RESEARCH ARTICLE OPEN ACCESS

Membrane Proteome-Wide Screening of Autoantibodies in CIDP Using Human Cell Microarray Technology

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Neurol Neuroimmunol Neuroinflamm 2024;11:e200216. doi:10.1212/NXI.0000000000200216

Abstract

Background and Objectives

Autoantibody discovery in complex autoimmune diseases is challenging. Diverse successful antigen identification strategies are available, but, so far, have often been unsuccessful, especially in the discovery of protein antigens in which conformational and post-translational modification are critical. Our study assesses the utility of a human membrane and secreted protein microarray technology to detect autoantibodies in chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).

Methods

A cell microarray consisting of human embryonic kidney-293 cells expressing >5,000 human proteins was used. First, a validation step was performed with 4 serum samples from patients with autoimmune nodopathy (AN) to assess the ability of this technology to detect circulating known autoantibodies. The ability of the cell microarray technology to discover novel IgG autoantibodies was assessed incubating the array with 8 CIDP serum samples. Identified autoantibodies were subsequently validated using cell-based assays (CBAs), ELISA, and/or tissue immunohistochemistry and analyzed in a cohort of CIDP and AN (n = 96) and control (n = 100) samples.

Results

Serum anti-contactin-1 and anti-neurofascin-155 were detected by the human cell microarray technology. Nine potentially relevant antigens were found in patients with CIDP without other detectable antibodies; confirmation was possible in six of them: ephrin type-A receptor 7 (EPHA7); potassium-transporting ATPase alpha chain 1 and subunit beta (ATP4A/4B); leukemia-inhibitory factor (LIF); and interferon lambda 1, 2, and 3 (IFNL1, IFNL2, IFNL3). Anti-ATP4A/4B and anti-EPHA7 antibodies were detected in patients and controls and considered unrelated to CIDP. Both anti-LIF and anti-IFNL antibodies were found in the same 2 patients and were not detected in any control. Both patients showed the same staining pattern against myelinating fibers of peripheral nerve tissue and of myelinating neuron-Schwann cell cocultures. Clinically relevant correlations could not be established for anti-LIF and anti-IFNL3 antibodies.

Discussion

Our work demonstrates the utility of human cell microarray technology to detect known and discover unknown autoantibodies in human serum samples. Despite potential CIDP-associated

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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

The Article Processing Charge was funded by the authors.

Glossary

AN = autoimmune nodopathy; **CBA** = cell-based assay; **CIDP** = chronic inflammatory demyelinating polyradiculoneuropathy; **DRG** = dorsal root ganglia; **EPHA7** = ephrin type-A receptor 7; **HEK293** = human embryonic kidney-293; **IHC** = immunohistochemistry; **LIF** = leukemia-inhibitory factor; **MS** = multiple sclerosis; **OD** = optical density; **SCs** = Schwann cells.

autoantibodies (anti-LIF and anti-IFNL3) being identified, their clinical and pathogenic relevance needs to be elucidated in bigger cohorts.

Introduction

Autoantibodies play a crucial role in the pathogenesis of many autoimmune diseases, including neurologic ones, and are used as biomarkers for their diagnosis, characterization, prognosis, and treatment.[1] However, identifying clinically relevant autoantibodies in rare and heterogeneous autoimmune diseases is challenging.

The discovery of autoantibodies as diagnostic biomarkers for autoimmune diseases has been an important topic of research for decades. Diverse technologies and experimental approaches have been used.[2] Immunoprecipitation followed by antigen identification with mass spectrometry has been one of the most common strategies to identify novel protein antigens.[3-5] More recently, new autoantibody discovery technologies have emerged, such as protein microarrays and phage-immunoprecipitation techniques.[6,7] Clinically relevant and pathogenic autoantibodies often target conformational epitopes displayed in the cell surface. So far, high-throughput autoantibody screening technologies were limited by the non-natural conformation in which the antigens are presented. Human cell microarray technologies expressing a large set of membrane and secreted proteins have been developed to detect the on-target and off-target effects of monoclonal antibodies or therapies based in recombinant proteins.[8] This technique involves the arraying on slides of large libraries of mammalian expression vectors encoding human membrane and secreted protein cDNAs, over which human embryonic kidney-293 (HEK293) cells are grown with a transfection-enabling reagent to enable expression of the cDNA-encoded proteins on the cell surface. These arrays are then used for screening of reactivities against all cell-expressed proteins. These features make this technology suitable for autoantibody screening and capable to overcome the limitations of traditional techniques. While protein microarrays have been used for the identification of novel autoantibodies before,[9,10] human cell microarray technology has never been used.

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is a rare neuroimmunologic disorder of the peripheral nervous system, in which autoantibodies seem to play an important role on its pathophysiology.[11,12] Autoantibodies targeting cell adhesion molecules of the node of Ranvier were described in patients with CIDP leading to an emerging disease category: autoimmune nodopathy (AN).[13,14] These antibodies are present only in 5–10% of patients fulfilling CIDP diagnostic criteria and target proteins such as contactin 1 (CNTN1),[3,15] contactin-associated protein 1 (Caspr1),[16,17] neurofascin 155 (NF115),[18,19] or pan-neurofascin (pan-NF)[4] and are associated with specific clinical phenotypes that are different from typical CIDP. However, the target antigen(s) remain unknown in the majority of patients with CIDP.

In this context, the aim of our study was to evaluate the human cell microarray technology for autoantibody discovery, taking CIDP as a paradigmatic example of a rare and complex autoimmune disease in which a diverse set of potentially relevant antigens may play a role in its pathogenesis.

Methods

Experimental Design and Samples

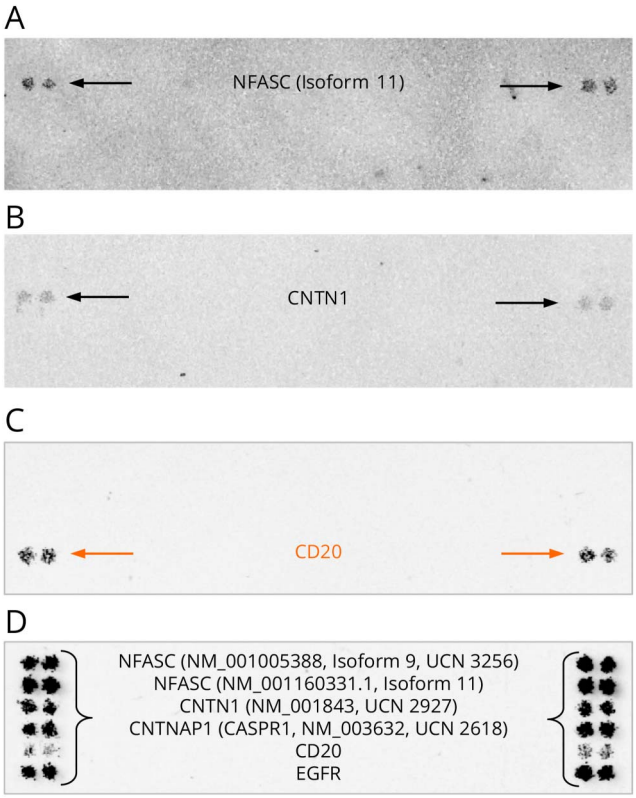
This study was designed in 3 steps: (1) validation of the human cell microarray technology in samples with known autoantibodies, (2) autoantibody discovery in CIDP samples, and (3) confirmatory experiments. In the first phase, 4 serum samples from AN patients with known antibodies (1 anti-NF155, 1 anti-NF140/186, 1 anti-CNTN1, and 1 anti-Caspr1) were included. In the discovery phase, a total of 8 CIDP patients' serum samples were included: 4 from patients with typical CIDP and 4 from patients with CIDP that showed IgG reactivity against neural structures but in which the target antigen was not known[20] (eFigure 1).

In the confirmatory phase, 96 serum samples were included. These samples included sera from 83 patients with CIDP (including the ones used in the discovery stage) and 13 patients with AN, all of them fulfilling the European Academy of Neurology/Peripheral Nerve Society Task Force 2021 revised diagnostic criteria[21] and followed in our center. The control group included 100 serum samples from healthy controls (HCs, n = 40), patients with multiple sclerosis (MS, n = 40), and patients with amyotrophic lateral sclerosis (ALS, n = 20), obtained from our biobank (Spanish Ministry of Health code C.0002365).

Standard Protocol Approvals, Registrations, and Patient Consents

Written informed consents were obtained from all patients for sample handling and data collection. Participation in the study

Figure 1 Prescreen of Autoimmune Nodopathy Samples With a Known Antibody



Prescreen of anti-NF155 (A) and anti-CNTN1 (B) CIDP serum samples against HEK293 cells overexpressing neurofascin (isoform 9 and 11), CNTN1, and Caspr1 and CD20 and EGFR. Screening of rituximab biosimilar (anti-CD20; C) was performed as a positive control and transfection control (D). CIDP = chronic inflammatory demyelinating polyradiculoneuropathy.

was conducted under a protocol approved by the Institutional Ethics Committee of the Hospital de la Santa Creu i Sant Pau (code of approval IIBSP-NAI-2022-88).

Human Cell Microarray Technology

Discovery of IgG autoantibodies was performed with the Retrogenix cell microarray technology.[8] This approach included an initial prescreen step to assess sample background reactivity and dilution selection. Then, the 4 AN serum samples were added to slides of fixed HEK293 cells overexpressing NF155, NF186, CNTN1, and Caspr1 (as problem samples) and CD20 and EGFR (as positive and transfection controls, respectively). After this prescreen step, the 8 CIDP serum samples without known autoantibodies were screened against fixed HEK293 cells/slides expressing duplicate 5,861 human plasma membrane proteins and cell surface–tethered human-secreted proteins, plus 371 human heterodimers. All transfection efficiencies exceeded the minimum quality threshold. An AlexaFluor647 anti-human IgG Fc detection antibody was used to detect IgG binding to the slides. Reactivities found were confirmed by a second analysis that consisted of a new screening of all the samples (4 AN and 8 CIDP) against HEK293 cells expressing only the proteins that had shown reactivity.

Reactivities were classified as specific and nonspecific and divided by intensity (very weak, weak, medium, and strong). Reactivities that were considered relevant were the ones that were specific when appeared in duplicate and when intensity was weak or higher.

Cell-Based Assays

Reactivities obtained in the discovery phase were validated by cell-based assay (CBA) and/or ELISA (eTable 1).

In CBA, mammalian expression vectors encoding human LIF, ATP4A+ATP4B, NPR3, or TNFSF4 were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen, CA). Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.3% TritonX-100 if needed, and blocked. ICC experiments were performed using patients' sera and appropriate primary and secondary antibodies (eTable 1).

ELISA

Maxisorb 96-well ELISA plates (Thermo Fisher Scientific, NUNC, Denmark) were coated overnight with LIF, EPHA7, PIP, IFNL1, or IFNL3 recombinant proteins (eTable 1). Wells were blocked with 5% non-fat milk in PBS 0.1% Tween20 for 1 hour, incubated with diluted sera for 1 hour, and then incubated with peroxidase conjugated rabbit anti-human IgG secondary antibodies for 1 hour at room temperature. ELISA was developed with tetramethylbenzidine solution (BioLegend, CA), and the reaction was stopped with 25% sulfuric acid. Optical density (OD) was measured at 450 nm in a Multiscan ELISA reader. Samples were considered positive by ELISA when they had a ΔOD higher than the average healthy control ΔOD plus 2 standard deviations. To calculate the antibody titers, the ELISA was performed with different serum concentrations (range 1/100–1/24300).

Peripheral Nerve and Stomach Tissue Immunohistochemistry

Sera from patients showing positivity against LIF were used to perform nerve tissue immunohistochemistry (IHC) on macaque peripheral nerve preparations. IHC was also performed on mouse stomach preparations to assess gastric mucosal antibodies in sera from patients showing positivity against the ATP4A/4B complex.

Macaque peripheral nerve slides and mouse stomach slides (Inova Diagnostics, San Diego) were blocked with 5% normal goat serum in PBS, followed by incubation with patients' sera at 1/40 for peripheral nerve tissue and 1/80 for stomach tissue. To study the staining pattern, peripheral nerve tissue slides were also incubated with rabbit anti-human S100 antibodies (ab52642, Abcam) at 1/50 to stain Schwann cells (SCs). Monkey-adsorbed goat anti-human IgG AF488 (Southern Biotech, AL) and goat anti-rabbit IgG AF594 were used as secondary antibodies in peripheral nerve tissue and goat anti-human IgG AF488 in mouse stomach slides, all at a 1/500 concentration. Finally, slides were mounted with Fluoromount and examined by 2 independent

Table Clinical and Laboratory Features, and Protein Reactivities of Patients With CIDP Without a Known Antigen Screened in Human Cell Microarray

	Sex	Age at onset (y)	Time from onset to sample	Treatment at the time of sample	IgG reactivity against neural structures	Protein reactivities found in human cell microarray	Maximum reactivity intensity detected
Patient 1	Female	30	14 y	IVIg, steroids	—	No specific hits identified	—
Patient 2	Male	51	1 y	IVIg, steroids	—	Ephrin type-A receptor 7	Weak
Patient 3	Male	67	2 y	IVIg, steroids	—	Atrial natriuretic peptide receptor 3	Weak
						Potassium-transporting ATPase subunit beta	Weak
						Ephrin type-A receptor 7	Weak
						Potassium-transporting ATPase alpha chain 1+ potassium-transporting ATPase subunit beta	Medium
Patient 4	Female	31	12 y	IVIg	—	Ephrin type-A receptor 7	Weak
Patient 5	Male	55	10 y	IVIg	Monkey cerebellum	No specific hits identified	—
Patient 6	Male	62	6 y	IVIg, steroids	Human Schwann cell line	Ephrin type-A receptor 7	Weak
Patient 7	Female	63	Onset	None	Human Schwann cell line	Tumor necrosis factor ligand superfamily member 4	Weak
						Interferon lambda 1	Weak
						Interferon lambda 2	Weak
						Interferon lambda 3	Weak
						Prolactin-inducible protein	Weak
						Leukemia inhibitory factor	Weak/ Medium
Patient 8	Male	51	8 y	None	Rat dorsal root ganglion neurons	Ephrin type-A receptor 7	Weak

Abbreviations: CIDP = chronic inflammatory demyelinating polyradiculoneuropathy; IVIg = IV immunoglobulin.

observers who were not blinded for diagnosis. Images were obtained with an Olympus BX51 Fluorescence Microscope (Olympus Corporation, Tokyo, Japan).

Myelinating Neuron-Schwann Cell Coculture Immunocytochemistry

Dorsal root ganglia (DRG) neuron and SC mixed cultures were prepared from E16 rat embryos as reported elsewhere.[22]

Live cocultures were then incubated overnight with patients' sera diluted 1/100 in a myelinating medium at 37°C. Cells were then fixed for 20 minutes with 4% PFA, permeabilized for 10 minutes with methanol at −20°C, and blocked for 1 hour with 5% normal goat serum in PBS. Primary antibody incubation with anti-myelin basic protein (808401, Biolegend) and anti-Caspr1 (ab34151, Abcam) at 1/300 was performed for 1 hour at room temperature. After washing, goat anti-human IgG AF488, goat anti-rabbit IgG AF594, and goat anti-mouse IgG AF647 were used as secondary antibodies at a 1/500 concentration.

Coverslips were mounted with Fluoromount and examined by 2 independent observers who were not blinded for diagnosis. Images were obtained with an Olympus BX51 Fluorescence Microscope.

Data Availability

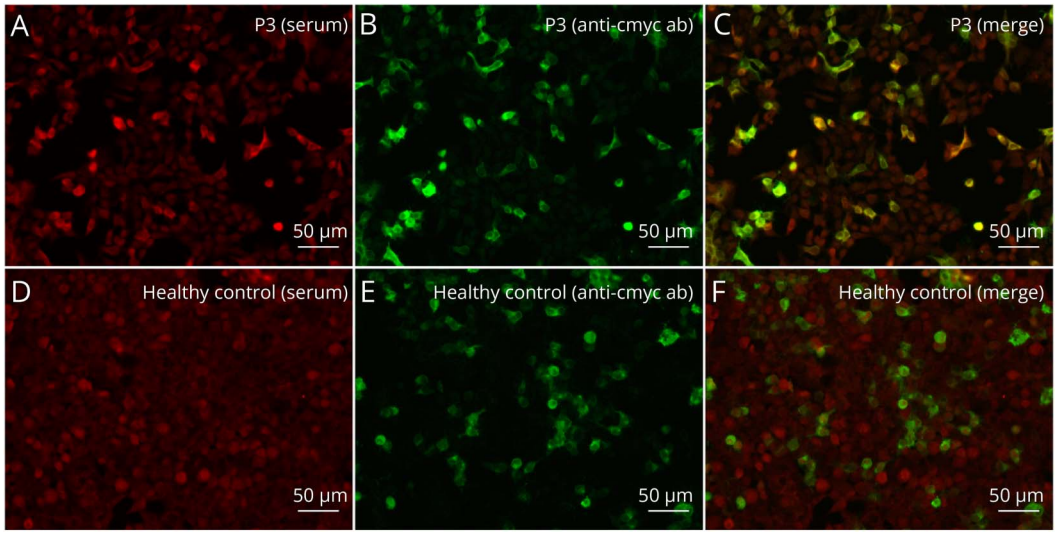
Anonymized data not published within this article will be made available by request from any qualified investigator.

Results

Human Cell Microarray Technology Validation Phase

To assess the ability of the cell microarray technology to identify known autoantibodies, serum samples of patients with AN were incubated with slides of fixed HEK293 cells overexpressing NF155, NF186, CNTN1, and Caspr1 and CD20 and EGFR. Sera with anti-NF155 and anti-CNTN1 antibodies showed strong and specific reactivity with NF155 and medium and specific reactivity with CNTN1, respectively

Figure 2 Immunocytochemistry of Anti-ATP4A/4B Antibodies



HEK293 cells were cotransfected with myc-DDK–tagged vectors ATP4A and ATP4B, double-stained with serum IgG (A, C) and with commercial antibody against c-myc (B, D). Patient 3 IgG binds to transfected cells (A), and colocalizes with c-myc (B); in contrast with the healthy control (D), that does not show any reactivity against ATP4A/4B-transfected cells.

(Figure 1). No reactivities were found on incubation of anti-NF140/186 and anti-Caspr1 sera.

Autoantibody Discovery Phase

To then assess the ability of the cell microarray technology to discover novel autoantibodies, sera from 8 patients with CIDP were incubated with the human cell microarray. In total, 31 hits (potential antigens) were identified. A second analysis screened all 12 serum samples (CIDP and AN) against HEK293 cells expressing these 31 hits plus NF186, NF155, CNTN1, and Caspr1. In this step, AN serum samples behaved as in the validation phase; 3 of the 4 AN samples also reacted against Ephrin type-A receptor 7 (EPHA7).

Protein hits confirmed in the second analysis with the human cell microarray in the 8 CIDP samples with unknown autoantibodies are summarized in the Table.

Confirmatory Experiments

All confirmed hits presented in the Table were included in the confirmatory experiments with ELISA or CBA. If a specific hit was confirmed (by ELISA or CBA) in the original sample where it was detected, 96 serum samples from patients with CIDP and AN (CIDP-AN cohort) and 100 control samples were then tested against the confirmed hit.

Unspecific Autoantibodies

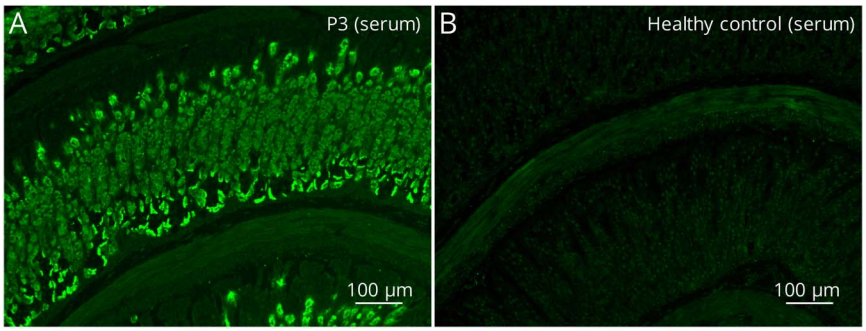
Anti-EPHA7 antibodies were detected in 5 of 8 patients in the discovery phase (Table). ELISA was used to confirm the presence of antibodies against EPHA7, and only one of these patients was positive (patient 3 in the Table). From all the patients with CIDP-AN (n = 96) and controls (n = 100), one more patient with CIDP was positive for anti-EPHA7 antibodies by ELISA (2/96) and 1 serum from the control group (1/100, 1 HC). This reactivity was then ruled out as disease-specific, and no more confirmatory experiments were performed.

Antibodies targeting the ATP4A/4B complex detected in patient 3 were confirmed by ICC of ATP4A/4B-cotransfected HEK293 cells (Figure 2). No reactivity was found against HEK293 cells transfected with ATP4A or ATP4B alone. We analyzed the presence of anti-ATP4A/4B antibodies by ICC in all patients with CIDP-AN (n = 96) and controls (n = 100). One patient with AN (anti-NF155+; 2/96) and 3 controls (3/100, 2 HCs and 1 patient with MS) were positive for anti-ATP4A/4B antibodies. None of the patients positive for anti-ATP4A/4B had gastric symptoms. Antibodies targeting ATP4A/4B have recently been associated with atrophic gastritis because this complex is present in gastric parietal cells.[23,24] For this reason, we performed IHC of mouse stomach slides and found that all patients and controls (n = 5) with antibodies against the ATP4A/4B complex showed reactivity against gastric parietal cells (Figure 3). Although this reactivity was confirmed, it was considered unrelated to CIDP.

Autoantibodies Detected Only in Patients With CIDP

Patient 7 showed reactivity against the leukemia-inhibitory factor in the discovery phase. This reactivity was confirmed by ELISA. An additional patient with anti-LIF antibodies was identified by ELISA (2/96) while none of the control sera tested reacted against LIF (0/100). Anti-LIF antibodies were additionally confirmed in these 2 patients with an in-house LIF-CBA (Figure 4). Patient 7 (anti-LIF+ patient I) did not have any previously known antibody while the other patient (anti-LIF+ patient II) was also positive for anti-Caspr1 antibodies.

Figure 3 Anti-ATP4/4B–Positive Patient Reactivity Against Stomach Tissue



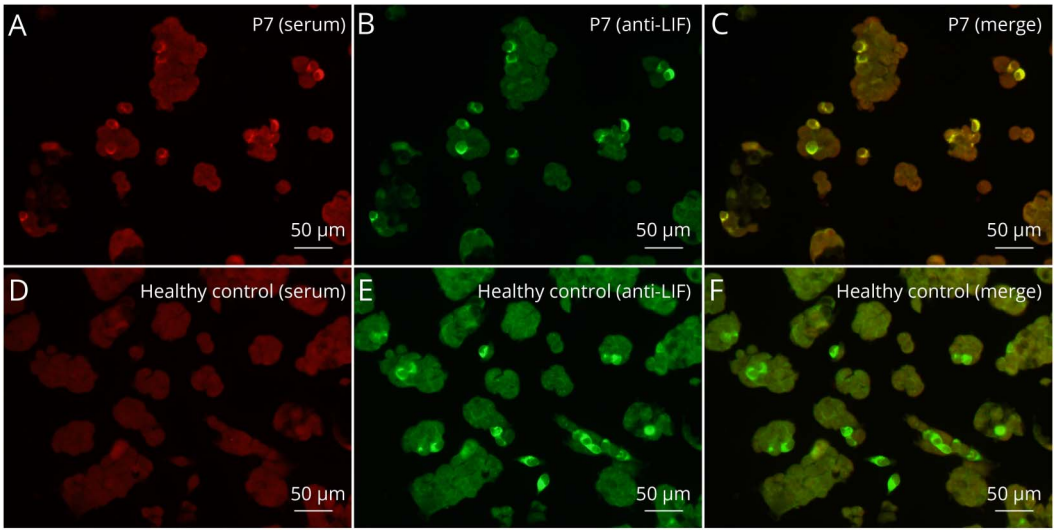
Mouse stomach preparation stained with patient 3 serum in IgG (A) and healthy control (B). Patient 3 with anti-ATP4A/4B antibodies reacts against gastric parietal cells (A) while a healthy control negative for anti-ATP4A/4B does not (B).

Both patients were positive for anti-LIF in the first sample (at disease onset) when treatment had not been initiated. Anti-LIF IgG titers were 1/8,100 for anti-LIF patient I and 1/900 for anti-LIF patient II. Anti-LIF antibody reactivity disappeared in both patients in samples collected on successful treatment. The role of LIF protein in the nervous system has been studied previously, and it seems to play a role in demyelination/remyelination.[25,26] Thus, we decided to study the reactivity of these patients' sera against peripheral nerve tissue. Sera from both anti-LIF+ patients showed strong IgG reactivity against the macaque peripheral nerve with a pattern of staining that suggested binding to myelin 27 (Figure 5). We also found that both patients showed reactivity against myelin in myelinating DRG/SC cocultures (Figure 6). Samples collected after treatment (that were negative for anti-LIF) showed no reactivity against myelin.

Anti-LIF+ patients did not have common clinical features. Anti-LIF patient I presented with an acute ataxic neuropathy that subsequently relapsed 3 months later, fulfilling CIDP criteria. Anti-LIF patient II presented with a sensory motor neuropathy with proximal and distal weakness and was diagnosed with typical CIDP. Clinical features of both patients are summarized in eTable 2.

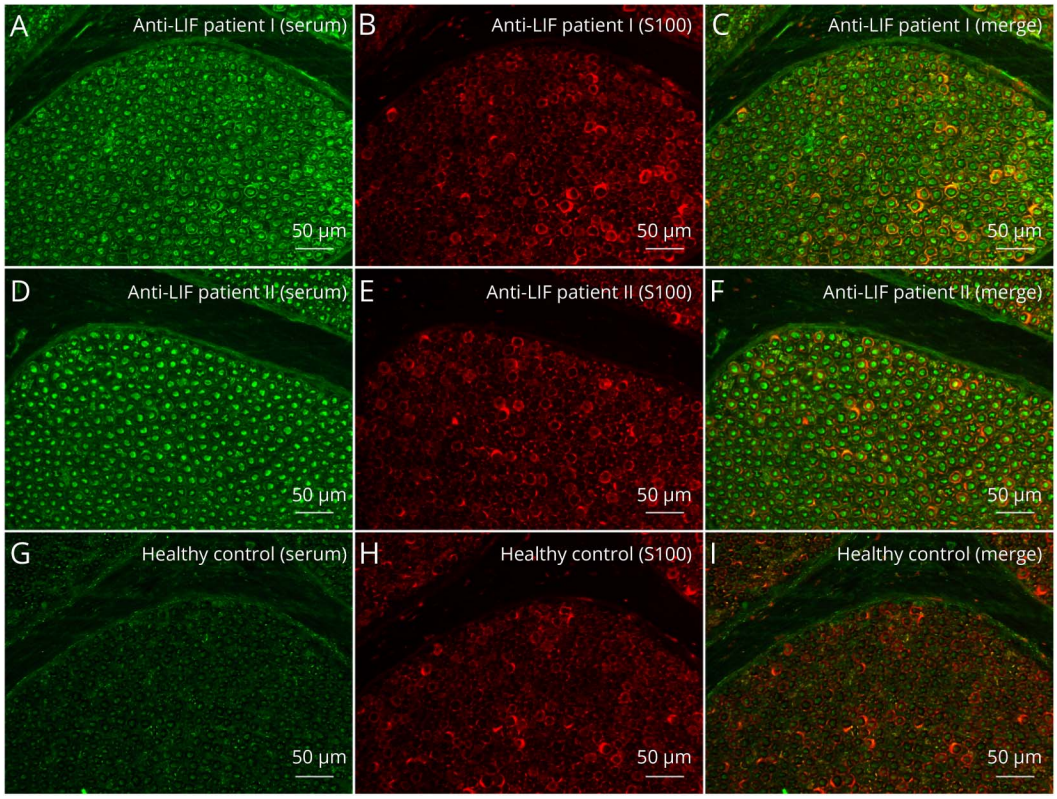
The same 2 patients who reacted against LIF also had antibodies against interferon lambda 1 and 3. Reactivity against IFNL1 and IFNL3 detected in patient 7 in the discovery phase was confirmed by ELISA. We did not test reactivity of patient 7 against IFNL2 because IFNL2 and IFNL3 share over 95% of the sequence.[28] Sequence identity between IFNL1 and IFNL2 and between IFNL1 and IFNL3 were 72.25% and 73.30%, respectively, suggesting that these hits were identified as a result of the binding of an autoantibody targeting a common epitope. Therefore, we used only the IFNL3 ELISA to test the entire cohort of patients with CIDP-AN and controls. When testing the full cohort of patients and controls, one more patient tested

Figure 4 Immunocytochemistry of Anti-LIF Antibodies



HEK293 LIF-transfected cells, double-stained with serum (A, C) and with commercial antibody against LIF (B, D). Patient 7 IgG binds to transfected cells (A), and colocalizes with LIF (B); in contrast with the healthy control (D), that does not show any reactivity against LIF-transfected cells. LIF = leukemia inhibitory factor.

Figure 5 Anti-LIF+ Patients' Reactivity Against Peripheral Nerve Tissue



Macaque peripheral nerve sections double stained in green with anti-LIF+ patient I serum in IgG (A), anti-LIF+ patient II (D), and healthy control (G) and in red with S100 (B, E, H). LIF = leukemia-inhibitory factor.

positive for anti-IFNL3 antibodies by ELISA (2/96) while none of the controls showed reactivity (0/100). It is of interest that the patients testing positive for anti-IFNL3 were the same patients who tested positive for anti-LIF.

Nonconfirmed Reactivities

All other reactivities found in the discovery phase were not confirmed (either by CBA or ELISA).

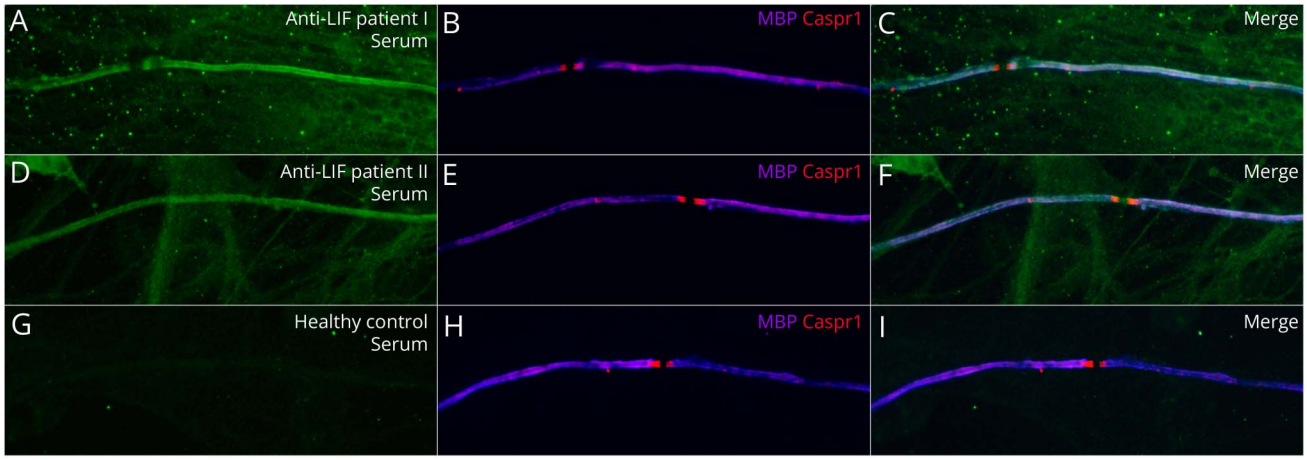
Discussion

Our work, using CIDP as a paradigmatic example of a rare and heterogeneous autoimmune disease without a known antigen, validates the ability of the human cell microarray technology to detect novel autoantibodies. First, using patients with AN, we demonstrated that the technology is able to detect previously known autoantibodies. Then, we demonstrated that novel autoantibodies can be discovered with the human cell microarray because we found 9 potential reactivities, and confirmation was possible in six of them. Although the 6 confirmed reactivities were proven with other different techniques, supporting their presence in serum of the tested patients, only anti-LIF and anti-IFNL were observed in the CIDP-AN cohort (and not in controls). The clinical and pathogenic relevance to CIDP of these antibodies is unclear and needs to be elucidated in larger cohorts.

Autoantibody discovery techniques have changed substantially in the past few decades. The human cell microarray technology has appeared as a new technique to screen for on-target and off-target reactivity of monoclonal antibodies, recombinant proteins, or other drugs against membrane and secreted proteins. The principal goal of this technology is to represent the maximum coverage of the human membrane proteome in a more physiologic environment. Our study provides a proof of principle that this technology is useful to screen human sera for autoantibody discovery purposes in autoinmune diseases, including neuroinmmune disorders.

In our work, 4 sera from patients with AN were used to validate the technique. Specific reactivity against the known antigen was shown in the samples harboring anti-NF155 and anti-CNTN1 antibodies. No other reactivities were found, apart from EPHA7 that was considered unspecific. However, samples harboring anti-Caspr1 and anti-NF140/NF186 antibodies did not show reactivity. First of all, a recent inter-laboratory validation study[29] found that performance of the CBA is decreased when sera are incubated with fixed cells (in comparison with live CBA). The human cell microarray technology expresses the proteins in fixed cells; this may have played a role in the absence of reactivity in anti-Caspr1 and anti-NF140/186 serum samples as well. As recently described,[17] detection of anti-Caspr1 antibodies by CBA is

Figure 6 Anti-LIF+ Patients' Reactivity Against Myelinating Neuron-Schwann Cell Cocultures



Neuron-Schwann cell cocultures triple stained in green with anti-LIF+ patient I serum in IgG (A), anti-LIF+ patient II (D), and healthy control (G) and in red with anti-Caspr1 and in purple with anti–myelin basic protein (MBP) (B, E, H). LIF = leukemia-inhibitory factor.

improved when Caspr1 and CNTN1 (that form a complex) are cotransfected, compared with transfection of Caspr1 only. Human cell microarray used in our work expressed Caspr1 or CNTN1 transfected alone, and that could explain the absence of reactivity when screening the anti-Caspr1+ serum sample. Along the same lines, we found several samples reacting against the ATP4A and ATP4B subunits of the gastric proton pump. The reactivity of these antibodies was significantly improved again when both subunits were cotransfected. Although some heterodimers are represented in the human cell microarray, these 2 findings suggest that future versions of these technologies should aim to include a growing list of multimeric protein complexes to make them even more efficient.

Sera harboring antibodies against NF140/186 bind to a common epitope of the 3 neurofascin isoforms (NF186, NF155, NF140). Apart from the fixed cells issue explained before, the titers of the AN patient with antiNF140/186 were low (1/100). These 2 reasons probably account for the lack of reactivity in this sample and again support the need of technologies that mimic the physiologic expression of the antigens. However, despite only 2 of the 4 known autoantibodies were detected with the human cell microarray technology, this experiment proves that at least some disease-specific autoantibodies can be discovered with the technology with limited off-target hits.

In addition to the known reactivities, we were able to confirm (by CBA, ELISA, or both) 6 of the 9 reactivities that were identified in the autoantibody discovery experiments, supporting the ability of the human cell microarray technology to uncover novel autoantibodies with a relatively clean and restricted set of identified reactivities despite its high-throughput nature. The specificity and cleanness of massive screening technologies is crucial when aiming to identify pathogenic antibodies in rare autoimmune diseases.

After confirming the reactivities found, we tried to assess their relevance by analyzing their presence in a larger cohort of patients with CIDP-AN and controls. As explained before, reactivity against EPHA7 was considered unspecific because it was found in patients' sera and one control. Autoantibodies against ATP4A and ATP4B have been described previously in atrophic gastritis[23,24] because this proton pump is present in the parietal cells of the gastric mucosa. In our work, 2 patients and 3 controls were positive for anti-ATP4A/4B antibodies, and IHC of the mouse stomach confirmed the reactivity against parietal cells. These findings supported that, despite being real autoantibodies and confirmed the reports of the gastroenterology literature, they are unrelated to CIDP. In addition, and maybe of relevance for the assessment of atrophic gastritis, our findings support the use of CBA with cotransfected ATP4A/4B to detect these antibodies in patients with atrophic gastritis and anti-parietal cell antibodies.

Antibodies targeting LIF have never been described before. LIF is a pleiotropic cytokine from the interleukin-6 family that acts on different cell types.[30] It exerts a broad range of functions on diverse organ systems by activating the JAK/STAT transcription pathway. Its role in both central and peripheral nervous systems has been studied,[25,26,31] and it seems to be related with the demyelination-remyelinating process that is also central in CIDP pathophysiology. In our cohort, one patient with CIDP and one with AN had anti-LIF antibodies, demonstrated by 2 different techniques (ELISA and CBA), while none of the controls had these antibodies. Considering both the described functions and the apparent specificity of the antibodies, we decided to study extensively these 2 patients. Both anti-LIF+ patients showed the same pattern of staining in peripheral nerve tissue, suggesting binding to the

peripheral nerve myelin, which could be potentially due to the presence of LIF antibodies. However, determining whether anti-LIF antibodies were responsible for the anti-myelin reactivity in these 2 patients was not possible in our study because of technical limitations related to poor performance of commercially available anti-LIF antibodies. Moreover, one of the anti-LIF–positive patients was also positive for Caspr1, an antibody with pathogenic potential, supporting that the anti-LIF could be an epiphenomenon in that patient.[32] Because of that, although anti-LIF antibodies are present only in our CIDP-AN cohort and not in controls, and positive patients have the same pattern of reactivity in nerve tissue, the role of anti-LIF antibodies in CIDP and their pathogenicity need to be further elucidated.

Finally, anti-interferon lambda reactivity was also confirmed. The type III interferon family, also known as the interferon lambda (IFNL) system, initiates the JAK/STAT transcription activation pathway.[33] Antibodies against interferons are widely described in the context of susceptibility to infections and autoimmunity.[34,35] However, there is only one study published on anti-IFNL antibodies (associated with persistence of respiratory symptoms after COVID-19).[36] In our work, we found 2 patients with anti-IFNL3 antibodies that, interestingly, were the same ones that were positive for anti-LIF. IFNL and LIF proteins do not share a significant proportion of their sequences, suggesting that the antigenic epitope is not the same for both autoantibodies or does not relate to their primary peptide sequence. Both IFNL and LIF activate the JAK/STAT pathway, and this might be related to the fact that they appear together, probably as an epiphenomenon. However, these findings need further studies on larger cohorts of patients to be clarified.

One of the main limitations of our study at the time of screening was the number of samples we were able to screen in the discovery phase with the human cell microarray technology. The human cell microarray technology is optimized and marketed for drug and monoclonal antibody profiling (and not for autoantibody discovery). The cost of screening each single sample precluded the profiling of an entire cohort of patients with CIDP. So, although we selected patients to optimize antibody discovery success, we have likely left out other interesting cases and cannot assume that the antigenic reactivities of these 8 patients reflect those of the entire cohort. That same limitation was the reason behind our choice of screening IgG antibodies only, despite the fact that IgM autoantibodies are also frequently found in other autoimmune neuropathies. Furthermore, because the 8 samples screened in this first phase were chosen according to specific criteria (typical CIDP or reactivity against neural structures), there is a selection bias that certainly affects the generalization of the screening results.

Another limitation of the technique is that it uses fixed cells. This may influence the reactivity against certain proteins (as illustrated with the NF140/186-positive sample) but also against some proteins or protein complexes in which the glycosylated moiety or other post-traductional modifications are relevant for the reactivity. Indeed, our study starts with the assumption that the antigens yet to be discovered in CIDP are proteins and that the antibodies are of the IgG isotype. The existence of other chronic, demyelinating neuropathies in which antibodies of the IgM isotype targeting gangliosides or glycans are found might argue against this approach in CIDP.[37]

Our study provides the proof of concept that the human cell microarray technology is useful as a novel autoantibody-screening technology that could be used in other autoimmune diseases, including neurologic ones. Despite potential CIDP-associated autoantibodies (anti-LIF and anti-IFNL) being identified, their clinical and pathogenic relevance in CIDP needs to be further elucidated.

Acknowledgment

The authors acknowledge the Department of Medicine at the Universitat Autònoma de Barcelona. The authors also thank all our patients for their support and collaboration. M. Caballero-Ávila, C. Lleixà, E. Pacual-Goñi, L. Martín-Aguilar, N. Vidal, C. Tejada-Illa, R. Collet-Vidiella, R. Rojas-García, E. Cortés-Vicente, J. Turon-Sans, E. Gallardo, M. Olivé, A. Vesperinas, A. Carbayo and L. Llansó are members of the European Reference Network for Neuromuscular Diseases.

Study Funding

This work was supported by UCB Pharma and by Fondo de Investigaciones Sanitarias (FIS), instituto de Carlos III, Spain, under grant PI22/00387.

Disclosure

M. Caballero-Ávila was supported by a personal Rio Hortega grant CM21/00101. L. Martín-Aguilar was supported by a personal Juan Rodés grant JR21/00060. E. Pascual-Goñi was supported by a personal grant from the GBS-CIDP foundation. A. Carbayo was supported by a personal Rio Hortega grant CM21/00057. L. Querol received research grants from Instituto de Salud Carlos III-Ministry of Economy and Innovation (Spain), CIBERER, Fundació La Marató, GBS-CIDP Foundation International, UCB, and Grifols; received speaker or expert testimony honoraria from CSL Behring, Novartis, Sanofi-Genzyme, Merck, Annexon, Alnylam, Biogen, Janssen, Lundbeck, ArgenX, UCB, LFB, Octapharma and Roche; serves at Clinical Trial Steering Committee for Sanofi-Genzyme and Roche and is Principal Investigator for UCB's CIDP01 trial. L. Querol was supported by a personal clinical intensification grant INT20/00080. J. Freeth and J. Soden were former employees for Retrogenix, a company that was acquired by Charles River. S. Dawson is a Charles River employee. All other authors report no disclosures relevant to the manuscript. Go to Neurology.org/NN for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* September 5, 2023. Accepted in final form January 19, 2024. Submitted and externally peer reviewed. The handling editor was Associate Editor Marinos C. Dalakas, MD, FAAN.

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Appendix *(continued)*

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