

The indirect immunofluorescence assay autoantibody profiles of myositis patients without known myositis-specific autoantibodies

M. Casal-Dominguez^{1,2}, I. Pinal-Fernandez¹⁻³, K. Pak¹, A. Marin-Sanchez⁴,
M.T. Sanz-Martinez⁴, A. Baucells⁵, Y. Hosono¹, L. Christopher-Stine²,
A.L. Mammen^{1,2}, for the Johns Hopkins Myositis Center Group

¹National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD; ²Johns Hopkins University School of Medicine, Baltimore, MD, USA;

³Faculty of Health Sciences and Faculty of Computer Science, Multimedia and Telecommunications, Universitat Oberta de Catalunya, Barcelona; ⁴Vall d'Hebron Hospital, Barcelona;
⁵Sant Pau Hospital, Barcelona, Spain.

Abstract Objective

The indirect immunofluorescence assay (IIFA) is used to screen for the presence of autoantibodies. Our objective was to determine the prevalence and clinical features of IIFA positive myositis patients without known myositis-specific autoantibodies (MSA).

Methods

Sera from healthy comparators (HC) and patients with dermatomyositis (DM), inclusion body myositis (IBM), and polymyositis (PM) with no detectable MSA were tested by IIFA on HEp-2 cells. The pattern of positivity was classified according to the International Consensus on Antinuclear Antibody Patterns. The prevalence and frequency of each IIFA pattern were compared between the different groups.

Results

Sera from 100 HC, 71 DM, 53 IBM, and 69 PM subjects were included in the study. The IIFA was positive in 35% HC compared to 66% DM ($p<0.001$), 49% IBM, and 64% ($p<0.001$) PM sera. Among IIFA positive sera, the staining was moderate or intense in 43% HC compared to 79% DM ($p<0.001$) but just 54% IBM, and 52% PM sera. IIFA positivity was predominantly nuclear in all groups (all >69%). The most common pattern in myositis patients was fine speckled with no differences between groups. In general, IIFA positive and negative DM patients showed similar clinical features and disease activity.

Conclusion

Half of MSA-negative DM patients have moderate/strong IIFA positivity, predominantly with a fine speckled pattern. In contrast, MSA-negative PM, IBM, and healthy comparators are more often weakly positive for IIFA. These findings suggest that unidentified autoantibodies are more likely to exist in DM patients than in the other myositis groups.

Key words

indirect immunofluorescence assay, autoimmune myopathies, myositis-specific autoantibodies, immunofluorescence pattern

Maria Casal-Dominguez, MD, PhD*

Iago Pinal-Fernandez, MD, PhD*

Katherine Pak, MD

Ana Marin-Sanchez, BS

Maria T. Sanz-Martinez, BS

Andres Baucells, MD

Yuji Hosono, MD, PhD

Lisa Christopher-Stine, MD, MPH

Andrew L. Mammen, MD, PhD

*These authors contributed equally to this project.

Members of the Johns Hopkins Myositis Center group:

Harlan Michelle, Jemima Albayda,

Eleni Tiniakou, Julie Paik,

Christopher Mecoli, Sonye

K. Danoff and Tom Lloyd.

Please address correspondence to:

Andrew L. Mammen

or Maria Casal-Dominguez,

Muscle Disease Unit,

Laboratory of Muscle Stem Cells and Gene Regulation,

National Institute of Arthritis and

Musculoskeletal and Skin Diseases,

National Institutes of Health,

50 South Drive, Room 1141,

Building 50, MSC 8024,

Bethesda, MD 20892, USA.

E-mail: andrew.mammen@nih.gov

maria.casal-dominguez@nih.gov

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Introduction

Antinuclear antibodies were first described as a heterogeneous group of antibodies targeting components of the cell nuclei (1) using frozen sections of animal organs as a substrate (2). Currently, the gold standard technique for antinuclear antibody detection is indirect immunofluorescence (IIFA) on Hep-2 cells (3, 4), an epithelial cell line derived from a human laryngeal carcinoma (5). Importantly, IIFA also detects antibodies targeting cytoplasmic components and the spindle apparatus (6). Thus, IIFA can be used to detect autoantibodies against all cellular components expressed in Hep-2 cells (7-9). To harmonise IIFA testing and interpretation, a group of experts from different fields and countries created the International Consensus on ANA Patterns (ICAP), designating and describing three major groups of staining categories: nuclear, cytoplasmic, and mitotic (10). Subsequent meetings of the group established a total of 29 distinct IIFA patterns (7). The detection of antibodies by IIFA has diagnostic utility for several autoimmune diseases (11-13).

The idiopathic inflammatory myopathies (IIMs) are a heterogeneous group of diseases characterised by muscle weakness and inflammatory infiltrates on the muscle biopsy. In addition to muscle involvement, the lung, skin, and joints may also be affected (14). The most common types of IIM are dermatomyositis (DM), immune-mediated necrotising myopathy (IMNM), sporadic inclusion-body myositis (IBM), overlap myositis (including antisynthetase syndrome), and polymyositis (PM) (14-16). Importantly, ~70% of IIM patients have myositis-specific autoantibodies (MSAs) targeting nuclear and cytoplasmic proteins (17, 18) (19). However, it is unknown whether the remaining 30% of patients have an as of yet unknown MSA.

When tested by IIFA, between 50 to 80% of patients with myositis are positive (20). However, the prevalence of such positivity has not been systematically studied in myositis patients who are negative for known MSAs. Thus, the objective of the present study is to determine the prevalence and pattern

of IIFA positivity in healthy comparators and MSA-negative DM, IBM, and PM patients.

Material and methods

Patients and healthy comparators

MSA negative patients enrolled in the Johns Hopkins Myositis Center Longitudinal Cohort study between 2006 and 2015 were enrolled in the study. Patient serum samples were screened for anti-HMGCR autoantibodies by enzyme-linked immunosorbent assay. Line blotting (EUROLINE myositis profile) and immunoprecipitation from S³⁵-labelled HeLa cell lysates were used to screen for anti-SRP, -Mi2, -NXP2, -TIF1g, -MDA5, -Jo1, -PL7, -PL12, -EJ, -OJ, -SAE, -Ku and -Pm/Scl autoantibodies (21, 22). All the IBM patients were tested for anti-NT5c1A antibodies by ELISA (cN-1A [Mup44, NT5C1A], IgG semiquantitative, EA 1675-4801 G. EUROIMMUN). Each patient was classified as DM, PM, or IBM based on Bohan and Peter (23) and Griggs (24) criteria, respectively. In addition, 100 sera from healthy individuals were used as a comparator group.

Strength was evaluated by the examining physician using the Medical Research Council scale. This scale was transformed to Kendall's 0-10 scale for analysis as previously described (25). Serial strength measurements for each patient were made by the same physician. For analyses, right- and left-side measurements for arm abduction and hip flexion strength were combined and the average was used for calculations (possible range 0-10). Serum creatine kinase (CK) levels were included for the longitudinal analysis if obtained within 6 weeks before or after strength testing. Skin manifestations specific for DM (*i.e.* heliotrope rash or Gottron's sign), symptoms of esophageal involvement, and antisynthetase syndrome-associated clinical features (*e.g.* mechanics hands, Raynaud's phenomenon, arthritis, fever) were documented both retrospectively at the onset of the disease and prospectively at each visit. Interstitial lung disease was defined through a multidisciplinary approach as recommended by the American Thoracic Society (26).

HEp2 indirect immunofluorescence testing

Both myositis patient and comparator sera were tested by HEp2 cell IIFA. Serum diluted 1:80 in phosphate-buffered saline (PBS) was overlaid onto fixed Hep-2 cells (Kallestad Hep-2 Cell Line Substrate, Bio Rad, Inc, Redmond, WA) for 30 minutes at room temperature. Each slide was rinsed once with a stream of PBS and then washed with PBS for 10 minutes. After that, the slides were overlaid with 25 microliters of Fluorescein isothiocyanate (FITC) conjugated antiserum to human immunoglobulins (IgG, IgA, IgM, Kallestad FITC conjugate, ref #30446) and incubated for 20 minutes at room temperature. The slides were rinsed briefly with PBS and incubated with 3 drops of Evan's blue counterstaining diluted in 150 ml of PBS for 10 minutes. After that, slides were drained briefly, a coverslip was placed over each slide. The serum dilution 1:80 was chosen as a compromise dilution that will allow us to detect the Hep-2 cells IIFA patterns clearly and has shown a relatively low rate of positivity in healthy comparators (13.4%) (27-30).

Imaging acquisition and reading

The slides were read using a fluorescence microscope (Leica 6000) at X20, X40, and X100 power. The images were randomised and a database was created to perform a blind reading of the IIFA patterns (10). Three different Immunologists (AM, MTS, and AB) read the slides and classified them into negative or positive. If positive, they indicated the Hep-2 cells IIFA pattern according to the ICAP consensus (7). For the analysis of the different patterns, we considered only those patients who had IIFA patterns agreed upon by at least two readers.

Standard protocol approvals

The study received the approval of the Johns Hopkins and National Institutes of Health Institutional Review Boards.

Statistical analysis

Dichotomous variables were expressed as percentages and absolute frequencies, and continuous features were

Table I. Indirect immunofluorescence prevalence and intensity in the different myositis subsets compared to the healthy comparators.

	HC (n=100)	DM (n=71)	IBM (n=53)	PM (n=69)
ANA positive	35% (35)	66% (47)***	49% (26)	64% (44)***
Weak	57% (20)	21% (10)***	46% (12)	48% (21)
Moderate	17% (6)	38% (18)*	31% (8)	27% (12)
Intense	26% (9)	40% (19)	23% (6)	25% (11)
Moderate or intense	43% (15)	79% (37)***	54% (14)	52% (23)

*p<0.05, **p<0.01, ***p<0.001.

Dichotomous variables were shown as % (n) and compared using the Chi-squared or Fisher's exact tests, as appropriate. Each group was compared with the healthy comparators.

HC: healthy comparator; DM: dermatomyositis; IBM: inclusion body myositis; PM: polymyositis.

Table II. Indirect immunofluorescence pattern in the different myositis subsets compared to the healthy comparators.

	HC (n=35)	DM (n=47)	IBM (n=26)	PM (n=44)
Nuclear	89% (31)	91% (42)	69% (18)	77% (34)
Cytoplasmic	18% (6)	14% (6)	40% (10)	33% (14)
Mitotic	0% (0)	0% (0)	10% (2)	5% (2)

*p<0.05, **p<0.01, ***p<0.001.

Dichotomous variables were shown as % (n) and compared using the Chi-squared or Fisher's exact tests, as appropriate. Each group was compared with the healthy comparators.

HC: healthy comparator; DM: dermatomyositis; IBM: inclusion body myositis; PM: polymyositis.

Table III. Nuclear indirect immunofluorescence pattern in the different myositis subsets compared to the healthy comparators.

	HC (n=31)	DM (n=42)	IBM (n=18)	PM (n=34)
Fine speckled	39% (12)*	64% (27)*	61% (11)	56% (19)
Homogeneous	29% (9)**	10% (4)*	6% (1)	0% (0)***
Nuclear large/coarse speckled	13% (4)	10% (4)	6% (1)	3% (1)
Centromere	6% (2)	0% (0)	0% (0)	6% (2)
Nucleolar homogeneous	6% (2)	7% (3)	6% (1)	18% (6)
Nucleolar clumpy	3% (1)	2% (1)	6% (1)	6% (2)
Topo I speckled	3% (1)	0% (0)	0% (0)	0% (0)
Nuclear envelope punctate	0% (0)	2% (1)	11% (2)	6% (2)
Nuclear envelope smooth	0% (0)	0% (0)	6% (1)	0% (0)
Dense fine speckled	0% (0)	2% (1)	0% (0)	0% (0)
Discrete nuclear dots multiple	0% (0)	2% (1)	0% (0)	3% (1)
Nucleolar envelope punctate	0% (0)	0% (0)	0% (0)	3% (1)

*p<0.05, **p<0.01, ***p<0.001.

Dichotomous variables were shown as % (n) and compared using the Chi-squared or Fisher's exact tests, as appropriate. Each group was compared with the healthy comparators. HC: healthy comparator; DM: dermatomyositis; IBM: inclusion body myositis; PM: polymyositis.

reported as means and standard deviations (SD). Pairwise comparisons for categorical variables between groups were made using the chi-square test or Fisher's exact test, as appropriate. Student's t-test was used to compare continuous variables among groups. CK, a highly positively skewed variable, was expressed as median, first, and third quartile for descriptive purposes. All statistical analyses were performed us-

ing Stata/MP 14.1. A 2-sided *p*-value of 0.05 or less was considered significant with no correction for multiple comparisons.

Results

Sera from 193 MSA-negative myositis patients (71 DM, 53 IBM, and 69 PM) (mean age 51.3 years [SD 16.6 years], 60% female, 74% white, 12% black, and 13% other races) and 100

Table IV. Comparison of clinical features during follow-up in IIFA-positive vs. IIFA-negative dermatomyositis patients.

	IIFA+ (n=47)	IIFA- (n=24)	p-value	Total (n=71)
Muscle weakness	91% (43)	92% (22)	1.0	92% (65)
Myalgia	15% (7)	21% (5)	0.5	17% (12)
DM-specific skin involvement	98% (46)	96% (23)	1.0	97% (69)
Sclerodactyly	0% (0)	0% (0)	1.0	0% (0)
Raynaud's phenomenon	43% (20)	50% (12)	0.6	45% (32)
Telangiectasias	2% (1)	4% (1)	1.0	3% (2)
Ulcers	0% (0)	0% (0)	1.0	0% (0)
Carpal tunnel	2% (1)	0% (0)	1.0	1% (1)
Livedo reticularis	2% (1)	4% (1)	1.0	3% (2)
Mechanics hands	26% (12)	21% (5)	0.7	24% (17)
Calcinosis	9% (4)	12% (3)	0.7	10% (7)
Subcutaneous oedema	34% (16)	21% (5)	0.2	30% (21)
Puffy hands	0% (0)	8% (2)	0.1	3% (2)
Interstitial lung disease	19% (9)	21% (5)	1.0	20% (14)
Pulmonary hypertension	0% (0)	0% (0)	1.0	0% (0)
Dyspnea	62% (29)	58% (14)	0.8	61% (43)
Cough	21% (10)	21% (5)	1.0	21% (15)
Gastroesophageal reflux disease	45% (21)	54% (13)	0.4	48% (34)
Dysphagia	51% (24)	54% (13)	0.8	52% (37)
Arthritis	21% (10)	29% (7)	0.5	24% (17)
Arthralgia	70% (33)	46% (11)	0.05	62% (44)
Fever	11% (5)	17% (4)	0.5	13% (9)
Sicca syndrome	4% (2)	0% (0)	0.5	3% (2)
Pericarditis	0% (0)	0% (0)	1.0	0% (0)
Glomerulonephritis	0% (0)	0% (0)	1.0	0% (0)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

Dichotomous variables are shown as %(n) and compared using the Chi-squared or Fisher's exact tests, as appropriate. IIFA: indirect immunofluorescence.

Table V. Comparison of muscle strength and muscle enzyme levels in IIFA-positive vs. IIFA-negative dermatomyositis patients.

	IIFA+ (n=47)	IIFA- (n=24)	p-value	Total (n=71)
Mean hip flexor strength	8.7 (1.6)	8.9 (1.9)	0.6	8.8 (1.7)
Hip flexors strength at last visit	8.7 (1.6)	9.0 (1.5)	0.6	8.8 (1.5)
Mean arm abductor strength	9.3 (1.0)	9.4 (1.2)	0.8	9.3 (1.1)
Arm abductors strength at last visit	9.2 (1.3)	8.8 (2.4)	0.5	9.0 (1.7)
Median CK	109 (58-280)	135 (55-204)	0.8	110 (58-280)
Maximum CK	155 (81-813)	168 (77-395)	0.6	155 (77-758)
Mean aldolase	8.8 (4.6)	7.1 (2.5)	0.1	8.2 (4.0)
Maximum aldolase	12.2 (12.3)	7.8 (2.9)	0.1	10.6 (10.1)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Strength values were expressed as means (SD) and CK as medians (Q1-Q3). Bivariate comparisons were made using Student's t-test for the strength and Wilcoxon rank-sum test for CK. IIFA: indirect immunofluorescence.

healthy comparators (mean age 52.4 years [SD 14 years], 41% female, 61% white, 24% black, and 14% other races) were included in the study. Using a fixed dilution of 1:80, thirty-five percent of the healthy comparators were IIFA positive compared to 66% of DM ($p<0.001$) 64% of PM ($p<0.001$), and 49% of IBM patients (Table I). Forty-five percent of the IBM patients were anti-NT5c1A positive and there was no

association between this autoantibody and the IIFA positivity (54% of positive and 37% of negative IIFA IBM patients had anti-NT5c1A autoantibodies [$p=0.2$]).

Most IIFA-positive DM patients had moderate or intense IIFA positivity (79%, $p<0.001$) compared to 52% with PM, 54% with IBM and 43% of healthy comparators. In contrast, very few DM patients showed weak IIFA-

positivity (21%, $p<0.001$) compared to 48% of PM, 46% of IBM, and 57% of healthy comparators (Table I).

In most IIFA-positive samples, the pattern was nuclear both for myositis patients and healthy comparators (all $>69\%$). In contrast, 40% of IIFA-positive IBM and 33% of IIFA-positive PM patients had a cytoplasmic pattern but most of them had weak cytoplasmic staining; however, this was not significantly different compared to the rest of the subjects included in the study. Finally, mitotic patterns were rarely noted in any of the groups (Table II). The most common IIFA pattern in myositis patients was the nuclear fine speckled, which was significantly more prevalent in DM than in the comparators (64% vs. 39%, $p=0.03$) (Table III).

The clinical features, the severity of the muscle weakness and muscle enzyme levels were not significantly different between IIFA-positive and IIFA-negative DM patients (Tables IV and V). Less than one-third of the 14 PM patients with a cytoplasmic IIFA pattern showed the characteristic clinical features of the antisynthetase syndrome (29% had ILD, 29% Raynaud's phenomenon, 21% arthritis, 7.1% fever, and 0% mechanic's hands).

Discussion

In this cross-sectional study of a large cohort of MSA-negative myositis patients, we found that those with DM had a higher prevalence and higher intensity of IIFA-positivity than patients with PM or IBM. We also demonstrated that the nuclear fine speckled pattern was the most common IIFA pattern in MSA-negative myositis patients. Taken together, our results suggest that previously unidentified autoantibodies may be present in MSA-negative myositis patients and that these are likely to target nuclear proteins.

Surprisingly, IBM patients had a relatively high prevalence of IIFA positivity (49%). However, most of these had a weak intensity, suggesting that these autoantibodies may be less relevant than those found in other types of myositis. Also, half of the IBM patients were positive for anti-NT5c1A autoantibodies but there was no association

between this autoantibody and the IIFA positivity.

Finally, in PM patients, the prevalence of IIFA-positivity was high (64%), but the staining intensity was often weak. Previous studies have reported that 50 to 80% of IIM patients, including those with MSAs, are IIFA-positive and the most common pattern is the nuclear speckled (20). In our study, we found a similar proportion of IIFA-positive patients among our cohort of MSA-negative myositis patients (61%). Also, we confirmed that the nuclear fine speckled was the most common pattern among autoantibody-negative myositis patients.

The strengths of our study include (a) the large sample of MSA-negative myositis patients verified by multiple testing platforms and healthy comparator groups, (b) the uniform testing methodology, (c) the blinded testing, and (d) the availability of clinical information from patients enrolled in the same single-center cohort. However, our study has several limitations. First, it is possible that our MSA screening methods (*e.g.* the line blot test) were not sufficiently sensitive and that some MSA-positive patients could have been included in the study. Additionally, the screening did not include testing for rare antisynthetase autoantibodies (*e.g.* like anti-KS, -Zo and -Ha) or myositis-associated autoantibodies such as those recognising mitochondrial proteins. Also, as we used a semiquantitative evaluation of the IIFA intensity at a fixed dilution of 1:80 and not a quantitative evaluation of the fluorescence, this may have decreased our ability to find significant differences in some of the less prevalent IIFA patterns. Finally, the positivity rate in healthy comparators was 35%, which is higher than previously reported for healthy comparators at a 1:80 dilution (31-33). In this study, we optimised the technique to obtain high-resolution images to improve the evaluation of the IIFA patterns; this may, in part, explain the unexpectedly high rate of positivity in healthy controls. Moreover, it is widely recognised that the positivity rate is highly variable depending on other factors, including the cell culture conditions or the slide preparation (34).

In this regard, the secondary antibody used in the Kallestad HEp-2 IIFA assay recognised not only IgG but also IgA, and IgM; this could help to explain the high positivity rate we observed relative to some other studies in which only IgG IIFA was detected.

These limitations notwithstanding, our study shows that most MSA-negative DM patients have prevalent and intense IIFA positivity and that additional nuclear autoantigens likely remain to be discovered.

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(MDI), Quantitative Muscle Testing (QMT), Myositis Functional Index-2 (FI-2), Myositis Activities Profile (MAP), Inclusion Body Myositis Functional Rating Scale (IBMFRS), Cutaneous Dermatomyositis Disease Area and Severity Index (CDASI), Cutaneous Assessment Tool (CAT), Dermatomyositis Skin Severity Index (DSSI), Skindex, and Dermatology Life Quality Index (DLQI). *Arthritis Care Res* (Hoboken) 2011; 63 (Suppl. 11): S118-57.

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