





BRIEF REPORT

Anti-Cytosolic 5'-Nucleotidase 1A Autoantibodies Are Absent in Juvenile Dermatomyositis

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Objective. To assess anti-cytosolic 5'-nucleotidase 1A (anti-cN-1A) autoantibodies in children with juvenile dermatomyositis (DM) and healthy controls, using 3 different methods of antibody detection, as well as verification of the results in an independent cohort.

Methods. Anti-cN-1A reactivity was assessed in 34 Dutch juvenile DM patients and 20 healthy juvenile controls using the following methods: a commercially available full-length cN-1A enzyme-linked immunosorbent assay (ELISA), a synthetic peptide ELISA, and immunoblotting with a lysate from cN-1A-expressing HEK 293 cells. Sera from juvenile DM patients with active disease and those with disease in remission were analyzed. An independent British cohort of 110 juvenile DM patients and 43 healthy juvenile controls was assessed using an in-house full-length cN-1A ELISA.

Results. Anti-cN-1A reactivity was not present in sera from juvenile DM patients or healthy controls when tested with the commercially available full-length cN-1A ELISA or by immunoblotting, in either active disease or disease in remission. Additionally, in the British juvenile DM cohort, anti-cN-1A reactivity was not detected. Three Dutch juvenile DM patients had weakly positive results for 1 of 3 synthetic cN-1A peptides measured by ELISA.

Conclusion. Juvenile DM patients and young healthy individuals did not show anti-cN-1A reactivity as assessed by different antibody detection techniques.

INTRODUCTION

Autoantibodies detected in idiopathic inflammatory myopathies can be divided into myositis-specific autoantibodies (MSAs) and myositis-associated autoantibodies. MSAs have a high disease specificity, which can be used to confirm a subtype of myositis, are frequently related to a specific clinical phenotype and, in some cases, are associated with disease activity or severity (1–3).

Anti-cytosolic 5'-nucleotidase 1A (anti-cN-1A) autoantibodies are present in a large subset of inclusion body myositis (IBM) patients but not in adults with other forms of myositis. Although anti-cN-1A was initially classified as an MSA (4,5), the relatively frequent seropositivity in adults with Sjögren's syndrome and systemic lupus erythematosus has raised questions about the specificity of anti-cN-1A autoantibodies (6). A recent study showed anti-cN-1A autoreactivity in 27% of patients with juvenile dermatomyositis

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(DM) (7). The use of different methods of detection in various cohorts hampers direct comparisons of sensitivity and specificity of anti-cN-1A autoantibody reactivity. In order to assess the presence of anti-cN-1A autoantibodies in juvenile DM and healthy controls, we used 3 different methods of antibody detection and substantiated the results in an independent juvenile DM cohort.

PATIENTS AND METHODS

Patients. Anti-cN-1A autoreactivity was tested in 34 Dutch patients with juvenile DM (22 with active disease and 12 with disease in remission, randomly selected from the Dutch juvenile DM biomarker study [8]) and in 20 healthy controls. Nine of the 22 juvenile DM patients with active disease were retested when their disease was in remission. Two juvenile DM patients who were initially tested during active disease were retested during a flare. Juvenile DM diagnosis was based on the Bohan and Peter criteria for definite or probable juvenile DM (9,10). Inactive disease was defined according to the updated Paediatric Rheumatology International Trials Organisation criteria (11,12). Demographic and disease-related parameters from the moment of serum sampling are presented in Table 1. Samples were stored for up to 9 years at -80°C , anonymized, and the results of enzyme-linked immunosorbent assay (ELISA) and immunoblotting were assessed by researchers who were blinded with regard to sample identification.

An independent British cohort was used to validate the data, consisting of 110 juvenile DM patients and 43 healthy subjects ages ≤ 16 years (randomly selected from the national registry and described elsewhere [3]); parameters are summarized in Table 1. Serum samples were stored for up to 19 years at -80°C . Ethical approval was obtained (regional METC no. 15-191, 11-499/C and MREC 1/3/22).

Full-length cN-1A ELISA. The anti-cN-1A ELISA based on recombinant full-length cN-1A antigen was performed using a commercially available kit (EA 1675-4801G) according to instructions of the manufacturer (Euroimmun Medizinische Labordiagnostika AG). The development and validation of this ELISA has been described elsewhere (13). Results were evaluated semiquantitatively as a ratio (optical density [OD] at 450 nm of the sample/OD₄₅₀ of the calibrator [cutoff]); a ratio of ≥ 1 was deemed positive.

Anti-cN-1A reactivity was determined in the independent British cohort using an in-house ELISA at the University of Bath, using 0.4 $\mu\text{g}/\text{ml}$ recombinant cN-1A protein (TP32461, cytosolic 1A [NT5C1A] expressed in HEK 293; OriGene) per well, serum samples diluted 1:250, with goat anti-human IgG (Sigma; dilution 1:30,000) as a secondary antibody. Each plate contained positive and negative controls. A cutoff of 5 SD above the mean of negative controls was deemed positive.

Peptide ELISA. Details on the development and test characteristics of the cN-1A peptide ELISA have been published elsewhere (4,14). Briefly, 3 synthetic peptides of 23 amino acids derived from the sequence of cN-1A were used as target antigens in this ELISA, referred to as peptides 1, 2, and 3. We used rabbit anti-human IgA, IgG, IgM, kappa, and lambda (Dako P0212; 1:2,000 dilution) as a secondary antibody. Each plate contained a positive control (serum from an IBM patient with anti-cN-1A autoantibodies) and, for each serum sample, background reactivity was determined without a coated peptide. The serum background value was subtracted from the peptide values. Sera were considered to be positive for anti-cN-1A when OD₄₅₀ values were 3 SD above the mean of negative controls.

Preparation of cell lysates and Western blotting. A stably transfected cN-1A-expressing Flp-In T-REx HEK 293 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal calf serum (FCS), 15 $\mu\text{g}/\text{ml}$ blasticidin, and 300 $\mu\text{g}/\text{ml}$ hygromycin. Expression of cN-1A was induced by adding 1 $\mu\text{g}/\text{ml}$ doxycycline to the medium. A Flp-In T-REx HEK 293 control cell line was cultured in DMEM with 10% FCS, 15 $\mu\text{g}/\text{ml}$ blasticidin, and 100 $\mu\text{g}/\text{ml}$ zeocin. Cells were harvested 24 hours after induction, and lysates were prepared in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer by sonication for 10 minutes, with 30 second intervals. After heating for 5 minutes, proteins were separated in 12% SDS-PAGE gels (lysate from 1 T75 flask loaded per 10-cm gel).

After electrophoresis, the separated proteins were blotted on a Protran nitrocellulose blotting membrane (GE Healthcare Life Science), stained with ponceau S (0.1% ponceau S in 5% acetic acid), and cut into 3-mm strips. The blot strips were blocked in blocking buffer (5% nonfat dry milk, 5% sheep serum in phosphate buffered saline-Tween [PBST]) for 1 hour at room temperature and incubated with sera (from juvenile DM patients and healthy controls) diluted 1:100 in blocking buffer (1 hour at room temperature). Serum from an anti-cN-1A-positive IBM patient and a commercial rabbit anti-NT5C1A antibody (Atlas HPA050283) were used as positive controls (both diluted 1:5,000). Blots were washed 3 times with blocking buffer and incubated for 1 hour with the secondary antibody (IRDye 800CW goat anti-human IgG or IRDye 800CW goat anti-rabbit IgG), diluted 1:5,000 in blocking buffer. Blots were washed twice with PBST and once in PBS before visualization of bound antibodies using a Li-Cor Odyssey imager.

Anti-cN-1A reactivity was analyzed in sera by the incubation of 2 blots in parallel, 1 containing lysate from cN-1A-expressing cells and another containing lysate from the control cells lacking detectable levels of cN-1A, in order to account for possible background staining or staining of other proteins. Sera were considered positive for anti-cN-1A when a band appeared at the proper position on the blot strip containing cN-1A but was absent on the control blot.

Table 1. Demographic and disease-related parameters at the time of serum sampling*

	Dutch cohort			British cohort	
	Active juvenile DM (n = 22)	Inactive juvenile DM (n = 12)	Healthy controls (n = 20)	Juvenile DM (n = 110)	Healthy controls (n = 43)
Baseline data					
Age at diagnosis, median (IQR) years	4.8 (3.7–9.0)	6.4 (4.2–8.3)	–	7.4 (4.2–10.6)	–
Age at serum sampling, median (IQR) years	5.4 (3.8–11.7)	12.7 (9.4–15.2)	11.0 (7.3–16.0)	9.4 (5.7–13.8)	13.4 (10.9–14.8)
Female sex	13 (59.1)	7 (58.3)	14 (70)	71 (64.5)	25 (58)
Time from disease onset to sampling, median (IQR) months	0 (0–2.5)	75.3 (39.9–106.5)	–	46.9 (11.3–132.1)	–
Autoimmune comorbidity	0	0	–	1	–
Other autoantibodies†					
ANA positive	11 (52.4)	4 (36.4)	–	–	–
ENA positive	1 (5)	2 (20)	–	–	–
MSA positive	4 (19)	2 (40)	–	52 (47)	–
Anti-NXP-2 positive	1 (5)	1 (8.3)	–	22 (20)	–
Anti-TIF1γ positive	2 (9)	0	–	14 (13)	–
Anti-PL-12 positive	1 (5)	0	–	0	–
Anti-Jo-1 positive	0	1 (8.3)	–	1 (1)	–
Anti-MDA-5 positive	0	0	–	7 (6)	–
Anti-HMGCR positive	0	0	–	3 (3)	–
Anti-Mi-2 positive	0	0	–	2 (2)	–
Anti-PL-7 positive	0	0	–	1 (1)	–
Anti-SAE positive	0	0	–	1 (1)	–
Anti-SRP positive	0	0	–	1 (1)	–
Disease activity‡					
CMAS, median (IQR) (range 0–52)	28 (12.3–44.5)	52 (51.3–52.0)	–	45 (31.5–52.0)	–
PhGA, median (IQR) (range 0–10)	6 (2.6–7.0)	0 (0)	–	2.15 (0.7–5.1)	–
CK, median (IQR) IU/liter§	374 (112.5–3,222.8)	118 (97.5–142.3)	–	110.5 (64.0–824.5)	–
Medication					
Steroids only	–	–	–	4 (4)	–
Steroids + other immunomodulatory drug(s)	6 (27)	–	–	36 (33)	–
Other immunomodulatory drug(s) only	1 (5)	4 (33)	–	11 (10)	–
None	15 (68)	8 (67)	–	14 (13)	–

* Except where indicated otherwise, values are the number (%). IQR = interquartile range; anti-TIF1γ = anti-transcriptional intermediary factor 1γ; anti-SAE = anti-small ubiquitin-like modifier-1 activating enzyme; anti-SRP = anti-signal recognition particle.

† In the Dutch cohort, there were missing data on the following: antinuclear antibody (ANA) testing in 1 juvenile dermatomyositis (DM) patient with active disease and 1 with inactive disease, extractable nuclear antigen (ENA) testing in 2 juvenile DM patients with active disease and 2 with inactive disease, myositis-specific antibody (MSA) testing in 1 juvenile DM patient with active disease and 7 with inactive disease. No data were available on ANA and ENA testing in the British cohort. In the Dutch cohort, MSA (Euroimmun DL 1530-6401-4 G) and ENA (Euroimmun DL1590-6401-3 G) were tested by line blot assay. ANA was tested by immunofluorescence on HEp-2 cells. In the British cohort, MSA was tested by immunoprecipitation with confirmation by enzyme-linked immunosorbent assay for anti-melanoma differentiation-associated protein 5 (anti-MDA-5), anti-nuclear matrix protein 2 (anti-NXP-2), and anti-hydroxymethylglutaryl-coenzyme A reductase (anti-HMGCR).

‡ In the Dutch cohort, there were missing data on the Childhood Myositis Assessment Scale (CMAS) in 6 juvenile DM patients with active disease and on the physician global assessment (PhGA) in 3 juvenile DM patients with active disease.

§ Reference value for serum creatine kinase (CK) level <170 IU/liter.

Statistical analysis. We used descriptive statistics with IBM SPSS Statistics 25 and GraphPad Prism for visualization.

Patient and public involvement statement. Patients were not involved in the design of this study, but participants in the Dutch juvenile DM biomarker study and the British juvenile DM cohort and biomarker study were informed about results of the study by regular updates via the national patient organizations.

RESULTS

Anti-cN-1A autoantibodies were not detected in juvenile DM patients or healthy controls by the full-length cN-1A ELISA (Figure 1) or the full-length cN-1A-containing cell lysate immunoblotting assay (data not shown) (see Supplementary Figure 1 for a representative example, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.41660/abstract>). The

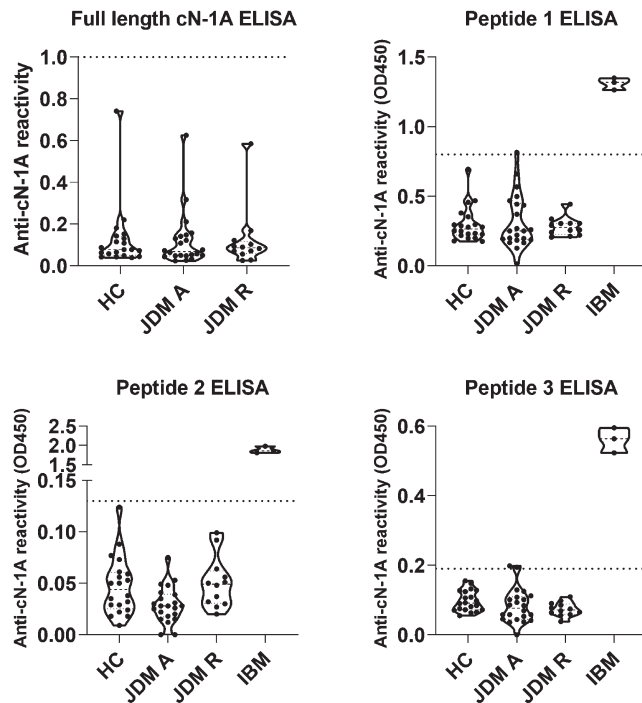


Figure 1. Results of full-length and peptide cytosolic 5'-nucleotidase 1A (cN-1A) enzyme-linked immunosorbent assays (ELISAs). Dotted lines show cutoff values. The cutoff value for the synthetic peptide ELISA was calculated based on the data for healthy adult control samples (mean + 3SD). Positivity for cN-1A was defined as a value of >0.80 for peptide 1, >0.13 for peptide 2, and >0.19 for peptide 3. Each each plate contained a positive control (inclusion body myositis [IBM] patient). Symbols represent individual subjects (healthy controls [HCs; n = 20]; IBM patient [n = 1]; juvenile DM patients with active disease [JDM A; n = 22]; juvenile DM patients with disease in remission [JDM R; n = 12]). OD450 = optical density of 450 nm.

peptide ELISA showed no anti-cN-1A reactivity in healthy controls, whereas weak, borderline reactivity against synthetic cN-1A peptides was detected in 3 juvenile DM patients with active disease (1 patient showed reactivity against peptide 1, and 2 patients showed reactivity against peptide 3) (Figure 1). In the independent validation cohort, we did not detect anti-cN-1A autoantibodies in the juvenile DM patients or healthy controls.

Comparison of repeated sampling in patients with active disease followed by disease in remission showed no anti-cN-1A reactivity in any of the 3 detection methods, either in active or inactive juvenile DM in 8 of 9 patients. The remission sample from 1 patient showed weak anti-cN-1A reactivity in the peptide ELISA (peptide 2), while the active disease sample was negative. Two juvenile DM patients who were initially tested during active disease were retested during a flare, and they were negative for anti-cN-1A autoantibody reactivity according to all 3 methods of detection at both moments. The low number of juvenile DM patients with weak anti-cN-1A reactivity made it impossible to make a reliable

comparison of clinical features between patients who were positive for anti-cN-1A and those who were negative.

DISCUSSION

Anti-cN-1A autoantibodies were not detected by full-length cN-1A ELISA or immunoblotting in juvenile DM patients or healthy juvenile controls, a finding that was substantiated in a large independent cohort. The absence of anti-cN-1A autoantibodies in juvenile DM was observed both in samples from patients with active disease and in those with disease in remission. In 3 of 34 Dutch juvenile DM patients (8.8%), weak anti-cN-1A reactivity was found using the peptide ELISA. Very low level anti-cN-1A reactivity has previously been detected in up to 5% of disease control groups using the same cN-1A peptide ELISA (13).

Our conclusions contrast with those of a recent study by Yeker et al (7), in which a large juvenile DM cohort was assessed by immunoblotting with lysates of transfected HEK 293 cells expressing cN-1A. Anti-cN-1A reactivity was found in 83 of 307 juvenile DM patients (27%), 11 of 92 healthy controls (12%), 11% of polymyositis patients, 35% of patients with overlap syndromes, and 27% of juvenile idiopathic arthritis patients. The presence of anti-cN-1A autoantibodies was related to more severe disease in juvenile DM. However, our results are consistent with the findings of a study that used an addressable laser bead immunoassay with a full-length human recombinant protein in a cohort of 40 juvenile DM patients (15), showing anti-cN-1A reactivity in none of these patients. An intermediate percentage (2 of 12 patients; 17%) of anti-cN-1A reactivity was observed in an Asian cohort of juvenile DM patients using a full-length recombinant ELISA, with confirmation by immunoprecipitation (16).

It remains to be established whether the differences observed between juvenile DM cohorts reflect heterogeneity of anti-cN-1A production among cohorts or are due to the different assays applied. Generally, studies using immunoblotting to detect anti-cN-1A antibodies have higher sensitivity and lower specificity than those that use the full-length cN-1A ELISA. The full-length cN-1A ELISA might miss linear epitopes, reducing sensitivity. However, specificity is higher than with the peptide ELISA, as the secondary antibody targets the IgG isotype only. The large differences in sensitivity and specificity of anti-cN-1A autoantibody detection between the various methods are summarized by Amlani et al (15). A head-to-head comparison of the different methods of anti-cN-1A antibody detection in a large international cohort should be performed to establish a well-validated gold standard. In clinical practice, high specificity of anti-cN-1A autoantibodies in the context of idiopathic inflammatory myopathies is more important than high sensitivity, as the presence of anti-cN-1A autoantibodies can provide additional evidence for a diagnosis of IBM instead of another idiopathic inflammatory myopathy that would require immunosuppressive therapy.

In conclusion, using a combination of detection methods, anti-cN-1A autoreactivity was not detected in juvenile DM patients or healthy subjects. An international gold standard for anti-cN-1A antibody testing should be established, as the large variation in specificity of anti-cN-1A autoantibody detection hampers its use in clinical practice.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Rietveld had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Rietveld, Wienke, van Engelen, van Royen-Kerkhof, Saris, Tansley, Pruijn.

Acquisition of data. Rietveld, Wienke, Visser, Vree Egberts, Schlumberger, van Engelen, van Royen-Kerkhof, Lu, Wedderburn, Saris, Tansley, Pruijn.

Analysis and interpretation of data. Rietveld, Wienke, Visser, Schlumberger, van Engelen, van Royen-Kerkhof, Lu, Wedderburn, Saris, Tansley, Pruijn.

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