Cytosolic 5′-Nucleotidase 1A Autoimmunity in Sporadic Inclusion Body Myositis

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Objective: We previously identified a circulating autoantibody against a 43 kDa muscle autoantigen in sporadic inclusion body myositis (IBM) and demonstrated the feasibility of an IBM diagnostic blood test. Here, we sought to identify the molecular target of this IBM autoantibody, understand the relationship between IBM autoimmunity and muscle degeneration, and develop an IBM blood test with high diagnostic accuracy.
Methods: IBM blood samples were screened using mass spectrometry and a synthetic human peptidome. Plasma and serum samples (N=200 patients) underwent immunoblotting assays, and results were correlated to clinical features. Muscle biopsy samples (n=30) were examined by immunohistochemistry and immunoblotting. Exome or whole genome sequencing was performed on DNA from 19 patients.
Results: Both mass spectrometry and screening of a 413,611 human peptide library spanning the entire human proteome identified cytosolic 5′-nucleotidase 1A (cN1A; NT5C1A) as the likely 43 kDa IBM autoantigen, which was then confirmed in dot blot and Western blot assays using recombinant cN1A protein. Moderate reactivity of anti-cN1A autoantibodies was 70% sensitive and 92% specific, and high reactivity was 34% sensitive and 98% specific for the diagnosis of IBM. One to 3 major cN1A immunodominant epitopes were identified. cN1A reactivity by immunohistochemistry accumulated in perinuclear regions and rimmed vacuoles in IBM muscle, localizing to areas of myonuclear degeneration.
Interpretation: Autoantibodies against cN1A are common in and highly specific to IBM among muscle diseases, and may provide a link between IBM's dual processes of autoimmunity and myodegeneration. Blood diagnostic testing is feasible and should improve early and reliable diagnosis of IBM.

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Sporadic inclusion body myositis (IBM) is an autoimmune and progressive degenerative muscle disease.[1,2] Its natural history is onset in middle and late adulthood with slow progression to disability, and its cause is unknown. Unlike the other major forms of myositis, polymyositis (PM), dermatomyositis (DM), and autoimmune necrotizing myopathy (NM), IBM is refractory to treatment, including immune modulation.

The pathogenic mechanisms underlying IBM muscle loss are poorly understood.[3] IBM autoimmunity has been widely viewed as T-cell mediated, after studies in the mid 1980s demonstrated cytotoxic T-cell–mediated

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pathology[4–7] in IBM muscle. Extensive studies over decades of IBM muscle T-cell clonality have failed to identify antigens driving IBM autoimmunity.[8]

After pivotal IBM pathological studies[4] reported sparse or absent CD19- and CD20-positive B cells in IBM muscle, a view that IBM had no significant antibody-mediated component emerged. However, a series of studies over the past decade, stimulated by microarray identification of immunoglobulin transcripts unique to the B-cell lineage in IBM muscle, led to the identification of an antigen-stimulated B-cell response.[9–12] The recognition of B-cell autoimmunity in IBM muscle then led to the discovery of an IBM circulating autoantibody with high sensitivity (53%) and specificity (100%) for IBM among 50 patients with autoimmune muscle diseases.[13]

Here we identify the target of this autoantibody as the cytoplasmic 5′-nucleotidase 1A (cN1A; NT5C1A), define its abnormal distribution in IBM muscle, and report development of a serum assay with high clinical diagnostic accuracy for the diagnosis of IBM among patients with muscle diseases.

Subjects and Methods

Patients, Blood, and Muscle Samples

All patient samples were collected after informed written consent was obtained and under protocols approved by the Partners Human Research Committee Institutional Review Board overseeing Brigham and Women's Hospital human research activities.

Plasma and serum samples from 200 patients were studied with the following diagnoses: IBM (n=47; mean age=67 years), PM (n=26; mean age=53 years), NM (n=14; mean age=71 years), DM (n=36; mean age=51 years), myasthenia gravis (n=13; mean age=49 years), muscular dystrophy (n=10; mean age=58 years; 4 with myotonic dystrophy, 4 with limb-girdle muscular dystrophy, 1 with myofibrillar myopathy, and 1 with distal myopathy with rimmed vacuoles), other muscle diseases (n=19; mean age=56 years), and healthy volunteers (n=35; mean age=39 years, range=19–66 years). Diagnostic criteria for IBM were European Neuromuscular Centre (ENMC) criteria for probable (n=11) or definite IBM (n=36),[14] except for 6 patients who did not meet the ENMC requirement of "sporadic" disease because they had an affected relative (what has been called familial inclusion body myositis [f-IBM][15,16]). All f-IBM patients had weakness of finger flexors and mononuclear inflammatory infiltrates with invasion of non-necrotic muscle fibers, and except for lack of sporadic disease, 5 met ENMC definite and 1 met ENMC probable criteria. Diagnostic criteria for PM were as previously used.[17] Diagnostic criteria for necrotizing myopathy included an immunoresponsive myopathy with muscle biopsy demonstrating multifocal myofiber necrosis in the absence of endomysial inflammation and invasion of non-necrotic myofibers. Criteria for DM were the presence of a characteristic skin rash, subacute predominantly proximal weakness, and muscle biopsy showing perifascicular atrophy or perivascular and perimysial inflammation without endomysial inflammation. Blood samples were collected over an 8-year period of time, frozen, and stored at −80°C. Healthy control samples used for analysis of peptidome data were from subjects self-reported to be free of autoimmune disease.

Muscle samples from 30 patients were studied using immunohistochemistry (IBM, n=15; PM, n=5; DM, n=5; normal, n=5) and used for lysates in Western blots.

Clinical variables measured were age (years) at time of blood collection; duration of symptoms (years), defined as patient report of impairment of hand use or gait at the time of blood collection; time (years) from blood draw to diagnosis of suspected or confirmed IBM; finger flexor strength, defined as the weakest deep or superficial finger flexor, and knee extension strength, defined on the weakest side, both measured using a modified manual muscle testing scale score (0–10; eg, Medical Research Council score 5−=9, 4+=8, 4=7); antinuclear antibody (ANA) positivity, defined as >1:80; and positive anti-Ro and anti-La antibody titers.

Human Muscle Lysates, 2-Dimensional Gel Electrophoresis, and Mass Spectrometry

Whole muscle lysates from patient biopsies were prepared as described in the Supplementary Methods.

Two-dimensional (2D) gel electrophoresis was performed in pairs using the ZOOM IPGRunner Combo Kit (Cat. No. ZM0002; Life Technologies, Grand Island, NY) and required ZOOM products following manufacturer protocol for the first isoelectric focusing dimension. The second dimension was carried out using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, with 1 gel transferred to nitrocellulose membrane and the other fixed (in solution containing ethanol and acetic acid) and stained in Simply Blue SafeStain (Cat. No. LC6060; Life Technologies) as previously described.[13] Immunoblotting was carried out on the whole membrane, and the spots specifically reactive to IBM plasma, compared with normal, were extrapolated to the stained gel, cut, and submitted for mass spectrometry analysis as previously described.[13,18,19]

Phage Immunoprecipitation Sequencing Screening and Analysis

The T7-Pep library was prepared as described previously.[20] Screening and analysis were performed as described in the Supplementary Methods.

cN1A Peptides and Protein

Full-length, purified recombinant N-terminal His-tagged cN1A protein (NCBI RefSeq NP_115915.1) was obtained (GenScript USA, Piscataway, NJ), and three 36-amino acid cN1A peptides and a control cN1B/NT5C1B peptide were synthesized commercially (Elim Biopharmaceuticals, Hayward, CA) and used in a dot blot assay as described in the Supplementary Methods.

Exome and Whole Genome Sequencing

Deep sequencing of blood DNA was performed by whole genome sequencing using a sequencing-by-ligation technology[21]

(Complete Genomics, Mountain View, CA) for 4 patients with IBM (and 1 healthy sibling) and exome sequencing using an Illumina Hi-Seq sequencer (Axeq Technologies, Rockville, MD) performed for these 4 plus an additional 15 patients with IBM.

Immunohistochemistry

Immunoperoxidase and fluorescent immunohistochemistry were performed using rabbit polyclonal anti-cN1A (1:400 dilution, Cat. No. ab75101; Abcam, Cambridge, MA) and mouse monoclonal anti–TAR DNA-binding protein 43 (TDP-43; 1:100 dilution, mouse anti-human TARDBP monoclonal antibody, isotype IgG1, Cat. No. 60019-2-Ig; Proteintech Group, Chicago, IL), as described in the Supplementary Methods.

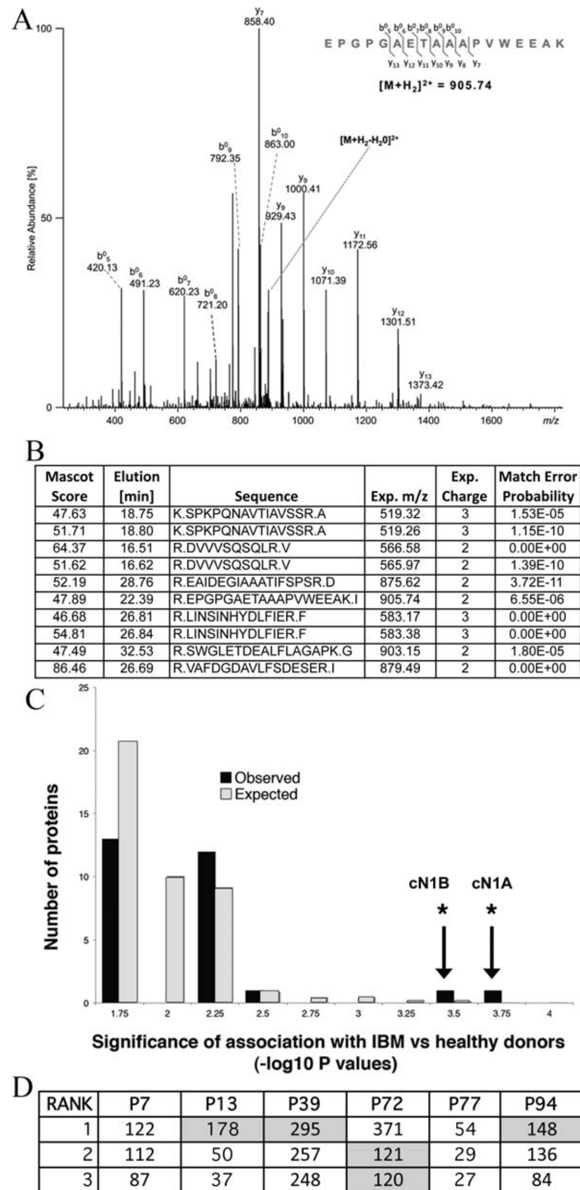
Results

Identification of cN1A as a Candidate Autoantigen

We previously reported the presence of plasma autoantibodies in patients with IBM directed against an approximately 43 kDa muscle autoantigen of unknown identity.[13] We adopted 2 independent approaches to identifying this autoantigen (Fig 1). First, we used mass spectrometry on trypsin digested gel spots, after 2D gel separation of human muscle lysates were probed with a single human IBM plasma sample (sample identifier P13). These studies yielded 7 potential autoantigen candidates within the range of 40 to 48 kDa (Supplementary Table).

We next used 6 IBM samples (5 plasma and 1 serum) to screen a comprehensive human peptidome phage library. This T7 peptidome phage display library is composed of 413,611 36-residue overlapping peptides spanning all open reading frames in the human genome, and was screened using a phage immunoprecipitation sequencing methodology as previously described.[20] All proteins were then tested for a correlation between peptide enrichment and IBM status (using 73 healthy sera as controls). cN1A and cN1B were the only proteins associated with IBM, with a false discovery rate of <10% (see Fig 1C). Four of the 6 IBM samples (including sample P13, which was used in the mass spectrometry approach described above) enriched ≥1 cN1A peptides with extremely high confidence. At least 1 peptide from cN1A was among the top 3 most significantly enriched peptides in all 4 patients with reactivity (see Fig 1D).

BLASTP searches against the human protein nr database revealed no significant homology (maximum scores<40) in the human proteome of these peptides to any human proteins other than cN1A. cN1A was also among the candidate autoantigens identified in the mass spectrometry experiments and is an approximately 43 kDa protein that is much more highly expressed in



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Mascot Score	Elution [min]	Sequence	Exp. m/z	Exp. Charge	Match Error Probability
47.63	18.75	K.SPKPQNAVTIAVSSR.A	519.32	3	1.53E-05
51.71	18.80	K.SPKPQNAVTIAVSSR.A	519.26	3	1.15E-10
64.37	16.51	R.DVVVSQSQLR.V	566.58	2	0.00E+00
51.62	16.62	R.DVVVSQSQLR.V	565.97	2	1.39E-10
52.19	28.76	R.EAIDEGIAAATIFSPSR.D	875.62	2	3.72E-11
47.89	22.39	R.EPGPGAETAAAPVWEEAK.I	905.74	2	6.55E-06
46.68	26.81	R.LINSINHYDLFIER.F	583.17	3	0.00E+00
54.81	26.84	R.LINSINHYDLFIER.F	583.38	3	0.00E+00
47.49	32.53	R.SWGLETDEALFLAGAPK.G	903.15	2	1.80E-05
86.46	26.69	R.VAFDGDAVLFSDESER.I	879.49	2	0.00E+00

D

RANK	P7	P13	P39	P72	P77	P94
1	122	178	295	371	54	148
2	112	50	257	121	29	136
3	87	37	248	120	27	84

FIGURE 1: Identification of cytoplasmic 5′-nucleotidase 1A (cN1A) as a candidate autoantigen. (A, B) cN1A tryptic peptides identified by a mass spectrometry–based proteomic approach. A representative spectra from the 7 distinct cN1A peptides detected is shown. Mass spectrometry was performed on a 2-dimensional gel spot corresponding with a reactive Western blot spot probed with inclusion body myositis (IBM) patient plasma sample P13. Exp.=experimental. (C) cN1A peptides identified by phage immunoprecipitation sequencing peptidome screening. Permutation analysis of protein enrichments associated with IBM. "Observed" bars indicate the number of proteins associated with IBM at a given probability value by Fisher's exact test. "Expected" bars show the number of proteins expected to have an association at least as strong as that due to chance alone (as determined by permutation analysis). *False discovery rate<10%. (D) Of 413,611 peptides, an cN1A peptide ranked in the top 3 most enriched peptides in 4 of the 6 IBM samples (scores represent $-\log_{10}$ probability values; cN1A peptide scores are highlighted in gray; boxes without highlights represent scores for peptides unrelated to cN1A). P7, P13, P39, P77, P72, P94=IBM samples.

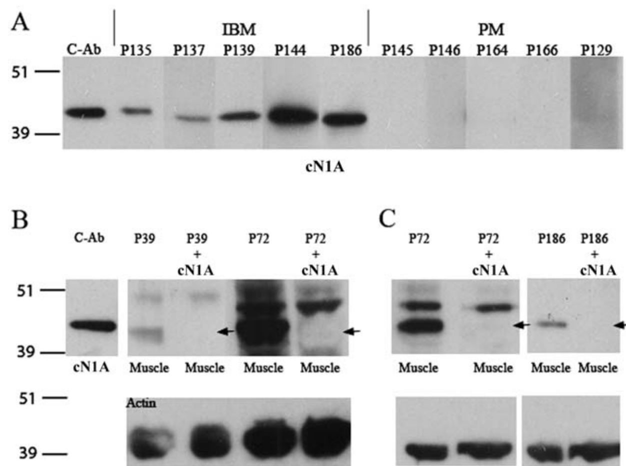


FIGURE 2: Confirmation that inclusion body myositis (IBM) sera and plasma autoantibodies are reactive to both recombinant cytoplasmic 5′-nucleotidase 1A (cN1A) and a 43 kDa muscle protein. (A) Western blot probing cN1A protein with patient serum. Seventy nanograms of recombinant cN1A protein was loaded in each lane, fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotted with the indicated antibodies. This blot was performed in a single experiment, with each patient sample (labeled top row, eg, P135) incubated with a single cut strip and strips then realigned and imaged together. The first lane was probed with a commercial anti-cN1A antibody (C-Ab) against cN1A protein, verifying C-Ab reactivity to cN1A. The remaining lanes were probed with serum samples from 5 patients with IBM and 5 patients with polymyositis (PM) as indicated. All patients with IBM and none with PM have strong reactivity to cN1A at approximately 43 kDa. (B, C) Blocking by preabsorption of plasma and serum with recombinant cN1A. Loss of 43 kDa muscle bands *(arrows)* with recombinant cN1A preabsorption of patient plasma P39 and P72, and serum P186 indicate the presence of patient autoantibodies reactive against both cN1A and the 43 kDa muscle protein.

human skeletal muscle than in any other tissue examined.[22] Taken together, these data suggested that cN1A is an IBM autoantigen.

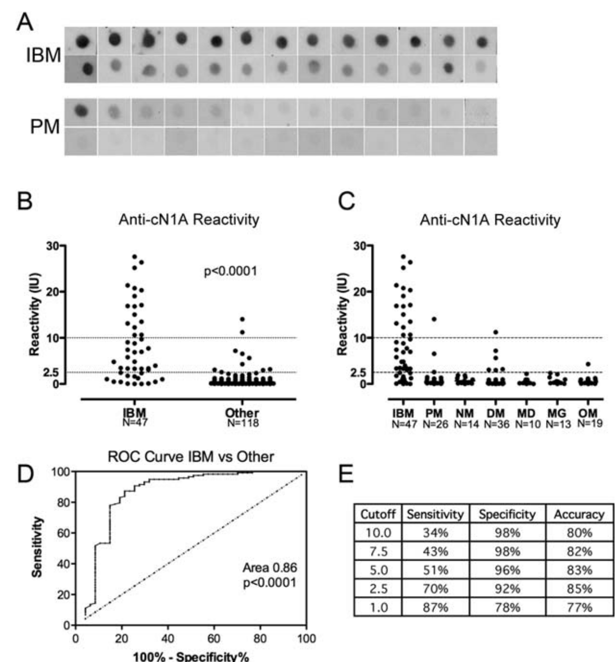
Confirmation of cN1A as an IBM Autoantigen

To determine whether cN1A is an IBM autoantigen, we obtained recombinant full-length N-terminal His-tagged cN1A protein produced in *Escherichia coli*. A commercial antibody detected this recombinant cN1A by immunoblotting. Seventeen IBM samples, including all 13 of the previously reported 43 kDa band reactive IBM samples,[13] but none of 16 PM samples, reacted strongly to recombinant cN1A protein in Western blots (Fig 2A), confirming that cN1A is an IBM autoantigen.

Blocking experiments in which patient plasma and sera were incubated with recombinant cN1A and then used to probe blots of skeletal muscle protein showed selective loss of 43 kDa reactivity (see Fig 2B, C), indicating that the 43 kDa bands seen in Western blots were due to anti-cN1A autoantibodies with reactivity against both native conformation recombinant cN1A and a denatured 43 kDa muscle protein, strongly suggesting that this 43 kDa muscle protein is cN1A. Some patient samples had 2 or 3 bands at approximately 43 kDa, with only 1 of these eliminated in blocking experiments.

Anti-cN1A Dot Blot Assay Has High Sensitivity and Specificity for IBM

We developed a quantitative dot blot assay for anti-cN1A autoantibodies and screened serum and plasma samples from 200 people (165 patients: 47 with IBM, 118 with other muscle diseases; and 35 healthy subjects) against full-length cN1A protein (Fig 3A). We defined the



Cutoff	Sensitivity	Specificity	Accuracy
10.0	34%	98%	80%
7.5	43%	98%	82%
5.0	51%	96%	83%
2.5	70%	92%	85%
1.0	87%	78%	77%

FIGURE 3: Diagnostic performance of an anti–cytoplasmic 5′-nucleotidase 1A (cN1A) protein autoantibody dot blot assay. (A) Dot blot assay image of the 26 most anti-cN1A–reactive inclusion body myositis (IBM) and the 26 most reactive polymyositis (PM) samples. Images organized from most reactive to least, left to right. (B) At an upper limit of normal of 10 intensity units (IU; *dashed line;* 3 standard deviations above the mean of 35 normal subjects), 34% (16 of 47) of IBM samples are abnormal compared with 1.7% (2 of 118) of non-IBM muscle diseased samples. (C) Comparison of IBM with other muscle diseases by category. NM=necrotizing myopathy; DM=dermatomyositis; MD=muscular dystrophy; MG=myasthenia gravis; OM=other muscle disease. (D) Receiver operating characteristic (ROC) analysis for IBM against other muscle disease samples. (E) Diagnostic sensitivity and specificity at various upper limit threshold values for IBM and other muscle disease samples. For example, an upper limit of 2.5 IU detects 70% of IBM patients, with 92% specificity and 85% accuracy.

A

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>gi|14210538|ref|NP_115915.1| cytosolic 5'-nucleotidase 1A [Homo sapiens]
MEPGQPREPQEPREPGPGAETAAAPVWEEAKIFYDNLAPKKKPKSPKPQNAVTIAVSSRALFRMDEEQQIYTEQGVEEYVRYQLEHENEPFSPG
Peptide-1:                                                             RALFRMDEEQQIYTEQGVEEYVRYQLEHENEPFSPG
Peptide-2:                            AKIFYDNLAPKKKPKSPKPQNAVTIAVSSRALFRMD

PAFPFVKALEAVNRRLRELYPDSEDVFDIVLMTNNHAQVGVRLINSINHYDLFIERFCMTGGNSPICYLKAYHTNLYLSADAEKVREAIDEGIA
AATIFSPSRDVVVSQSQLRVAFDGDAVLFSDESERIVKAHGLDRFFEHEKAHENKPLAQGPLKGFLEALGRLQKKFYSKGLRLECPIRTYLVTA
Peptide-3:            SQLRVAFDGDAVLFSDESERIVKAHGLDRFFEHEKA

RSAASSGARALKTLRSWGLETDEALFLAGAPKGPLLEKIRPHIFFDDQMFHVAGAQEMGTVAAHVPYGVAQTPRRTAPAKQAPSAQ
Peptide-13:                                                  HVAGAQEMGTVAAHVPYGVAQTPRRTAPAKQAPSAQ
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B

Protein	Peptide	Label	P72	P94
NT5C1A	RALFRMDEEQQIYTEQGVEEYVRYQLEHENEPFSPG	Pep-1	21	19
NT5C1A	AKIFYDNLAPKKKPKSPKPQNAVTIAVSSRALFRMD	Pep-2	3	826
NT5C1A	SQLRVAFDGDAVLFSDESERIVKAHGLDRFFEHEKA	Pep-3	2	1
NT5C1A	KIRPHIFFDDQMFHVAGAQEMGTVAAHVPYGVAQTP	Pep-12	54	585
NT5C1A	HVAGAQEMGTVAAHVPYGVAQTPRRTAPAKQAPSAQ	Pep-13	17	878
NT5C1B	DGDAVLFSDESEHFTKEHGLDKFFQYDTLCESKPLA	Pep-15	158	4

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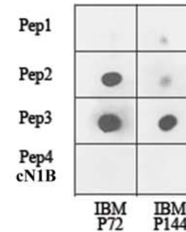

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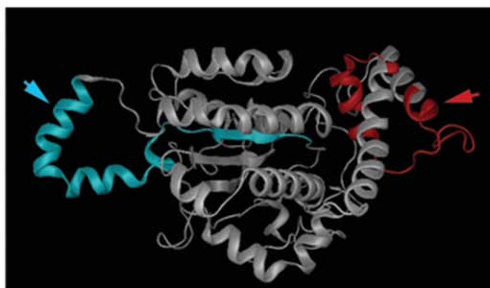

FIGURE 4: Epitope spreading of anti–cytoplasmic 5′-nucleotidase 1A (cN1A) autoimmunity in inclusion body myositis (IBM). (A) Alignment of peptide probes against full-length cN1A protein. (B) Evidence of epitope spreading in peptidome immunoprecipitation sequencing data was present with 2 IBM samples reactive against multiple nonoverlapping cN1A peptides, as well as an cN1B peptide (Pep-15) with high homology to cN1A. Rankings of scores among 413,611 peptides for each samples are shown (eg, the #2 ranking peptide for sample P72 is Pep-3). Sample P72 reacts strongly to 5 peptides, of which 3 are nonoverlapping; sample P94 reacts to 2 nonoverlapping cN1A peptides and to an cN1B peptide with high sequence homology to an cN1A subsequence. (C) Peptide dot blot assay shows epitope spreading for samples P72 and P144. Pep-4 is a negative control cN1B peptide with no sequence homology to cN1A. (D) Predicted structure of cN1A protein with epitopes Pep-2 shown in red and Pep-3 shown in green.

normal upper limit of pixel intensity units (IU) as the dot blot densitometry mean plus 3 standard deviations for the 35 healthy subjects, and scaled data to an arbitrary normal upper limit of 10 IU. Comparisons of plasma and serum samples from the same patients showed no differences in reactivity.

The cN1A protein dot blot assay showed high diagnostic sensitivity and specificity for IBM compared to all other muscle diseases. Highly elevated reactivity (>10 IU) of anti-cN1A autoantibodies was present for 34% (16 of 47) of patients with IBM compared to 1.7% (2 of 118) of patients with non-IBM muscle diseases, resulting in a diagnostic sensitivity of 34% and specificity of 98.3% (see Fig 3B). Among patients with muscle diseases by category, high positivity was present for 34% of IBM, 4% (1 of 26) of PM, 0% (0 of 14) of NM, 2.7% (1 of 36) of DM, 0% (0 of 13) of myasthenia gravis, 0% (0 of 10) of muscular dystrophy, and 0% (0 of 19) of other muscle diseases (see Fig 3C).

Receiver operating characteristic analysis showed area under the curve of 0.85 with $p<0.0001$ (see Fig 3D), demonstrating high diagnostic performance of this assay at a range of thresholds. Optimal accuracy occurred at reactivity of >2.5 IU and was present for IBM with a diagnostic sensitivity of 70%, specificity of 92%, and accuracy of 85% (see Fig 3E).

cN1A Epitope Spreading

Epitope spreading, defined as the diversification of epitope specificity from the initial focused, dominant epitope-specific immune response to subdominant epitopes on that protein,[23] appears to be present for cN1A in IBM. Peptidome library screening data indicated reactivity for at least 2 nonoverlapping peptides in 2 samples

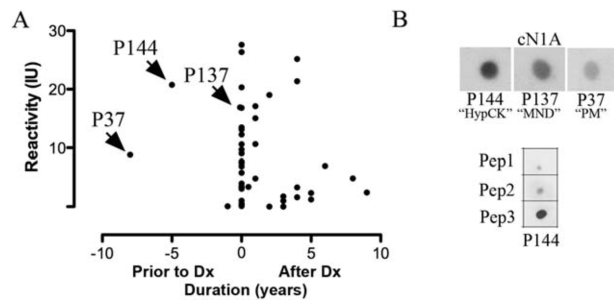

FIGURE 5: Presymptomatic cytoplasmic 5′-nucleotidase 1A (cN1A) autoantibody reactivity. (A) Three patients with blood samples drawn 1 month to 8 years prior to the diagnosis (Dx) of inclusion body myositis (IBM) demonstrated positivity before clinical features of IBM developed. Duration is measured between blood draw and IBM diagnosis, with negative duration indicating blood drawn before diagnosis. (B) Misdiagnoses of asymptomatic serum creatine kinase elevation (HypCK; Patient P144), polymyositis (PM; Patient P37), and motor neuron disease (MND; Patient P137). All 3 had anti-cN1A reactivity, and 1 (P144) had epitope spreading present 5 years before diagnosis. Patient P144 was asymptomatic, evaluated for asymptomatic elevated serum creatine kinase with normal strength on 4 biyearly examinations until 2 years later, when mild finger flexor weakness developed, and another 3 years passed until the diagnosis of IBM was made. IU=intensity units.

and 3 nonoverlapping peptides in 1 of 6 IBM samples. Screening by dot blot assay of all 47 IBM samples using a set of 3 peptides (Pep-1, Pep-2, and Pep-3) showed 6% (3 of 47) of samples had reactivity to 2 nonoverlapping cN1A peptides (Fig 4).

An immunodominant epitope near the N-terminus was suggested by the reactivity against the overlapping peptides Pep-2 (amino acid positions 30–65) and Pep-1 (amino acid positions 59–94). A second epitope may be present given the strong reactivity seen against Pep-3 (amino acid positions 204–239), but this was not systematically explored in our entire cohort. A third epitope at the C-terminus (Pep-13, amino acid positions 334–368) also showed reactivity in peptidome screening data (see Fig 4B).

cN1A has not yet been crystallized, and so its structure is currently unknown. In an effort to understand the relationship between these epitopes' locations and the predicted structure of cN1A, we utilized the I-TASSER protein structure prediction server.[24] The most likely structure according to this analysis localizes Pep-2 and Pep-3 to solvent-exposed loops on opposing faces of the protein (see Fig 4D).

Relationship of cN1A Autoimmunity to Clinical Features

We considered clinical features and their relationship to cN1A autoimmunity in the cohort of 47 IBM patients (Supplementary Fig 1). Anti-cN1A antibody reactivity in IBM was not correlated with age ($r=-0.08$, $p=0.61$), duration of symptoms ($r=-0.11$, $p=0.47$), strength of weakest finger flexor ($r=0.07$, $p=0.66$), or strength of weakest knee extension ($r=0.21$, $p=0.17$; all tests Spearman rank correlation). Anti-cN1A reactivity was not associated with ANA positivity ($p=0.15$) or anti-Ro or anti-La positivity ($p=0.23$; Mann–Whitney). Anti-cN1A IBM positivity was not confounded by other autoimmune diseases: 4 patients of 33 with anti-cN1A reactivity of >2.5 IU had a recognized other autoimmune disease (2 with Sjögren syndrome, 2 with psoriasis). A serum monoclonal antibody detectable by immunofixation was present in only 1 of 15 patients tested who had high anti-cN1A reactivity.

Two of the blood samples with high anti-cN1A reactivity had been obtained 5 and 8 years before the diagnosis of IBM was made. These patients were thought to have asymptomatic elevated serum creatine kinase (hyperCKemia) or PM by neuromuscular specialists (Fig 5).

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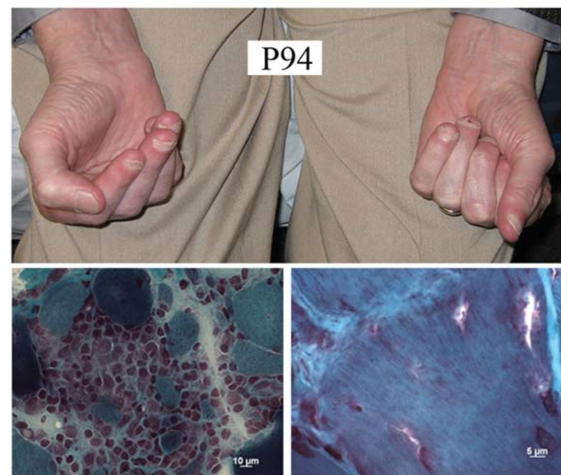

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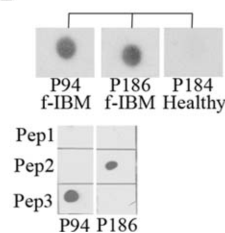

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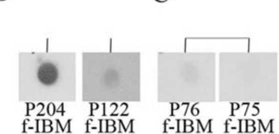

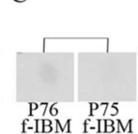

FIGURE 6: Familial inclusion body myositis (f-IBM) and autoimmunity. (A) Sibship of 3 brothers, none twins. (A1) Brother P94 with characteristic finger flexor weakness and muscle pathology (Gomori trichrome) showing endomysial inflammation and rimmed vacuoles. (A2) The 2 brothers with IBM had strong anti–cytoplasmic 5′-nucleotidase 1A (cN1A) reactivity (P94 21 intensity units [IU]; P186 19 IU), with distinct immunodominant epitopes; their 3rd brother P184 did not have anti-cN1A reactivity. (B) Two unrelated patients with f-IBM had high anti-cN1A reactivity. (C) Two nontwin sisters with f-IBM. P76 had negative anti-cN1A reactivity (2.4 and 1.2 IU).

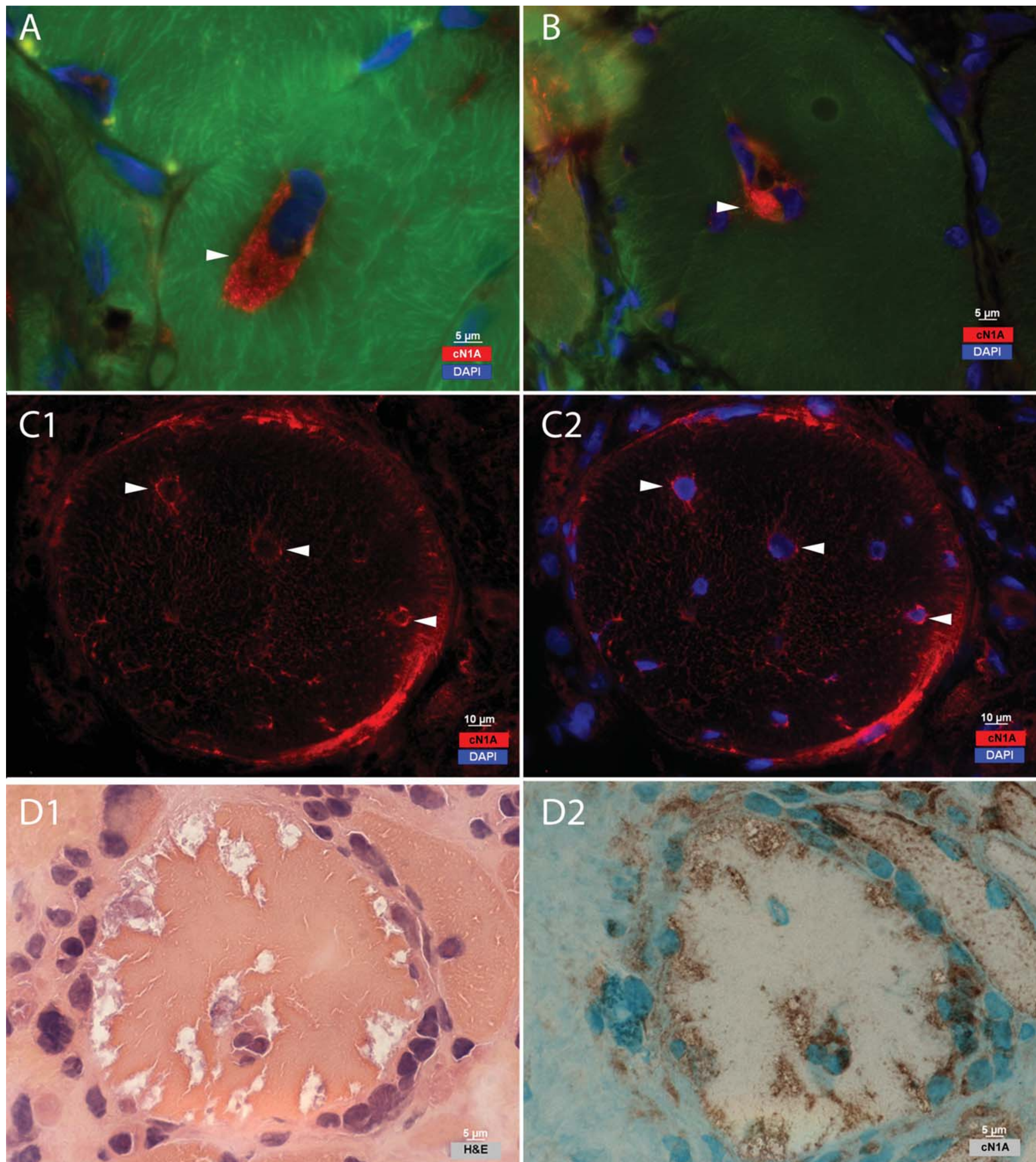

FIGURE 7: Perinuclear accumulation of cytoplasmic 5′-nucleotidase 1A (cN1A) immunoreactivity in inclusion body myositis (IBM) myofibers. Immunofluorescent stained sections from muscle biopsies from 3 different IBM patients. cN1A (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue). (A) Large accumulations of cN1A immunoreactivity *(arrowhead)* around DAPI-stained myonuclei. (B) A central vacuole is surrounded by an cN1A-immunoreactive accumulation *(arrowhead)* and 2 myonuclei. (C) cN1A immunoreactivity lining myonuclei. (D) Hematoxylin and eosin (H&E)-rimmed vacuoles with cN1A immunoreactivity seen on adjacent section.

Familial Sporadic Inclusion Body Myositis

We studied 3 brothers, 2 of whom had sporadic inclusion body myositis and 1 of whom did not. This entity has been called f-IBM.[15,16] The 2 affected brothers, but not the unaffected brother, had high anti-cN1A reactivity directed against distinct antigenic epitopes (Fig 6A). Two unrelated patients with f-IBM had elevated anti-cN1A reactivity; blood

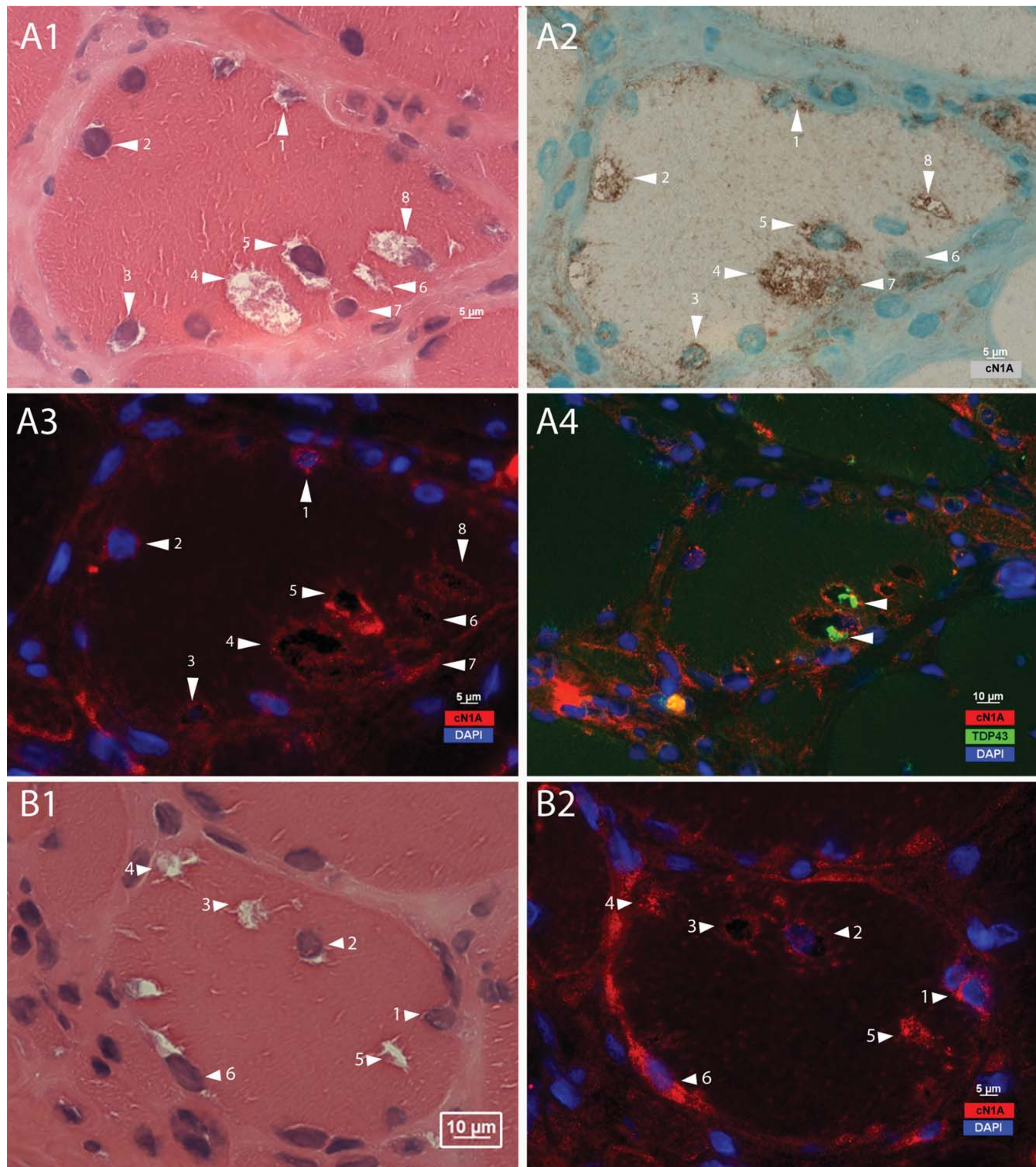

FIGURE 8: Rimmed vacuoles and cytoplasmic 5′-nucleotidase 1A (cN1A) immunoreactivity in various stages of myofiber degeneration. (A) Serial adjacent 10μm sections of inclusion body myositis muscle sections stained (A1) with hematoxylin and eosin, (A2) for cN1A, (A3) for cN1A and nuclei (4′,6-diamidino-2-phenylindole ([DAPI]), and (A4) for cN1A, TAR DNA-binding protein 43 (TDP-43), and nuclei (DAPI). Numbered arrowheads allow tracking of myofiber regions across the 4 serial sections. Myofibers show various stages of rimmed vacuole formation including perinuclear vacuolization accompanied by cN1A immunoreactivity, usually with a visible DAPI-stained myonucleus still present; myonuclear degeneration with cN1A immunoreactivity rimmed vacuole and loss of all DAPI signal; and late stage vacuoles with cN1A immunoreactivity deposits. TDP-43 immunoreactivity vacuole deposits did not overlap with cN1A signal (additional data not shown). (B) Another myofiber viewed in 2 serial sections.

samples from their affected family members were not available (see Fig 6B). Two sisters with f-IBM had negative anti-cN1A reactivity (see Fig 6C). cN1A autoimmunity appears to be present in some patients with f-IBM and may tend to be concordant among siblings.

cN1A Immunohistochemistry

To examine the distribution of cN1A in IBM muscle, immunoperoxidase and fluorescent immunohistochemistry were performed using a commercial antibody. No previous studies of this antibody in human skeletal muscle have been published. We confirmed that this antibody reacted to native conformation cN1A, the form of cN1A we sought to detect in tissue sections, through immunoblot blocking experiments.

cN1A commercial antibody reactivity was sometimes found in perinuclear accumulations and lining the rims of vacuoles in IBM (Fig 7). Other inflammatory myopathy and normal muscle did not show perinuclear cN1A reactivity (Supplementary Fig 2). Examination of serial sections stained with hematoxylin–eosin and anti-NT5C1A/cN1A antibody showed the accumulation of cN1A reactivity in perinuclear regions colocalizing with granular intravacuole deposits and vacuole rims (Fig 8). Although prominent sarcoplasmic TDP-43 aggregates did not colocalize with cN1A reactivity, colocalization was often present in rimmed vacuoles (Supplementary Fig 3).

cN1A Sequencing in IBM

No cN1A nonsynonymous variants were present in high-quality exome data (average coverage >50×) or whole genome data (average coverage >75×) from any of 19 patients with IBM. Twelve of these patients had anti-cN1A autoantibody reactivity (>2.5 IU). Because 83% of the epitope sequence Pep-3 also mapped to cN1B without gaps (chr2: 18,757,489–18,757,578), we also searched for nonsynonymous variants in this region of NT5C1B/cN1B and found no protein-altering variants.

Discussion

IBM is a poorly understood autoimmune and degenerative disorder. After key studies of immune cell phenotypes in IBM muscle were published in the 1980s,[4–7] IBM autoimmunity has largely been viewed as T-cell mediated. At least 15 published studies have examined T-cell receptor sequences in IBM muscle since 1993, but none has identified antigens driving IBM autoimmunity. In contrast, it has been widely believed that B-cell autoimmunity is unimportant in IBM. After microarray muscle profiling studies in 2002 identified a brisk B-cell immunoglobulin transcriptional response,[9] an antigen driven B-cell response in IBM muscle was defined. This was characterized by abundant CD138 plasma cells,[10] immunoglobulin gene rearrangements characteristic of clonal expansion of B cells in response to local antigen stimulation,[11,12] and the presence of ectopic lymphoid structures within muscle containing clonal populations of B cells.[12]

We sought to identify antigens responsible for this B-cell response by probing human muscle with IBM blood samples. In 2011, these studies identified a plasma and serum autoantibody reactive on Western blots to an approximately 43 kDa human muscle protein in 52% (13 of 25) of IBM samples and 0% (0 of 25) of other autoimmune muscle disease samples.[13] In the present study, we report the use of proteomic approaches to identify the protein cN1A as a candidate IBM autoantigen. Subsequent Western and dot blot assays confirmed that the presence of cN1A autoantibodies is a common and highly specific feature of IBM. A contemporaneous study has independently arrived at the same conclusion.[25] The presence of >1 approximately 43 kDa band in some patients, noted previously[13] and again here, suggests that some patients have autoantibodies to other autoantigens or to modified versions of cN1A. Although the refractoriness of IBM to immune therapies has led to uncertainty about its status as an autoimmune disorder, the identification of the cN1A autoantigen and demonstration of multiple cN1A epitopes firmly establishes specific antigen-driven autoimmunity in IBM.

cN1A is a cytosolic 5′-nucleotidase that is most abundant in skeletal muscle.[22] It catalyzes nucleotide hydrolysis to nucleosides, and is involved in a variety of functions, including regulation of deoxynucleotide pools formed during the degradation of nucleic acids.[26] Several enzymes involved in DNA repair have 5′-nucleotidase sequence motifs and activity, suggesting that 5′-nucleotidases may be involved in DNA repair metabolism.[27]

The apparent perinuclear and vacuole rim accumulations of cN1A reactivity have important potential implications for understanding the relationship between IBM autoimmunity and myofiber degeneration, although further confirmation studies are needed exploring the nature and distribution of human skeletal muscle cN1A in IBM. Rimmed vacuoles are a distinctive feature of IBM pathology, and appear to be derived from myonuclei, given rimmed vacuole localization of unique nuclear components, such as nuclear membrane proteins emerin and lamin A/C,[28] histones,[29] and DNA repair enzymes.[30] The apparent localization of cN1A reactivity to rimmed vacuoles and perinuclear regions suggests that cN1A might be involved in nucleic acid metabolism occurring during myonuclear degradation. Altered nucleic acid metabolism in IBM muscle is likely, given the accumulation of a single-stranded DNA binding protein[31] and the sarcoplasmic mislocalization of TDP-43 from IBM myonuclei.[32,33] These data raise the possibility that

IBM anti-cN1A autoimmunity may be a consequence of IBM myonuclear degeneration.

No autoantibody testing is currently available for IBM despite the availability and usefulness of myositis autoantibody testing in the clinical management of DM and PM.[34–36] The anti-cN1A autoantibody assay reported here in 165 patients with muscle disease has optimal accuracy of 85% at moderate reactivity (>2.5 IU), where it detects 70% of patients with IBM, with specificity of 92%. Combined with even modest amounts of clinical information, such as the absence of a typical DM skin rash or highly elevated serum creatine kinase, the moderate reactivity range may provide optimal sensitivity and specificity for IBM diagnosis.

The anti-cN1A assay has potential to improve the care and management of patients with suspected IBM. The IBM initial misdiagnosis rate is estimated at 30[37] to 54%,[38] contributing to median delays in diagnosis of 4.9 to 5.2 years. The positivity of IBM samples did not correlate with duration of symptoms of IBM muscle weakness. Eight patients had high reactivity of blood samples at symptom durations less than the median time to diagnosis of IBM (range = −5 to 3 years). Two patients in particular had high anti-cN1A reactivity years before development of symptoms and diagnosis of IBM despite careful neuromuscular follow-up. Together these data suggest that blood testing will identify patients with IBM early on in their disease course.

Anti-cN1A autoantibody testing may be particularly valuable in distinguishing IBM from PM, because the clinical boundary between IBM and PM can be difficult to establish.[39] PM is the most common misdiagnosis, and in 1 series, 16 of 43 (37%) patients with biopsy features of PM had clinical features of IBM.[17] Such patients, called PM/IBM, meet ENMC criteria for probable IBM, and almost certainly have IBM based on the pattern of weakness and refractoriness to therapy.[17] In our series, 11 patients had PM/IBM, and 64% of them had high anti-cN1A reactivity of >10 IU. More generally, in our 73 patients with IBM or PM, high anti-cN1A reactivity indicated a 9× greater likelihood of having IBM than PM, and moderate reactivity (>2.5 IU) indicated a 6× greater likelihood of having IBM than PM. These data suggest that this assay could be of substantial benefit in the differential diagnosis of IBM and PM.

An anti-cN1A autoantibody assay also has potential to reduce the number of muscle biopsies performed in patients with suspected IBM. Almost all patients with suspected IBM undergo muscle biopsy, and many have 2 or more biopsies performed before the correct diagnosis is established. The calculated diagnostic accuracy of biopsy for IBM from a series of 107 patients[17] with IBM and PM (64 with IBM, 27 with PM, and 16 with PM/IBM) is 85% (64 true positives, 27 true negatives, 0 false positives, and 16 false negatives). Anti-cN1A blood testing at 2.5 IU or greater reactivity provided the same diagnostic accuracy of 85% in our patients with muscle diseases and is less invasive than biopsy. An IBM blood test of comparable accuracy to muscle biopsy could be of substantial clinical value. Additionally, improved diagnosis of IBM might reduce the practice of ineffective treatment of IBM patients with corticosteroids.

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Potential Conflicts of Interest

H.B.L., M.S., S.J.E., S.A.G.: inventors of related intellectual property owned and managed by Brigham and Women's Hospital. A.A.A.: consultancy, MedImmune, Biogen.

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