# CHAPTER III

# MATERIALS AND METHODS

## 1. Studied populations

### 1.1 Patients

Seventy-six patients (74 females and 2 males) with systemic lupus erythematosus (SLE) and thirty-four patients (33 females and 1 male) with rheumatoid arthritis (RA) were included in this study. The mean age of SLE and RA patients were 36 years (range 16-56 years), and 48 years (range 20-76 years) respectively.

All patients were randomly selected from the Rheumatology Clinic of Chulalongkorn hospital. SLE was defined by the presence of four or more of the revised criteria for SLE of the American Rheumatism Association , (ARA) 1982 (62) (Table 3 ) and rheumatoid arthritis by the revised criteria for RA of the American Rheumatism Association (ARA), 1987 (92). (Table 4)

### 1.2 Control subjects.

One hundred healthy individuals (50 females and 50 males), randomly selected from normal blood donors of the National Blood Centre, were included as control, the mean age of which was 41 years (range 17-55 years).

## 2. Reference C4 allotypes.

Reference sample of known C4 allotypes was kindly supplied by Dr. Koichi Suzuki, Department of Legal Medicine, Osaka Medical school

Japan and Dr. Preeyachit Charoenwonge, Department of Microbiology, Faculty of Medicine, Chulalongkorn University. (Table 5)

3. Sample Collection and Treatment.

Fresh EDTA plasma, collected into 10 mM $Na_2$EDTA solution (Appendix) were rapidly separated by centrifugation (1,500-2,000 rpm) at 4°C for 10 minutes, divided into small aliquots and stored at -80°C until use.

3.1 Preparation of enzyme treated sample.

Two types of enzymes have been used through out this study. Neuraminidase enzyme from *Clostridium perfringens* (Sigma type VIII, 10 units) was dissolved in 200 ul of 0.1 M phosphate buffer pH6.8/5mM $Na_2$ EDTA, whereas carboxypeptidase B from porcine pancreas (Sigma type I) with enzyme activity of 2000 unit in 2.5 ml of 0.1 M NaCl was employed. Each enzyme was aliquoted and stored at - 80°C until use (41,93,94).

Sample for C4 allotyping was pretreated with neuraminidase alone or neuraminidase and carboxypeptidase B enzyme at a concentration of 10 mU and 160 mU/ul of plasma respectively at 4°C for 18 hrs.

Only neuraminidase was utilized for pretreatment of sample in hemolytic function assay.

1. Immunofixation electrophoresis.

Plasma was electrophoresed in agarose Seakem ME (FMC Bioproduct Marine Colloids Division, Rockland,) after which different bands of complement protein were detected directly by overlaying of the gel surface with specific antiserum to form immunoprecipitates. The agarose gel was washed to remove other proteins and the precipitates are stained (10,93,95,).

1.1 Agarose gel Preparation.

Twelve millilitres of 1.6% (w/v) agarose gel Seakem ME were dissolved in distilled water by boiling and were mixed with 12 ml hot gel buffer plus 60 ul of 0.2 M EDTA solution pH 7.4.

The hot agarose gel solution was then poured into a vertical moulding chamber (Figure 6),consisting of two 12x22 $cm^2$ glass plates and a plastic U-frame (1 mm thick) to get a 11x20x0.1 $cm^3$ gel.

Since this was a thin gel, it was better to leave the agarose gel in vertical cassette in order to ensure uniform gel thickness, particularly along the border.

The firm gel was then kept for at least overnight at $4^oC$ in a humid chamber in order to increase the mechanical strength of the gel.

1.2 Sample Application

Before sample application, the gel surface was freed of excess fluid by a gentle touch of whatman filter paper (Whatman NO1) applied briefly on the part of the gel intended for sample application. The gel surface was then covered with a thin application foil with 1.5 mm wide slits on which 5 ul of each sample was applied. Hemoglobin was included as a migration marker in each run. Ten minutes was allowed for complete diffusion into the gel before the application foil was removed.

1.3 Agarose gel electrophoresis

After sample application, the plate was immediately transfered to the electrophoresis chamber (Multiphor II LKB) prefilled with a minimum of one litre of electrode buffer (appendix) in each trough. About 5 ml of water was spread along one side on the surface of the cooling bed (12.5x26 $cm^2$). The gel plate was at first placed down with one long side in contact with water and the other side slowly lowered until completely rested on the cooling surface. Several sheets (5-6 sheets) of filter paper (Whatman NO 1) or thick cotton sheet served as contact wicks between the gel and buffer. The gel was then covered with a second glass plate to avoid droplet of water condensation on the gel.

Electrophoretic separation was carried out with 65 mA constant current for 4 hrs. at $4^{o}c$ by a circulated cooling water

system (Multitemp, LKB). By then, hemoglobin, the migration marker, had migrated about 10 cm towards the anode.

### 1.4 Immunofixation Method.

After electrophoresis, the gel plate was immediately transfered to a moist chamber. Cellulose acetate strips (0.9x5 $cm^2$) were soaked with specific antiserum, rabbit anti humam C4 (DAKO, dilution 1:4). With a pair of tweezers to avoid contamination, the completely soaked cellulose acetate strip was then applied onto the agarose gel covering the area of interest. Care should be taken to remove air bubbles trapped between the cellulose acetate strip and the gel surface. The gel plate was incubated in the moist chamber at room temperature for 3 hrs. to allow antibody diffusion into the agarose gel.

The cellulose acetate strips were then carefully removed, the gel pressed with absorbent paper, and washed in 0.15 M sodium chloride for 1 hr. After the last washing in water, the gel was completely dried in a hot stream of air and stained in Coomassie brilliant blue for 1 hr. followed by decolorization.

## 2. Immunoblot.

Immunoblotting is a technique employed to facilitate the examination of protein characteristics. This method required minute ammounts of reagent for detection, yielded high resolution and provided the possibility to determine the immobilised proteins on nitrocellulose sheets by either immunochemical reaction or protein

staining. Consequently, in our study for C4 allotypes, Immunoblotting was mainly used , and the conventional immunofixation method was only utilized when verification of some C4 allotypes was necessary.

## 2.1 Electroblotting.

The method of semi-dry electroblotting for C4 allotype was initially suggested by Udomsopagit (96) and followed that that of Kyhse-Anderson (97), in which proteins or charged molecules were transfered from gel to nitrocellulose sheets using wetted filter paper as buffer reservior in a special electroblotting appraratus.

This method required only 1 ul sample volume for C4 allotyping. Thus, one microliter of plasma was appied on the 0.5 mm wide slit of an application foil (LKB), and allowed to diffuse for 5 minutes into the gel. Electrophoretic separation of protein were then proceeded as mentioned.

Electrotransfer was performed in a horizontal, semi-dry electroblotting apparatus (Sartatoblot) consisting of two graphite electrodes. Electrophoresed gel was first soaked in transfer buffer (Anode buffer NO II). The graphite plates, (anode and cathode) were rinsed with distilled water, before six layers of filter paper (Whatman NO I) soaked in anode buffer NO I (appendix) were placed on the lower anode plate. On top of that three pieces of filter paper soaked in anode buffer NO II were laid, followed by a nitrocellulose membrane (pore size 0.45 micron, Biorad) prewetted with the same buffer. The electrophoresed gel was laid on top of the nitrocellulose

membrane and covered with nine layers of filter paper soaked in cathode buffer (appendix).

This sandwich or "TRANS-UNIT" (Figure 7) was quickly assembled to avoid diffusion of protein antigen. Great care was taken to remove all air bubbles causing uneven transfer, by wetting the gel with a few drop of transfer buffer and squeezing out air bubble with a glove protected finger. Electrotransfer was then achieved with a constant current of 0.8 $mA/cm^2$ gel at room temperature for 1 hr before immunostaining.

2.2 Immunostaining.

The blotted nitrocellulose membrane was removed, incubated with 5% nonfat-dry milk in washing buffer (appendix) at 37°C for 1 hr. to block the free protein binding site and minimize background before rinsing shortly in washing buffer. This membrane was then immunostained by incubating with a primary antibody, rabbit anti human C4 at an optimal dilution previously determined (1:750) at 37°C for 1 hr. After incubation, the membrane was washed five times, 10 minutes each in fresh washing solution to remove excess unbound antiserum followed by incubation at 37°C with a secondary antibody, peroxidase conjugated swine antirabbit Ig at a optimal dilution (1:900) for 1 hr. The nitrocellulose membrane was then shaken in washing buffer three times, 10 minutes each to wash off excess unbound antirabbit Ig, after which freshly prepared substrate solution, diaminobenzidine (DAB) were added and incubated at room temperature. This reaction was carried out in the dark and terminated when yellow-

brown colour was visible (about 30 min). The membrane was finally washed with distilled water, dried and stored in the dark before reading. Photography was taken as early as possible for record.

3. Hemolytic Gel Function Assay.

Hemolytic gel function technique was an additional test to distinguish or further define the C4 variants that had intermediate mobilities between or overlapping those of the common variant C4A and C4B. As a rule C4B variants exhibit 5 to 10 times hemolytic activity to C4A variants (41).

Antibody - sensitized sheep erythrocytes and artificially produced C4-deficient guinea pig serum were incorporated in gel and layered onto an agarose gel after completion of C4 electrophoresis. Bands of hemolysis are selectively produced from C4B variants whereas those of C4A seen by immunofixation produce little or no functional activity under these conditions (93,95).

3.1 Preparation of Antibody-Sensitized Sheep Erythrocyte.

Ten millilitre sheep erythrocytes in Alsever's solution were centrifuged, 2000 rpm at 4°C for 10 minutes. The supernatant plasma and the buffy coat were removed by aspiration and the packed cell washed four times (10 minutes each) with veronal buffer saline (VBS-EDTA) solution (appendix) followed by another three 10 minutes washing with gelatin-veronal buffer saline solution (GVBS+$Mg^{2+}$+$Ca^{2+}$) (appendix). The supernatant fluid after the second and third wash

should be colourless, indicating that no spontaneous cell lysis has occurred.

One hundred microlitres of 5% red cell suspension in GVBS+$Mg^{2+}$+$Ca^{2+}$ were added to 2.9 ml deionized water and the optical density (O.D.) of the lysate was measured in a spectrophotometer (wave length 541). An O.D. reading of 0.385 corresponded to $1x10^9$ sheep erythrocyte per millitre.

Rabbit anti - sheep red blood cells , hemolysin was titrated for the highest dilution of maximum lysis, twice the concentration of which was used for sensitization. Sensitized erythrocytes ( $5x10^8$ cell/ml) was incubated at $37^oC$ for 1 hr. and stored at $4^oC$ for a maximum of one week.

### 3.2 Preparation of Hydrazine Treated Guinea Pig Serum.

Hydrazine treated guinea pig serum was used as C4-deficient serum. One part of 0.15 M hydrazine sulfate to 4 parts pooled undiluted guinea pig serum were incubated at $37^oC$ for 1 hr (41,93,95).

### 3.3 Hemolytic gel Assay.

For preparation of hemolytic gel , 7.5 ml of antibody - sensitized sheep red blood cells were washed twice with GVBS+$Mg^{2+}$+$Ca^{2+}$ buffer. The packed cells were then added to 24 ml of warm 0.6%(w/v) agarose gel (Seakem ME) in GVBS+$Mg^{2+}$+$Ca^{2+}$ at $60^oC$ in a waterbath. Half a mililiter of hydrazine-treated guinea pig serum was added

just before moulding. The moulded hemolytic gel prepared in a similar fashion as electrophoretic gel was cooled at 4°C overnight.

Hemolysis was achieved by overlaying the hemolytic gel on the eletrophoretic gel after electrophoresis for about 1 hr at 37°C or until band of lysis was observed. The overlaying gel was then withdrawn, fixed in 1% glutaraldehyde in saline and air-dried.

4. C4 Nomenclature.

Characteristic bands were identified corresponding to the gene products of the two loci C4A and C4B. Each of the alleles at each locus is identified by the relative mobilities of its bands compared to reference C4 allotypes, C4 A3,3 B1,1, included on each run. This identification followed the C4 nomenclature devised from the IV$^{th}$ International Workshop for the Genetics of Complement (10) (Figure 8). Known reference C4 allotypes in Table 5 were also included for comparison when necessary.

5. Assignment of C4 Null Alleles.

After immunoblotting, the nitrocellulose membranes were subjected to densitometric evaluation with a Laser Densitometer, Single Dimension Gel Scan XL Program 1986 (LKB), wave length 633 nm , for the assignment of C4 null alleles (Figure 9). Complete absence of C4A or C4B bands was considered to indicate homozygous deficiency while the heterozygous deficiency were decided on the relative densities of the C4A and C4B band. This could be done by determining

the total areas under the C4A and C4B curves were and hence deriving the A/B ratio.

A ratio of 0.2-0.6 indicates heterozygous deficiency at the C4A locus whilst a ratio of 1.5-2.4, at the C4B locus. A ratio between 0.7-1.2 indicates an equal number of allele at both loci which may include individual heterozygous null allele at both loci (76).

6. Statistical Analysis.

HLA antigen frequencies or phenotype frequencies (P.F.) are simple counts of each antigen generally convertd to percent in a specific population. For practical proposes, this can be convert to "allele frequency" or "gene frequency" (G.F.) by the use of a simple formula (98).

$$\text{Gene frequency} = 1 - \sqrt{1 - \text{P.F}}$$

The evaluation of differences in the frequency of complement phenotypes and the significance of association between each allotypes of control subjects and patients were determined by the 2x2 contingency tables and Yates' corrected Chi-squared test. Fisher's exact test was utilized when the number in each cell less than 5. Relative risk (R.R) considered as a measure of disease - allele association were calculated as odd ratios.

$$\text{R.R} = \frac{\text{Patients with antigen}}{\text{Normals with antigen}} \times \frac{\text{Normals without antigen}}{\text{Patients without antigen}}$$

Table 3. The 1982 revised criteria for classfication of SLE*

| Criterion | Definition |
|---|---|
| 1. Malar rash | Fixed erythema, lat or raised, over the malar eminences, tending to spare the nasolabial folds |
| 2. Discoid rash | Erythematous raised patches with adherent keratotic scaling and follicular plugging: atrophic scarring may occur in older lesions |
| 3. Photosensitivity | Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation |
| 4. Oral ulcers | Oral or nasopharyngeal ulceration, usually painless, observed by a physician |
| 5. Arthritis | Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion |
| 6. Serositis | (a) Pleuritis-convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion. OR<br>(b) Pericarditis-documented by ECG or rub or evidence of pericardial effusion |
| 7. Renal disorder | (a) Persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation not performed<br>(b) Cellular casts-may be red cell, hemoglobin, granular, tubular, or mixed |
| 8. Neurologic disorder | (a) Seizures-in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance. OR<br>(b) Psychosis-in the absence of offending drugs or known metabolic derangements, e.g. uremia. ketoacidosis, or electrolyte imbalance |
| 9. Hematologic disorder | (a) Hemolytic anemia-with reticulocytosis. OR<br>(b) Leukopenia-less than 4,000/$mm^3$ total on 2 or more occasions OR<br>(c) Lymphopenia-less than 1,500/$mm^3$ on 2 or more occasions OR<br>(d) Thrombocytopenia-less than 100,000/$mm^3$ in the absence of offending drugs |
| 10. Immunologic disorder | (a) Positive LE cell preparation OR<br>(b) Anti-DNA: antibody to native DNA in abnormal titer OR<br>(c) Anti-Sm: presence of anitbody to Sm nuclear antigen OR<br>(d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pullidum* immobilization or fluorescent treponemal antibody absorption test |
| 11. Antinuclear antibody | An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome |

* The proposed classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person shall be said to have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

Table 4. The 1987 revised criteria for the classification of rheumatoid arthritis (traditional format)*

| Criterion | Definition |
|---|---|
| 1. Morning stiffness | Morning stiffness in and around the joints, lasting at least hour before maximal improvement |
| 2. Arthritis of 3 or more joint areas | At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints |
| 3. Arthritis of hand Joints | At least 1 area swollen (as defined above) in wrist, MCP, or PIP joint |
| 4. Symmetric arthritis | Simultaneous involvement of the same joint areas (as defined in 2) on both sides of body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry) |
| 5. Rheumatoid nodules | Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician |
| 6. Serum rheumatoid factor | Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in < 5% of normal control subjects |
| 7. Radiographic changes | Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify) |

*For classification purposes, a patient shall be said to have rheumatiod arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite, or probable rheumatoid arthritis *is not* to be made.

PIPs = proximal interphalangeal joints; MCPs = metacarpophalangeal joints; MTPs = metatarsophalangeal joints

**Table 5. Reference or Known C4 Allotypes Plasma in This Study**

| Code NO. | C4 allotypes | Source |
|---|---|---|
| 120386 | C4 A 3,3 B 1,1 | Dr. Koichi Suzuki |
| 121186 | C4 A 3,QO B 1,1 | |
| 870304 | C4 A 3,3 B QO,QO | |
| 870305 | C4 A 3 B 5 | |
| 121486 | C4 A 4,3 B 2,1 | |
| 870622 | C4 A 4,3 B 21,1 | |
| 121886 | C4 A 4,3 B 5,2 | |
| CHA 017 | C4 A 3,3 B 1,1 | Dr. Preeyachit Charoenwonge |
| CHA 023 | C4 A 3,QO B 1,QO | (The 3rd ASIA-OCEANIA |
| CHA 039 | C4 A 3,3 B 2,1 | Histocompatibility Workshop |
| CHA 061 | C4 A 3,3 B 2,2 | Conference 1986 ) |
| CHA 019 | C4 A 3,QO B 4,1 | |
| CHA 046 | C4 A 3,3 B 5,5 | |
| CHA 021 | C4 A 3,2 B 2,1 | |
| CHA 077 | C4 A 4,QO B 2,1 | |
| CHA 065 | C4 A 4,3 B 2,2 | |
| CHA 078 | C4 A 5,3 B 1,1 | |
| CHA 080 | C4 A 5,2 B 1,1 | |
| CHA 034 | C4 A 6,3 B 1,1 | |
| CHA 010 | C4 A 6,3 B 2,1 | |

Not base on family study.

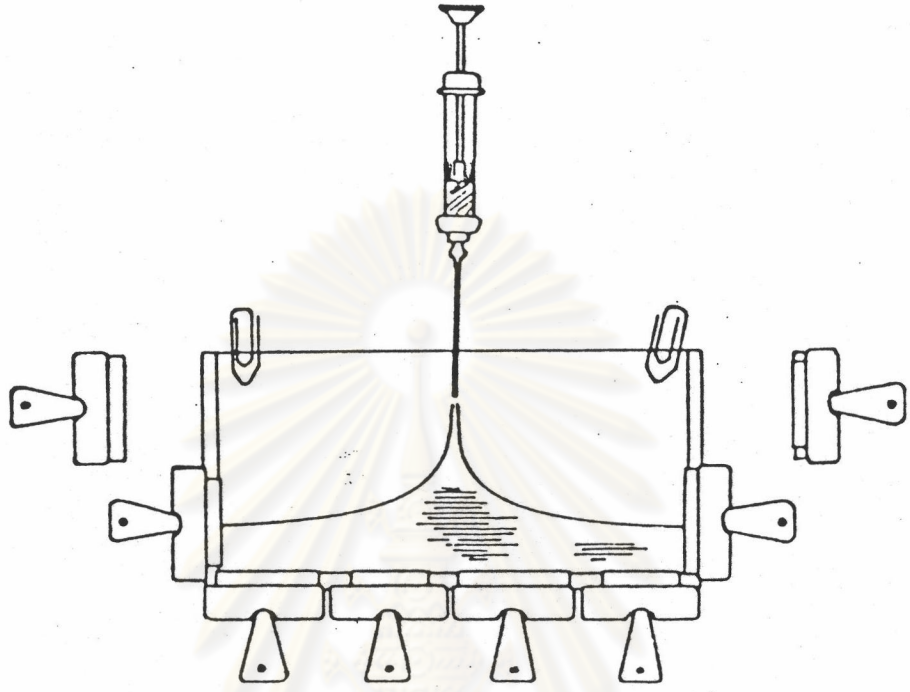

Figure 6 Moulding chamber for the thin gel.

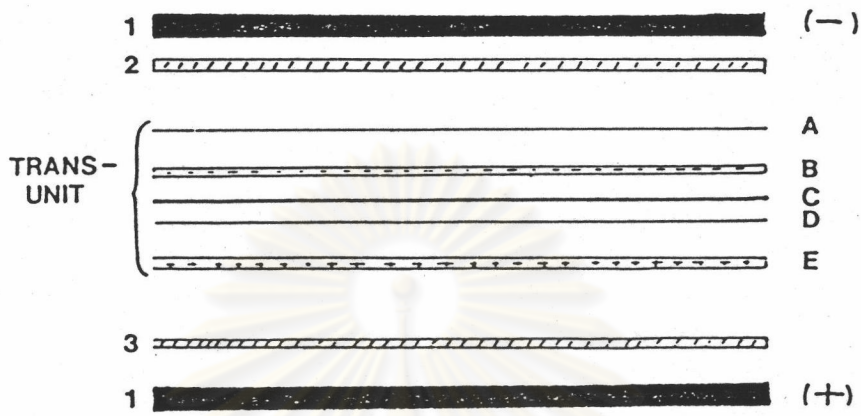


Figure 7 Assembly of one TRANS-UNIT in the electroblotting apparatus (1) Graphite electrodes; (2) Six layers of filter paper soaked in cathode buffer; (3) Six layers of filter paper soaked in anode buffer No. I. The TRANS-UNIT : (A) dialysis membrane; (B) three layers of filter paper soaked in cathode buffer; (C) gel; (D) nitrocellulose membrane; (E) three layers of filter paper soaked in anode buffer No. II.

From : Kyhse-Anderson et al, J. Biochem. Biophys. Method, 10,203-209,1884

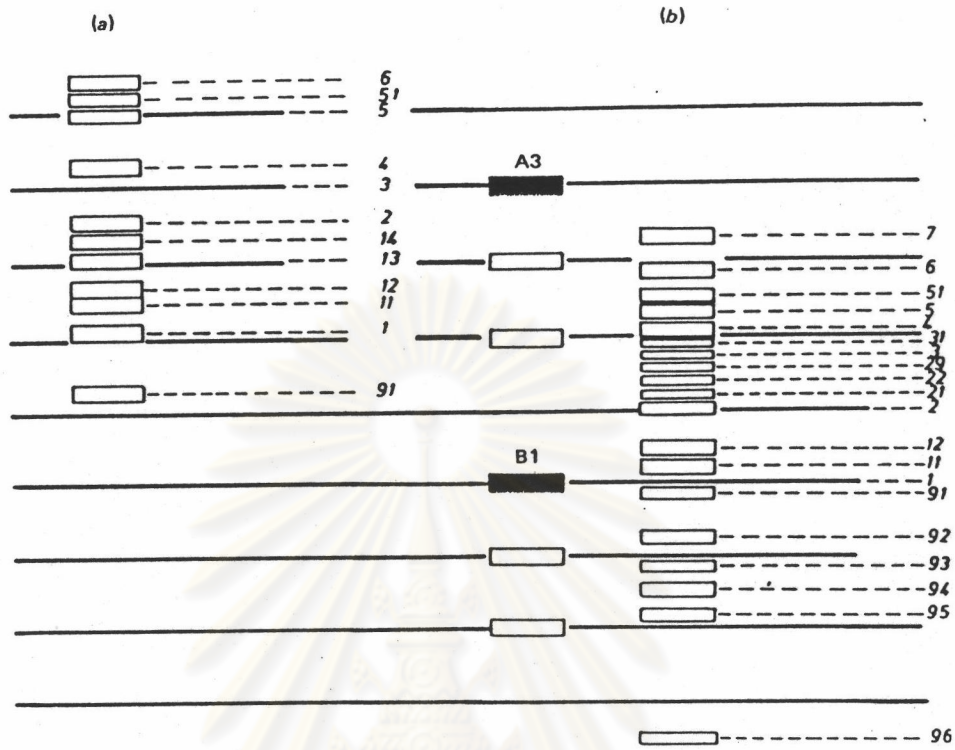


Figure 8 A diagrammatric summary that shown relative position of electrophoretic human C4 allotypes, C4A(left) and C4B (right) Although C4A3,C4B1 is shown as it actually appears (a heavy anodal band and two lighter cathodal bands for each allotypes), the other allotypes are indicated by the position of only the most anodal band.

From : Mauff et al, Immunobiology, 164, 184 - 191, 1983

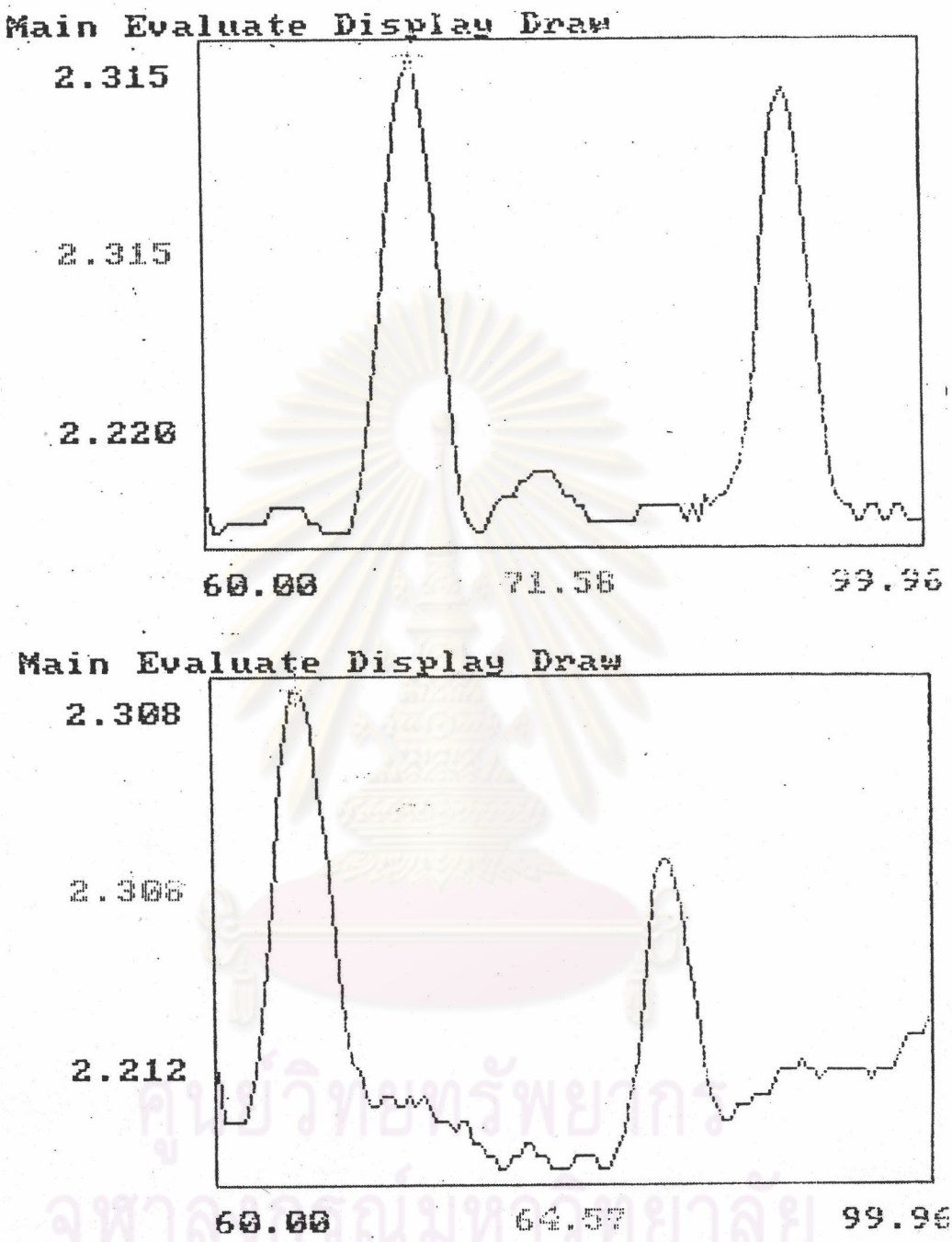


Figure 9 Densitometric pattern of C4 allotype without null alleles (upper) and C4 allotype with null alleles (Heterozygous) (lower).