CHAPTER 5

RESULTS

Development of ELISA method for the detection of antinucleosome antibodies in SLE patients

An ELISA, indirect technique, was developed for the detection of antinucleosome antibodies in Thai SLE patients. The antigen used in this assay was the nucleosomes prepared from chicken erythrocyte nuclei and subsequently digested by Micrococcal nuclease.

Preparation of nucleosomes for antigen in ELISA

Nucleosomes were isolated by digesting chromatin from chicken erythrocyte cell nuclei with Micrococcal nuclease. The samples of the chromatin were fractionated by gel filtration chromatography on a Sephacryl S-300 column. Nucleosome fractions were collected by spectrophotometry after determination of DNA at OD_{260}. Major peak of nucleosomes was obtained. Since nucleosome was the large molecule and unable to enter the bead pores, therefore they could passed directly through the column. After determination by 1.5% agarose gel electrophoresis and SDS-PAGE, the fractions in peak 1 were found to be composed of nucleosomes.

To prove that the fractions in peak 1 was nucleosome, the nucleosomal DNA was examined by phenol extraction and ethanol precipitation respectively, and monitored by 1.5% agarose gel electrophoresis in Tris borate-EDTA buffer pH 8.3, and nucleosomal core proteins were examined by SDS-PAGE.

The presence of nucleosomal DNA

After extraction of DNA from nucleosome fractions by phenol extraction and ethanol precipitation, respectively, the precipitate was analyzed by electrophoresis. Gel was examined and photographed under UV transillumination. The result showed that nucleosomes prepared from chicken erythrocyte nuclei consisted of nucleosomal DNA banding at 154-bp (figure 10).

The presence of nucleosomal histones

Nucleosomal histones were separated by electrophoresis in a 15% polyacrylamide gel. Protein bands were revealed by soaking the gel in Coomassie Brilliant Blue R-250 staining solution and destaining solution, respectively. The SDS-PAGE analysis showed that the nucleosomes had the core histone bands at molecular masses of 16.5k (H3), 15k (H2A), 13.5k (H2B), and 12k (H4), indicating that this preparation yielded "intact" mononucleosome core particles (figure 11).

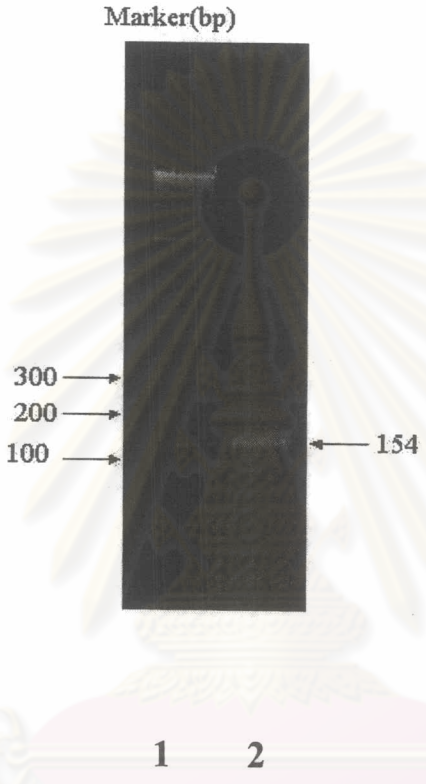


Figure 10: The presence of nucleosomal DNA was examined by phenol extraction and ethanol precipitation respectively, and monitored by agarose gel electrophoresis, applying on 1.5% agarose, 44.5 mM Tris borate buffer, and 1 mM EDTA at pH 8.3. Lane 1 showed DNA markers. The correct composition of the nucleosomes using agarose gel electrophoresis was shown the presence of nucleosomal DNA at 154 bp as shown in lane 2.

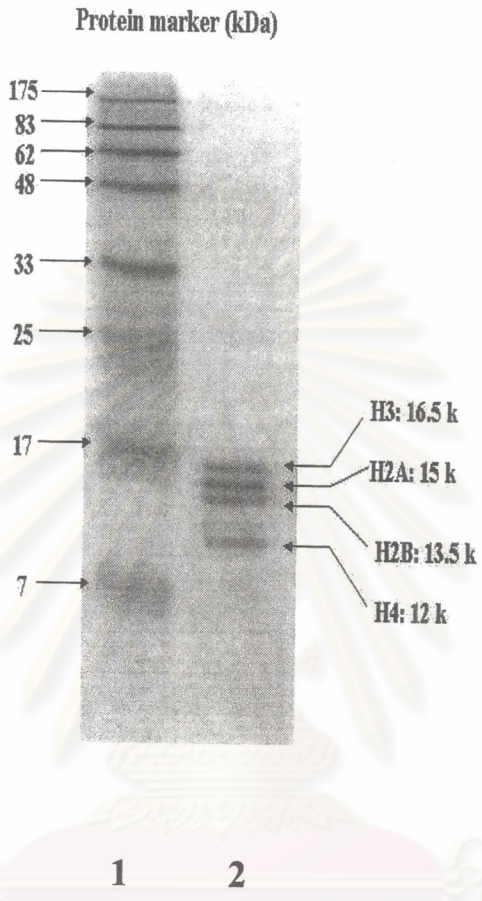


Figure 11: SDS-PAGE of nucleosomal core proteins. In order to examine the composition of nucleosome, SDS-PAGE was used to show the presence of nucleosomal histones consist of H2A, H2B, H3 and H4. The SDS-PAGE method was described by Laemmli (113), using 15% separating and 5% stacking gel. Lane 1 showed protein markers. Nucleosomal histones on SDS-PAGE banding at molecular masses of 12k (H4), 13.5k (H2B), 15k (H2A), and 16.5k (H3) as shown in lane 2.

Determination of the optimal conditions for antinucleosome indirect ELISA

To examine the optimal conditions for antinucleosome antibody using an indirect ELISA in SLE patients, the checkerboard titration was performed (figure 12).

Various concentrations of nucleosomes (2.5, 5, 7.5, and 10 μg/ml) and rabbit antihuman IgG-peroxidase conjugate (1:2000, 1:3000, 1:4000, and 1:5000 dilutions) were titrated against positive control serum (1:100 dilution), negative control serum (1:100 dilution) and PBS (reagents control) by an indirect ELISA. As shown in table 7, concentration of 5 μg/ml for nucleosomes and dilution of 1:4000 for the peroxidase-conjugated antihuman IgG produced better results and were chosen for subsequent tests.

Determination of optimal conditions for anti-dsDNA indirect ELISA system

To determine the optimal conditions for antinucleosome antibody by an indirect ELISA in SLE patients, the checkerboard titration was performed (figure 13).

Various concentrations of dsDNA (2.5, 5, 7.5, and 10 μg/ml) and rabbit antihuman IgG-peroxidase conjugate (1:2000, 1:3000, 1:4000, and 1:5000 dilutions) were titrated against positive control serum (1:100 dilution), negative control serum (1:100 dilution) and PBS (reagents control). As shown in table 8, concentration of 5 μg/ml for nucleosomes and dilution of 1:4000 for the peroxidase-conjugated antihuman IgG produced better results and were chosen for subsequent tests.

Table 7: Checkerboard titration to determine the optimal concentration of reagents in antinucleosome ELISA test. Various concentrations of nucleosomes (2.5, 5, 7.5, and 10 μg/ml) and rabbit antihuman IgG-peroxidase conjugate (1:2000, 1:3000, 1:4000, and 1:5000 dilutions) were titrated against positive control serum (1:100 dilution), negative control serum (1:100 dilution) and PBS (reagents control) by an indirect ELISA. The results are expressed as optical density (OD) at absorbance 492 nm.

Nucleosome (μg/ml.)	Conjugate 1:2000			Conjugate 1:3000			**Conjugate 1:4000**			Conjugate 1:5000		
	+	-	PBS	+	-	PBS	+	-	PBS	+	-	PBS
2.5	1.462	0.125	0.051	1.361	0.107	0.050	1.291	0.101	0.047	1.171	0.096	0.046
5	1.683	0.111	0.051	1.576	0.110	0.053	**1.452**	**0.107**	**0.046**	1.292	0.104	0.044
7.5	1.989	0.111	0.051	1.793	0.104	0.049	1.667	0.098	0.049	1.567	0.102	0.051
10	1.976	0.108	0.052	1.857	0.103	0.052	1.712	0.093	0.048	1.566	0.096	0.048

Table 8: Checkerboard titration to determine the optimal concentration of reagents in anti-dsDNA ELISA test. Various concentrations of dsDNA (2.5, 5, 7.5, and 10 µg/ml) and rabbit antihuman IgG-peroxidase conjugate (1:2000, 1:3000, 1:4000, and 1:5000 dilutions) were titrated against positive control serum (1:100 dilution), negative control serum (1:100 dilution) and PBS (reagents control). The results are expressed as optical density (OD) at absorbance 492 nm.

dsDNA (µg/ml.)	Conjugate 1:2000			Conjugate 1:3000			**Conjugate 1:4000**			Conjugate 1:5000		
	+	-	PBS	+	-	PBS	+	-	PBS	+	-	PBS
2.5	1.934	0.104	0.060	1.775	0.097	0.052	1.500	0.089	0.050	1.440	0.090	0.050
5	2.097	0.109	0.045	1.909	0.104	0.044	**1.607**	**0.109**	**0.045**	1.571	0.103	0.043
7.5	2.166	0.117	0.048	1.939	0.100	0.046	1.659	0.107	0.047	1.661	0.100	0.045
10	2.192	0.125	0.055	1.942	0.122	0.053	1.711	0.101	0.050	1.769	0.111	0.051

Precision of indirect ELISA test for antinucleosome antibody

A within-plate precision was determined using patient sera prepared by diluting in PBS-T -10% fetal calf serum. Precision for sera was calculated by running 90 wells in one microtiter plate and overall precision was estimated using the same patient sera analysed on separate microtiter plates. The results showed that the coefficient of variation (CV) of indirect ELISA for antinucleosome antibodies in within plate analysis was 8.2% and in overall precision assay was 15.4%.

Precision of indirect ELISA test for anti-dsDNA antibody

For anti-dsDNA ELISA, a within-plate precision was determined using sera from patient prepared by diluting in PBS-T-10% fetal calf serum. Precision for sera was evaluated by running 90 wells of the same microtiter plate. For overall precision, it was calculated using the same patient sera analysed on separate microtiter plates. The data revealed that the coefficient of variation (CV) of indirect ELISA for anti-dsDNA antibodies in within-plate and overall precision assay was 5.4% and 14.8%, respectively.

Detection of antinucleosome and anti-dsDNA antibodies in SLE patients

Enzyme-linked immunosorbent assay, indirect technique, using the optimal conditions of nucleosomes and dsDNA at 5 μg/ml and 1:4000 dilution of peroxidase-conjugated antihuman IgG as obtained by checkerboard titration, were used for the detection of antinucleosome antibody in this study. The threshold for positivity of antinucleosome and anti-dsDNA antibodies was defined as 3SD above the mean value in controls (0.225 and 0.287 OD units for antinucleosome and anti-dsDNA antibody positivity, respectively).

From 65 SLE patients and 115 healthy controls, as shown in table 9, the results showed that the mean of antinucleosome antibodies reactivity, revealed as optical density, was significantly higher in SLE patients versus normal controls ($p < 0.0001$). The similar result was found in anti-dsDNA antibodies, it demonstrated that mean of anti-dsDNA antibodies were statistically higher in SLE patients compared with healthy controls ($P < 0.0001$).

Table 9: The mean antinucleosome and anti-dsDNA antibody reactivity in 65 SLE patients and in 115 healthy controls. Antinucleosome and anti-dsDNA antibodies were measured by indirect enzyme-linked immunosorbent assay (indirect ELISA). Values are the mean ± SD optical density, measured by enzyme-linked immunosorbent assay. P values are versus the control group, by Student's t-test.

Antibody	SLE patients (n = 65)	Healthy controls (n = 115)	P value
Antinucleosome	0.451 ± 0.447	0.138 ± 0.029	<0.0001
Anti-dsDNA	0.378 ± 0.364	0.153 ± 0.044	<0.0001

Prevalence of antinucleosome antibody in SLE patients

From 65 SLE patients, 34 (52.3%) were considered positive (OD range 0.251-1.673) for antinucleosome antibodies. In contrast to healthy controls, none of these were present the antinucleosome antibody activity, $P < 0.0001$ (OD range 0.088-0.225) (figure 12).

Next we studied whether antinucleosome antibody activity was associated with the disease activity. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used for defining the active SLE from inactive patients. From this index score, it revealed that 45 in 65 (69.2%) patients had active SLE (SLEDAI > 5) and the other 20 (30.8%) SLE patients had inactive disease (SLEDAI 5). In 45 active SLE, 29 patient sera (64.4%) were positive for antinucleosome antibody. Further analysis in SLE patients who had inactive stage, 5 of 20 (25%) were shown antinucleosome antibody activity (figure 13), indicating that active SLE patients had significantly higher positivity (64.4%) of these antibodies than those with inactive disease (25%) as shown in figure 17 ($P = 0.008$).

Figure 12: The antinucleosome antibody reactivity in 65 SLE patients and in 115 healthy controls as measured by indirect enzyme-linked immunosorbent assay (indirect ELISA). Horizontal bar shows the mean + 3SD optical density of healthy controls. P value is versus the control group, by chi-square test.

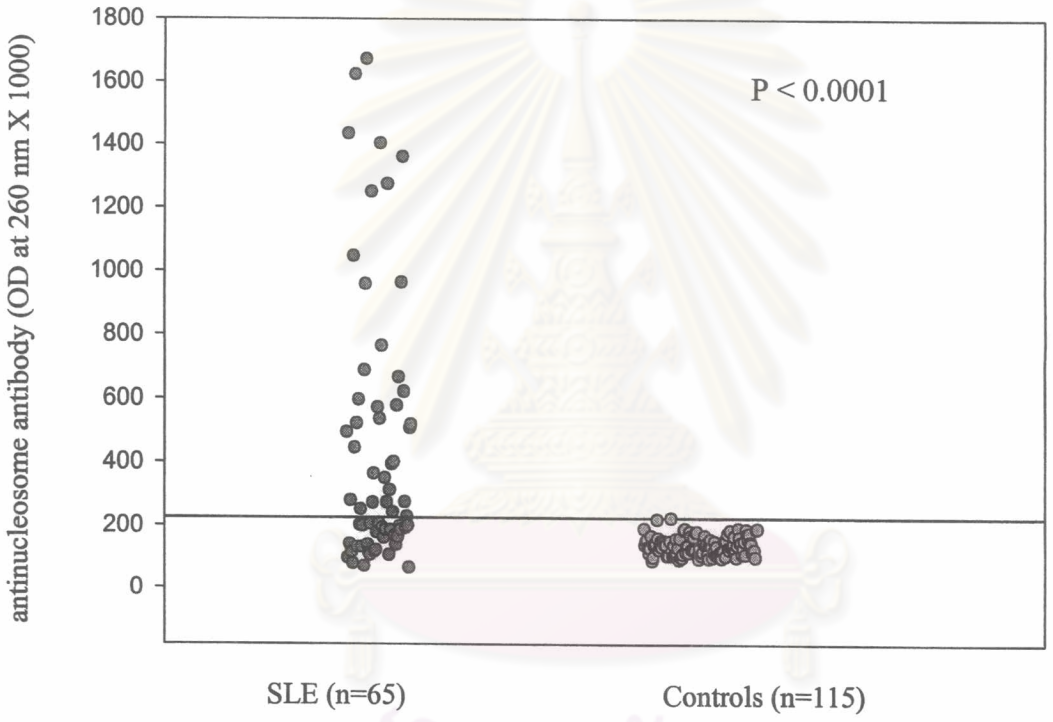
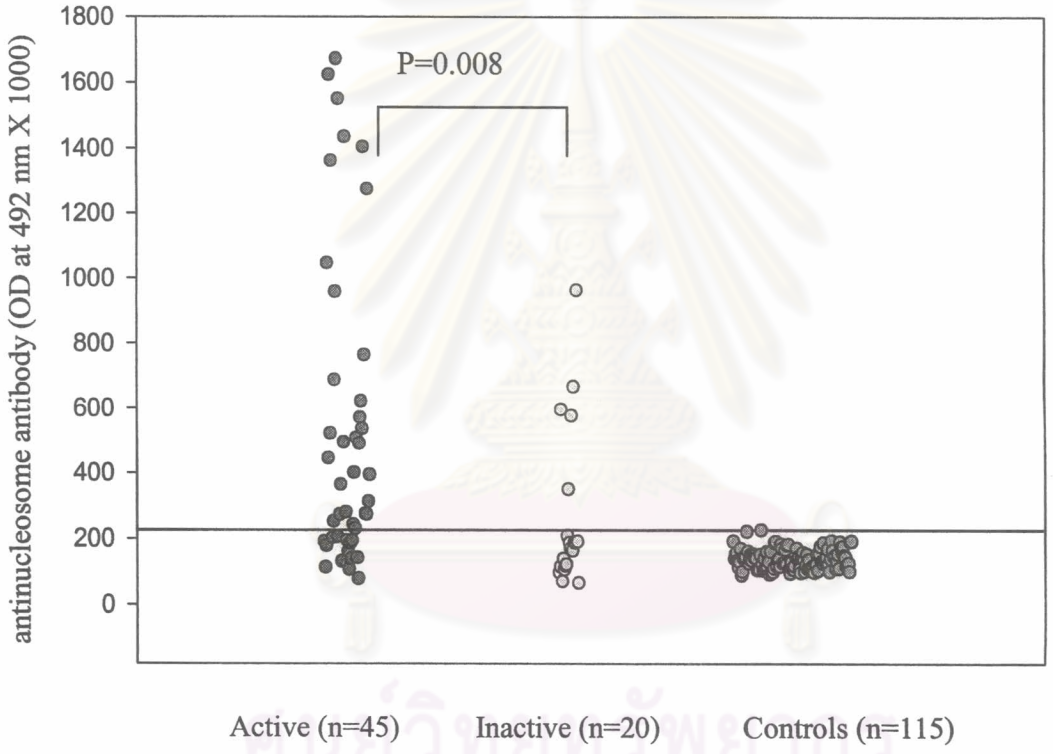


Figure 13: The antinucleosome antibody reactivity in SLE patients with active and inactive group as measured by indirect enzyme-linked immunosorbent assay (indirect ELISA). Horizontal bar shows the mean + 3SD optical density of healthy controls, P value is active versus inactive SLE, by chi-square test.



Prevalence of anti-dsDNA antibody in SLE patients

For the detection of anti-dsDNA antibody in SLE patient sera, the results demonstrated that 24 (36.9%) of 65 patients were found to be positive (OD range 0.298-1.491) in the anti-dsDNA ELISA. In 115 healthy controls, none of healthy persons were considered to positive for dsDNA antibody, $P < 0.0001$ (OD range 0.082-0.274) (figure 14).

Next we examined the prevalence of anti-dsDNA antibody in 2 SLE subgroups (active and inactive SLE). We found that the prevalence of anti-dsDNA antibody in active SLE patients was 46.7% (21 of 45). In the inactive group, 3 of 20 (15%) SLE patients were revealed anti-dsDNA positivity (figure 15), suggesting that active SLE patients had significantly higher positivity (46.7%) of these antibodies than those with inactive disease (15%) as shown in figure 17 ($P = 0.031$).

Figure 14: The anti-dsDNA antibody reactivity in 65 SLE patients and in 115 healthy controls as measured by indirect enzyme-linked immunosorbent assay (indirect ELISA). Horizontal bar shows the mean + 3SD optical density of healthy controls.
P value is versus the control group, by chi-square test.

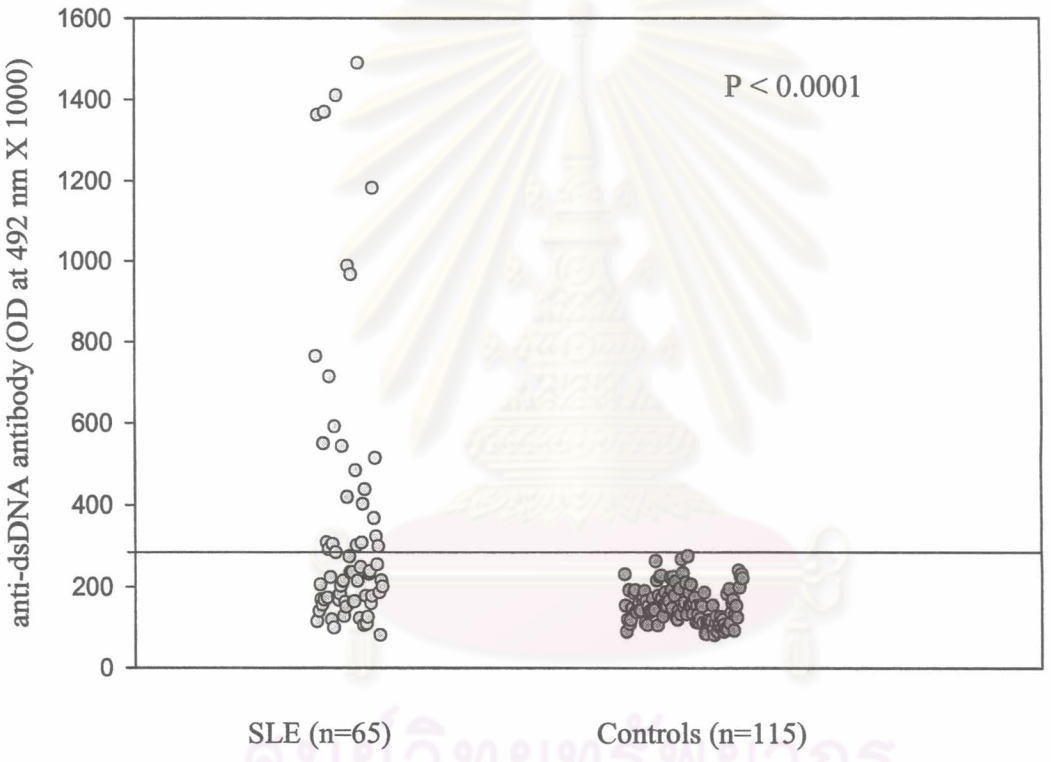
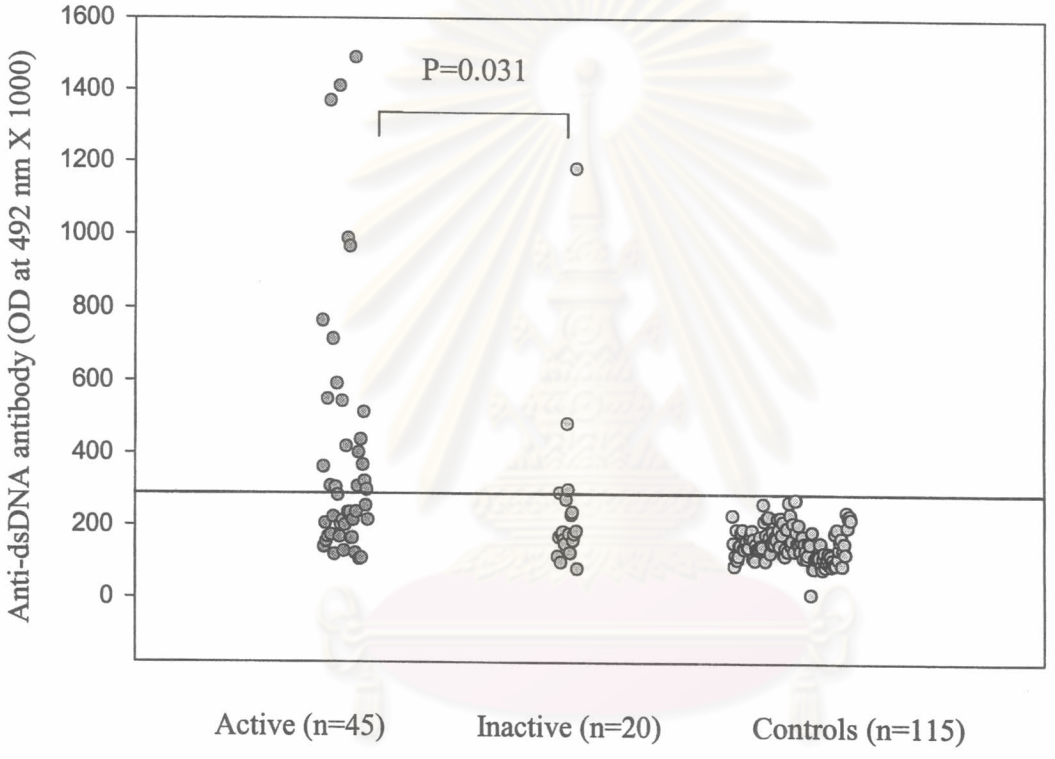


Figure 15: The anti-dsDNA antibody reactivity in SLE patients with active and inactive group as measured by indirect enzyme-linked immunosorbent assay (indirect ELISA). Horizontal bar shows the mean + 3SD optical density of healthy controls, P value is active versus inactive SLE, by chi-square test.



Levels of C3 complement component in SLE patients

We also studied other laboratory parameters for diagnosis of SLE i.e., complement C3 and C4 factors. Levels of C3 were measured directly by nephelometry using the diagnostic kit (Behring AG, Marburg, Germany). The result was evaluated by comparison with a standard of known concentration and the normal values for C3 was 76-171 mg%.

In 65 SLE patients, the results showed 19 (29.2%) sera had low complement C3 levels (range 22.9 – 74 mg%). Of these SLE patients with low C3 levels, 18 (40%) were in the active SLE (n = 45). Only 1 (5%) SLE patient sera showed low value of C3 in the inactive group (table 10).

Levels of C4 complement component in SLE patients

Next, we studied complement C4 levels as detected by nephelometry. The normal values for C4 was 10-40 mg% calculated from reference curve. In 65 SLE patients, the results present 8 (12.3%) sera were considered had low C4 levels (range 6.0 – 8.7 mg%). Additionally, all of these were in the active group (8/45; 53%) as shown in table 10.

Table 10: Association of low complement component C3 and C4 levels in SLE. The results are expressed as the percentage of low complement levels in each group.

Complement	SLE patients (n = 65)	
	Active SLE (n = 45)	Inactive SLE (n = 20)
Low C3 (n = 19)	18/45 (40%)	1/20 (5%)
Low C4 (n = 8)	8/45 (53%)	0/20 (0%)

Association of antinucleosome with anti-dsDNA antibodies and complement C3, C4 levels

To access the antinucleosome antibodies in SLE patients, we found that the prevalence of antinucleosome antibodies in both overall (52.3%) and active SLE (64.4%) was higher than those of anti-dsDNA antibodies (36.9% and 46.7% in overall and active SLE; P = 0.052 and P = 0.096, respectively) as shown in figure 16 and 17.

From the 34 SLE sera, who had antinucleosome antibody activity, we found that 18 (52.9%) patients exhibited concomitant anti-dsDNA antibody activity. Interestingly, the other 16 of 34 sera (47.1%) were shown to react with nucleosomes detected by ELISA without positivity to dsDNA measured by the similar assay. In 16 antinucleosome-positive sera without anti-dsDNA, 13 (81.3%) sera were detected in active SLE, only 3 (18.7%) sera were found in patients with inactive SLE (table 11). However 6 of 24 SLE patient sera (25%), which had anti-dsDNA antibody activity without exhibited reactivity to nucleosomes, were present.

Figure 16: Prevalence of antinucleosome and anti-dsDNA antibodies in 65 SLE patients versus 115 healthy controls by using indirect ELISA. The results are expressed as percentage of positivity of antinucleosome or anti-dsDNA antibody. P value is determined by chi-square test.

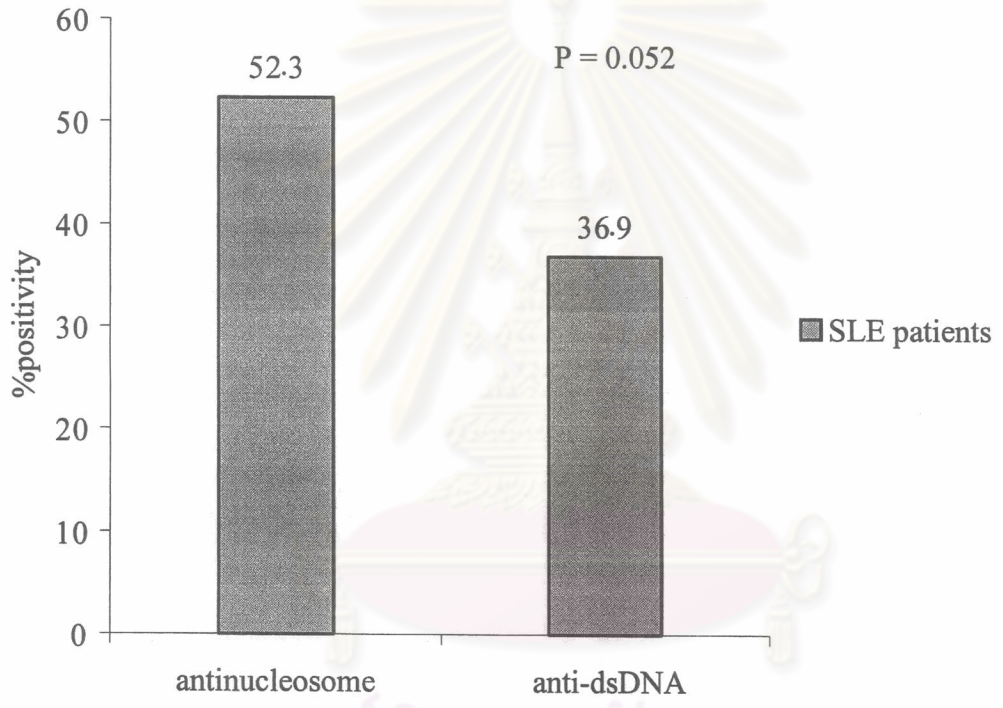


Figure 17: Association of antinucleosome and anti-dsDNA antibody with active and inactive SLE patients. In active SLE consisted of 45 patients and 20 SLE patients in inactive group. Antinucleosome and anti-dsDNA antibodies were detected by enzyme-linked immunosorbent assay (ELISA), indirect technique. The results are expressed as the percentage of antibody positivity. P values are determined by chi-square test.

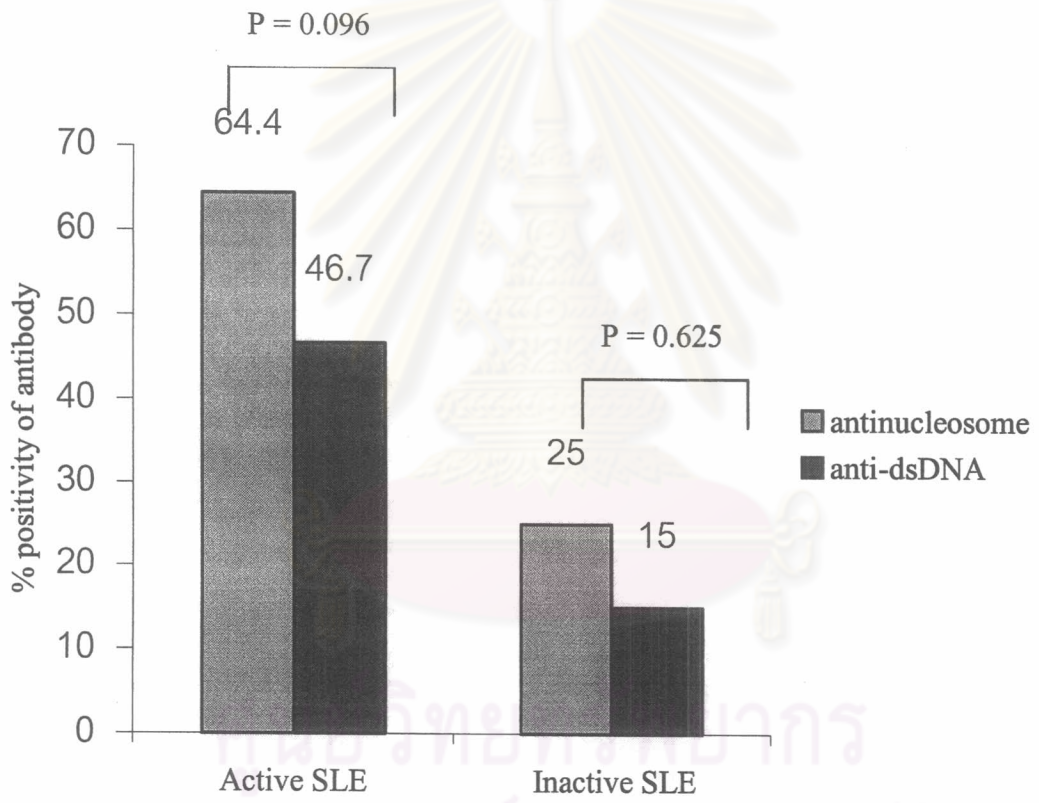


Table 11: Association of the presence of antinucleosome antibodies (with or without anti-dsDNA) with disease stage in 34 SLE patients. The results are expressed as the percentage of antibody positivity.

Positivity	Antinucleosome/Anti-dsDNA N = 18 (52.9%)		Antinucleosome (without anti-dsDNA) N = 16 (47.1%)	
	Active SLE	Inactive SLE	Active SLE	Inactive SLE
	16 (88.9%)	2 (11.1%)	13 (81.3%)	3 (18.75%)

In overall SLE, 16 (47.1%) of 34 sera with antinucleosome positivity and 11 (45.8%) of 24 anti-dsDNA positive sera were found to have low C3 levels. Of note, all sera which had low C4 (100%) were shown antinucleosome antibody activity. In contrast to anti-dsDNA positive sera, 6 of 8 (75%) sera with low C4 were shown anti-dsDNA antibody activity (table 12:A). In active SLE, the data revealed 15 (51.7%) and 8 (27.6%) of 29 antinucleosome- positive sera had low complement C3 and C4, respectively. In contrast to inactive SLE, only 1 (20%) of 5 antinucleosome-positive SLE sera were found to had low C3, of note, none of the sera in this group were revealed low complement C4 factor as demonstrated in table 12:B.

To analyze the presence of anti-dsDNA in active SLE, the data revealed 11 (52.4%) and 6 (28.6%) of 21 anti-dsDNA-positive sera had low complement C3 and C4, respectively. In contrast to inactive SLE, none of the 3 anti-dsDNA-positive sera were found to had low complement C3, C4 factor.

Table 12: Association of antinucleosome and anti-dsDNA antibodies with complement component C3, C4 levels in SLE patients. The results are expressed as the percentage of antibody positivity.

Antibody	Low Complement levels	SLE	
		Active SLE	Inactive SLE
Antinucleosome 16/34 (47.1%)	C3	15/29 (51.7%)	1/5 (20%)
Antinucleosome 8/34 (23.5%)	C4	8/29 (27.6%)	0/5 (0%)
Anti-dsDNA 11/24 (45.8%)	C3	11/21 (52.4%)	0/3 (0%)
Anti-dsDNA 6/24 (25%)	C4	6/21 (28.6%)	0/3 (0%)

Correlation between Antinucleosome antibody activity and SLEDAI

To evaluate the correlation between antinucleosome antibody activity (the result was expressed as OD unit) detected by indirect ELISA and the SLEDAI score, the simple regression analysis was used as shown in figure 18. Antinucleosome antibody activity in SLE was shown to be significant correlated with SLEDAI (r = 0.33, P = 0.007).

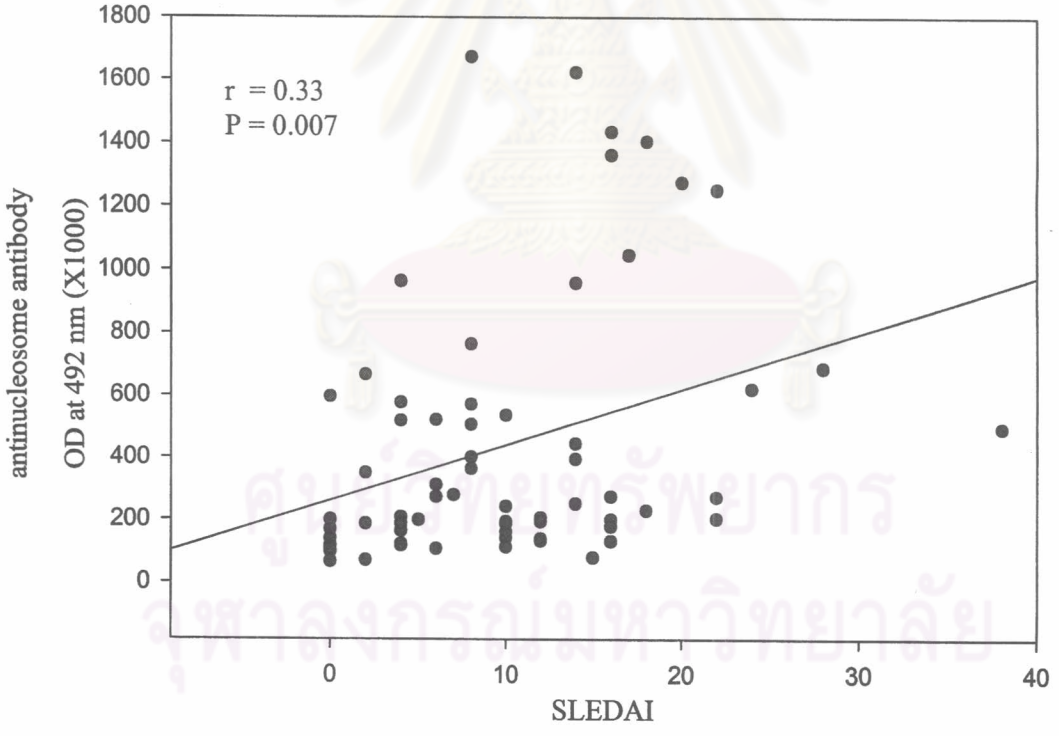


Figure 18: Correlation between antinucleosome antibody activity and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in 65 SLE patients. Correlation analysis is performed using linear regression.

Correlation between antinucleosome and anti-dsDNA antibody activities

Further examined the correlation between these 2 antibodies, the data demonstrated antinucleosome antibodies significantly correlated with anti-dsDNA antibodies in SLE patients ($r = 0.82$, $P < 0.0001$) as illustrated in figure 19.

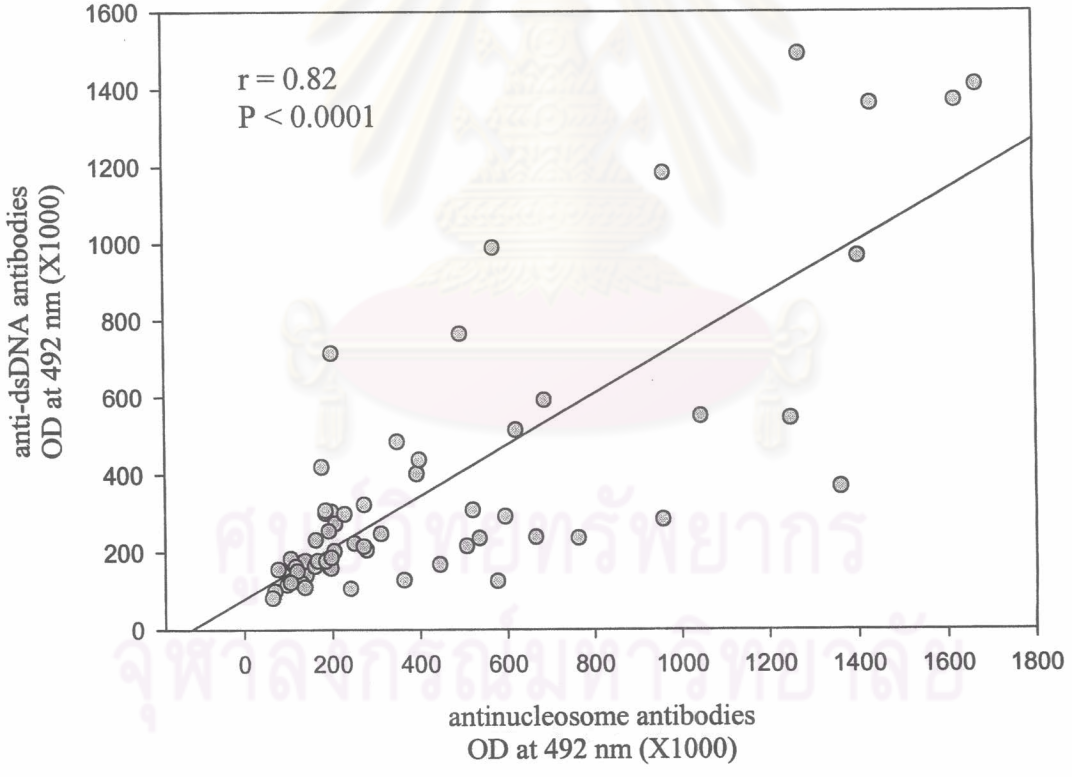


Figure 19: Correlation between antinucleosome and anti-dsDNA antibody activities. Antinucleosome and anti-dsDNA antibodies were measured by enzyme-linked immunosorbent assay. Correlation analysis is performed using linear regression.

Antinucleosome antibody activity and complement C3 levels

We further examined the correlation between antinucleosome antibody activity and complement C3 levels in SLE patients. Levels of C3 factor were measured directly by nephelometry. According to C3 levels by nephelometry (normal values was 76-171 mg%), we found that low C3 levels were statistically associated with antinucleosome antibody activity ($r = -0.48$, $P < 0.0001$) (figure 20).

Antinucleosome antibody activity and complement C4 levels

Further analysis, we determined the correlation between antinucleosome antibody activity and complement C4 levels in 65 SLE patients. According to normal values for C4 was 10-40 mg%, low complement C4 levels were found to be no associated with antinucleosome antibody activity ($r = -0.23$, $P = 0.063$) figure 21.

Figure 20: Correlation between C3 levels and antinucleosome antibody activity. C3 levels of 65 patients with SLE were assessed by nephelometry assay. Antinucleosome antibodies were measured by enzyme-linked immunosorbent assay. Correlation analysis is performed using linear regression.

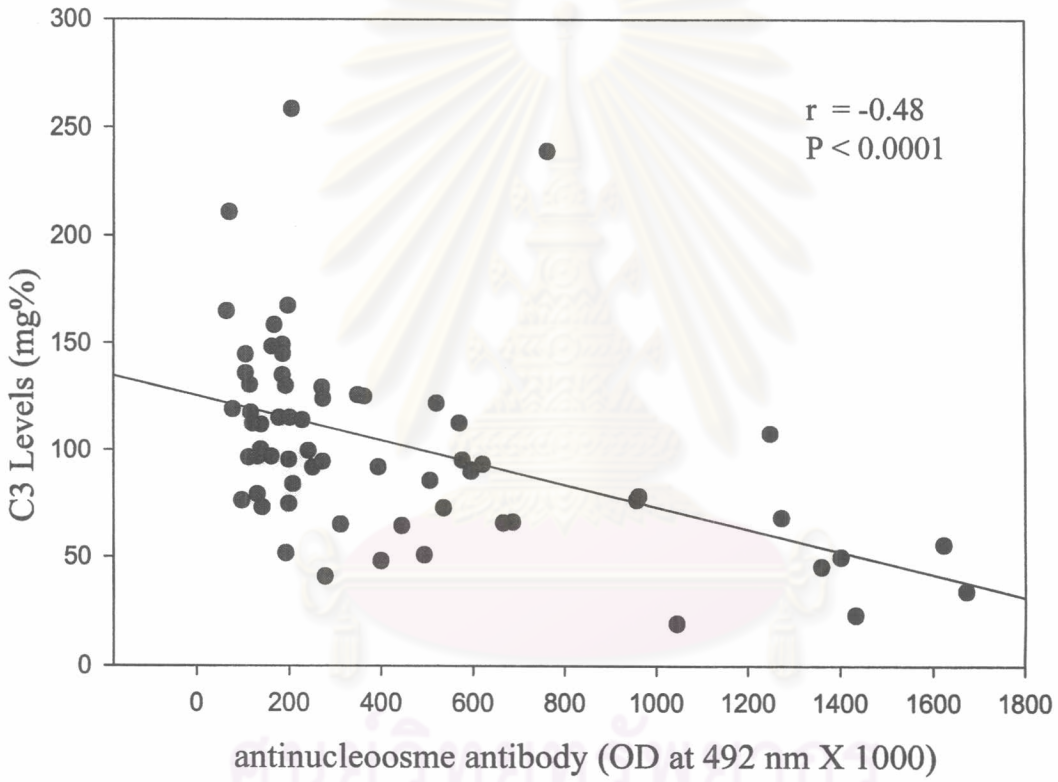
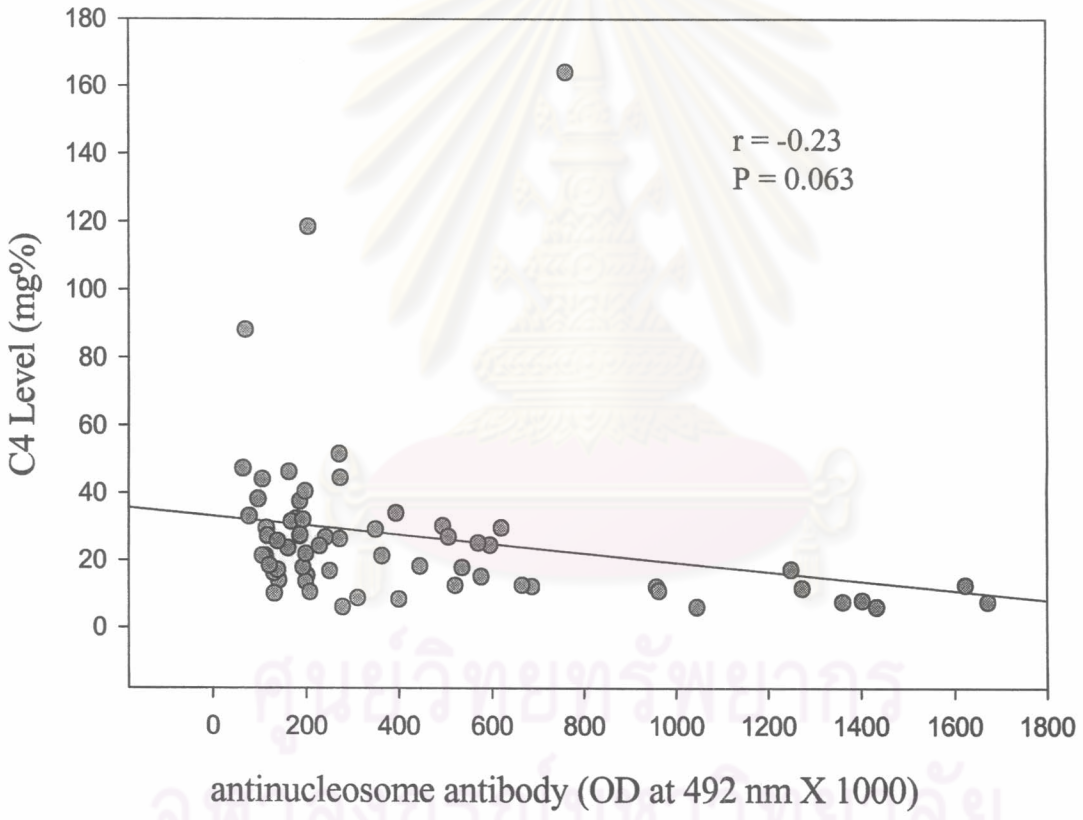


Figure 21: Correlation between C4 levels and antinucleosome antibody activity. Sixty-five patients with SLE were examined for correlation between C4 levels and antinucleosome antibody activity. C4 levels were assessed by nephelometry assay. Antinucleosome antibodies were measured by enzyme-linked immunosorbent assay. Correlation analysis is performed using linear regression.



Correlation between Anti-dsDNA antibody activity and SLEDAI

To evaluate the correlation between anti-dsDNA antibody activity (the result was expressed as OD unit) detected by indirect ELISA and the SLEDAI score, the simple regression analysis was used as shown in figure 22. Anti-dsDNA antibody activity in SLE was shown to be significant correlated with SLEDAI (r = 0.37, P = 0.002).

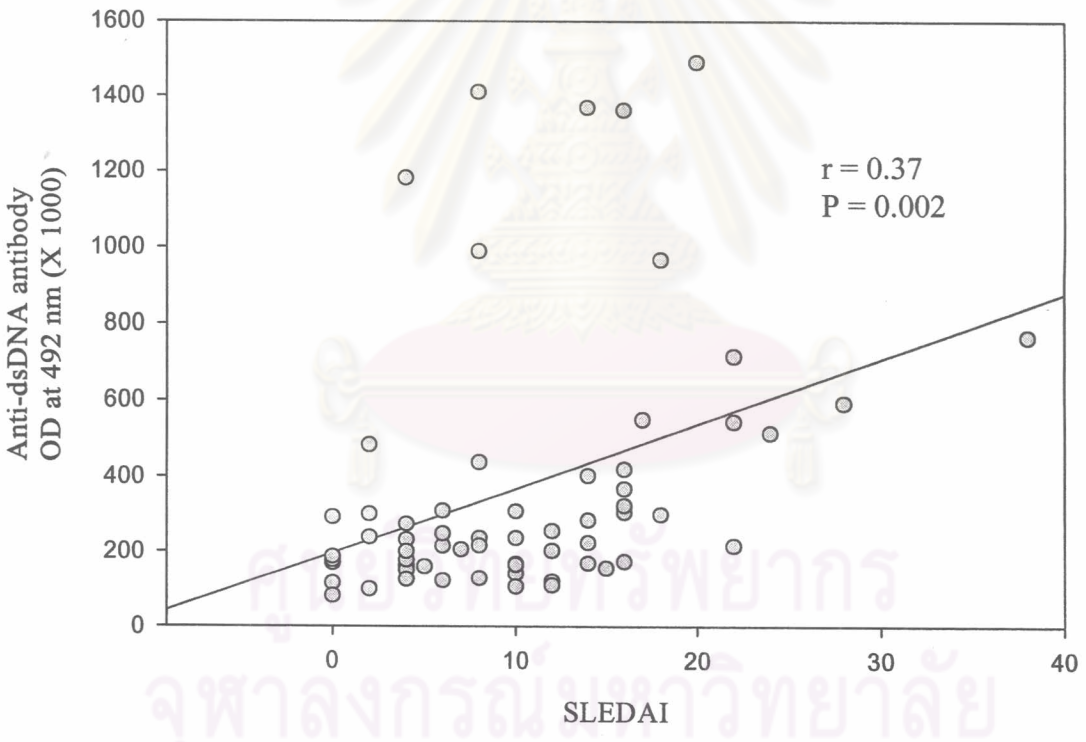


Figure 22: Correlation between anti-dsDNA antibody activity and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in 65 SLE patients. Correlation analysis is performed using linear regression.

Anti-dsDNA antibody activity and complement C3 levels

To examine the correlation between anti-dsDNA antibody activity and complement C3 levels in 65 SLE patients. The normal value for C3 was 76-171 mg%. According to C3 levels by nephelometry, we revealed that low C3 levels were statistically associated with anti-dsDNA antibody activity ($r = -0.42$, $P = 0.001$) using indirect ELISA as shown in figure 23.

Anti-dsDNA antibody activity and complement C4 levels

Further analysis, we evaluated the correlation between anti-dsDNA antibody activity and complement C4 levels in SLE patients. According to normal values for C4 was 10-40 mg%, we found that low C4 levels were statistically associated with anti-dsDNA antibody activity ($r = -0.26$, $P = 0.037$) using indirect ELISA as illustrated in figure 24.

Figure 23: Correlation between C3 levels and anti-dsDNA antibody activity. C3 levels were assessed by nephelometry assay. Anti-dsDNA antibodies were measured by enzyme-linked immunosorbent assay. Correlation analysis is performed using linear regression.

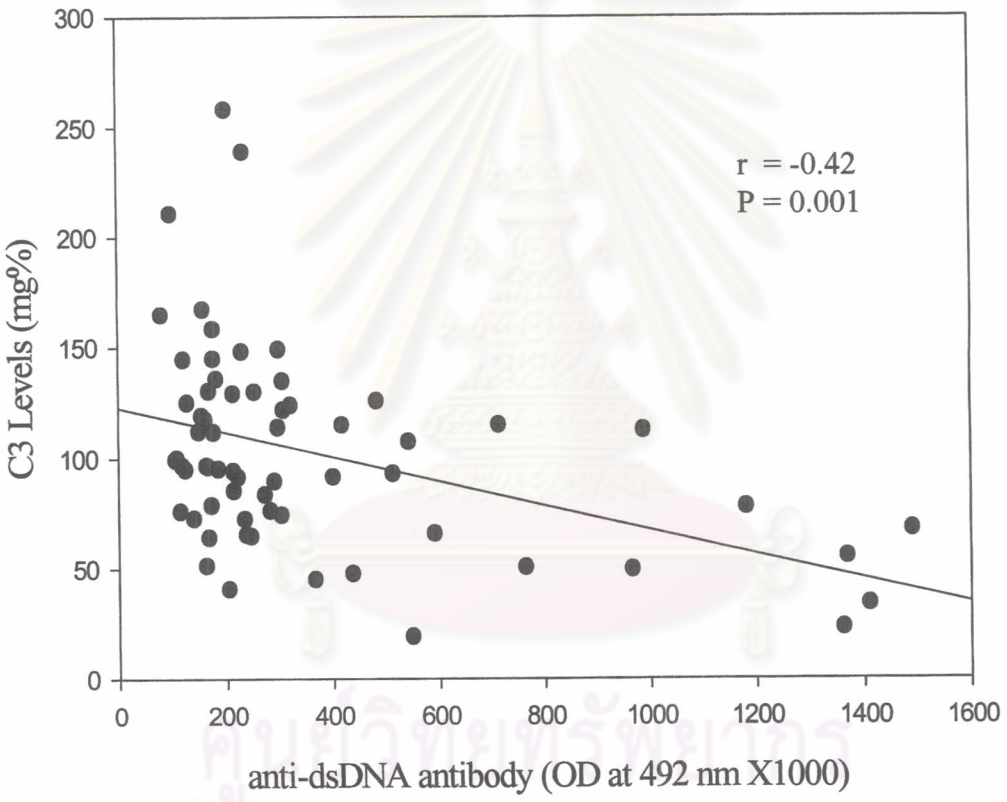


Figure 24: Correlation between C4 levels and anti-dsDNA antibody activity. C4 levels were assessed by nephelometry assay. Anti-dsDNA antibodies were measured by enzyme-linked immunosorbent assay. Correlation analysis is performed using linear regression.