and smaller in IgG2 (Lefvert et al., 1987; Rødgaard et al., 1987). An IgG1-dominated, IgG2-poor antibody profile is thought to be typical of a T-cell-dependent humoral response against protein antigens (Barrett and Ayoub, 1986). The question of class could also be answered in theory by examining the sequences of immunoglobulin genes in pathogenic B cells. For example, Cardona et al. (1994), by fusing patient B cells with a mouse-human heterohybridoma cell line, screening the supernatants of resulting hybridomas by TE671 cell ELISA (TE671 is a rhabdomyosarcoma cell line which endogenously expresses the alpha, beta and delta subunits of the AChR and can be made to express complete adult AChR by transfection with the epsilon subunit - Beeson et al., 1996), and limiting dilution, obtained 14 stable clones, of which 5 produced IgM and 9 IgG (2 IgG1, 4 IgG2, 1 IgG3, 1 IgG4, and one unspecified). More recently, Rose et al. (2022) described 6 AChR-specific immunoglobulins of which 3 were IgG1, none was IgG2, 1 was IgG3, and 2 IgG4. These results confirm that anti-AChR immunoglobulins of all four subclasses exist, but draw attention to some of the disadvantages of studying single B cells as opposed to serum. Firstly, there is the question of anatomy - both these studies of single B cells used B cells from peripheral blood, but it is possible that the B cells responsible for producing pathogenic antibodies reside elsewhere, for example the bone marrow, the thymus, or in tertiary lymphoid organs. Secondly, there is the question of B cell subtype - each method of B cell isolation has its own bias regarding which type of B cell is targeted. For example, the MACACS method used by Rose et al. is biased towards memory B cells (Callegari et al., 2022), and these may not faithfully reflect the type of B cells that actually produce the pathogenic antibodies. Thirdly, there is the question of numbers. The human anti-vaccine antibody response is thought to involve of the order of 50-400 clonotypes per individual (Wine et al., 2015), and (assuming that the antibody response is somewhat similar in the autoimmune MG context) while this diversity will be evenly sampled by serum studies, the

numbers of AChR-specific B cells so far obtained in single cell studies, with many fewer than fifty published sequences in the entire literature, are so small that only limited inference about the original population of antibodies from which they were taken can be drawn. Finally it should be noted that these studies concern what class of antibody is found in MG, not what class of antibody causes the problem.

Somatic Hypermutation

It is currently not possible to determine the sequences of soluble serum antibodies with enough precision to measure somatic hypermutation, and therefore what we know about this parameter is derived entirely from the analysis of B cell cDNA sequences. The mutational profile across the entire B cell memory repertoire is similar between patients with MG and healthy individuals (Vander Heiden et al., 2017), with around 3% of bases mutated in IgM heavy chains, and 4-7% in IgG and IgA, depending somewhat on the V gene family and the donor. Naive B cells, almost by definition, have zero somatic hypermutation (Klein et al., 1998). Immunoglobulin gene sequences from single IgG B cells with established specificity for AChR have been consistently mutated (see Table 1). Cardona et al. (1995) analyzed the immunoglobulin gene sequences of four of the AChR-specific B cells they had previously described (Cardona et al., 1995) and report mutation frequencies of 5.7 - 8 %. The

Table 1. Properties of patient derived antibodies from each of four publications. Coumn "source" rports the tissue from which the B cells were taken. Column "% nt mut" is the percentage of nucleotides in the VH genes 5′ of the CDR3, the calculatioin of which may vary slightly between publications. The column PTMG indicates whether the antibody induced cmyasthenic signs in a passive transfer model (yes: behavioral signs and complement activation; EMG: electromyographic signs; "combined"- in combination with another antibody.). Blank fields indicate that the data are not provided or not applicable.

author< (year)	source	mAb ID	subclass	% nt mut	subunit epitope	MIR	PTMG?
Kamo (1982)	thymus				not gamma		EMG
Cardona (1994)	blood						
		M1	1	8	alpha		
		M2	2				
		M3	3				
		M4	2				
		M5	2	8			
		M6	2	7.8	alpha		
		M7	4	5.7			
		M8	1				
Makino (2017)	blood	B12L		mutated	alpha	yes	yes
		3B1					
		1G3					
Vrolix (2014)	thymus	131	1	mutated	gamma		
Rose (2022)	blood	2M18	1	5.9	epsilon		
		5H10	1	4.5	delta		
		3I3	3	5.1	beta		
		5D2	4	5.7	beta		
		6J2	4	7.5	beta		combined
		1J7	1	13.2	alpha	yes	combined

antibodies reported by Vrolix et al. (2014), and Makino et al. (2017) were also mutated. Rose et al. (2022) saw mutation frequencies from 4.5 -13.2 % in the six IgG antibodies they described. From these results it appears that the level of somatic hypermutation in the immunoglobulin genes of AChR-specific IgG B cells from patients with MG is typical of the memory B cell pool. Here too, it should be born in mind that the cells that were sequenced may not be typical of the cells that make the antibodies, but the observed mutation pattern can reasonably be interpreted to imply that these B cells developed their affinity for AChR in the context of an antigen-driven, T cell dependent germinal reaction. This raises the question of what the triggering/driving antigen(s) might be (see Table 1).

Epitope specificity

Early efforts to isolate AChR-reactive B cells were directed towards obtaining monoclonal antibodies, to better understand the relationship between serum antibodies and disease (Kamo et al., 1982; Cardona et al., 1994). These included why anti-AChR titers and disease course are so weakly correlated, and why some murine anti-AChR cause disease when passively transferred, while others do not (Cardona et al., 1994). Broadly, the question was "what makes an anti-AChR antibody pathogenic?". Animal experiments conducted in the nineteen eighties suggested that antibodies targeting a small region on the alpha subunit (known as the main immunogenic region or MIR, because of its immunodominance in rats immunized against *Torpedo* AChR - Tzartos and Lindstrom, 1980) are the pathogenic ones. An obvious question was whether this conclusion could be extended to human patients, but the non-availability of patient-derived monoclonal antibodies meant that this question was mostly addressed using studies of patient sera. Sophianos and Tzartos (1989) looked at whether Fab fragments of rat monoclonals directed against the MIR could protect AChR on TE671 cells from internalization-mediated depletion by patient sera. The results showed clearly that they could, while a control rat monoclonal targeted against the beta subunit could not. This result, however, is far from demonstrating that anti-MIR activity is responsible for pathogenicity in patients, because it looked only at internalization and not at, for example complement activation, and (ii) internalization is dependent on cross-linking which is more extensive when induced by anti-alpha antibodies (which have two binding sites per receptor, rather than the single binding site offered by the other subunits). A number of groups subsequently tackled this question in vivo, where several pathomechanisms are expected to operate, and it was demonstrated that monovalent (Fab or IgG4) versions

of a MIR-binding antibody can protect an animal against intact IgG1 monoclonals that would otherwise induce severe myasthenic signs (Panastasiou et al., 2000; Losen et al., 2017). These experiments still did not reveal which kinds of antibodies are pathogenic in patients, because they were conducted with an experimentally constructed antibody as the pathogenic agent, rather than with patient serum. When Namkamura et al. (2018) examined the ability of a Fab fragment of the MIR-targeting mAb35, in the polyclonal autoantibody context of experimental autoimmune myasthenia gravis (EAMG), they found that although the Fab could attenuate the antigenic modulation and complement-activating effects of EAMG serum in vitro, it offered no protection against the passive transfer of such serum in vivo.

A more direct approach would be to isolate anti-AChR antibodies from patients, and determine which antibodies are pathogenic, and which not. Table 1 summarizes reports of AChR-binding antibodies isolated from patients by immortalization with EBV, using hybridoma technology, and more recently by single cell molecular cloning. The earliest reported isolation of a monoclonal human anti-AChR antibody was achieved by immortalizing B cells from a patient's thymus with Epstein Barr virus, and limiting dilution (Kamo et al., 1982). The resulting antibody precipitated AChR from innervated human muscle, suggesting that it targeted a non-gamma subunit. The antibody also induced a reduction in the muscle action potentials evoked by sciatic nerve stimulation, which could be partially rescued by edrophonium chloride administration. This was a good demonstration that patient-derived anti-AChR antibody could cause myasthenic signs without other serum components, but very little information was provided about the characteristics of the antibody. Information about class, subunit specificity, and immunoglobulin sequence was provided by Cardona et al. (1994, 1995) for the anti-AChR antibodies that they isolated, but pathogenicity, other than the potential to mediate antigenic modulation in vitro, was not reported. Using EBV immortalization, the Maastricht group isolated a B cell from the thymus of a patient whose IgG was directed against the gamma subunit (Vrolix et al., 2014; Saxena et al., 2017). These authors reported that the anti-gamma antibody induced neither antigenic modulation nor myasthenic signs by in vivo passive transfer. Makino et al. (2017) sorted memory and plasmablast cells from patients and a healthy donor, and labeled antigen-specific cells with recombinant extracellular domain (ECD) of the human nAChR α-subunit directly conjugated with phycoerythrin. They prepared recombinant IgG antibodies from these cells, and tested them by ELISA or by flow cytometry with AChR-expressing cells. Even without pre-screening the

memory B cells with fluorescent antigen, the authors were able to obtain several recombinant monoclonal antibodies from each of five patients that were AChR-specific by the criterion of binding to recombinant ECD in an ELISA assay. However these antibodies all failed the subsequent test of binding specifically to AChR expressed on live cells. This finding, although reported as more of a nuisance by the authors, is significant because methods relying on denatured proteins (antigen arrays and ELISA, to name but two) are commonplace, and may well be misleading in the context of autoantibody research because there is some evidence that pathogenically relevant autoantibodies are likely to be antigen-conformation-dependent, at least in animal models (Krolick et al., 1994). After pre-screening the B cells with a fluorescently labeled alpha ECD, the authors were able to isolate from 6 donors 8 AChR-specific mAbs that passed the more stringent test of AChR-dependent binding to live cells. Among these was one highly mutated antibody, B12L, that competed with mAb35, and induced myasthenic pathology after transfer into rats. This strategy was clearly an effective one for isolating a pathogenic antibody, but not suitable for screening for a wide variety of potentially pathogenic antibodies. The use of the soluble single subunit extracellular alpha domain as a bait antigen not only restricts the screen to alpha-specific antibody, it also rules out those antibodies whose epitope spans more than one subunit, or those whose conformational epitope is dependent on the interaction between the subunits. Rose et al. (2022) used a different technique, named membrane antigen capture activated cell sorting (MACACS) to isolate AChR-specific B cells and, like Makino et al., cloned the immunoglobulin genes from single cells to prepare recombinant antibodies. Resulting monoclonals were discovered that recognized each of the four adult subunits (alpha, beta, delta and epsilon), and, as expected, the anti-alpha monoclonal was the strongest activator of complement in vitro, although none of the antibodies was as strong as the B12L antibody described by Makino et al. (2017), and none of the antibodies induced myasthenic signs when injected into rats at 4 mg/kg. Unexpectedly, several combinations of antibodies were significantly stronger complement activators in vitro than the individual antibodies, and this was also seen in vivo, where 2 mg/kg each of an anti-alpha and an anti-beta induced clear myasthenic signs, while 4 mg/kg of either given alone did not.

From these results, the postulate derived from animal experiments with animal-derived antibodies that anti-alpha antibodies (and in particular antibodies that react with the MIR) are critical for inducing myasthenic pathology currently can be considered valid with patient-derived antibodies, with the caveat that the only tests of "pathogenicity" we have are either in vitro, or else in animal models, and may differ from the situation in patients. However, the observation that combinations of antibodies show emergent properties that were not predicted from the behavior of single antibodies may require some reevaluation of our model of how anti-AChR antibodies induce pathology. The interaction between two independent anti-AChR antibodies is clearly not an absolute requirement for the induction of pathology, because the single anti-MIR antibody B12L described by Makino et al. (2017) alone induces pathology in rats in a manner very similar to the well-studied pathogenic rat monoclonals such as mAb35. Resolution of this difference will require the isolation of a broader range of patient-derived antibodies, and more systematic assessment of their key properties, notably affinity and fine epitope specificity.

What are the phenotypes of pathogenic B cells?

If the pathogenic agent is considered to be soluble anti-AChR antibodies in circulation, then they may well be derived principally from plasma cells, and it might be argued that none of the patient-derived monoclonal antibodies isolated (which very likely all came from memory B cells) came from a directly pathogenic B cell. On the other hand, since memory and plasma cells are thought to derive from germinal centers that produce both (Elsner and Shlomchik, 2020), information derived from one B cell subtype concerning the specificity and affinity of the immunoglobulins involved is likely to be relevant to the entirety of the AChR-targeted humoral attack. Because memory B cells are thought to differentiate into antibody-secreting plasma cells upon secondary antigen exposure (Kurosaki et al., 2015), it is also possible that the memory B cells themselves are a step in the pathogenic cascade. This possibility is supported by the partial efficacy of CD20-depleting therapies in anti-AChR MG (Brauner et al., 2020), which would be expected to deplete memory but not plasma cells. Assuming then that the memory B cells in the blood to which we have access are in some way representative of the pathogenic population, what information could we usefully gain about them? One question is whether their phenotypes and functions are like the "effector-like" memory B cells that develop in response to infections or vaccines, or whether they can (also) exert a "regulatory" or immunosupressive phenotype (Catalán et al., 2021). A second parameter of interest is their age. Very long-lived memory B cells have particular characteristics that could be used to distinguish them from recently generated counterparts (Chappert et al., 2022), and particularly among newly diagnosed patients, this would have implications for the origin of the disease. Valuable insights into these characteristics could be gained

by state-of-the-art single cell techniques, but unfortunately the original phenotypes of the cells are destroyed, or at the least radically disturbed by the processes used thus far to identify them, including hybridoma formation, EBV immortalization, and MACACS. The technique described by Makino et al. (2017), which only requires labeling the cells with antigen is potentially the least destructive, but is restricted to those B cells that recognize a soluble single subunit, at least in the implementation described. The three other techniques have the advantage that they can be used to screen for B cells whose antigen is dependent on the intact structure of the membrane-expressed AChR. It is possible that some hybrid technique exploiting the best features of more than one of the published methods will be required to obtain this kind of non-sequence information about the pathogenic B cell population.

What was the triggering antigen?

The observations about antibody class and somatic hypermutation discussed above suggest that pathogenic anti-AChR antibodies are the result of a T-cell-dependent B cell response against a protein antigen. This raises the question of what this antigen might be. Both the facts that patient-derived antibodies are found against all four subunits, and that they commonly recognize the human AChR but not the closely related rat AChR (Rose et al., 2022) suggest that the antigen must be something very like the human AChR. In the field of myasthenia research, as in studies of other autoimmune diseases, the notion of "molecular mimicry" (i.e., the idea that an antigen from a pathogen is similar enough to the target autoantigen that the immune immune response against the pathogen gets specifically transferred to the autoantigen) is periodically discussed (e.g., He et al., 2018;), and re-surfaced, not surprisingly in view of the immense numbers of infected people and the resources devoted to detecting and documenting the infections, during the SARS-CoV-2 pandemic of 2020-2022 (Ramdas et al., 2022). Molecular mimicry offers a plausible source of initiating antigen in cases of Guillain Barré syndrome associated with *Campylobacter jenuni* infection, because adequately powered studies have demonstrated an epidmeological connection between the pathogen and the autoimmune syndrome (McCarthy and Giesecke, 2001), and an experimentally supported mechanistic model exists to explain the connection (Yuki et al., 2004). However, no such level of evidence supports the hypothesis that a similar mechanism might be involved in MG.

It might of course, be simply the AChR itself that is the initiating B cell antigen. If this were the case, it would demand that even the germline versions of the mutated AChR-specific antibodies would recognize the AChR, which

will hopefully become clear as more antibodies are isolated and characterized. That this can happen has been clearly demonstrated in the context of MG with autoantibodies against muscle-specific kinase (Fichtner et al., 2020). Examining three monoclonal antibodies from two patients, these authors demonstrated that although germline versions of these antibodies had significantly (100-fold or more) lower affinity for the autoantigen, they nonetheless demonstrated clearly specific binding. Even the lower affinities of the germline versions were in the nanomolar Kd range that is thought to be relevant for mediating antigen capture and B cell activation (Abbott et al., 2018).

The major question would then be how such B cells could get T cell help for a self protein, and if this could be answered, we might be a long way towards understanding autoimmunity in general. Our favored hypothesis in this regard is the notion of membrane antigen co-capture (Sanderson et al., 2017). If, on the other hand, the affinity of the germline BCR is too weak to enable capture of the mature AChR, other mechanisms must be envisaged that would generate antigens different enough to be immunogenic, but similar enough to lead to autoimmunity. Some possibilities are discussed by Vincent et al. (1998). This line of enquiry would be greatly facilitated by the availability of more patient-derived antibodies, above all members of expanded, mutated clones. Rose et al. (2022) described two members of a single AChR-binding clone, and this offers the particular opportunity to investigate whether, with additional mutations acquired, affinity for the AChR is increased, as would be predicted if the AChR itself is the driving antigen, or decreased, as is predicted by some other models, for example the idea of molecular mimicry (Burnett et al., 2018).

Summary

The study of single, patient-derived, AChR-specific B cells can yield information that is not available from studies of sera. So far, this has been limited to the study of antibodies derived from such B cells, in particular their epitope specificity, their mutational status, and their ability to induce pathology in passive transfer paradigms, and the results have mostly been consistent with hypotheses developed from studies of sera and animal models. So far unexplored is the study of the phenotypes of these pathogenic B cells, outside of their immunoglobulin products.

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