Quantification of antineural antibodies in autoimmune neurological disorders

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More than 50 different neurological pathologies have a confirmed or suspected autoimmune etiology affecting an estimated number of 75 million people worldwide. Autoantibodies are a useful diagnostic marker for most autoimmune diseases even if their pathological role is not evident, and several tests for their detection are commercially available. However, for autoimmune diseases involving the nervous system, lack of clear information on the identity of antineural antibody targets and the presence of many rare diseases have hampered the development of specific diagnostic assays. This review focuses on the actual knowledge on confirmed and suspected autoimmune diseases that target the CNS and the diagnostic relevance of corresponding antineural autoantibodies.

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CONTENTS

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Autoimmune diseases are provoked by an incorrect response to the body by the immune system that mistakes self-tissues for nonself and mounts an inappropriate attack with production of autoantibodies. This response can be directed at a particular organ (organ-specific autoimmunity) or to several tissues or organs (systemic autoimmunity).

The physiological antibody repertoire contains natural autoantibodies, usually polyreactive, of low titer and affinity [1] that are present even in newborns [2]. Unwanted autoreactive B-cell clones are controlled and eliminated in germinal centers of secondary lymphoid tissues [3]. However, B cells can escape established tolerance, for example, owing to genetic alterations or because of environmental events, and start to produce autoantibodies that finally contribute to the damage of tissues. Three main factors are involved in developing autoimmunity: environment, genetics and gender.

Environmental factors in autoimmunity

Among the environmental causes for the activation of autoreactive B and T cells, there is the phenomenon of molecular mimicry. In this case, the specific immune response triggered by an invading pathogen results in the propagation of T-cell clones, or antibodies, which cross-react with self-tissues due to similarity between the pathogen's antigen structure and the structure of self tissues [4]. Other environmental factors are represented by proteins from bacteria or viruses, known as superantigens, which do not undergo processing by antigen-presenting cells. They interact with a large number of T and B cells, independently of their antigen specificity, inducing a widespread B-cell activation and antibody secretion, even at very low concentrations.

Autoimmune reactions may also result from an environmentally induced modification by xenobiotics, including irradiations [5], drug-induced lupus [6], toxic oil syndrome [7], tryptophan ingestion [8], pesticides [9] and mercury [10]. Another important trigger of autoimmune pathologies is given by deficiency of some minerals in the diet [11] or intolerance to particular components of ingested food, such as gluten in celiac disease [12].

Finally, autoreactive B cells can be generated when an initial humoral or cellular immune response, against a defined epitope of an autoantigen, diversifies with time and is directed to other epitopes, on the same molecule or to other nearby molecules, which are

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not implicated initially. This process can induce or amplify an autoimmune response and is termed 'epitope spreading' or 'determinant spreading'.

Genetic factors in autoimmunity

The correlation between genes and autoimmunity is very complicated because mutations or polymorphisms in the genome of individuals usually have no effect, but in combination with other genes or environmental factors, they become pathogenic. Moreover, autoimmunity is not inherited in a normal Mendelian way but has a complex and often unknown mode of inheritance and finally, populations with the same disorder may carry different susceptibility alleles [13]. However, studies based on families and twins show the heritability of some autoimmune diseases [14,15].

To better understand the link between genes and autoimmunity, different animal models have been studied, such as non-obese diabetic mice [16], which develop insulin-dependent diabetes mellitus (IDDM), the F1 hybrid of New Zealand black , white and MRL-lpr mice used for studying systemic lupus erythematosus (SLE) [17], the experimental allergic encephalomyelitis (EAE) as a model of multiple sclerosis (MS) [18], the experimental allergic orchitis or autoimmune ovarian dysgenesis as models of the homonymous pathologies [19,20]. The use of animal models is an advantage as the environment is controlled and therefore the frequency of the disease in the progeny is mainly correlated with genes.

The human MHC encodes for the HLA genes and is associated with more than 100 diseases, including many autoimmune diseases, such as diabetes, rheumatoid arthritis (RA) and psoriasis. In particular, the presence of specific class I or class II HLA alleles are risk factors for autoimmune disorders [21,22]; moreover, other non-HLA genes have been identified in the HLA region that are correlated with autoimmunity [21]. Recently, the list of genes involved in autoimmunity has grown [23] with the addition of the protein tyrosine phosphatase 22 gene (*PTPN22*) variant in association with increased risk of IDDM, RA, SLE and Graves' disease (GD) [24]; the cytotoxic T-lymphocyte antigen-4 gene (*CTLA-4*) in association with GD [25], autoimmune thyroid disease [26] and others [23,27]; while some are still unconvincing because different studies report conflicting results (e.g., polymorphism in the programmed cell death *PDCD1* gene in association with SLE [28] RA [29] and GD [30]; a variant encoding the L503F amino acid substitution in *SLC22A4* in association with Crohn's disease [31]; the FcR-like receptor 3 gene (*FCRL3*) in association with RA and autoimmune thyroid in the Japanese population [32]).

Gender factor in autoimmunity

Approximately 80% of autoimmune patients are women, most frequently affected during the childbearing years and some of these diseases affect African, American Indian and Latin women more than Caucasian women [33]. Among autoimmune diseases with involvement of the nervous system, the highest frequency of incidence in women, with respect to men, is found in SLE, Sjogren's syndrome and MS. For some of them, a correlation with hormones has been demonstrated. In particular, estrogen (both endogenous and exogenous) and prolactin are immunostimulators linked with worsening in SLE, evidence also supported by the possible tendency of lupus flare during pregnancy and remission after menopause [34]. Estrogen has also been implicated in myasthenia gravis (MG) [35] and RA [36] and prolactin in systemic sclerosis [37]. For other pathologies, such as Sjogren's syndrome and MS, the implication of hormones is not so evident but, as the incidence in females is higher, correlations between genes located on the sex chromosomes and diseases are suspected. Importantly, autoantibodies, irrespective of whether they are connected to autoimmune disease or not, are more common in women, although this difference disappears at higher age, when both men and women have a higher frequency of autoantibodies, at least antinuclear antibodies [38]. Thus, age, in addition to gender, should also be taken into account when interpreting findings of autoantibodies.

Criteria to determine autoimmunity

Pathologies must fulfill six criteria to be safely considered as autoimmune [39]:

- Presence of circulating autoantibodies against a specific organ (organ specific) or unspecific antigens (systemic disorder)
- Presence of infiltrating autoreactive lymphocytes
- Presence of specific autoantigen(s)
- Efficacy of immunosuppressive therapy or plasmapheresis
- Passive transfer of the disease in experimental animals
- Induction of the disorder in experimental animals

On the basis of these stringent criteria, not so many pathologies can be considered as truly autoimmune and, if we focus on CNS diseases, the number decreases. In fact, most of the pathologies have not been systematically investigated considering all six criteria and, therefore, should fall into the category of 'suspected autoimmune'.

The first criterion to be met is the presence of circulating autoantibodies. This alone, however, is not sufficient since autoantibodies can be generated in response to tissue damage. If we consider the CNS as the target organ of autoimmunity, we have to keep in mind that circulating autoantibodies must pass several hurdles to enter in contact with their antigen in the CNS. These antibodies must first cross the blood–brain barrier (BBB) and then penetrate the dense intercellular matrix between the capillary and the cell bearing the target antigen. Moreover, antibodies specific for synaptic antigens must penetrate the narrow astrocyte ensheathed structure of the synaptic cleft. Finally, if pathology depends on complement fixation, this cascade of more than 20 proteins must accumulate or be produced locally [40] in sufficient quantities to enable complement activation. It is probably because of this multiplicity of barriers that CNS disorders involving humoral immune attack are relatively uncommon. Nevertheless, in the last decades, the intrathecal production of various antineural autoantibodies ranging from antimyelin basic protein (anti-MBP) to antitissue transglutami-

Table 1. List of intrathecally synthesized antibodies and correlated pathologies based on literature.

Intrathecal antibodies	Pathology	Ref.
Anti-GAD	SPS	[74,75]
	SPS with cerebellar ataxia	[76]
	Cerebellar ataxia	[77,78]
Anti-NF proteins	MS	[79,80]
	Alzheimer's disease	[41]
	Parkinson's disease	[41]
	Neuroborreliosis	[81]
Anti-Hu	Paraneoplastic encephalomyelitis	[42,82]
Anti-Yo	Paraneoplastic encephalomyelitis	[82,83]
Antiganglioside	NPSLE	[84]
Anti-SSA (anti-Ro)	Primary Sjogren's syndrome with CNS involvement	[85]
Anti-TG2	Not specified	[86]
Anti-MBP	MS	[41,87,88]
	Optic neuritis	[41]
Anti-MOG	MS and other neurological diseases	[89]
Anti-MAG	MS	[90]
Antimyelin lipid	MS	[91]
Anti-GFAP	Alzheimer's disease	[41]
	Parkinson's disease	[41]
Anti-β-amyloid peptide	Alzheimer's disease	[41]
	Parkinson's disease	[41]
Anti-Nogo-A	MS	[92]
Antithyroid	Hashimoto's encephalopathy	[93]
Anti-P/Q type VGCC	Paraneoplastic cerebellar degeneration with lung cancer	[94]
Antitubulin	Alzheimer's disease	[41]
	Parkinson's disease	[41]
	Amyotrophic lateral sclerosis	[41]
	MS	[41]
	Optic neuritis	[41]
	SMN/CIDP	[41]
Anti-α-B-crystallin	Neuro-Behçet's disease	[95]
	MS	[95]
Anti-AN-2 (NG-2)	MS	[96]
Anti-DNA	MS	[97]
	SLE	[97]
Antioligodendrocyte-specific protein	MS	[98]
Antiproteasome	MS	[99]
Antimyelin proteolipid protein	MS	[100]
Antitransaldolase H	MS	[101]
Single case		
Anti-GAD	Autoimmune thyroiditis and diabetes with tremor of the mouth	[102]
	IDDM and drug-resistant epilepsy	[103]
	Nystagmus and muscle spasms	[104]
Anti-Ri	Anti-Ri syndrome	[105]
Anti-Zinc finger protein	Cerebellar degeneration	[106]
Antigliadin	Ramsay Hunt syndrome	[107]
Controversial		
IgG anti-NR2 glutamate receptor	SLE/NPSLE	[108,109]
Anticardiolipin	NPSLE	[110,111]

CIDP: chronic inflammatory demyelinating polyneuropathy; GAD: Glutamic acid decarboxylase; GFAP: Glial fibrillary acidic protein; IDDM: Insulin-dependent diabetes mellitus; MBP: Myelin basic protein; MS: Multiple sclerosis; NF: Neurofilament; NPSLE: Neuropsychiatric systemic lupus erythematosus; SLE: Systemic lupus erythematosus; SPS: Stiff person syndrome; SSA: ;TG2: Tissue transglutaminase; VGCC: Voltage-gated calcium channel.

nase (anti-TG2) has been demonstrated (TABLE 1). In some cases, the association of circulating antineural antibodies with the pathology is still unclear, whereas in others a clinical correlation with the severity of the pathology has been demonstrated and therefore their importance for diagnosis. This is the case for the anti-MBP in MS [41] or the anti-Hu in paraneoplastic encephalomyelitis [42]. In more general terms, the production of

antineural autoantibodies in the cerebrospinal fluid (CSF) should be considered as the sign of a specific organ attack and of a higher possibility for antibodies to cross-react with the target antigen in the CNS.

Importantly, although the BBB constitutes a barrier to humoral immunity, it is less of a barrier to cell-mediated immunity. Indeed, activated T cells can easily enter the CNS thanks to their ability to disrupt the BBB; such disruption is a critical step in mounting the cell-mediated autoimmune reactions that, for example, produce demyelination in MS [18]. If the existence of these barriers is the principal reason why pathogenic antibodies fail to produce a CNS disease, situations involving BBB deterioration should favor CNS diseases. Alteration of the BBB is a well-recognized consequence of trauma, but high levels of psychological stress have also been shown to compromise the BBB [43]. So a subset of idiopathic CNS disease could arise from unfortunate interactions with the environment that allow a reaction between circulating antibodies and the CNS that generally does not occur in healthy people by virtue of intact protective barriers.

The second criterion for autoimmunity to be met is the presence of autoreactive lymphocytes in the target organ. Infiltrating autoreactive B and T cells have been described in various organs, such as the kidney in lupus nephritis [44], gastric mucosa in celiac disease [45], or CNS in patients where intrathecal synthesis of autoantibodies has been demonstrated [41]. The presence of specific autoantigen(s) (third criterion for autoimmunity) has been determined in very few neurological and non-neurological diseases, such as amphiphysin for anti-GAD negative stiff-person syndrome (SPS) patients [46], tTG2 in celiac disease [47], epidermal transglutaminase in dermatitis herpetiformis [48] and, very recently, aquaporin-4 [49] for the neuromyelitis optica. Other autoantigens, even if characteristic of a specific pathology, have been described as autoantigen in more than one disease, such as glutamic acid decarboxylase (GAD) for SPS, IDDM, Satoyoshi syndrome [50], progressive cerebellar ataxia [51], drug-resistant epilepsy and myoclonus [52].

Immunosuppressive treatments (fourth criterion) are based on immunosuppressants (immunosuppressive drugs), intravenous immunoglobulin (IVIg) injections or plasmapheresis. Immunosuppressants are used in RA, MG, SLE, Crohn's disease and ulcerative colitis. They are also used after organ transplantations and in some allergic conditions (e.g., allergic asthma control). IVIg injection actions are based on blockade of Fc receptors, anticytokine effects, inhibition of complement activation, enhanced clearance of endogenous pathogenic autoantibodies via the FcRn receptor, neutralization of autoantibodies, neutralization of superantigens and downregulation of T- or B-cell function [53]. IVIg is the first-line therapy for Guillain–Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy and multifocal motor neuropathy, but is widely used in many other conditions, such as immunoglobulin deficiency, Kawasaki disease, MG, SPS, MS and Lambert Eaton syndrome [53]. Plasmapheresis treatment requires the extraction of blood from the patient, plasma is then removed by a cell separator and blood cells are returned to the patient while the plasma could be first treated and then returned to the patient or could be discarded and replaced by donor plasma or saline with added proteins. Plasmapheresis is used for the same pathologies that need an IVIg treatment. Efficacy of IVIg or plasmapheresis demonstrates that autoreactive Igs are involved in the pathologies and represents a well-accepted evidence of an autoimmune etiology.

Passive transfer of the pathology to animals (fifth criterion for autoimmunity) has actually been demonstrated for a few diseases. With this methodology, it is expected to induce the pathology in experimental healthy animals by injecting serum or purified antibodies from patients. The first demonstration of passive transfer of an autoimmune pathology in the nervous system was made by Toyka and collaborators in 1977 [54]. Since then, passive transfer became a well-recognized approach to demonstrate the pathogenicity of serum antibodies. Antiamphiphysin antibodies from a patient with breast cancer and SPS provoked stiffness when injected in rats [55]. Plasma or serum from patients with active RA induce inflammation and histological lesions in mice [56], autoantibodies from patients with IDDM with anti-voltage gated calcium channel activity induced overactive bladder, a common complication of diabetic autonomic neuropathy [57] and a very recent work demonstrated that anti-GAD antibodies from neurological patients, when injected *in vivo*, induced continuous motor activity and abnormal reflexes, adding new evidences to the hypothesis that SPS and cerebellar ataxia are antibody-mediated diseases [58]. With passive transfer of autoantibodies, a mood disorder, such as depression in SLE [59], could also be induced. A new interesting approach to this methodology is based on 'humanization of autoantigens'. Nishie *et al.* demonstrated that the passive transfer of bullous pemphigoid by human autoantibodies into wild-type mice does not induce skin lesions because the target antigen (collagen XVII) has a different amino acid sequence between mice and humans. On the contrary, the genetic modification of mice knocked down for mouse gene but rescued by the human ortholog, resulted in the passive transfer of the pathology [60].

Finally, the sixth criterion for defining a disease as autoimmune is the induction of the pathology after injection of the autoantigen or autoreactive lymphocytes. EAE is the widely used animal model for MS. It is obtained by immunization with myelin antigens or by transfer of myelin-specific T cells with different approaches [61,62]. Biozzi ABH mice immunized with the neurofilament NF-L protein develop spastic paresis and axonal degeneration similar to the clinical features described in MS [63].

In reviewing the literature of the last years, we have separated the pathologies, directly involving the CNS or the PNS, that are commonly accepted by the medical and scientific community as autoimmune (even if only MG respects the six criteria described previously [64]) (TABLE 2) from the ones that are only suspected as autoimmune because the scientific and medical community has dissenting opinions on their etiology

Table 2. List of commonly accepted autoimmune disorders of the CNS and PNS* .

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
CNS						
Acute disseminated encephalomyelitis	Rare in developed countries, more common in developing countries. Estimated to follow 1/1000 cases of measles	Anti-MOG	MOG	Possible to distinguish from multiple sclerosis	Yes	[112]
Bickerstaff's brainstem encephalitis (Bickerstaff's syndrome; brainstem encephalitis)	Rare	Anti-GQ1b ganglioside antibodies	GQ1b ganglioside	Yes	Yes	[113]
Brainstem encephalitis (paraneoplastic syndrome; paraneoplastic encephalomyelitis)	Rare	Anti-Hu (ANNA1, nELAV) antibodies	HuB, HuC, HuD	Yes, associated with SCLC	Yes	[114]
		Anticollapsin response mediator proteins CRMP5/CV2 antibodies	CRMP5	Yes	Yes	
		Antiamphiphysin antibodies	Amphiphysin	No	Yes	
		Anti-Ri (ANNA2, NoVA1) antibodies	Ri protein	Yes, associated with breast cancer	Yes	
		Anti-Ma2(Ta) antibodies	Ma2 protein	Yes, associated with testicular cancer	No	
CIDP	Rare, related to GBS but less common	Anti-β–tubulin in Asymmetric CIDP	Tubulin amino acids 301–314	Debated	No	[115–117]
		Anti-sulfated glucuronyl paragloboside (SGPG)	SGPG	No	Yes	[118]
		anti-MAG/SGPG	MAG/SGPG	No	Yes	[119]
		anti- Schwann cell	Schwann cell processes and neurites	No	No	[120]

*Most of the pathologies in this list are accepted as bona fine autoimmune disorders by the scientific and medical community even if they do not fulfill all the six criteria defining an autoimmune disease. AChR: Acetylcholine receptor; CIDP: Chronic imflammatory demyelinating polyradiculoneuropathy; CRMP: Collapsin response mediated protein; GAD: Glutamic acid decarboxylase; GBS: Guillain–Barré syndrome; GluR3: Type-3 glutamate receptor; MAG: myelin-associated glycoprotein; MBP: Myelin basic protein; MOG: Myelin oligodendrocyte glycoprotein; MuSK: muscle specific kinase; SCLC: small cell lung carcinoma; SGPG: Sulfated glucuronyl paragloboside; TULIP: Tubby-like protein; VGCC: Voltage-gated calcium channel; VGKC: Voltage-gated potassium channel.

Table 2. List of commonly accepted autoimmune disorders of the CNS and PNS* (cont.).

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
Limbic encephalitis (paraneoplastic limbic encephalitis)	Rare	Anti-Hu (ANNA1, nELAV) antibodies	HuB, HuC, HuD	No	Yes	[114]
		Anti-CRMP5/CV2	CRMP5	Yes, associated with lung cancer	Yes	
		Antiamphypisin	Amphypisin	Yes, associated with lung cancer	Yes	
		Anti-VGKC	VGKC	No	Yes	
		Anti-Ma2(Ta) antibodies	Ma2 protein	Yes, associated with testicular cancer	No	
Multiple sclerosis (disseminated sclerosis, encephalomyelitis disseminata)	One case in every 700 people in USA	Anti-MBP	MBP	No	Yes	[121]
		Anti-MOG	MOG	No	Yes	
		Antimyelin proteolipid protein	Myelin proteolipid protein	No	Yes	
Opsoclonus myoclonus syndrome (opsoclonus–myoclonus–ataxia, paraneoplastic opsoclonus–myoclonus ataxia, Kinsbourne syndrome, myoclonic encephalopathy of infants, dancing eyes–dancing feet syndrome, dancing eyes syndrome)	Rare, one case in every 10,000,000 people	anti-Hu (ANNA1, nELAV) antibodies	HuB; HuC; HuD	No	Yes	[122,123]
		Antineurofilament	neurofilament	No	Yes	
		Anti-Ri antibodies (ANNA2, NoVA1)	Ri protein	No	Yes	[124]
		Anti-Yo (PCA1, CDR1 = CDR34, CDR2 = P17, CD2 = CDR62, CZF) antibodies	Purkinje cells	No	Yes	
		Anti-Ma1	Ma1 protein	No	Yes	
		Anti-Ma2	Ma2 protein	No	No	
		Antiamphiphysin	Amphiphysin	No	Yes	
		Anti-CRMP-5/CV2	CRMP5	No	Yes	
		Anti-Zic2		No	No	

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Table 2. List of commonly accepted autoimmune disorders of the CNS and PNS* (cont.).

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
		Antineuroleukin		No	No	
Paraneoplastic cerebellar degeneration	Rare, less than 200,000 cases in the USA	Anti-Yo (PCA1, CDR1 = CDR34, CDR2 = P17, CD2 = CDR62, CZF) antibodies	Purkinje cells	Yes, associated with cancer of the ovary, breast, or other gynecological malignancies	Yes	[114,123]
		Anti-Tr antibodies	Purkinje cells	Yes, associated with Hodgkin disease and rarely with non-Hodgkin lymphoma	No	
		Anti-VGCC antibodies	VGCC	Yes, associated with SCLC	Yes	
		Anti-Hu (ANNA1, nELAV) antibodies	HuB, HuC; HuD	Yes, associated with lung cancer	Yes	
		Anti-CRMP-5/CV2	CRMP5	No, associated with lung cancer	Yes	
		Anti-Zic4	Zic family member 4	No, associated with lung cancer	No	
		Anti-Ma1	Ma1 protein	No, associated with lung cancer	Yes	
		Anti-Ma2	Ma2 protein	No, associated with lung cancer	No	
Paraneoplastic encephalomyelitis	Unknown	Anti-Hu (ANNA1, nELAV) antibodies	HuB, HuC; HuD	Yes associated with SCLC	Yes	[114,123]
		Anti-CRMP-5/anti-CV2	CRMP5	No	Yes	
		Antiamphiphysin	Amphiphysin	No	Yes	
Paraneoplastic retinopathies	Rare	Antirecoverin antibodies	Recoverin	No	Yes	[114,123,125]
		Antienolase antibodies	Enolase	No	Yes	
		Anti-TULIP-1	TULIP-1	No	No	

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Table 2. List of commonly accepted autoimmune disorders of the CNS and PNS* (cont.).

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
		Antiphotoreceptor cell-specific nuclear receptor	Photoreceptor cell-specific nuclear receptor	No	No	
		Anti-rod bipolar cell antibodies	Rod bipolar cell	No	No	
Rasmussen's encephalitis (Rasmussen's syndrome)	Rare, one case in every 1,000,000 individuals	Anti-GluR3	GluR3	Yes	Yes	[126]
Stiff person syndrome (stiff-man syndrome, stiff limb syndrome, stiff baby syndrome, stiff-trunk syndrome)	Rare, less than 200,000 cases in the USA	Anti-GAD	GAD	Yes	Yes	[127]
		Antiamphiphysin	Amphiphysin	Yes, associated with breast cancer	Yes	
		Antigephyrin	Gephyrin	No	No	
PNS and neuromuscular						
Antibody-mediated arthrogryposis	Rare	Antifetal AChR	Fetal AChR	Yes	Yes	[128]
CIDP	Rare, related to GBS but less common	Anti-β–tubulin in Asymmetric CIDP	Tubulin amino acids 301–314	Debated	No	[116,117]
		Anti-SGPG	SGPG	No	Yes	[118]
		Anti-MAG/SGPG	MAG/SGPG	No	Yes	[119]
		Anti-Schwann cell	Schwann cell processes and neurites	No	No	[120]
Guillain–Barré syndrome (acute inflammatory demyelinating polyradiculoneuropathy, acute motor axonal neuropathy)	Rare, 1–2 cases every 100,000 people in the USA	Antiganglioside antibodies	Gangliosides GM1, GM1b, GD1a, and GalNAc-GD1a	No	Yes	[129,130]
Lambert Eaton myasthenic syndrome	One case in every 100,000 individuals	Anti-P/Q-type VGCC	P/Q-type VGCC	Yes	Yes	[131]

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Table 2. List of commonly accepted autoimmune disorders of the CNS and PNS* (cont.).

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
Miller–Fisher syndrome (variant of Guillain–Barré syndrome)	Rare	Anti-GQ1b ganglioside	GQ1b ganglioside	Yes	Yes	[113]
Myasthenia gravis	20 cases in every 100,000 individuals in USA	Anti-AChR	Nicotinic AChR	Yes	Yes	[132]
		Anti-MuSK antibodies	MuSK	Yes for seronegative AChR patients	No	
Neuromyotonia (Isaacs' syndrome, Isaccs–Mertens syndrome, continuous muscle fiber activity syndrome, quantal squander syndrome)	Rare	Anti-VGKC	VGKC	Yes	Yes	[133]

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Table 3. List of disorders of the CNS and PNS with a suspected autoimmune etiology .

Suspected autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
CNS						
Autism	1–2 cases in every 1000 individuals	Antibrain antibodies	Brain neurons	No	No	[134]
		Antiserotonin binding site receptors antibodies	5-HT receptors	No	No	
		Antibrain endothelial cells	Brain endothelial cells	No	No	
Alzheimer's disease	1–2 cases every 68 individuals	Antibrain antibodies	Brain neurons	No	No	[65,135]
Bipolar disorder	5–6% of the world's population	Anti-GAD65	GAD65	No	Yes	[136]
		Antigastric parietal cells	H/K adenosine triphosphatase (ATPase)	No	Yes	
		Anti-TPO	TPO	No	Yes	
Cerebellar ataxia	1–2 cases every 100,000 individuals	Anti-GAD	GAD	No	Yes	[52]
Epilepsy	5–10 cases in every 1000 individuals	Anti-GAD	GAD	No	Yes	[52]
		Anti-VGKC antibodies	VGKC	No	Yes	[137]
		ANA	Cells' nucleus	No	Yes	[138]
		Antianticardiolipin antibodies	Anticardiolipin	No	Yes	
		Anti-GluR3 antibodies	GluR3	No	Yes	[139]
Gluten ataxia	Rare	Anti-Purkinje cells antibodies	Purkinje cells	No	Yes	[140]
Landau–Kleffner syndrome and Landau–Kleffner variant syndrome (infantile acquired aphasia, acquired epileptic aphasia, aphasia with convulsive disorder)	Rare, less than 200,000 individuals in the USA	Antibrain antibodies	Brain neurons	No	Yes	[141,142]
Pediatric autoimmune neuropsychiatric disorders associated with *Streptococcus* infection	Unknown	Antibasal ganglia antibodies	Basal ganglia antigens	No	No	[143]
Antistreptolysin O	Streptolysin O	Yes	Yes			
Schizophrenia	1% of the world's population	Antibrain antibodies	Brain neurons	No	No	[144]
		Antilymphocyte nuclei antibodies	Lymphocyte nuclei	No	No	

AChR: Acetylcholine receptor; ANA: Antinuclear antibody; GAD: Glutamic acid decarboxylase; GluR3: Type-3 glutamate receptor; TPO: Thyroid peroxidase; VGKC: Voltage-gated potassium channel.

Table 3. List of disorders of the CNS and PNS with a suspected autoimmune etiology (cont.).

Suspected autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
		Anti-DNA antibodies	DNA	No	Yes	
		Anticardiolipin antibodies	Cardiolipin	No	Yes	
		Antiheat-shock proteins antibodies	Heat shock proteins	No	Yes	
		Antigastric parietal cells antibodies	Gastric parietal cells	No	Yes	
		Antiplatelets antibodies	Platelets	No	Yes	
		Antineurotransmitter receptors antibodies	Neurotransmitter receptors	No	No	
Sydenham's chorea (Saint Vitus dance)	20–30% of patients with rheumatic fever	Antibasal ganglia antibodies	Basal ganglia antigens	No	No	[143]
		Antistreptolysin O	Streptolysin O	Yes	Yes	
Tourette syndrome	1–11 cases every 1000 individuals	Antibasal ganglia antibodies	Basal ganglia antigens	No	No	[145]
Antistreptolysin O	Streptolysin O	Yes	Yes			
PNS						
Dysautonomia including: autoimmune autonomic neuropathy, autonomic instability, mitral valve prolapse dysautonomia, multiple system atrophy (Shy–Drager syndrome), neurocardiogenic syncope, postural orthostatic tachycardia syndrome, pure autonomic failure.	Unknown	Antiganglionic AChR antibodies	Ganglionic AChR	No	Yes	[146,147]

AChR: Acetylcholine receptor; ANA: Antinuclear antibody; GAD: Glutamic acid decarboxylase; GluR3: Type-3 glutamate receptor; TPO: Thyroid peroxidase; VGKC: Voltage-gated potassium channel.

(TABLE 3). Moreover, we have listed accepted and suspected autoimmune disorders not directed to the CNS or PNS that can have a neurological or psychiatric evolution (TABLE 4). The inclusion of Alzheimer's and Parkinson's diseases in this review deserves a specific mention. Abnormal cellular and humoral immune response including elevated levels of interleukins, circulating immune complexes and autoantibodies have been described in both pathologies (reviewed in [65–67]). In addition, the intrathecal synthesis of autoantibodies has been detected [41]. Although the role of autoantibodies and autoimmunity in these pathologies remains unclear, it has recently been proposed that the aberrant immune functions observed in a subset of Alzheimer's disease patients may be consistent with the presence of a pathological autoimmune reaction that may favor massive neuronal cell death [41,65,66]. We have finally considered the diagnostic relevance of the presence of autoantibodies in such pathologies. For the large majority of cases, the serological test for autoimmunity is performed, in some cases to confirm the pathology, in others to eliminate this hypothesis and, in others, just because of the idiopathic features of the disorder. This implies a great diagnostic importance for autoantibody detection not only in clinical practice but also for research studies aimed at understanding the role of autoantibodies in the clinical evolution of the pathology.

In vitro assays for the diagnosis of autoimmune neurological disorders

A rapid and correct diagnosis in autoimmune pathologies is important not only because autoimmunity is a chronic and incurable condition but also because in case of an immune attack against the CNS, it is crucial to start the treatment before the neuronal damage occurs, after which there is no remedy. However, the possibility to diagnose autoimmunity in a patient with neurological symptoms using a commercial *in vitro* diagnostic assay is presently restricted to only a few classes of diseases:

- Neuropathologies with antiphospholipids (GBS, Miller–Fisher syndrome, multifocal motor neuropaties, antiphospholipid syndrome);
- Neurological disorders associated with systemic inflammatory diseases with antinuclear antibodies (SLE, mixed connective tissue disease, Sjögren's syndrome, RA) or antibodies against neutrophil cytoplasma (ANCA) (vasculitides), although it must be noted that there is no specific test to discriminate patients with a potential risk for neurological complications;
- Paraneoplastic syndromes that are often characterized by very specific antineural antibodies;
- The thyroid autoimmune diseases that, although not involving antibodies directly targeting the nervous system, may have indirect neurological complications (Hashimoto's syndrome, GD);
- Antibodies to the acetyl choline receptor are important for the diagnosis of MG;
- Antibodies against myelin components, such as myelin associated glycoprotein (MAG), may be helpful in certain autoimmune polyneuropathies.

In addition, a diagnostic assay for the Menière's syndrome (antitubulin antibody assay) has recently become available [201]. As these tests cover only 15–20 autoimmune diseases of the nervous system out of more than 50, it is clear that there is a tremendous need for development of novel tests for neuropathologies.

Various techniques are used for the detection of autoantibodies from patient's sera or CSF. If the recombinant antigen recognized by autoantibodies is available, the preferred screening method is the ELISA test that also allows identification of 'conformational epitopes' from recombinant proteins. These epitopes are very sensitive to denaturation and are generally lost in most preparations. The recombinant antigen can also be used for the 'dot line' or dot blot method, wherein the protein is transferred onto nitrocellulose simply by spotting it without denaturation. Both with ELISA and dot line, more than one antigen can be used so that more than one autoantibody can be detected. Another well-known technique for the detection of autoantibodies is the western blot. This technique allows the identification of specific antigens from a complex lysate and is used for example, for the detection of antibodies in paraneoplastic syndromes. Compared with ELISA and dotblots, preparation of the samples implies a denaturation of the antigens that are separated electrophoretically on a sodium dodecyl sulfate polyacrylamide gel before blotting on a nitrocellulose membrane. Therefore, if the antibody recognizes a conformational epitope this method will yield false-negative results.

Alternatively, polystirene beads coated with purified antigens can be incubated with patients' sera or CSF and the reaction is revealed with a secondary antibody conjugated with enzyme or fluorescence. This system has the advantage, in respect to other tests, that it is fully automated [68].

Finally for a very sensitive detection, immunoprecipitation (IP) or radio immune assay (RIA) are used especially for the detection of antibodies against membrane antigens that are impossible to be produced as full recombinant proteins, such as the voltage-gated potassium channel to diagnose the Lambert Eaton's syndrome, or the acetylcholine receptor to diagnose MG. With the IP assay, the complex antigen–antibody is precipitated from solution by capture with an antibody-binding protein attached to a solid support, such as an agarose bead, whereas in the RIA technique, antibodies are quantified as immune complexes after binding of radioactively-labeled secondary antibody.

Immunohistochemistry on tissue sections or cell lines is an old, but still valid alternative to these methods, particularly if the antigens are not known. This technique is also very useful if the searched antibodies react against an antigen with conformational epitopes especially if the antigen is a transmembrane protein. However, the major drawback of immunohistochemistry on tissues, or cells, is that reproducibility of the assay and interpretation of the results are strongly operator-dependent. Thus, only few highly qualified laboratories are able to reliably perform these tests.

Table 4. List of commonly accepted or suspected autoimmune disorders that can evolve with neurological or psychiatric complications.

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
With neurological complications						
Antiphospholipid antibody syndrome (antiphospholipid antibody syndrome; Hughes syndrome)	Rare, less than 200,000 individuals in the USA	aPL	Plasma proteins that bind to phospholipids	Debated	Yes	[148,149]
		ACA, aCL	Cardiolipin	Yes	Yes	
Celiac disease	One in 250 individuals	Anti-TG2	Tissue transglutaminase	Yes	Yes	[150]
		Antigliadin		Yes, but less specific than TG2	Yes	
		Antiendomysium		Yes, but less specific than TG2	Yes	
Crohn's disease	Seven in 100,000 individuals	Not autoantibodies but antibodies against *Saccharomyces cerevisiae* (ASCAs)	*S. cerevisiae*			[151,152]
		Antibodies against exocrine pancreas (PAbs)	Unknown	No	No	
Diabetes mellitus Type 1	0.3% of Caucasian populations	Anti-GAD 65	GAD 65	Yes	Yes	[153,154]
		Islet cell antibodies	Against the antigen present in the cytoplasm of the endocrine cells in pancreatic islets	Yes	Yes	
		Insulin autoantibodies	Insulin	Yes	Yes	
		Insulinoma associated 2 autoantibodies	Protein tyrosine phosphatase	Yes	Yes	
Hashimoto's thyroiditis (Hashimoto's disease, chronic lymphocytic thyroiditis) associated with Hashimoto encephalitis	0.3–1.5 cases every 1000 individuals. Hashimoto encephalitis rare	Antithyroid microsomal antibody	Thyroid microsomal	Yes	Yes	[155–157]
		Anti-TPO antibody	TPO	Yes	Yes	
		Antithyroglobulin antibody	Thyroglobulin	Yes	Yes	
Idiopathic thrombocytopenic purpura (immune thrombocytopenic purpura)	10 –125 cases in every 1,000,000 individuals	Antiplatelet antibodies	Platelets	No	Yes	[158]
		Antiplatelet membrane glycoproteins Iib-IIIa or Ib-IX	Platelet membrane glycoproteins Iib-IIIa or Ib-IX	No	Yes	
		ANA	Cells' nucleus	No	Yes	[159,160]
		Anti-SSA/Ro and nuclear RNP (nRNP) antigens	SSA/Ro	No	Yes	[159]

ACA (aCL): Anticardiolipin antibodies; ANA: Antinuclear antibody; aPL: Antiphospholipid antibodies; ENA: Extractable nuclear antigen; GAD: Glutamic acid decarboxylase; MOG: Myelin oligodendrocyte glycoprotein; MS: Multiple sclerosis; NPSLE: Neuropsychiatric systemic enrythematosus; SLE: Systemic lupus encephalomyelitis; TG2 (tTG): Tissue transglutaminase; TPO: Thyroid peroxidase.

Table 4. List of commonly accepted or suspected autoimmune disorders that can evolve with neurological or psychiatric complications.

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
Kawasaki disease (lymph node syndrome, mucocutaneous node disease, infantile polyarteritis and Kawasaki syndrome)	175 cases every 100,000 individuals in Japan	Antiendothelial cell antibodies	Endothelial cell	No	Yes	[161,162]
		aCL antibodies	Cardiolipin	No	Yes	[163]
Optic neuritis (Devic's disease, neuromyelitis optica)	6.4 cases in every 100,000 people in USA	Antiaquaporin 4 (AQP4)	Aquaporin 4	No, but suggested to distinguish from MS	No	[164,165]
Pernicious anemia may evolve in neuropathy	127 cases every 100,000 Caucasians	Antigastric intrinsic factor Anti-parietal cells	Gastric intrinsic factor Gastric parietal cells	No	Yes	[166]
Rheumatoid arthritis, may evolve in neuropathy or mononeuritis multiplex	1% of the USA adult population	Anti-Fc portion of IgG (rheumatoid factor)	Fc portion of IgG	Yes	Yes	[167]
		Anticitrullinated protein antibodies	Citrullinated protein	Yes	Yes	[167]
		Anticyclic citrullinated peptide	Cyclic citrullinated peptide	Yes	Yes	
		ANA	Cells' nuclei	Yes	Yes	
SLE may evolve in NPSLE	SLE 40–50 cases every 100,000 individuals in USA NPSLE 10% of SLE	ANA	Cells' nucleus	Yes	Yes	[168]
		Anti-ENA antibodies	ENA	Yes	Yes	
		aPL	Phospholipids	Yes	Yes	
		Anti-smith (anti-Sm)	Sm ribonucleoprotein	Yes	Yes	
		Anti-ribosomal P antibodies	Ribosomal P protein	Yes	Yes	[168,169]
		Anti-dsDNA antibodies	dsDNA	Yes	Yes	
		Anti-Ro (anti-SSA, SSA1, SSA, RO52, TRIM21)	Ribonucleoprotein	Yes	Yes	
		Anti-La (anti-SSB, LARP3)	La protein	Yes	Yes	
		Histone antibody (antihistone)	Histones	Yes	Yes	
Sjögren's syndrome	1–4 million people in the USA	ANA	Cells' nuclei	Yes	Yes	[170]
		Anti-Ro (anti-SSA, SSA1, SSA, RO52, TRIM21)	Ribonucleoprotein	Yes	Yes	
		Anti-La (anti-SSB, LARP3)	La protein	Yes	Yes	
		Anti-Fc portion of IgG (rheumatoid factor, RF, RhF)	Fc portion of IgG	Yes	Yes	

ACA (aCL): Anticardiolipin antibodies; ANA: Antinuclear antibody; aPL: Antiphospholipid antibodies; ENA: Extractable nuclear antigen; GAD: Glutamic acid decarboxylase; MOG: Myelin oligodendrocyte glycoprotein; MS: Multiple sclerosis; NPSLE: Neuropsychiatric systemic enrythematosus; SLE: Systemic lupus encephalomyelitis; TG2 (tTG): Tissue transglutaminase; TPO: Thyroid peroxidase.

Table 4. List of commonly accepted or suspected autoimmune disorders that can evolve with neurological or psychiatric complications.

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
Wegener's granulomatosis	8.5 cases every 1,000,000 individuals	Antineutrophil cytoplasmic antibodies (ANCAs; cANCA)	Antigens in the cytoplasm of neutrophil granulocytes	Yes	Yes	[171,172]
Antiperinuclear antineutrophil cytoplasmic antibody (p-ANCA)	p-ANCA	Yes	Yes			
With psychiatric complications						
Addison's disease	from 1/100,000 to 1/25,000–1/16,600 upon studies	Anti-21-hydroxylase autoantibodies	21-hydroxylase	Yes, to distinguish from infectious or genetic causes	Yes	[173]
Celiac disease (CD)	One in 250 individuals	Anti-TG2	TG2	Yes	Yes	[150]
		Antigliadin	Gliadin	Yes, but less specific than TG2	Yes	
		Antiendomysium	TG2	Yes, but less specific than TG2	Yes	
Crohn's disease	Seven in 100,000 individuals	Not autoantibodies but ASCAs	*S. cerevisiae*			[151] [174]
		PAbs	Unknown	No	No	
Diabetes mellitus Type 1 (T1D; IDDM)	0.3% of Caucasian populations	Anti-GAD 65	GAD 65	Yes	Yes	[153,175]
		Islet cell antibody	Antigen present in the cytoplasm of the endocrine cells in pancreatic islets	Yes	Yes	
		Insulin autoantibody		Yes	Yes	
		IA-2	Protein tyrosine phosphatase	Yes	Yes	
Graves' disease (Basedow's disease, Graves–Basedow disease)	Five in 10,000 individuals	Antithyroid-stimulating hormone receptor (TSH)	Receptor for TSH	not principal	Yes	[176,177]
		Antithyroglobulin antibodies	Thyroglobulin	not principal	Yes	
		Antithyroid hormones T3 and T4 antibodies	Hormones T3 and T4	not principal	Yes	
Hashimoto's thyroiditis (Hashimoto's disease, chronic lymphocytic thyroiditis) associated with Hashimoto encephalitis	0.3–1.5 cases every 1,000 individuals Hashimoto encephalitis rare	Antithyroid microsomal antibody	Thyroid microsomal	Yes	Yes	[155–157]
		TPO antibody	TPO	Yes	Yes	
		Antithyroglobulin antibody	Thyroglobulin	Yes	Yes	

ACA (aCL): Anticardiolipin antibodies; ANA: Antinuclear antibody; aPL: Antiphospholipid antibodies; ENA: Extractable nuclear antigen; GAD: Glutamic acid decarboxylase; MOG: Myelin oligodendrocyte glycoprotein; MS: Multiple sclerosis; NPSLE: Neuropsychiatric systemic enrythematosus; SLE: Systemic lupus encephalomyelitis; TG2 (tTG): Tissue transglutaminase; TPO: Thyroid peroxidase.

Table 4. List of commonly accepted or suspected autoimmune disorders that can evolve with neurological or psychiatric complications.

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
Primary biliary cirrhosis	5/100,000 worldwide	Antimitochondrial antibodies (AMAs)	Mitochondria	Yes	Yes	[178,179]
		ANA	Cells' nuclei	Yes	Yes	
SLE may evolve in NPSLE	SLE 40–50 cases in every 100,000 individuals in the USA NPSLE 10% of SLE	ANA	Cells' nucleus	Yes	Yes	[168]
		Anti-extractable nuclear antigen (Anti-ENA) antibodies	Extractable nuclear antigen	Yes	Yes	
		aPL	Phospholipids	Yes	Yes	
		Anti-Sm antibodies	Sm ribonucleoprotein	Yes	Yes	
		Antiribosomal P antibodies	Ribosomal P protein	Yes	Yes	[168,169]
		Anti-dsDNA antibodies	dsDNA	Yes	Yes	
		Anti-Ro (anti-SSA, SSA1, SSA, RO52, TRIM21)	Ribonucleoprotein	Yes	Yes	
		Anti-La (anti-SSB, LARP3)	La protein	Yes	Yes	
		Histone antibody (antihistone)	Histones	Yes	Yes	
		AECA	Endothelial cells	No	Yes	[180]
		Anti-Nedd5 antibodies	Nedd5	No	No	[181]
Suspected autoimmune pathologies with neurological complications						
Behçet's disease (Behçet syndrome, neuro-Behçet's disease)	Rare, less than 200,000 cases in the USA	Not specific		No		[182,183]
Chagas disease (American Trypanosomiasis; South American trypanosomiasis, New World trypanosomiasis)	16–18 million individuals are infected in 18 countries of Latin America	Antimyosin antibodies	Myosin	No	No	[184,185]
		Antisulfatide antibodies	Sulfatide	No	No	
Neurologic Lyme disease	Unknown	Anticytokeratin 10	Cytokeratin 10	No	No	[186]

ACA (aCL): Anticardiolipin antibodies; ANA: Antinuclear antibody; aPL: Antiphospholipid antibodies; ENA: Extractable nuclear antigen; GAD: Glutamic acid decarboxylase; MOG: Myelin oligodendrocyte glycoprotein; MS: Multiple sclerosis; NPSLE: Neuropsychiatric systemic enrythematosus; SLE: Systemic lupus encephalomyelitis; TG2 (tTG): Tissue transglutaminase; TPO: Thyroid peroxidase.

Table 4. List of commonly accepted or suspected autoimmune disorders that can evolve with neurological or psychiatric complications.

Accepted autoimmune pathologies	Frequency	Antibodies	Antigen	Diagnostic relevance of autoantibodies	Available kits	Ref.
Neurosarcoidosis	30 cases in every 100,000 individuals in the USA	Anti-MOG	MOG	No	Yes	[187,188]
Vogt–Koyanagi–Harada disease (Vogt–Koyanagi–Harada syndrome, uveoencephalitis, uveomeningitis, granulomatous panuveitis)	1–4% of all uveitis cases the incidence of uveitis is 15 cases every 100,000 individuals in the USA	Antimelanocytes	Melanocytes	No	No	[189]
Suspected autoimmune pathologies with psychiatric complications						
Alopecia universalis	One case in every 100,000 individuals	Antihair follicle antibodies	Hair follicles	No	No	[190,191]
Chronic fatigue syndrome	Four cases every 1000 adults in the USA and other industrialized countries	Antinuclear membrane antibodies	Nuclear membrane	No	Yes	[192,193]
		Antivimentin antibodies	Vimentin	No	Yes	
		Antilamin B1	Lamin B1	No	No	

ACA (aCL): Anticardiolipin antibodies; ANA: Antinuclear antibody; aPL: Antiphospholipid antibodies; ENA: Extractable nuclear antigen; GAD: Glutamic acid decarboxylase; MOG: Myelin oligodendrocyte glycoprotein; MS: Multiple sclerosis; NPSLE: Neuropsychiatric systemic enrythematosus; SLE: Systemic lupus encephalomyelitis; TG2 (tTG): Tissue transglutaminase; TPO: Thyroid peroxidase.

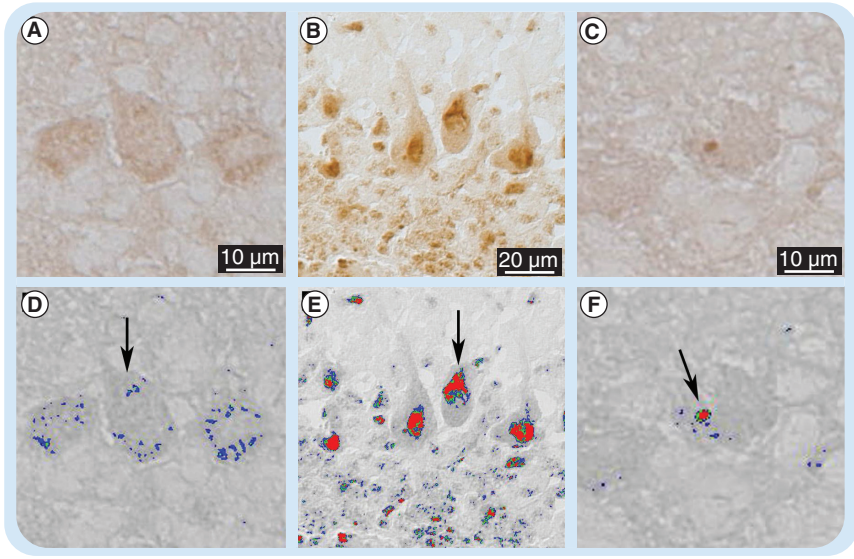

Figure 1. Densitometric analysis converting pictures in false colors. With our densitometric analysis on rat brain staining in immunohistochemistry, we convert normal RGB color pictures **(A, B, and C)** into pseudo-colors **(D, E, and F)** on the basis of a previously calculated cut off [73]. We can therefore, distinguish a borderline staining (**D**, arrow) from a positive or strongly positive staining (**E,D**, arrows).

Expert commentary

While at present the main efforts are directed towards the identification of single antigens, future avenues for the diagnosis of autoimmunity will certainly involve the design of multiple antigen platforms to screen in a single step a large amount of different autoantibodies. As the immunological manifestation of autoimmune diseases is often very complex, the evaluation of hundreds or even thousands of different antigens in one single step should give more information than the presently available tests. Considering multiple antigens may also allow understanding of individual differences in clinical manifestations of each autoimmune disease and, in perspective, should lead to personalized treatments. The autoantigens microarrays developed so far, have shown to have a sensitivity comparable with ELISA [2,69–71] but they are still not commercially available.

As the new antigen array technology is still developing, the authors wish to suggest the use of immunohistochemistry as a good entry test to diagnose autoimmunity at the first clinical inspection of a patient with neurological complaints. The reasons for this choice are threefold: a brain tissue section, covering different brain areas, contains basically all CNS antigens, including proteins, sugars, lipids and small molecules and represents, therefore, the screening test with the broadest sensitivity possible at the moment; all antigens found in the original tissue are in a native status that includes correct folding, proper distribution across the cell membranes, complex secondary and tertiary molecular interactions and post-translational protein modifications, all features that are impossible to achieve even in the presently available antigen arrays; and a specific regional or subcellular pattern of the autoantibody reactivity may allow identification of a specific pathology and correlations between the possible sites of action of the autoantibodies and a patient's symptoms.

For all these unique features, it is the opinion of the authors that immunohistochemistry may remain an unsurpassed entry test even in the future era of antigen microarrays. However, in order to make this old technology a state-of-the-art diagnostic tool, three aspects need to be optimized: maximal antigen preservation, reduction, if not elimination, of dependence from operator or laboratory conditions, and high discrimination power of the pathological reactivity versus natural immunity.

Immunohistochemistry is not just a single protocol and most treatments are used on tissues or cells before incubation with sera. Fixatives and paraffin embedding are normally used to preserve cells and tissues structure, but their use often alters proteins, masking some epitopes. To avoid this inconvenience, the method of fresh frozen, unfixed sections is preferred, allowing excellent antigen preservation, even if sections contain less morphological details and resolution of certain patterns is slightly lower than with other methods. Mild fixative procedures have been used successfully on sections obtained from fresh frozen tissues. These procedures, including alcohol and/or acetone fixation but not involving aldehydes, usually give better results than paraffin-embedded tissues [72]. However, even with these mild treatments, some sensitive conformational epitopes may be irremediably lost. To maximize native antigen preservation, we propose to refrain from any tissue treatment and use a very simple protocol, which is production of 10-µm cryostate sections from fresh frozen rat brains, followed by overnight dehydration of sections at room temperature.

One of the major limiting aspects in the use of immunohistochemistry to diagnose autoimmunity is that results may vary among different laboratories and interpretation of the staining is strongly operator-dependent. To overcome these basic problems, we recommend the use an automatic stainer for immunohistochemistry and we have developed a method for semiquantitative densitometric analysis [73]. The semiquantitative analysis is also a powerful method to discriminate a pathological reactivity with respect to the background produced by natural immunity. In a recent study, we have characterized the natural antineural humoral response and found that, although a strong reactivity is very rare, approximately 11% of healthy people have a basal, mild antineural reactivity that is perfectly in agreement with previous estimations made in different countries [73]. Thanks to that study, we have defined the baseline of natural antineural autoreactivity and used it as the cut-off for subsequent densitometric quantification of antineural reactivity of patients' sera on rat brain sections. A macro of the image analysis program that we designed for this purpose generates automatically pseudo-

colors (FIGURE 1), allowing an objective, error-free ranking of the serum reactivity against rat brain sections into four classes: negative, borderline, positive and strongly positive. The test, which is now available as a service from the University of Trieste (Italy), has a sensitivity of 81%, a specificity of 89% and a negative predictive value of 97% [73]. Thus, it represents an excellent exclusion test for the presence of antineural antibodies. These parameters have been calculated using 107 healthy adults' blood donors' sera, as healthy population, and sera from 16 subjects with autoimmune disorders including SLE, neuropsychiatric systemic lupus erythematosus, paraneoplastic cerebellar degeneration and SPS, as patient population (sera dilution 1:600). The antineural reactivity of sera has been detected on three rat brain areas as described previously [73].

Some of the patterns obtained with our method are partially available on the website [301] where we show different images of rat brain sections labeled with sera from patients with some frequent neurological disorders. It is important to underline here that, in principle, immunohistochemistry allows the detection of even those very rare autoimmune disorders for which the autoantigens are unknown.

Five-year view

It is likely that within 5 years, more neural autoantigens specific for autoimmune neurological disorders will be discovered. In theory, this would allow the development of novel diagnostic tests based on ELISA, beads or blot technologies. However, as most autoimmune neurological disorders are very rare diseases, identifying specific antigens in diseases affecting a few patients in the world is intrinsically difficult. Lack of a collection of consistent observations, wide geographic dispersion of patients and variability of the immune response are likely to jeopardize efforts to identify specific markers for any of these pathologies. These disorders are likely to remain orphan of specific diagnostic tools not only because of the inherent difficulty of identifying the autoantigens but also because of economical reasons. In fact, it is expected that no biotech company will take the risk to develop and bring to the market a diagnostic kit dedicated to a restricted number of patients. Thus, as outlined previously, only diagnostic platforms with a broad spectrum will be able to also incorporate the markers for these rare disorders. These platforms will consist of antigen arrays and the 'evergreen' immunohistochemistry that novel automated image analysis approaches are likely to revamp as a modern, state-of-the-art diagnostic tool for a first initial screening in the diagnostic procedure of basically any neurological disease with a suspected autoimmune basis.

It must also be noted that, from the therapy point of view, the field of autoimmune diseases is characterized by a relatively poor diversity of the available treatments so that the most diverse disorders are treated with the same drugs. The absence of specific diagnostic tools is considered particularly deleterious for the development of novel drugs. First, as patients are undiagnosed or misdiagnosed, their exact number cannot be evaluated correctly, and as a consequence, the market dimension for a pharmaceutical compound specifically addressed to these patients cannot be estimated correctly. Second, evaluating the efficacy of a drug in suppressing the production of a specific type of autoantibodie requires the possibility to identify such antibodies and monitor their titer in Phase II clinical trials. In absence of these diagnostic assays, clinical trials cannot be performed with antigen-based kits. Therefore, it is expected that a more generalized use of broad-spectrum diagnostic platforms able to faithfully detect the presence of even rare antineural antibodies, will boost pharmaceutical industries to develop novel and possibly more specific, drugs to treat these disorders.

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Key issues

- Autoimmunity is a complex phenomenon triggered by environmental, genetic and gender factors.
- Detection of autoantibodies in autoimmune disorders is a key diagnostic step for some autoimmune diseases.
- Autoantibodies may be predictive of autoimmunity and are useful for patients' treatments.
- Evolution of novel multiantigen platforms for diagnostic purpose is of maximum priority for autoimmune disorder especially the rare ones.
- Immunohistochemistry is still a powerful diagnostic test for the detection of a wide range of autoantibodies, including rare diseases.

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