CHAPTER 6

DISCUSSION

The nucleosome is emerging as the most reactive substrate among the nuclear antigens in SLE, since 48-80% of SLE patients were found to have antibody response to nucleosomes (1-6). In this study the histone-DNA-containing substrates, nucleosomes, were prepared for the detection of the antinucleosome antibody in SLE by indirect ELISA. The ELISA was chosen for detection of antinucleosome antibody in this study since it has been found to have high sensitivity, rapidity and simplicity.

The nucleosome is a large macromolecular complex comprised of histones and DNA, with a relative molecular mass of 250 kDa. Recent evidence obtained in murine models of SLE suggests that the nucleosome, a 154 base-pair DNA wrapped around a core histone octamer, is a preferential target for lupus autoantibodies and a putative autoantigen triggering the production of antibodies against its components, dsDNA and histones (7).

For a long time, "naked" dsDNA has been believed to be the major autoantigen in SLE. As a consequence, most of the studies on sensitivity and specificity have focused on anti-dsDNA antibodies. Nevertheless, dsDNA does not occur as such in vivo and also has poor immunogenicity in animal models. DNA outside the cell is generally present in the form of nucleosomes generated by apoptosis (7-9). Nucleosomes appear to be the particles that provide DNA in vivo, possibly making the DNA immunogenic when not properly cleared. Apoptosis defects are well known to be associated with certain animal models of lupus, and have also been discussed in connection with human SLE (9).

In order to investigate whether the nucleosome might play a key role in human lupus, we assessed, by mean of ELISAs, the serum antinucleosome antibody reactivity in SLE patients. Furthermore antinucleosome antibody was compared with other laboratory parameters i.e., anti-dsDNA antibody, C3 and C4 levels, and also with disease activity index, SLEDAI.

In the present study the nucleosomes were prepared from chicken erythrocyte nuclei. After fractionated by Sephacryl S-300 column chromatography, the nucleosome fraction was on the first peak as proven by SDS-PAGE and agarose gel electrophoresis. The result showed that nucleosomes prepared from chicken erythrocyte nuclei consisted of nucleosomal DNA banding at 154 base pairs and the core histone bands at molecular masses of 16.5k (H3), 15k (H2A), 13.5k (H2B), and 12k (H4) without the presence of H1 linker protein (31k) or any other contaminant proteins, indicating that this preparation yielded "intact" and highly purified mononucleosome core particles as previously described (10, 11). For the detection of anti-dsDNA antibody by indirect ELISA, we used highly purified commercial DNA as antigen.

In development of the indirect ELISA for antinucleosome and anti-dsDNA antibody, we performed the checkerboard titration for optimal condition. The concentration of 5 μg/ml for nucleosomes and dsDNA, and dilution of 1:4000 for the peroxidase-conjugated antihuman IgG produced better results and were chosen for subsequent tests in both assays. The ELISA system that we performed in this study did work very well with a clear background and low non-specific binding. Additionally the intra- and interassay coefficients of variation in this system were belonged to the acceptable range and, moreover, none of healthy control sera was found to positive for both autoantibodies as determined by indirect ELISA, suggesting that the assay which developed in the present study was a reliable and high specificity test. These may result from intact and highly purified nucleosomes as well as dsDNA, which used in this assay and the proper concentration of nucleosomes for coating antigen as obtained by checkerboard titration. Moreover Tween 20 and fetal bovine serum used in this assay were found to be efficient blocking agents, when compared to other blocking agent, in preliminary study, dry milk was used as blocking agent but high background was obtained (data not shown).

Since the nucleosome is a major nuclear antigen, we studied the frequency of antinucleosome antibody in 65 SLE patients (63 women and 2 men) who fulfilled at least 4 of the American College of Rheumatology (ACR) revised criteria for SLE, compared with 115 healthy controls (36 women and 79 men). Antinucleosome antibodies were presented in 34 (52.3%) of 65 lupus patients. In contrast to healthy controls, none of 115 normal controls were considered to have antinucleosome antibody activity ($P < 0.0001$). To analyze the anti-dsDNA antibody using indirect ELISA, the data demonstrated 24 (36.9%) of 65 SLE patients were found to be positive by the anti-dsDNA ELISA. Of the 115 healthy controls, none of them were revealed for anti-dsDNA positivity ($P < 0.0001$).

It remains concerns that anti-dsDNA and/or antihistone antibodies may crossreact with the nucleosome antigens used for the antinucleosome assay, and thus be measured as positive anti-nucleosome. However, it was demonstrated in previous studies that most antinucleosome positive sera showed persistent antibody reactivity with little reduction in intensity even after depletion of dsDNA-specific and/or antihistone specific antibodies using solid phase adsorption (3, 4).

The recent accumulated evidences have shown that in SLE, 48-80% lupus patients were shown to positive for antinucleosome antibodies (1-6). In this study, antinucleosome antibodies displayed a sensitivity of 52.3% in SLE, which is similar to that of previous report (2). The possibilities of wildly varying sensitivity in different studies might be due to the different population, sample size, test sensitivity and specificity etc.

In the present study, the results showed a higher frequency of antinucleosome antibodies (52.3%) than anti-dsDNA antibodies (36.9%) as investigated in other studies which revealed the similar results (1, 4, 12, 13). For specificity of anti-dsDNA ELISA, we used the cutoff for defining positive at mean + 3SD in this test. However, this result is rather low when compare to most of other studies who reported up to 80% for anti-dsDNA, although as low as 24% also has been reported (1-4, 12). The possibilities of variation in sensitivity for anti-dsDNA may similar to antinucleosome antibody as described above.

Our data also suggest that the production of anti-dsDNA may be associated with that of antinucleosome antibodies. Indeed, in the 24 anti-dsDNA positive SLE sera tested, anti-dsDNA activity was detected in 75% (18/24) concomitant with antinucleosome antibody activity. Interestingly, in anti-dsDNA negative sera, Amoura et al. (8) and others(12, 14) reported that 40-65% of this group showed antinucleosome antibody activity in SLE patients. In present study we found 16 of 34 sera (47.1%) were shown to react with nucleosomes detected by ELISA without positivity for anti-dsDNA antibody measured by the similar assay supporting the view that the antinucleosome antibody may be a good marker for anti-dsDNA negative SLE. However 6 of 24 SLE patient sera (25%), which had anti-dsDNA antibody activity without exhibited reactivity to nucleosomes, were present. According to the kinetic analysis of autoantibody production in lupus-prone mice, it demonstrated that the nucleosome-specific antibodies occur early in the disease and precede the formation of anti-dsDNA and antihistone antibodies (15). However the kinetic thereafter of these 3 autoantibodies was not clear. Moreover the studies have not been demonstrated whether the kinetic of autoantibody production in human SLE is similar in lupus mice, further longitudinal studies are required.

The recent accumulated evidence suggests that not only anti-dsDNA, but also antinucleosome, antibodies are related to the SLE pathogenic process (8). In this regard, our study, both patients with active SLE (n = 45) and patients with inactive SLE (n = 20) were analyzed, we found that, in 45 active SLE, 29 (64.4%) were positive for antinucleosome antibody. Further analysis in SLE patients who had inactive SLE, it revealed that 5 of 20 (25%) patient sera showed antinucleosome antibody positivity. Our data was similar to the previous reports (1, 4). One previous study (4) showed antinucleosome antibodies was much higher in active (100%) than those of inactive SLE patients (25%), although there was a higher frequency in active SLE than our study however the sample size (n = 12 in active SLE) which used in the previous report was smaller than this present study (4) (n = 45 for active SLE).

Moreover, in this study it demonstrated that antinucleosome antibody reactivity in SLE patients was found to have similar correlation to disease activity by using SLE Disease Activity Index (SLEDAI) ($r = 0.33$, $P = 0.007$) as anti-dsDNA ($r = 0.37$, $P = 0.002$) and strong correlation to anti-dsDNA antibody reactivity ($r = 0.82$, $P < 0.0001$). Our data are supported by many previous studies in which antinucleosome antibody were found to correlate with disease activity and anti-dsDNA (1, 2, 8, 12, 16). Further analysis for the isotype of antibody might be more meaningful, since it was demonstrated antinucleosome IgG3 subclass was present at high levels in patients with active SLE and no detected in those with inactive stage (22). Their levels also showed a positive correlation with disease activity and found to be closely associated with lupus nephritis. In contrast to IgG3 antinucleosome, no significant correlation was found between disease activity and IgG3 anti-dsDNA (22).

This finding, together with the high specificity of this test, which is positive in a restricted set of other connective tissue diseases (1), is encouraging in respect of the practical use of this parameter, antinucleosome antibody, for the early diagnosis of SLE. Apart from SLE, they were detected only in mixed connective tissue disease (MCTD) and systemic scleroderma and were absent in patients with inflammatory myopathies, primary Sjögren's syndrome, rheumatoid arthritis, primary anti-phospholipid syndrome, Wegener's granulomatosis, giant cell arteritis, Takayasu's arteritis, relapsing polychondosis, Behcet's disease and hepatitis C virus infection (1, 17, 18).

According to anti-dsDNA ELISA, we found that the prevalence of anti-dsDNA antibody in active SLE patients was 46.7% (21/45). In the inactive group, 3 of 20 (15%) inactive SLE patients had anti-dsDNA positivity. The higher frequency of antinucleosome antibodies in active SLE than those of anti-dsDNA antibodies were shown in this study as supported by the previous studies (1, 4).

Next we studied the correlation between antinucleosome and anti-dsDNA antibody with other activity markers; C3 and C4 levels. Measurement of C3, C4 complement components is widely used to assess disease activity in SLE (19). Decreased C3 levels, as well as C4 levels (20), have been reported to be associated with active disease in SLE as shown in our report. In our data antinucleosome

antibodies as well as anti-dsDNA antibodies were significantly correlated with C3 levels in SLE patients. When C4 levels were compared with antinucleosome antibody activity, no correlation was seen, however weak correlation between antinucleosome antibodies and C4 levels has been reported (14). Interestingly, all 8 sera with low C4 levels in this study had antinucleosome activity. Although anti-dsDNA antibodies showed the correlation with C4 levels but it was a weakly association.

Among 4 SLE disease markers studied in this report including antinucleosome, anti-dsDNA antibody, C3 and C4, we found that antinucleosome antibody was the most sensitive marker for SLE patients.

Further immunochemical, molecular, and cellular studies of antinucleosome antibody will clarify their etiological and pathological roles in origin of antinucleosome in human SLE. Although the accumulative evidences have demonstrated the nucleosome is a major immunogen in SLE, antinucleosome antibodies have had few applications in clinical practice. It should be emphasized that the determination of antinucleosome antibody as the earliest antibody in SLE contributes to the diagnosis of connective tissue diseases and it seem to be especially valuable in anti-dsDNA negative lupus. Use of antinucleosome antibody measurement as a marker of disease activity deserves further evaluation.