CHAPTER 4

MATERIALS AND METHODS

Study groups

1. Patients

Sixty-five patients from outpatient and inpatient service of King Chulalongkorn Memorial Hospital, who fulfilled at least 4 of the American College of Rheumatology (ACR) revised criteria for SLE (table 2), were studied. SLE patients consisted of 63 women; age range 16-62 years (mean 33 years) and 2 men; 19 and 28 years.

Clinical and biologic information was obtained at each patient visit, and this information was used to determine the score on the SLE Disease Activity Index (SLEDAI) by physician (table 6). This was a blinded study, technician had no any information in disease activity of each patient individuals before evaluation the correlation of antibody and disease activity. Active lupus was defined as a SLEDAI score > 5 as previously described (111). Forty-five of patients had active SLE and 20 had inactive SLE. Of the 45 patients with active disease, 1 with organic brain syndrome, 3 with visual disturbance, 5 with lupus headache, 1 with CVA, 10 with arthritis, 3 with myositis, 3 with urinary casts, 21 with hematuria, 28 with proteinuria, 22 with pyuria, 9 with new rash, 17 with alopecia, 4 with mucosal ulcers, 1 with pleurisy, 25 with low complement, 19 with increased DNA binding, 2 with fever, 2 with thrombocytopenia, 1 with leucopenia.

2. Controls

One hundred and fifteen healthy blood donors (36 women, age range 19-53 years; mean 33 years,and 79 men, age range 19-58 years; mean 34 years) from National Blood Bank Center; Thai Red Cross Society were carried out as control group.

Table 6: SLEDAI: DATA COLLECTION SHEET

Chart no.: __________ Date of Visit: ________________

M.D.: ________________________________ Patient's Name: ______________________________________

(Enter weight in SLEDAI Score column if descriptor present at the time of the visit or in the preceding 10 days.)

Weight	SLEDAI Score	Descriptor	Definition
8	______	Seizure	Recent onset. Exclude metabolic, infectious, or drug causes.
8	______	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Exclude uremia and drug causes.
8	______	Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least 2 of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious, or drug causes.
8	______	Visual disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serous exudate or hemorrhages in the choroid, or optic neuritis. Exclude hypertension, infection, or drug causes.
8	______	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	______	Lupus headache	Severe, persistent headache; may be migrainous, but must be nonresponsive to narcotic analgesia.
8	______	CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	______	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis.
4	______	Arthritis	More than 2 joints with pain and signs of inflammation (i.e., tenderness, swelling, or effusion).
4	______	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/aldolase or electromyogram changes or a biopsy showing myositis.
4	______	Urinary casts	Heme-granular or red blood cell casts.
4	______	Hematuria	> 5 red blood cells/high power field. Exclude stone, infection, or other cause.
4	______	Proteinuria	> 0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.
4	______	Pyuria	> 5 white blood cells/high power field. Exclude infection.
2	______	New rash	New onset or recurrence of inflammatory type rash.
2	______	Alopecia	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2	______	Mucosal ulcers	New onset or recurrence of oral or nasal ulcerations.
2	______	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	______	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
2	______	Low complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2	______	Increased DNA binding	> 25% binding by Farr assay or above normal range for testing laboratory.
1	______	Fever	> 38°C. Exclude infectious cause.
1	______	Thrombocytopenia	< 100,000 platelets/mm 3 .
1	______	Leukopenia	< 3,000 white blood cells/mm 3 . Exclude drug causes .

Total SLEDAI Score __________

Specimen collection

After informed consent were provided, 5 milliliters of blood samples were collected by venepuncture. Then, each serum was separated by centrifugation at 2,000 rpm, 10 minutes. All sera were aliquots and stored at -80°C until used.

Antigens

1.Nucleosomes

Nucleosomes were prepared as described previously(15), with slight modifications (figure 10).

Chicken erythrocytes were prepared from 1-2 ml of chicken blood by centrifuging for 10 minutes at 800 g at room temperature and the buffy coat was removed by suction. The erythrocyte pellet was washed 3 times with 10-fold excess in a 15 mM Tris buffer containing 15 mM NaCl, 60 mM KCl, 2mM EDTA, 0.5 mM EGTA, 0.15 mg/ml spermine, 0.5 mM spermidine, 0.34 M saccharose, 15 mM 2-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), for 10 minutes, 2,000 rpm at 4°C. Cells were lysed in washing buffer containing 0.5% Triton X-100, using 10 ml of buffer/ml of red blood cells. Incubated 5 minutes, 4°C and centrifuged for 10 minutes, 2,500 g at 4°C. Pellets were washed 2 times in a digestion buffer containing 50 mM Tris, 25 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.2 mM PMSF, using 10 ml of buffer/ml of red blood cells for 10 minutes, 2,000 rpm at 4°C. The pellet containing the nuclei was resuspended approximately in 1 ml of washing buffer/ml of starting volume of red blood cells and measured the concentration of DNA at OD 260 nm. Micrococcal nuclease (N-3755 Sigma, USA) was added in the concentration of 40 IU/mg DNA and incubated at 37°C for 30 minutes and terminated the reaction by addition of 0.2 M Na_2EDTA to a final concentration of 2mM.

Nuclei was pelleted at 2,500 g for 10 minutes, 4°C and resuspended in 1 ml of an extraction buffer/ml of starting volume of red blood cells. Then homogenized in tight-fitting dounce homogenizer. The homogenate was dialyzed against extraction buffer overnight at 4°C and centrifuged for 10 minutes, 27,000 g at 4°C. The supernatant was subjected to a gel filtration column (Sephacryl S-300; Pharmacia, Freiburg, Germany) equilibrated in 50 mM Tris, 0.25 mM EDTA, 0.02% NaN_3, and 0.2 mM PMSF, the volume should be in the range of 1-4% of the total bed volume of the column at flow rate 12 ml/h and collected the samples at 3 ml/fraction. Nucleosome fractions were collected by spectrophotometry after determination of the OD_{260} (figure 11).

Fractions corresponding to pure mononucleosomes were concentrated on Amicon PM-30 filters (Amicon, Lexington, MA), and were stored at 4°C for no longer than 2 weeks.

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Figure 10: Procedure of Nucleosome Preparation

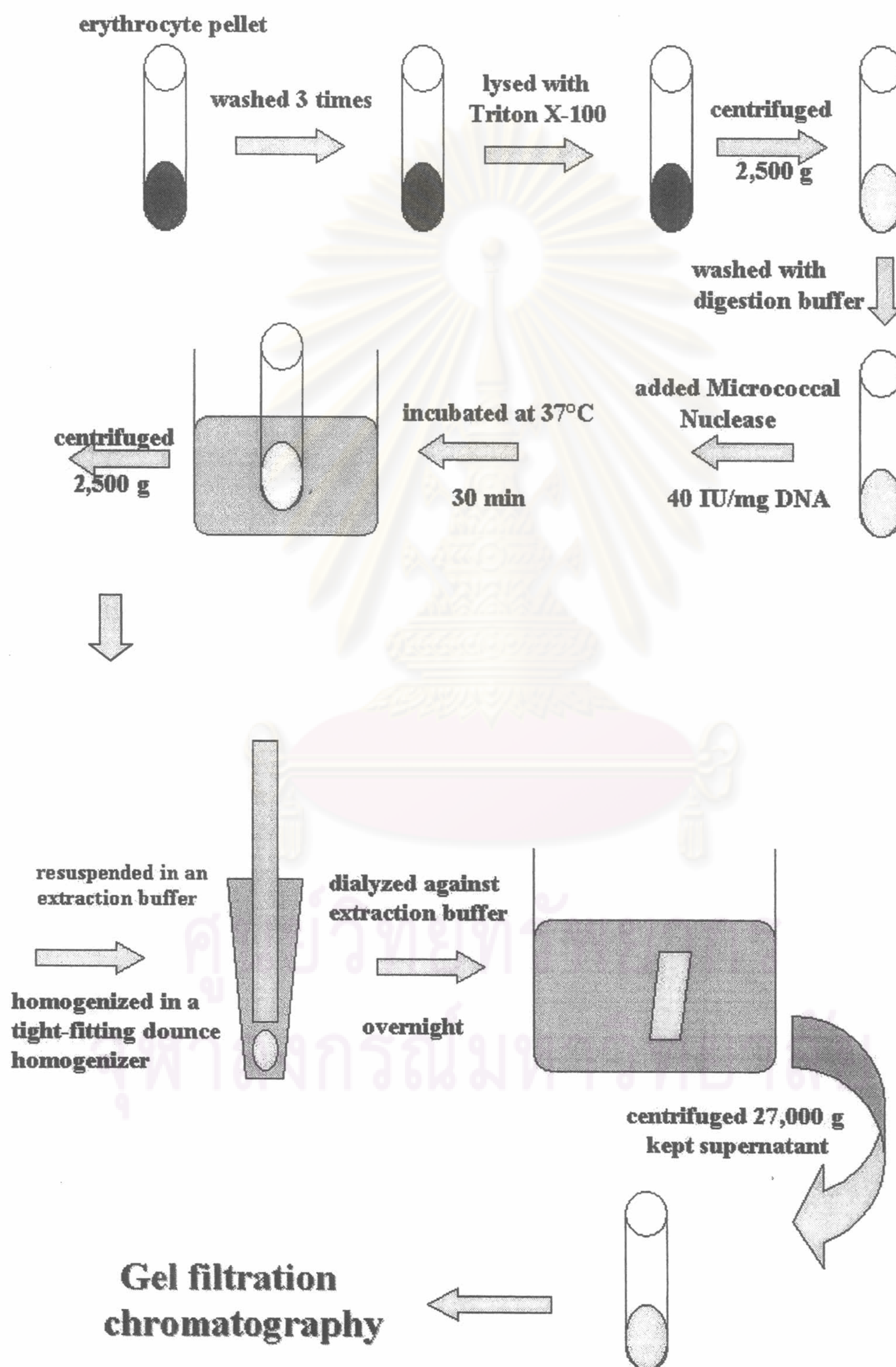
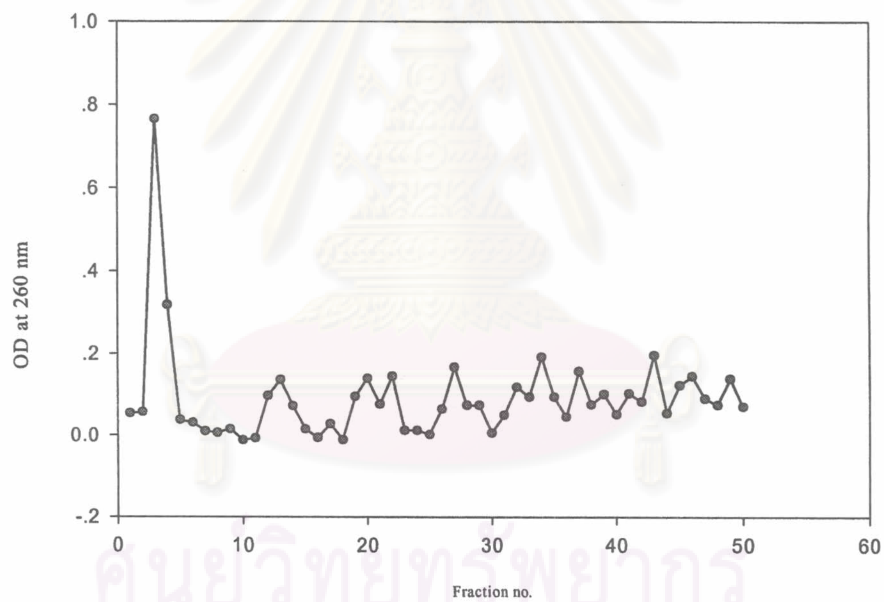


Figure 11: Elution profile of nucleosomes from a column chromatography on Sephacryl S-300. The fractions corresponding to nucleosomes were found in the first peak.



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Examination of nucleosomal DNA

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. The correct composition of the nucleosomes using agarose gel electrophoresis was shown the presence of nucleosomal DNA at 154 bp.

Phenol extraction and ethanol precipitation

The method was followed as described previously (112). Five hundred microliters of nucleosome-containing fractions were performed. Added 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1 ratio) in nucleosome-containing sample and vortexed 30-60 seconds. Centrifuged at 5000 rpm (Sigma model 2K15; Sigma, USA) for 5 minutes at room temperature and carefully removed the upper aqueous phase and transfered to a fresh centrifuge tube. Added an equal volume of phenol: chloroform: isoamyl alcohol and repeated the extraction a second time as described above. Added an equal volume of chloroform: isoamyl alcohol (24:1) to the aqueous phase. Mixed gently for 10 minutes to extract the remaining phenol and centrifuged at 5000 rpm for 5 minutes at room temperature. Removed the upper aqueous phase, added 0.2 volumes of 10 M ammonium acetate and then added 2 volumes of absolute ethanol. Capped the tube and mixed. Incubated in -20 °C freezer for 20 minutes and centrifuged the tube at 5000 rpm for 5 minutes at room temperature. A small precipitate on the bottom side of the tube was appeared. Washed the pellet with 70% ethanol, spinned 1 minute at 5000 rpm, discarded supernatant. Spined briefly and removed the remaining liquid carefully. Allowed the pellet to dry 30 minutes in fume hood, then resuspended in 50-200 µl Tris-EDTA buffer pH 8.0 per tube depending on the size of the pellet and stored at 4°C until used.

Examination of nucleosomal histones

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).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The 15% separating acrylamide gel with gentle mixing in order not to incorporate air was prepared. Immediately, the gel was pipetted into the prepared glass plate sandwich to a height of 6 cm., and was carefully overlayed with distilled water after pouring to give a smooth interface after polymerization. Then, the gel was solidified for 45 minutes at room temperature. After gel polymerization, the overlay-distilled water was discarded.

The 5% stacking acrylamide gel was prepared. The comb was placed between the glass plate-sandwich and the gel solution was added immediately to the top of the glass plate for 45 minutes at room temperature. After polymerization, the comb was removed by gently pulling in a vertical direction. Then, the wells were washed with distilled water and any small fragments of polyacrylamide gel and unpolymerized monomers were removed. Thereafter, the glass plate-sandwich was placed in the electrophoretic tank (Mini-Protean II cell, Bio-Rad, U.S.A.). The electrophoretic buffer was then added to the tank. Fifteen microliters of nucleosomes and 8 ul of protein marker (P7708S Prestained protein marker, Broad Range, New England Biolabs, USA) were loaded and the gel was electrophoresed with a constant voltage of 110 volts (Power supply model 2197 LKB BROMMA, Sweden) in stacking gel and resolving gel until the loading buffer appeared nearly the end of the gel.

When electrophoresis was completed, the stacking gel was cut. The separating gel was stained 2 hours in 0.25% Coomassie blue R (Coomassie

Brilliant Blue R-250., Bio-Rad, CA., cat. no. 161-0400). To observe the band, the gel was destained until the destaining solution was clear. The destaining reaction was stopped by washing in distilled water.

2. dsDNA

Lambda phage dsDNA was purchased from Roche (0.25 ug/ul, code. 0745782 Manheim, Germany).

Determination of antinucleosome and anti-dsDNA antibodies

Antinucleosome and anti-dsDNA antibody determination were assessed by indirect enzyme-linked immunosorbent assay (ELISA) (figure 12) as described previously (15) with slight modifications.

Figure 12: Principle of Indirect ELISA

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Checkerboard titration to determine optimal conditions for indirect enzyme-linked immunosorbent assay (ELISA) for antinucleosome and anti-dsDNA antibodies

When establishing a solid-phase assay, both nucleosomes or dsDNA coating concentration and the Ab conjugate dilution must be determined. A checkerboard titration (figure 13) was performed as follows.

Nucleosomes or dsDNA were diluted in carbonate-bicarbonate buffer pH 9.6, using the concentration range 2.5, 5, 7.5, and 10 μg/ml. One hundred microliters of Ag was added to the microtiter plate (Nunc-immuno plate Maxisorp, Nunc, Denmark) with a multichannel 8- or 12-well pipette tool. For anti-dsDNA ELISA, the microtiter plate was precoated with poly-L-lysine before using dsDNA as antigen. The plate was coated horizontally and incubated overnight at 4°C. The contents were then washed in phosphate buffer saline (PBS) -Tween 20, pH 7.4 using ELISA washer (Washer 400 Organon Teknika, Belgium) three or six times. Following this washing step, unreactive sites were blocked with 120 μl of blocking reagent, 10% fetal calf serum in PBS-Tween 20 pH 7.4, to the plate and incubated 2 h at room temperature. Washing was repeated as mentioned above and then added 100 μl of test samples including positive control, negative control, and buffer alone (blank alone) in a vertical fashion.

In this study, we used the positive control from SLE patients who had positive to ANA in high titer and, in preliminary study, had OD value more than 1.0 OD units in antinucleosome or anti-dsDNA ELISA. For negative control, we used sera from healthy control who was negative to ANA and had OD value less than 0.2 OD units in antinucleosome or anti-dsDNA ELISA. For this study, a 1:100 dilution of serum samples was used in an antinucleosome and anti-dsDNA indirect ELISA as described previously (15).

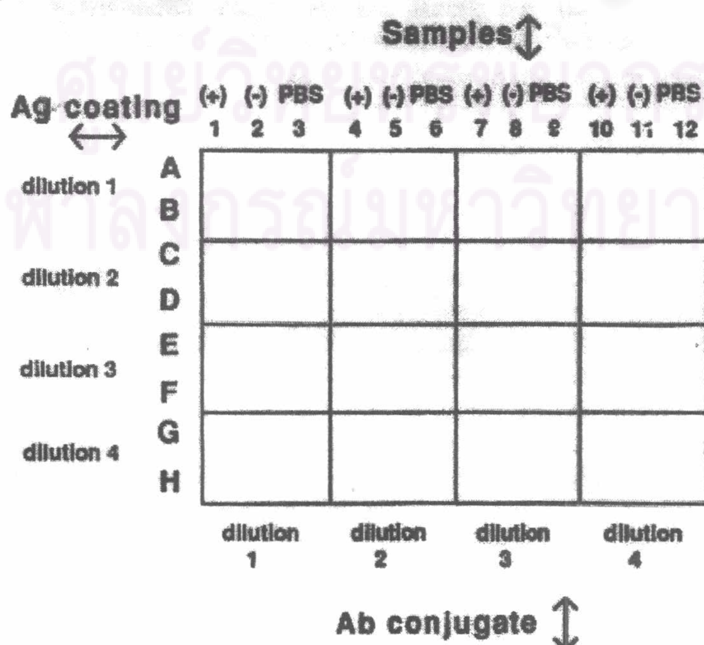
Following an incubation of 2 h at room temperature, the plate was washed with PBS-Tween 20. One hundred microliters of the peroxidase-conjugated rabbit immunoglobulin IgG (gamma-chain) using 1:2,000, 1:3,000, 1:4,000, and 1:5,000

dilutions were added vertically and incubated 2 h at room temperature. After washing step, 100 μl of *o*-diphenylenediamine substrate (Sigma, P3888) at the concentration of 0.4 mg/ml in citrate buffer with 30% H_2O_2 was added and incubated at room temperature in the dark for 5 minutes.

Finally, the reaction was stopped with 100 μl of 1 N sulfuric acid. The absorbance at 492 nm wavelenght was measured by Microelisa system reader (model 311.CO ,Organon Teknika, Belgium).

The optimal choice for these assays was a combination of Ag concentration and conjugate dilution which showed a PBS value of <0.05 OD units, a negative control value of <0.2 OD units, and a positive control value of >1.0 OD units.

Figure 13: Scheme for checkerboard titration to determine optimal Ag coating concentration and peroxidase-conjugated rabbit immunoglobulin of human IgG (gamma-chain) dilution. Four different Ag coating concentrations can be varied horizontally on the plate. Samples tested include positive control (+), negative control (-), and buffer alone (PBS). Four peroxidase-conjugated rabbit immunoglobulin of human IgG (gamma-chain) dilutions were varied in a vertical fashion.



Precision study

A within-plate precision was determined using SLE patient sera prepared by diluting sera in PBS-Tween 20-Fetal calf serum. Precision for sera was calculated by running 90 wells in one microtiter plate.

Overall precision was estimated using same patient sera analysed on seperate microtiter plates.

Mean and standard deviation were calculated. Coefficient of variation (CV) in percent was calculated by the following formula:

$$\% \text{ coefficient of variance} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

Determination of Antinucleosome antibodies

Antinucleosome antibody determination was assessed by indirect enzyme-linked immunosorbent assay (ELISA) as described previously (15).

One hundred microliters of the purified nucleosomes in coating buffer were coated on each microliter well plate (Nunc-immuno plate Maxisorp, Nunc, Denmark) at concentration of 5 µg/ml and incubated overnight at 4 °C. Plates were washed 4 times, by ELISA washer (Washer 400 Organon Teknika, Belgium), in phosphate buffer saline (PBS, pH 7.4) plus 0.05% Tween 20 and then incubated with 120 µl of 10% fetal calf serum (FCS) in PBS plus 0.05% Tween 20 for 2 hours at room temperature. After blocking reaction, the plate was washed again with 4 times of PBS plus 0.05% Tween 20. One hundred microliters of 1:100 patient sera diluted in 10% FCS in PBS plus 0.05% Tween 20 were added and incubated for 2 hours at room temperature. Four times of PBS plus 0.05% Tween 20 washing were proceeded and

100 µl (dilution 1:4,000) of peroxidase-conjugated rabbit immunoglobulin of human IgG (gamma-chain) (P214 Dako, Denmark) was added and incubated for 2 hours at room temperature. After 5 times washing, 100 µl of *o*-diphenylenediamine substrate (Sigma, P3888) at the concentration of 0.4 mg/ml in citrate buffer with 30% H_2O_2 was added and incubated at room temperature in the dark for 5 minutes.

Finally, the reaction was stopped with 100 µl of 1 N sulfuric acid. The absorbance at 492 nm wavelenght was measured by Microelisa system reader (model 311.CO, Organon Teknika, Belgium). Positive control using sera from SLE patients and negative control using sera from healthy blood donors were performed parallely in each experiment to evaluate the accuracy of the test. The OD of tested sera was substracted by OD of blank reagent before considered as positive or negative results.

The results were expressed as positive or negative for antinucleosome ELISA. The threshold value for positivity was defined as 3SD above the mean in the 115 sera (diluted 1:100) from healthy blood donors. The OD cutoff value for antinucleosome ELISA was .

Determination of anti-dsDNA antibodies

Anti-dsDNA antibody determination were assessed by indirect enzyme-linked immunosorbent assay (ELISA), as described previously (14, 15).

ELISA plates (Nunc-immuno plate Maxisorp, Nunc, Denmark) were precoated with poly-L-lysine (P-8920 Sigma, USA) and bovine serum albumin (BSA) (1:1). The mixture of protein were diluted in PBS, pH 7.4 to the final concentration of each protein was 5 µg/ml and placed 100 µl of the mixture in each well of ELISA plates. Incubated at 4 °C overnight and then plates were washed 2 times in PBS using ELISA washer (Washer 400 Organon Teknika, Belgium). To poly-L-lysine-BSA coated wells, added 100 µl of DNA diluted in PBS at the concentration of 5 µg/ml and

subsequently incubated overnight at 4 °C. The plates were then postcoated with 120 μl of 10% fetal calf serum (FCS) in PBS plus 0.05% Tween 20 for 2 hours at room temperature. After blocking reaction, the plate was washed again with 4 times of PBS plus 0.05% Tween 20. One hundred microliters of patient sera were diluted 1:100 in 10% FCS in PBS plus 0.05% Tween 20 and incubated for 2 hours at room temperature. Four times of PBS plus 0.05% Tween 20 washing were proceeded and 100 μl (dilution 1:4,000) of peroxidase-conjugated rabbit immunoglobulin of human IgG (gamma-chain) (P214 Dako, Denmark) was added and incubated for 2 hours at room temperature. After 5 times washing, 100 μl of *o*-diphenylenediamine substrate (P3888 Sigma, USA) at the concentration of 0.4 mg/ml in citrate buffer with 30% H_2O_2 was added and incubated at room temperature in the dark for 5 minutes.

Finally, the reaction was stopped with 100 μl of 1 N sulfuric acid. The absorbance at 492 nm wavelenght was measured by Microelisa system reader model 311.CO (Organon Teknika, Belgium). Positive control using sera from SLE patients and negative control using sera from healthy blood donors were performed parallely in each experiment to evaluate the accuracy of the test. The OD of tested sera was substracted by OD of blank reagent before considered as positive or negative results.

The results were expressed as positive or negative for anti-dsDNA ELISA. The threshold value for positivity was defined as 3SD above the mean in the 115 sera (diluted 1:100) from healthy blood donors. The OD cutoff value for anti-dsDNA ELISA was .

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C3 and C4 determination

Levels of C3 and C4 complements were measured directly by nephelometry using the diagnostic kit, N Antiserum to Human C3c (code No. OSAP, Behring AG, Marburg, Germany) and to Human C4 (code No. OSAO, Behring AG, Marburg, Germany).

Principle of the method

In an immunochemical reaction, complement factors (C3 or C4) contained in the human serum sample from immune complexes with specific antibodies. These complexes scattered a beam of light passed through the sample. The intensity of the scattered light was proportional to the concentration of the relevant complement factor in the sample. The reference curve was constructed by multi-point calibration using the N protein standard SL. The result was evaluated by comparison with a standard of known concentration.

For accuracy and precision control, the N Protein controls SL/M was used after first opening an antiserum vial as well as for each series of patient serum samples.

Procedure for Behring Nephelometers:

Patient samples were automatically diluted 1:20 with N diluent and measured. All steps were performed automatically by Behring Nephelometer (Marburg, Germany). The normal values for C3 76-171 mg%, for C4 10-40 mg%.

If the readings obtained were outside the assay range, the assay can be repeated using a higher or lower dilution of sample. The results were evaluated automatically by means of a logit-log function.

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Statistical analysis

Student's t-test was used for comparison of the means. The chi-square test was used to determine significant levels of correlations. The correlation analysis was analysed by linear regression.