Serological and Clinical Characterization of Anti-dsDNA and Anti-PM/Scl Double-Positive Patients

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ABSTRACT: Antibodies to double-stranded desoxyribonucleic acid (ds-DNA) and to the polymysitis/scleroderma (PM/Scl) complex are regarded as serological markers for systemic lupus erythematosus (SLE) and PM/Scl overlap syndrome, respectively. In a previous study, serum samples were identified that contained antibodies specific for both ds-DNA and PM/Scl. Fourteen of these sera were available for more detailed investigation including the autoantibody profile as determined by several methods including an addressable laser bead assay, Crithidia luciliae indirect immunofluorescence test (CLIFT) and a PM1-Alpha ELISA. Moreover, 300 samples from connective tissue disease patients and 30 PM/Scl positive samples were screened for anti-dsDNA+/PM/Scl+ specimens by CLIFT, dsDNA ELISA, and PM1-Alpha ELISA. We confirmed anti-dsDNA and anti-PM/Scl reactivity in 2/7 samples from the previous study. One sample had also anti-chromatin and anti-SS-A reactivity and the second sample was oligoreactive. In addition, 2/300 (0.7%) unselected samples from connective tissue disease patients were identified with anti-dsDNA and anti-PM/Scl reactivity. In a panel of PM1-Alpha positive samples (n = 30) collected regardless of the diagnosis of the patients, no anti-dsDNA reactivity was found. All anti-dsDNA+/anti-PM/Scl+ patients identified fulfilled sufficient criteria to be classified as definite SLE and also had at least one feature of systemic sclerosis (i.e., sclerodactyly and/or Raynaud’s phenomenon). Only 1/4 patients had clinical evidence...
of dermatomyositis. The combination of anti-dsDNA+/anti-PM/Scl+ in patients suffering from connective tissue disease is less frequently found than previously described when newer assays are used. Clinically, anti-dsDNA+/anti-PM/Scl+ patients may define a small subgroup of SLE patients with additional features of systemic sclerosis.

**KEYWORDS:** dsDNA; PM/Scl; SLE; autoantibody; peptide

**INTRODUCTION**

Antibodies to double-stranded desoxyribonucleic acid (dsDNA) and to the polymyositis/scleroderma (PM/Scl) complex are serological markers for systemic lupus erythematosus (SLE) and PM/Scl overlap syndrome, respectively. Antibodies targeting the PM/Scl complex are found in up to 55% of sera of PM/Scl patients but are also seen in isolated PM and Scl patients without clinical evidence of overlap syndrome. The PM/Scl complex was identified as the human counterpart of the yeast exosome and consists of 11–16 polypeptides with molecular masses ranging from 20 to 110 kDa. In previous studies, the human immune response targeting the PM/Scl complex has been reported to be predominantly directed against PM/Scl-75c and PM/Scl-100. Several other components of the human exosome are also recognized by anti-PM/Scl antibodies, but in lower frequency. The prime reactivity of anti-PM/Scl seropositive sera was localized to an N-terminal domain of PM/Scl-100 represented by amino acids 231–245 and mutational analysis identified the amino acids that contribute to autoantibody binding. Based on these observations and on secondary structure predictions, a local alpha-helical structure has been proposed for this major PM/Scl-100 epitope. The identified epitope called PM1-Alpha was used to develop an enzyme linked immunosorbent assay (ELISA) that was evaluated in a previous international multicenter study. The sensitivity of the PM1-Alpha peptide ELISA was superior to other assays to detect anti-PM/Scl antibodies. Autoantibodies to this peptide were detected in 55% of PM/Scl, 13.2% of Scl, 7.5% of PM, and 1.7% of unrelated controls. In a study of a panel of 36 clinically defined PM/Scl patients, there was a good agreement between ELISA based on the PM1-Alpha peptide and on the recombinant PM/Scl-100 expressed in insect cells ($R^2 = 0.82$). In a previous study, a high prevalence of anti-PM/Scl positive samples (42%) were found to contain antibodies to both dsDNA and PM/Scl, each of which are historically regarded as specific markers for SLE and PM/Scl, respectively. The present study was designed to verify the occurrence of a rare autoantibody combination (anti-dsDNA+/anti-PM/Scl+) and to use newer technologies and assays to investigate the clinical features of anti-dsDNA+/anti-PM/Scl+ positive patients.
MATERIALS AND METHODS

Serum Samples

Three panels of sera were analyzed: the first set of samples (panel 1) was taken from a previous published cohort and included seven PM/Scl+/dsDNA+ and seven PM/Scl+/dsDNA negative specimens. Panel 2 was 300 sera obtained from patients with connective tissue disease including SLE (n = 100), systemic sclerosis (SSc) (n = 40), PM (n = 30), and mixed connective tissue disease (n = 30) collected at the Faculty of Medicine, University of Calgary (Calgary, Canada) and at the Wroclaw University of Medicine (Wroclaw, Poland) all of which were screened for anti-dsDNA/anti-PM/Scl reactivity. Last, 30 anti-PM/Scl positive samples (panel 3) were collected from different centers and tested for anti-PM1-Alpha and anti-dsDNA reactivity. All samples were collected and treated in accordance to the local ethical board regulations and stored at −20°C until use.

Diagnostic Tests

Indirect immunofluorescence (IIF) was carried out on HEp-2 cells using a commercial kit (ImmunoConcepts, Sacramento, CA). Antibody titers were determined using 10-fold serial dilutions in phosphate buffered saline and the assay performed according the manufacturer’s instructions (a 1:20 sample dilution was used). Antibodies to dsDNA were determined by *Crithidia luciliae* indirect immunofluorescence test (CLIFT, ImmunoConcepts) and ds-DNA ELISA (Dr. Fooke Laboratorien GmbH, Neuss, Germany, Catalogue number 25005). For the detection of autoantibodies to the PM/Scl complex, the semiquantitative PM1-Alpha ELISA (Dr. Fooke Laboratorien GmbH, Catalogue number 25001) was used. The assay is based on a 16mer synthetic peptide derived from the known common reactive epitope of PM/Scl-100. All samples were measured in duplicate according to the instructions for use. The autoantibody profile of serum panel 1 was determined using several methods including an addressable laser bead assay (ALBIA; QUANTA Plex 8™, INOVA Diagnostics Inc., San Diego, CA) and a line assay with myositis associated antibodies (Myositis-Profil containing Mi-2, Ku, PM-Scl, Jo-1, PL-7, PL-12, Ro-52; Catalogue number DL 1530–1601 G; Euroimmun, Lübeck, Germany). The ALBIA profile allows for the semiquantitative detection of autoantibodies to chromatin, Jo-1, Rib-P, RNP, Scl-70, Sm, SS-A (Ro), and SS-B (La). The assay was performed according to the manufacturer’s instructions as previously described. For further information see also http://www.inovadx.com/detailfiles/708910.pdf.
TABLE 1. Clinical, serological, and demographic features of anti-dsDNA/anti-PM1-alpha double-positive samples

<table>
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<th>SSc</th>
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<td>Ma, Ph, U, Re, H</td>
<td>RP</td>
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</tr>
<tr>
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<td>F</td>
<td>Re, D, Se, A</td>
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<td>Scl-70</td>
</tr>
<tr>
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<td>54</td>
<td>H</td>
<td>F</td>
<td>A, Ph, Ma, Re</td>
<td>RP</td>
<td></td>
</tr>
</tbody>
</table>

A = arthritis; D = discoid rash; f = female; H = haemolytic anemia; Ma = molar rash; Ph = photosensitivity; Re = glomerulonephritis; RP = Raynaud’s phenomenon; Sc = sclerodactyly; Se = serositis; U = oral ulcers.

Serological follow-up patient 245/04 was done as follows. ANA were determined by IIF on HEp2 cells, ENA by line immunoassay, dsDNA by CLIFT, and anti-cardiolipin antibodies (IgG, IgM) by ELISA (all from Euroimmun).

RESULTS

In panel 1, we confirmed anti-dsDNA seropositivity in 3/7, anti-PM/Scl reactivity in 3/14, and anti-dsDNA and PM/Scl seropositivity in 2/7 samples (TABLE 1). One sample had also anti-chromatin and anti-SS-A reactivity and the second sample was oligospecific (anti-chromatin, anti-Rib-P, anti-Sm, anti-RNP, anti-Scl-70). In both double-positive samples (6, 14) no clear nucleolar staining could be observed by IIF on HEp-2 cells (FIG. 1). Two additional sera in the study of 300 unselected samples (panel 2) could be identified with anti-dsDNA and anti-PM/Scl reactivity. In a cohort of anti-PM/Scl positive serum samples (n = 30) collected in different centres regardless of the diagnosis of the patients, no anti-dsDNA reactivity was observed by ELISA. All four anti-dsDNA+/anti-PM/Scl+ patients fulfilled sufficient criteria of the American College of Rheumatology (ACR)19 to be classified as definite SLE and each had at least one clinical feature (sclerodactyly or Raynaud’s phenomenon, RP) of SSc. Only one of these four double-positive patients had clinical evidence of myositis (dermatomyositis, DM). The clinical, serological and demographic data of the patients are shown in TABLES 1 and 2.

Three of the anti-dsDNA/anti-PM/Scl double-positive patients (n = 4) were clinically and serologically followed. Patient 245/04, a female patient who first presented with allergy and Quincke oedema, also suffered from RP and muscle weakness at disease onset which responded well to steroids (without elevation of muscle enzymes). Since April 2004 she was diagnosed as SLE according to the ACR criteria (polyserositis, anemia, thrombocytopenia, transient proteinuria, positive ANA, and anti-dsDNA). Later on she developed some features
FIGURE 1. IIF staining patterns on HEp-2 cells. IIF analysis of a prototype anti-dsDNA and anti-PM/Scl positive serum show the typical staining pattern of anti-dsDNA and anti-PM/Scl antibodies, respectively. In both anti-dsDNA+/anti-PM/Scl+ samples (6, 14) no clear nucleolar staining pattern is visible. Original magnification X400. (In color in Annals online.)

of antiphospholipid syndrome. At diagnosis a homogenous staining of HEp-2 cells was found in IIF at a titer of 1:10,000. Anti-dsDNA and anti-Scl-70 antibodies were detected, while anti-CL (IgG and IgM) were negative. Clinically, no features of SSc was noted. The patient was treated with steroids and Arechin (Chloroquine). At the patient’s second visit in August 2006 clinical remission was observed (SLEDAI 2). ANA showed a homogenous staining pattern (1:1000), but all ENA, dsDNA and anti-CL were negative. Anti-PM1-Alpha was not tested as no serum was available for testing. The other two patients showed no clinical evidence of PM or SSc (6, 14) during clinical observation.
**DISCUSSION**

Historically, anti-PM/Scl antibodies and anti-dsDNA antibodies were reported as specific autoantibody markers for the PM/Scl overlap syndrome and SLE, respectively.\(^1\,^2\) Anti-PM/Scl antibodies were detected by IIF on HEp-2 cells, immunodiffusion (ID) assays using calf thymus extract and/or by immunoblot (IB) using human cell extracts.\(^4\,^5\) Antibodies to dsDNA can be detected using a variety of methods, but the CLIFT and FARR assay are considered as the most specific assays.\(^20\) The detection of anti-PM/Scl antibodies by IB and IIF is difficult because the reactivity of the antibodies, particularly PM/Scl-75, in cell extracts is notoriously weak in the IB assay due to the importance of conformational epitopes for strong autoantibody binding.\(^21\) Although anti-PM/Scl antibodies have been reported to produce a typical staining of the nucleoli, the identification of anti-PM/Scl seropositivity by IIF is a difficult task and requires intense laboratory experience with detailed training in the interpretation of IIF patterns.\(^22\) The detection of anti-PM/Scl by screening IIF may also vary depending on the manufacturer of the HEp-2 kit and the secondary antibody reagents used (unpubl. obs.). In addition, other autoantibodies, such as antibodies to fibrillarin and RNA polymerase (RNAP), also stain the nucleoli making the specific assignment of autoantibody specificity an even bigger challenge.\(^23\) The diagnosis of PM/Scl overlap syndrome, SSc, and PM may be improved by providing an antigen array that includes different nucleolar antigens such as fibrillarin, RNA polymerase I, NOR-90/UBF, Th/To, nucleolin, B23 (nucleophosphmin), Ku, and PM/Scl for the more specific analysis of sera that produce a nucleolar IIF pattern.\(^24\) In this context, it is noteworthy that commercial test systems for the detection of anti-fibrillarin (Mikrogen, Munich, Germany) and anti-RNAP antibodies (MBL, Nagoya, Japan) recently became available.

Vandergheynst and colleagues identified 14 patients with anti-PM/Scl seropositivity by IIF, ID, or IB.\(^25\) In these patients the most frequently observed clinical features (85% of the patients) were pulmonary interstitial disease and arthralgia or arthritis. Of interest, there was a relatively high incidence of renal disease in the anti-PM/Scl seropositive group (3/14). Two of the patients with renal crisis were diagnosed as PM/Scl overlap syndrome and one as SSc. Moreover, none of the 14 anti-PM/Scl positive patients had cancer or died after a mean follow-up of 6.1 years.\(^25\) Neither patient had SLE nor was anti-dsDNA detected in their sera.

In the study of Warner and Greidinger anti-PM/Scl antibodies were detected by ID using calf thymus nuclear antigen in combination with a PM/Scl prototype serum and anti-dsDNA antibodies by CLIFT using an in-house assay.\(^18\) In the present study we used an ELISA with a PM/Scl-100 derived peptide termed PM1-Alpha\(^15\,^17\) for the detection of anti-PM/Scl antibodies and CLIFT and ELISA for the detection of anti-dsDNA antibodies. The native PM/Scl antigen employed by Warner and Greidinger comprised several B cell epitopes.\(^18\) In
<table>
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<td>A</td>
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Samples collected for the present study

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<td>/</td>
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**NOTE:** aab = autoantibody; A = arthritis; Bx = muscle biopsy consistent with myositis; D = discoid rash; DM = dermatomyositis; DP = digital pits; H = haemolytic anaemia; I = immunological abnormalities; Ma = malar rash; nDNA = native DNA; N = neuropsychiatric lupus; Ph = photosensitivity; Pu = pulmonary fibrosis; Re = glomerulonephritis; Rib-P = ribosomal P protein; RNP = ribonucleoprotein; RP = Raynaud’s phenomenon; Sc = sclerodactyly; Se = serositis; U = oral ulcers; We = proximal muscle weakness.
contrast, the PM1-Alpha assay used in the current study was based on a single 16mer peptide comprising the major PM/Scl-100 epitope. Therefore, the difference in the anti-PM/Scl results between the original study and the current one might be attributed to the recognition of different PM/Scl epitopes as already suggested.

The variation in the anti-dsDNA antibody results is more difficult to explain as in both investigations nDNA antibodies were detected by CLIFT. One might speculate that there is a difference in the sensitivity or specificity between the in-house CLIFT test used by Warner and Greidinger and the commercial CLIFT assay (ImmunoConcepts) as the in-house method was performed at a 1:10 dilution and the commercial test at 1:20. It is noteworthy that the anti-dsDNA titers of the anti-PM/Scl positive samples were significantly lower than that of a matched anti-dsDNA positive patient cohort without anti-PM/Scl reactivity (mean 64.1 versus 712.5). Another explanation may be a putative loss in autoantibody reactivity of the specimens due to long-term storage (up to 30 years). Although the sample were stored frozen at $-20^\circ C$ it cannot be conclusively proven that the samples may have been subjected to freezing and thawing cycles, which could reduce the autoantibody reactivity in these samples.

The clinical relevance of anti-PM/Scl antibodies in anti-dsDNA positive specimens remains unclear. Warner and Greidinger concluded that in anti-dsDNA$^+$ patients anti-PM/Scl positivity has limited clinical relevance. This is in agreement with the observation of anti-PM/Scl antibodies in patients without clinical PM or Scl. In contrast, Borrows et al. described the de novo development of anti-PM/Scl antibodies, dysphagia, and muscle weakness in an SLE patient with anti-dsDNA antibodies and concluded that anti-PM/Scl antibodies had clinical relevance in this patient. A plausible explanation for the high prevalence of PM/Scl and dsDNA double-positive specimens in the Missouri cohort compared to the PM/Scl positive samples from the panel 3 cohort in the present study (42% versus 0%) could also be due to differing referral patterns. At the time the samples were collected, the University of Missouri was a major referral center particularly for patients with putative lupus overlap syndromes. In contrast, the samples from panel 3 were mainly from unselected patients with SSc, PM, DM, or related diseases referred by a spectrum of clinicians that included family physicians, internists and various specialists including neurologists and rheumatologists. Furthermore, unlike the routine test algorithm of most clinical diagnostic laboratories, all ANA positive samples at the University of Missouri were assayed for anti-PM/Scl antibodies. In contrast, most laboratories only assay for anti-PM/Scl antibodies if specifically requested as part of clinical investigation of possible PM or SSc. In a previous study, anti-Scl-70 antibodies, historically known as highly specific marker for SSc have been reported in SLE patients with a prevalence of up to 25%. Two patients of our study with anti-dsDNA and anti-PM/Scl reactivity had also antibodies to Scl-70.
In our study all patients fulfilled at least four of the ACR SLE criteria and had RP and 1/4 patients had clinical evidence of DM. As certain autoantibodies have been reported to precede the underlying systemic rheumatic disease for many years, it remains unclear if the anti-PM/Scl reactivity in SLE patients predict the long-term progression to PM or SSc. Follow-up of three anti-dsDNA/anti-PM/Scl double-positive patients revealed muscle weakness (without elevated muscle enzymes) in one patient (245/04) which responded well to steroid therapy, an observation which is in good agreement to cases reported in literature. No clinical evidence of SSc and/or PM was observed in the two other patients (6, 12) indicating that anti-PM/Scl antibodies have limited or no clinical value in these patients. Longitudinal studies of anti-dsDNA/anti-PM/Scl positive patients are mandatory to shed more light on the clinical impact on anti-PM/Scl antibodies in SLE patients with anti-dsDNA reactivity.

CONCLUSION

The rare combination of anti-dsDNA+/anti-PM/Scl+ occurs in patients suffering from connective tissue disease but appear to be less frequent than previously described when state of the art detection methods are used. Clinically, anti-dsDNA+/anti-PM/Scl+ patients may define a subgroup of patients with SLE and additional features of SSc (sclerodactyly and/or RP).

LIST OF ABBREVIATIONS

aab = autoantibody; anti-CL = anti-cardiolipin; ACR = American College of Rheumatology; ALBIA = addressable laser bead immunoassay; ANA = antinuclear antibodies; CLIFT = Crithidia luciliae indirect immunofluorescence test; DM = dermatomyositis; ELISA = enzyme-linked immunosorbent assay; IB = immunoblot; ID = immunodiffusion; IIF = indirect immunofluorescence; PM = polymyositis; RA = rheumatoid arthritis; RU = relative units; Scl = scleroderma; SLE = systemic lupus erythematosus; SSc = systemic sclerosis.

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REFERENCES


