Ann Rheum Dis. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:
Ann Rheum Dis. 2016 April ; 75(4): 696–701. doi:10.1136/annrheumdis-2014-206691.

Disease-specificity of autoantibodies to cytosolic 5'-nucleotidase 1A in sporadic inclusion body myositis versus known autoimmune diseases

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Abstract

Objectives—The diagnosis of inclusion body myositis (IBM) can be challenging as it can be difficult to clinically distinguish from other forms of myositis, particularly polymyositis (PM). Recent studies have shown frequent presence of autoantibodies directed against cytosolic 5'-nucleotidase 1A (cN-1A) in patients with IBM. We therefore, examined the autoantigenicity and disease-specificity of major epitopes of cN-1A in patients with sporadic IBM compared with healthy and disease controls.

Methods—Serum samples obtained from patients with IBM (n=238), polymyositis (PM) and dermatomyositis (DM) (n=185), other autoimmune diseases (n=246), other neuromuscular diseases (n=93) and healthy controls (n=35) were analysed for the presence of autoantibodies using immunodominant cN-1A peptide enzyme-linked immunosorbent assays (ELISAs).

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Results—Autoantibodies directed against major epitopes of cN-1A were frequent in IBM patients (37%) but not in PM, DM or non-autoimmune neuromuscular diseases (<5%). Anti-cN-1A reactivity was also observed in some other autoimmune diseases, particularly Sjögren's syndrome (SjS; 36%) and systemic lupus erythematosus (SLE; 20%).

Conclusions—In summary, we found frequent anti-cN-1A autoantibodies in sera from IBM patients. Heterogeneity in reactivity with the three immunodominant epitopes indicates that serological assays should not be limited to a distinct epitope region. The similar reactivities observed for SjS and SLE demonstrate the need to further investigate whether distinct IBM-specific epitopes exist.

Keywords

Autoantibodies; Cytosolic 5'-nucleotidase 1A (cN-1A); Disease-specificity; ELISA; Idiopathic inflammatory myopathy

INTRODUCTION

Idiopathic inflammatory myopathies (IIMs) comprise a diverse group of inflammatory muscle diseases with an insidious onset characterised by chronic muscle weakness, inflammation of skeletal muscle, electromyographic abnormalities, and increases in muscle enzymes [1, 2]. Two of the three forms of IIM, polymyositis (PM) and dermatomyositis (DM), are well recognised as autoimmune diseases. They exhibit an array of autoantibodies directed against ubiquitous intracellular antigens that can be either myositis-specific (MSAs) or myositis-associated (MAAs) [3]. In contrast, the pathogenesis of sporadic inclusion body myositis (IBM) has been a question of debate and many consider it to be a degenerative myopathy with secondary inflammation rather than a primary autoimmune disease [4]. However, the recent discovery of an autoantibody (anti-Mup44) directed against cytosolic 5'-nucleotidase 1A (cN-1A) with frequent–reactivity in the sera of IBM patients [5, 6] has prompted a reconsideration of this paradigm.

IBM is supposedly the most common acquired muscle disease in adults over the age of 50 years [7, 8], characterised clinically by an insidious onset of muscle weakness and muscle atrophy that slowly leads to severe disability [9]. Unlike PM and DM, IBM is largely refractory to treatment with immunosuppressive, immunomodulatory or other therapies [10, 11] which has contributed to the notion of IBM as a primarily degenerative disorder with a secondary immune component. Pathologically, IBM is characterised by a combination of degenerative features (rimmed vacuoles and abnormal protein accumulations) that partly resemble the histopathological features of other neurodegenerative diseases and which include aggregates of TAR DNA-binding protein 43, as found in frontotemporal dementia and amyotrophic lateral sclerosis, Aβ-42 and tau, as found in Alzheimer's dementia [12-14] and the presence of p62 in rimmed vacuoles [15]. However, muscle-specific autoimmune features such as cytotoxic T-cell infiltration and clonal expansion of lymphocytes, which are common in IBM muscle biopsies, support a role for autoimmunity in these patients [16] as does association of the autoimmune-prone HLA-B8-DR3 ancestral haplotype with sporadic IBM [17, 18]. Antinuclear antibodies (ANA) and antibodies against extractable nuclear antigens (ENA) have been detected in up to 30% of IBM patients [19]. Detection of cN-1A

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directed autoantibodies in IBM patients in recent studies [5, 6] provides interesting new insights into IBM pathophysiology and which may lead to the development of a disease-specific serological diagnostic test for IBM.

The enzyme cN-1A catalyses the conversion of adenosine monophosphate (AMP) into adenosine and phosphate, and it is highly expressed in skeletal muscle where it may be involved in muscle contraction [20, 21]. In an earlier study [5] using immunoprecipitation techniques we observed frequent anti-cN-1A reactivity in IBM sera and that was remarkably strong compared with other IIM autoantibodies, thus revealing the potential of anti-cN-1A antibodies as a disease-specific biomarker for IBM. Therefore, further characterisation of anti-cN-1A autoantibodies and their diagnostic role in IBM is clearly required. The aim of the current study was to determine the specificity of serum anti-cN-1A autoantibodies for IBM using an enzyme-linked immunosorbent assay (ELISA) format with three synthetic peptides containing cN-1A autoepitopes previously identified by overlapping peptide microarray analyses [5]. In addition we investigated differences in cN-1A epitope recognition by IBM sera compared with sera from other IIM patients and from a range of other neuromuscular and autoimmune diseases.

METHODS

Patients and serum samples

We included serum samples from a large group of well characterised IBM patients (n=238) and 35 healthy controls with no known history of autoimmune or (neuro)muscular disease. The sera of the IBM group were gathered from the following participating European institutes: Radboud University Medical Centre, Nijmegen; Leiden University Medical Centre, Leiden (the Netherlands); University of Manchester on behalf of UKMYONET and University College London (United Kingdom); Ghent University Hospital, Ghent (Belgium); and Karolinska Institutet, Stockholm (Sweden). All patients fulfilled the European Neuromuscular Centre [22] or MRC 2010 [23] criteria. Disease control sera were obtained from patients with DM and PM (n=185), scleroderma (n=44), PM/scleroderma overlap (n=12), multiple sclerosis (MS) (n=40), SjS (n=22), SLE (n=44), rheumatoid arthritis (RA) (n=44), type 1 diabetes (T1D) (n=40), and other neuromuscular diseases (NMD) (n=93) [5, 24]. These were collected in Nijmegen except for PM/Scleroderma overlap (Czech Republic) and PM/DM (Nijmegen and Manchester). Serum from healthy subjects was obtained from the Sanquin Blood Supply Foundation (Nijmegen, the Netherlands). Written or verbal informed consent was obtained from most of the patients from whom sera were used and all patient information was decoded to maintain confidentiality. The study protocol was in accordance with the Helsinki Declaration and all procedures were approved by the local ethics committees.

cN-1A peptides

Three 23 amino acid synthetic peptides derived from the sequence of cN-1A were used as target antigens in the ELISA. These peptides were identified as major epitope regions in our previous study [5] and contained the sequences: Peptide 1: PVWEEAKIFYDNLAPKKKPKSPK; Peptide 2: SERIVKAHGLDRFFEHEKAHENK; and

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Peptide 3: AHVPYGVAQTPRRTAPAKQAPSA (all peptides contain aminohexanoic acid-Lys(biotin)-amide at the C-terminus).

Peptide ELISA

Optimal ELISA conditions were determined using checkerboard titrations (data not shown) and the OD450 values for serially diluted serum samples at optimal dilutions were plotted (Supplementary Figure S1). Biotinylated peptides (~30 ng per well) in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) were immobilized on pre-blocked Streptawell High Bind microplates (Roche, Mannheim, Germany) for 1 hour at 37°C. After washing 3 times with 200 µl PBS/0.1% Tween-20 (PBST), 50 µl of 400-fold diluted patient serum in 1% BSA/PBS/0.05% Tween-20 was added, followed by incubation for 1 h at 37 °C. Subsequently, wells were washed 5 times with 300 µl PBST and incubated with 50 µl 2,000-fold diluted rabbit anti-human Ig (Dako, P0212) in 1% BSA/PBS/0.05% Tween-20 for 1 h at 37 °C. Finally, after washing 5 times with 200 µl PBST, bound antibodies were visualized by adding 50 µl TMB/UP (Thermo Scientific) and the reaction was stopped by the addition of 50 µl 2 M H_2SO_4 after 5 min. Signals were quantified by determining optical densities (OD) at 450 nm. Each plate contained at least one positive control and 5 negative control sera.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM 5 software (San Diego, California) or SPSS software version 20.0 (Chicago, IL).

RESULTS

Based upon the reactivity of anti-cN-1A-positive IBM sera with a set of overlapping cN-1A peptides (15-mers, immobilized on microarrays) [5], three 23-mer peptides corresponding to the most frequently targeted regions of cN-1A were synthesized for ELISA analyses (Figure 1) and optimised using checkerboard titrations. ROC curves were produced (Supplementary Figure S2) to compare IBM sera positive in immunoprecipitation experiments (reported previously [5]) against all disease controls excluding SLE and SjS. The OD450 value corresponding to the highest Youden index ($sensitivity + specificity - 1$) at which ≥98% specificity was achieved was chosen for each peptide. Sera were assessed as reactive if they were above the established cut-off value for at least one of the peptide antigens. Subsequently, the differences in reactivity between the different IBM cohorts (Figure 2) and between IBM and other disease controls (Figure 3) were investigated. No major differences were observed between the recognition of the cN-1A peptides among the IBM samples from different centres. The frequency of anti-cN-1A reactivity varied from 34% to 44% among the different IBM cohorts (Figure 2).

The frequency by which cN-1A peptides are recognized by patient sera is summarised in Table 1. For the IBM patients we observed 37% reactivity for at least one of the cN-1A peptides. Anti-cN-1A autoantibody reactivity was observed in just 4% of PM or DM patients (n=185) of which 7 were PM patients and one a DM patient. In sera from other disease controls not more than 5% of sera from patients with PM/Scleroderma overlap (n=12), other

neuromuscular diseases (n=93), scleroderma (n=44), rheumatoid arthritis (n=44), multiple sclerosis (n=40), or type 1 diabetes (n=40), showed reactivity. However, we did observe frequent reactivity in sera from patients with SjS (36%; n=22) and SLE (20%; n=44). Anti-cN-1A reactivity did not correlate with the presence of other autoantibodies (Supplementary Tables S1 and S2) in IBM, SLE or SjS nor with IgG content (r^2=0.07, p=0.23).

There was also heterogeneity across the groups in terms of peptide specificity. Of the reactive IBM sera autoantibodies targeted not only single peptides – peptide 1 (23%), 2 (25%) or 3 (11%) – but also two (25%) or three peptides (16%) (Figure 4). Similarly, when we compared peptide reactivity in each of the other disease groups, we found high heterogeneity in reactivity for individual and combination peptides. However, reactivity to peptide 3 alone was only observed in IBM or PM sera, while the combination of peptides 2 and 3 was observed infrequently and only in patients with IBM (5%) and not in any of the disease control groups.

Among the IBM patients, the average age at onset of disease did not differ significantly between the seronegative (median age = 64yrs; IQR. = 15; n=148) and seropositive (median age = 66yrs; IQR = 17; n=84) groups. More male IBM patients (41%) were reactive with one or more peptides than female IBM patients (34%) but this difference was not statistically significant (p=0.18).

DISCUSSION

In this study we used a newly developed cN-1A peptide ELISA to investigate the prevalence and significance of anti-cN-1A autoantibodies in the sera of patients with IIM versus other autoimmune and neuromuscular diseases. We used synthetic peptides containing three immunodominant epitope regions of cN-1A to detect these autoantibodies. Screening of a large group of IBM patients revealed that 37% had serum anti-cN-1A autoantibodies directed against at least one of the three epitopes. These results correlate well both with our own previous immunoprecipitation experiments [5] showing high concentrations of cN-1A autoantibodies in 33% of IBM sera, and with the results of a study showing cN-1A autoantibody reactivity in 34% of IBM sera using dot blot assays [6]. Furthermore, we found that cN-1A autoantibodies were much more prominent in IBM than PM or DM with only 4.3% of all PM/DM patients showing reactivity to cN-1A, 7 with PM and 1 with DM. Although IBM may initially be misdiagnosed as PM, the clinical characteristics of 3 of the PM patients with cN-1A autoreactivity (clinical phenotype data for other patients was not available) showed that they did not exhibit the IBM phenotype.

In contrast to the frequent reactivity observed in 37% of IBM sera, less than 5% of sera from patients with scleroderma, PM/Scl overlap, MS, T1D, and RA or non-autoimmune neuromuscular diseases contained anti-cN-1A autoantibodies. However, frequent anti-cN-1A autoantibody reactivity was detected in patients with SjS (36% of patients) or SLE (20% of patients). The significance of anti-cN-1A autoantibodies in these patient groups is unclear and will require further investigation. Interestingly, an increased incidence of SjS with IBM is supported by the literature (See for example [25, 26]).

The peptides we designed for use as antigens in our ELISA are linear sequences containing 23 amino acids which were previously shown to contain the most frequently targeted regions of cN-1A by autoantibodies in microarray and in dot blot assays [5, 6]. In the peptide ELISAs, we observed high heterogeneity in serum reactivity within each disease group, with some showing reactivity to just a single peptide and others showing reactivity to two or all three peptides. Among the three peptides, peptide 3, located at the C-terminus, was least frequently the single target of autoantibodies, particularly among the disease controls. We also noted that some of the sera testing positive for cN-1A autoantibodies during immunoprecipitation experiments [5] were not reactive to any of the three epitopes using peptide ELISAs. Therefore, it is likely that cN-1A autoantibody reactivity is not restricted to the three linear epitopes used in the ELISA assays but that additional immunodominant epitopes are yet to be identified and will likely include conformational or discontinuous epitopes.

This notion was supported by the finding that reactivity to the combination of peptides 2 and 3 was observed only in some of the IBM patients but not in any of the disease controls. In contrast, sera from patients with SLE predominantly targeted peptide 1 alone or in combination with peptide 2 and sera from patients with SjS predominantly targeted peptides 1 or 2 in isolation. However, the numbers of SLE and SjS patients tested were relatively small so these results require confirmation in studies of larger groups. Furthermore, similar percentages of anti-cN-1A positive patients were observed in our previous immunoprecipitation experiments and the current peptide ELISAs, but the correlation between the IP positive and peptide reactive sera was low (Supplementary Figure S3). This also strongly supports the existence of conformational autoepitopes in addition to the linear epitopes.

In summary, we developed an ELISA useful to measure anti-cN-1A autoantibodies in serum. Using this assay we were able to confirm the results of earlier studies [5, 6, 27] showing that cN-1A is a major autoantigen in IBM and that anti-cN-1A autoantibodies represent a new serological marker for IBM, particularly in differentiating IBM from other neuromuscular diseases, including PM. The clinical utility should not be severely reduced by the presence of anti-cN-1A autoantibodies in SLE and SjS, as in clinical practice there will generally be little difficulty differentiating a patient suffering with IBM from one suffering with SLE or SjS. However, in cases of comorbidity this biomarker may be less informative. The results obtained here from larger patient cohorts have allowed confirmation of three distinct linear epitopes recognised by circulating antibodies. Thus, due to the heterogeneous anti-cN-1A response in established IBM, serological assays should not be limited to a distinct epitope region. Despite anti-cN-1A autoantibodies occurring in both IBM and in other autoimmune diseases, particularly SLE and SjS, the pattern of reactivity to distinct (combinations of) epitopes differed. Further research is now in progress to establish whether more IBM-specific cN-1A epitopes exist, and which could facilitate the future development of more IBM-specific serological assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

Part of this study was supported by the Prinses Beatrix Spierfonds (project no. W.OR12-15).

We would like to acknowledge Dr Maryam Dastmalchi and Snjolaug Arnardottir for collecting clinical data and blood samples and Eva Jemseby for handling of a serum biobank in the rheumatology research laboratory, Karolinska Institutet.

The UKMYONET adult cohort was funded by Arthritis Research UK Programme Grant 18474. Further funding has been provided through the UK Myositis Support Group, Association Francaise Contre Les Myopathies (AFM), The European Union Sixth Framework Programme (project AutoCure; LSH-018661), European Science Foundation (ESF) in the framework of the Research Networking Programme European Myositis Network (EUMYONET), the Swedish Research Council and The regional agreement on medical training and clinical research (ALF) between Stockholm County Council and Karolinska Institutet".

This report includes independent research funded by the National Institute for Health Research Manchester Musculoskeletal Biomedical Research Unit. The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health, UK.

The funding agencies played no role in study design, collection, analysis or interpretation of the data, preparation of the manuscript or the decision to submit the article for publishing.

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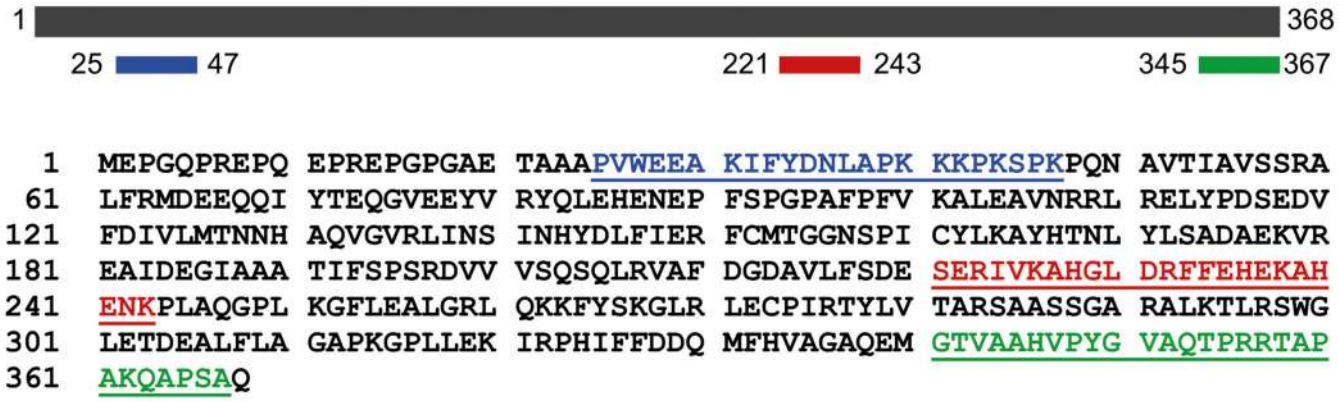

Figure 1.

cN-1A peptide sequences of the synthetic peptides used in ELISA assays.

Schematic representation of the cN-1A polypeptide (top) and amino acid sequence of cN-1A (bottom). The positions of the three peptide sequences used in ELISA are highlighted in blue, red and green, respectively.

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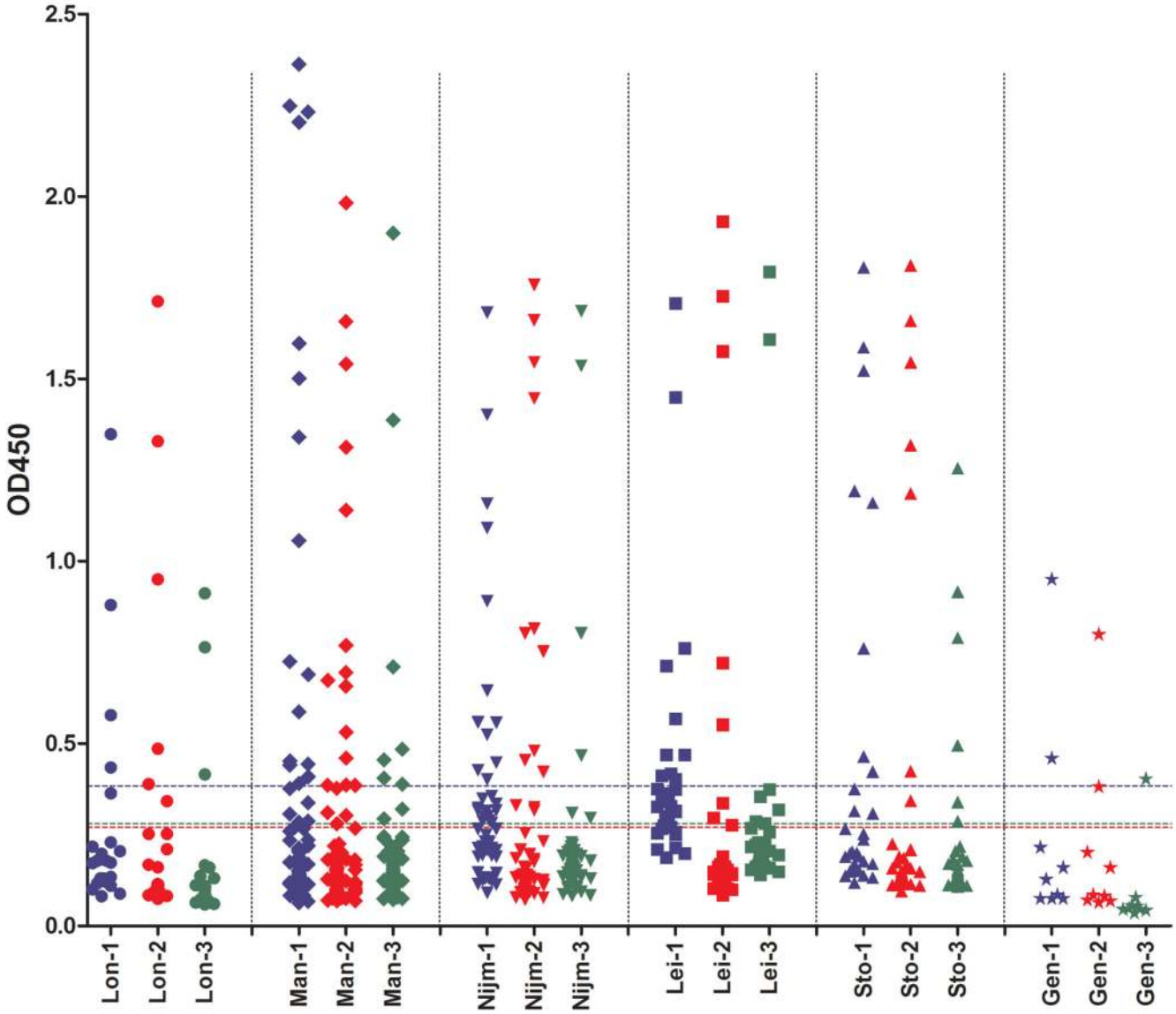

Figure 2.

Reactivity of IBM sera from various cohorts with cN-1A peptides.

The presence of anti-cN-1A antibodies in samples from 6 IBM cohorts was analysed in ELISA using the three cN-1A peptides specified in Figure 1. Lines represent cut-off values for each peptide (colour coding as in Figure 1). Lon: London, England (n = 24), Man: Manchester, England (n = 89), Nijm: Nijmegen, the Netherlands (n = 52), Lei: Leiden, the Netherlands (n = 32), Sto: Stockholm, Sweden (n = 32), Gen: Ghent, Belgium (n = 9). Numbers following cohort abbreviation represent peptide numbers (1: PVWEEAKIFYDNLAPKKKPKSPK; 2: SERIVKAHGLDRFFEHEKAHENK and 3: AHVPYGVAQTPRRTAPAKQAPSA). Please note that samples from Manchester were provided on behalf of UKMyoNet.

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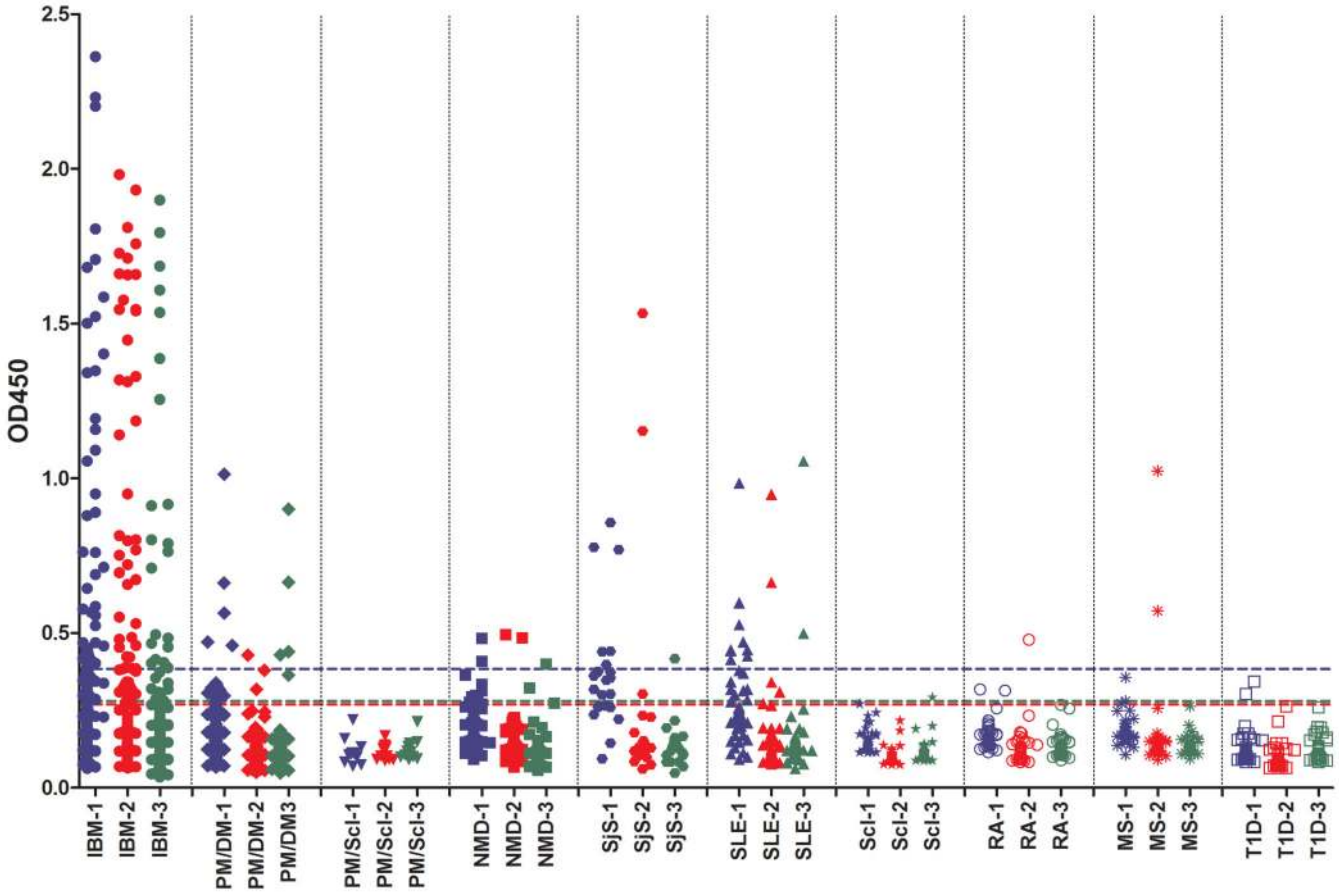

Figure 3.

Reactivity of patient sera with cN-1A peptides.

The presence of anti-cN-1A antibodies in samples from different disease groups was analysed in ELISA using the three cN-1A peptides specified in Figure 1. Lines represent cut-off values for each peptide (color coding as in Figure 1). IBM: sporadic IBM, PM/DM = polymyositis/dermatomyositis, PM/Scl = polymyositis/scleroderma overlap syndrome, NMD = other neuromuscular diseases, SjS = Sjögren's syndrome, SLE = systemic lupus erythematosus, Scl = scleroderma, RA = rheumatoid arthritis, MS = multiple sclerosis, T1D = type 1 diabetes. Numbers following disease abbreviation represent peptide numbers (1: PVWEEAKIFYDNLAPKKKPKSPK; 2: SERIVKAHGLDRFFEHEKAHENK and 3: AHVPYGVAQTPRRTAPAKQAPSA).

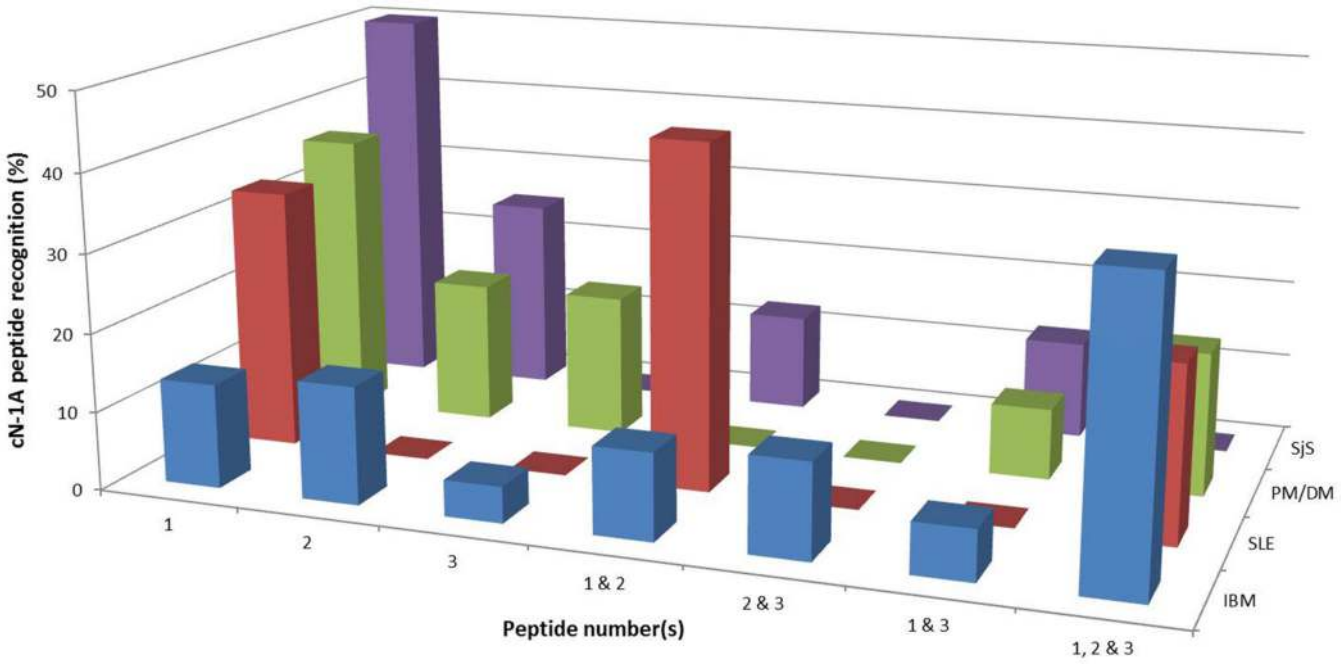

Figure 4.

Reactivity to (combinations of) cN-1A peptides.

Reactivity to either individual cN-1A peptides or combinations of peptides was calculated as a percentage of the total number of reactive sera for inclusion body myositis (IBM; blue), systemic lupus erythematosus (SLE; red), polymyositis/dermatomyositis (PM/DM; green) and Sjögren's syndrome (SjS; purple).

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Table 1
Sensitivity and specificity of anti- cN-1A autoantibodies

Sera	Number	anti-cN-1A reactivity [a]	
		No.	%
Inclusion body myositis	238	88	37
Polymyositis/Dermatomyositis	185	8	4
Polymyositis/Scleroderma overlap	12	0	0
Neuromuscular diseases	93	4	4
Sjögren's syndrome	22	8	36
Systemic lupus erythematosus	44	9	20
Scleroderma	44	1	2
Rheumatoid arthritis	44	1	2
Multiple sclerosis	40	2	5
Type 1 diabetes	40	0	0
Disease controls [b]	*458*	*16*	*3*

[a] Reactivity with at least one of the three cN-1A peptides higher than cut-off;

[b] Disease controls: total of all disease control groups except IBM, SLE and SjS.