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# Analysis of Antinuclear and Anti-double Stranded DNA Antibodies in Sera of Systemic Lupus Erythematosus Patients in Malaysia

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**Abstract:** An analysis of the profile of antinuclear (ANA) and anti-dsDNA antibodies in sera of SLE patients and related diseases revealed similarities and differences between ANA-immunofluorescence test (IF-ANA), anti-dsDNA using hemagglutination test (HA-dsDNA) and immunofluorescence test (IF-dsDNA) in detecting positive sera. Of 1620 sera examined, 179 (11.0%) were positive for ANA when tested under non-stringent condition (titre≥1:10) but only 71 (4.4%) when tested under stringent condition (titre≥1:80). Most of the sera from non-SLE patients were negative when tested under the stringent condition. However, Mixed Connective Tissue Disease (MCTD), Discoid Lupus Erythematosus (DLE) and scleroderma exhibited high percentage of ANA positive cases and high titre antibodies. Analysis of anti-dsDNA antibodies in 85 sera from confirmed SLE and 27 non-SLE cases revealed that the different screening method detected antibodies to different autoantigens or different form of the same antigens. The total number of sera detected by HA-dsDNA for SLE and non-SLE sera were 80.7 and 63.0%, respectively. The corresponding values for IF-dsDNA were 73.7 and 0.0%, showing that this test has diagnostic value for SLE. This was supported by the greater reduction in the percentage of positive sera of treated SLE patients when screened with IF-dsDNA method (from 73.7 to 17.9%) as compared to HA-dsDNA (from 80.7 to 71.4%). The implication of this reduction on search for SLE therapeutics was discussed.

**Key words:** SLE, antinuclear antibodies, ANA, anti-dsDNA, syntonic lupus erythematosus

## INTRODUCTION

Systemic Lupus Erythematosus (SLE) is an autoimmune disease which shows different clinical manifestations. At the early phase of the disease, the clinical symptoms observed include fever, reduced body weight, malaise and lethargy[1]. During the active stage, SLE can affect several important organs such as kidney, joints, skin and central nervous system including the brain[2,3]. Other manifestations of the disease include Raynaud syndrome, discoid lupus, serositis, neurological disorder and kidney failures which may be due to nephritis syndrome[4].

Among the several indicators of SLE is the presence of antinuclear antibodies (ANA) in peripheral blood. ANA are heterogeneous and include autoantibodies to nuclear DNA, histone or non-histone proteins, nucleolar antigens and several other antigens[5-8]. However, in addition to SLE patients, ANA are also found in patients of other autoimmune diseases such as Mixed Connective Tissue Disease (MCTD), Rheumatoid Arthritis (RA), scleroderma and Discoid Lupus Erythematosus (DLE)[9]. The higher frequency and high titres of ANA present in the blood enable workers to distinguish autoimmune disease from other rheumatologic diseases.

Due to the heterogeneity of ANA, the presence of these antibodies is useful in the prognosis of SLE. Confirmatory tests based on the presence of other antibodies, which are more specific to SLE, are subsequently carried out, including detection of anti-dsDNA and anti-Smith antibodies[10,11]. Recently, Dahle *et al.*[12] conducted a comprehensive experiment and then discussed the value of immunofluorescence microscopy for the detection of antinuclear antibodies (IF-ANA). They concluded that, despite its limitations, IF-ANA should continue to be used in routine diagnosis. In the present study, data on the profiles of ANA and anti-dsDNA antibodies in sera of Malaysian patients with rheumatologic diseases were obtained and analysed in the context of the finding of Dahle *et al.*[12].

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## MATERIALS AND METHODS

**Serum samples:** For the ANA assay a total of 1620 sera were selected from individuals known or suspected to have the autoimmune or related diseases. The sera were obtained in 1988-1990. For the anti-dsDNA test, a total of 112 sera were used, of which 85 were from confirmed SLE patients and 27 were from patients with non-SLE autoimmune diseases.

**Immunofluorescence test for ANA:** The substrates used for the immunofluorescent tests for ANA (IF-ANA) were cryostat sections of rat liver tissues[13,14]. The liver sections were prepared according to Beck[15] with modifications. The tissue was cut into small squares (3x3x2 mm) frozen in liquid nitrogen and then kept at -70°C. Sectioning of the frozen tissues was done using the Cryocut equipment (American Optical). Fourteen sections of 4 μm thickness were arranged on a glass slide and then stored frozen at -20°C (or -40°C). Immediately before use, the slides were fixed with acetone for 5-10 min.

The procedure for IF-ANA test as described by Cleymaet and Nakamura[16] was used to assay ANA levels in the 1620 serum samples. The sera were first diluted to 1/10 and when they were found positive, further dilutions of up to 1/640 (in PBS) were used. The sera were flooded onto slides and after 10 min, the slides were washed twice with PBS (pH 7.2) followed by the treatment for 30 min with fluorescent-conjugated sheep anti-human immunoglobulin. After washing, the slides were overlaid with glycerol and then covered with cover slips. Positive and negative reactions as well as the patterns of the nuclear fluorescence were observed using a fluorescent microscope.

**Anti-dsDNA antibody assay:** Two methods of assay were used, that is Hemagglutination Assay (HA) and Immunofluorescence Assay (IF). In HA test for anti-dsDNA antibody (HA-dsDNA), sera for the test were first heated for 30 min at 56°C to inactivate complement. This was followed by serial 2-fold dilution with PBS (pH 7.2) in a microtiter plate, beginning at 1:5 dilution. To each of the wells containing 25 μL diluted sera, was added 75 μL of sensitised chicken erythrocytes. This treated microtiter plate shaken in a microshaker and then incubated at 4°C for 2 h. Visual examinations were carried out to obtain the scores of negative (-), not determined (±), weak (+), intermediate (2+) and strong (3+). Sera with titer of 1:80 or greater were considered positive for anti-dsDNA antibody.

For the immunofluorescence anti-dsDNA antibody (IF-dsDNA) assay, serum samples from SLE patients were diluted to 1:10 and then flooded onto smears of *Crithidia luciliae* on glass slides. The glass slides were incubated at room temperature for 30 min, washed with PBS (pH 7.2) and then blotted dry with soft tissue paper. The slides were then treated with FITC and further incubated for 30 min. After washing, the slides were fixed and covered with a coverslip and observed for fluorescence under a fluorescent microscope[17,18].

## RESULTS AND DISCUSSION

While it is not meant to represent the overall picture of the Malaysian population, the number of samples was large enough to give an indication of the possible trend of profiles of the ANA and anti-dsDNA antibodies in the Malaysian patients with autoimmune diseases.

**Profile of ANA in sera:** The immunofluorescence test detected ANA in various autoimmune syndromes with titers ranging from 1:10 to greater than 1:640 (Table 1). Three forms of ANA patterns were observed. Of the 180 samples, 53% (95) were homogenous, 25.6% (46) were speckled and 21.2% (38) were peripheral. None of the 180 ANA positive sera was of the nucleolar type. Dahle *et al.*[12] reported 62% were homogenous, 22% were speckled and 15% were nucleolar. The differences could be due to the use of rat liver cells in this study as compared to HEp-2 used their study.

Table 1 shows the distribution of positive sera among the 1620 sera used in the study. Using a non-stringent criterion 179 (11%) of the Sera were positive for ANA. Sera from confirmed SLE patients were highest (82.4%). This was followed by MTDC (80%), DLE (30%) and scleroderma (27.8%). Other types of diseases recorded percentages of positives ranging 0.7-16%. The frequency of occurrence of ANA in the sera was highest in non-organ specific rheumatologic diseases, with SLE, which is the extreme of the non-organ specific rheumatologic diseases, being the highest.

The detection of ANA in non-SLE patients when a non-stringent criterion was used made IF-ANA test worthless in routine diagnosis[12]. Hence, a more stringent criterion (a titer of 80 was considered positive) was used. When this cut-off titre was used only few types of diseases were positive for ANA (Table 1). The cut-off ANA titre of 80 may be taken as similar to 100 of Dahle *et al.*[12], since it was found that Hep-2 or Vero cells were more sensitive than the rat liver tissues used in this study[19] Besides, the majority of the sera of patients with the above diseases showed higher ANA titers, some reaching 640. For example, 26 of

Table 1: Profile of antinuclear antibodies (ANA) of 1606 sera from patients with different forms of autoimmune disorders

| | Number of sera with different titers | | | | Number (%) of positives | |
|---|---|---|---|---|---|---|
| Diseases | Total | 10-40 | 80-160 | 320-640 | Not stringent[a] | Stringent |
| SLE (confirmed)[a] | 85 | 30 (35.3) | 14 (16.5) | 26 (30.6) | 70 (82.4) | 40 (47.1) |
| SLE (unconfirmed) | 161 | 19 (11.8) | 7 (4.3) | 8 (5.0) | 34 (21.1) | 15 (9.3) |
| MCTD | 5 | 0 | 0 | 4 (80.0) | 4 (80.0) | 4 (80.0) |
| Scleroderma | 18 | 0 | 1 (5.5) | 4 (22.0) | 5 (27.8) | 5 (27.8) |
| DLE | 10 | 2 (20.0) | 1 (10.0) | 0 | 3 (30.0) | 3 (10.1) |
| Facial erythema | 35 | 2 (5.7) | 1 (2.9) | 0 | 3 (8.6) | 3 (2.9) |
| Collagenosis | 131 | 4 (3.1) | 0 | 0 | 4 (3.1) | 0 |
| RA (confirmed) | 50 | 2 (4.0) | 6 (12.0) | 0 | 8 (16.0) | 0 |
| RA (not confirmed) | 63 | 4 (6.3) | 0 | 0 | 4 (6.3) | 0 |
| Nephritic syndrome | 52 | 5 (9.6) | 1 (1.9) | 0 | 6 (11.5) | 0 |
| Raynaud syndrome | 9 | 1 (11.1) | 1 (11.1) | 0 | 2 (22.2) | 0 |
| AIHA | 21 | 2 (1.0) | 0 | 0 | 2 (1.0) | 0 |
| ITP | 20 | 2 (1.0) | 0 | 0 | 2 (1.0) | 0 |
| Glomerulonephritis | 25 | 2 (0.8) | 0 | 0 | 2 (0.8) | 0 |
| Kidney failure | 27 | 2 (0.7) | 0 | 0 | 2 (0.7) | 0 |
| Polyarthritis | 261 | 7 (2.6) | 0 | 0 | 7 (2.7) | 0 |
| Miscellaneous | 647 | 20 (3.1) | 1 (0.2) | 0 | 21 (3.6) | 1 (0.2) |
| Total | 1620 | 104 (6.4%) | 33 (2.0%) | 42 (2.6%) | 179 (11.0%) | 71 (4.4%) |

[a]Non-stringent criteria means a titer of 1:10 or higher taken as positive; Stringent criteria means 1:80 or higher as positive

85 SLE sera and 4 of 5 MCTD sera tested were positive at titer of ≥320. Thus, the term abnormally high ANA titre can be used fittingly to describe a criteria for SLE.

The significance of ANA-negative sera of SLE patients has been the subject of discussion by several workers. Although the percentage of ANA occurrence in SLE patients is high, the use of ANA alone as the criteria for identifying SLE is inadequate[11]. The absence of positive ANA test may result in miss detection during the screening stage. Moreover, at non-active stage or at the early phase of active stage, the titers are low or even negative for ANA. It is believed that the sera, which are negative for SLE, are at the non-active stage of the disease[7]. The occurrence of low levels of ANA in normal individuals[13] and patients with other autoimmune diseases has been one of the arguments against IF-ANA as a screening test

**Profile of anti-dsDNA antibodies in sera:** In the HA and IF assays for the detection of anti-dsDNA antibodies, a titer of 20 or greater was considered positive. The profile of antinuclear and anti-DNA antibodies are shown in Table 2. The 85 sera was grouped into eight categories (i.e A-H) according to whether they were positive or negatives in the ANA, HA-dsDNA and IF-ds-DNA assays. The sera were further categorized into untreated and treated, according to whether the sera were obtained from patients not yet being treated and those under treatments. Group A sera, which represented 40% of the 85 SLE sera, contained antibodies detectable by all the three tests. The second category was those sera positive for ANA and anti-dsDNA by HA but not IF-ds-DNA (24.7%). The other categories were not as prevalent and ranged from 2.4 to 8.2%. There were 12 sera which were negative for ANA but positive for anti-dsDNA by HA test (F, 8.2%), IF test (G, 2.4%) or both (E, 3.5%). Besides, there were 7 sera which were positive for ANA but negative for dsDNA by both the HA and IF assays.

On the other side of the scale, sera in group H were hose that were negative for all the three tests. The absence (for non-stringent criterion) or low occurrence of 1.8% (in stringent criterion) support the requirement for more than one test for identification of SLE cases. The presence of variable proportions of sera for groups B – G may be due to the presence of different types or forms of targets for the binding of autoantibodies.

Among the 27 none-SLE patients with rheumatologic diseases, 17 (62.9%) were positive for anti-dsDNA antibodies when detected with HA, but none were tested positive by the IF method. The absence of IF-dsDNA positive sera (Table 3) supports the conclusion that anti-dsDNAs are diagnostic for SLE[20,21] However, specificity of the anti-dsDNA test is crucial for proper detection, since false positive can occur. Table 3 shows that 63% of the selected non-SLE sera were positive in HA-dsDNA test.

One observation that is worth mentioning from this study is the decrease in positives for anti-dsDNA antibodies in treated SLE patients (Table 2 and Table 3). When detection was made using IF-dsDNA the decrease was from 73.7 to 17.9%, as compared to 80.7 to 71.4% when HA-dsDNA was used. The difference in the decreases observed with IF-dsDNA and HA-dsDNA suggests that the two tests are relying on different antigen-antibody reactions.

The contrasting antibody profiles in the sera from untreated and treated patients may have significance in the search for SLE therapeutics. At present none of the

Table 2: Different groupings of sera according to the results of detection by IF-ANA, HA- anti-dsDNA and IF-dsDNA tests

| | | | | Number (percentage) of positive sera | | | | |
|---|---|---|---|---|---|---|---|---|
| | Results of tests | | | ANA≥10 | | ANA≥80 | | |
| Grouping | ANA | HA | IF | Untreated | Treated | Untreated | Treated | Non-SLE |
| A | + | + | + | 34(59.6) | 4(14.3) | 27(47.4) | 4(14.3) | 0(0) |
| B | + | + | - | 11(19.3) | 11(39.0) | 5(8.8) | 7(25.0) | 9(33.3) |
| C | + | - | + | 9(15.8) | 0(0) | 6(10.5) | 1(3.6) | 0(0) |
| D | + | - | - | 3(5.5) | 3(10.7) | 2(3.5) | 2(7.1) | 4(14.8) |
| E | - | + | + | 1(1.8) | 0(0) | 7(12.3) | 0(0) | 0(0) |
| F | - | + | - | 0(0.0) | 6(21.4) | 7(12.3) | 8(28.6) | 8(29.6) |
| G | - | - | + | 1(1.8) | 0(0) | 2(3.50) | 0(0) | 0(0) |
| H | - | - | - | 0(0.0) | 4(14.3) | 1(1.8) | 5(17.9) | 6(22.2) |
| Total | | | | 57 | 28 | 57 | 28 | 27 |

Table 3: Analysis of sera profiles according to matching between ANA and dsDNA tests of sera from 85 SLE and 27 non-SLE patients

| | | Number (percentage) of positive sera (minimum titre ANA titre of 80 ) | | |
|---|---|---|---|---|
| Results of tests | | Untreated (57 sera) | Treated (28 sera) | Non-SLE (27 sera) |
| **Matching ANA-dsDNA results** | | | | |
| Matching PosANA-PosHA-dsDNA | (A+B) | 32(56.1) | 12(42.9) | 9(33.3) |
| Matching NegANA-NegHA-dsDNA | (G+H) | 3(5.3) | 5(17.9) | 6(22.2) |
| Matching PosANA-PosIF-dsDNA | (A+C) | 33(57.9) | 5(17.9) | 0(0) |
| Matchibg NegANA-NegIF-dsDNA | (F+H) | 8(14.0) | 13(46.2) | 14(51.9) |
| **Total positives and negatives** | | | | |
| Total positive HA-dsDNA | (A+B+E+F) | 46(80.7) | 20(71.4) | 17(63.0) |
| Total negative HA-dsDNA | (C+D+G+H) | 42(73.7) | 5(17.9) | 0(0) |
| Total positive IF-dsDNA | (A+C+E+G) | 11(19.3) | 8(28.6) | 10(37.0) |
| Total negative IF-dsDNA | (B+D+F+H) | 15(26.7) | 23(82.1) | 27(100) |

agents used in SLE treatment are developed specifically for SLE[21]. However, non-detection of antibodies in treated patients may be useful in proteomics studies of SLE. The use of monoclonal binding studies, combinatorial chemistry along with the use of microarray studies from temporal gene expressions and the applications of proteomics knowledge perhaps could further hasten the discovery of specific targets which are responsible for the generation of the autoantibodies and their ligands which can block the autoimmune response.

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