MATERIALS AND METHODS

A. Subjects

1. Healthy controls

Thirteen healthy control subjects in this study were blood bank donors and graduate students. They were age-matched with the patient groups and consisted of 13 females with the age range from 18 to 44 years old. All the control subjects were in a good health at the time of blood collection.

2. Systemic lupus erythematosus patients

Thirteen female patients with active SLE from Immunology Clinic, Maharaj Nakorn Chiang Mai Hospital were studied. All SLE patients were identified based on the revised criteria of the American Rheumatism Association for diagnosis of SLE (Tan et al., 1982 ; see Table 2). They had the age ranging from 15 to 47 years old. Twelve had never received corticosteroid or other immuno-suppressive drugs. Except one had received prednisolone 50 mg/day for 7 months, and her blood was drown 24 hours after the last steroid dose.

B. Cells preparation for assay system

1. Preparation of peripheral blood mononuclear cells

Human PBMC were isolated from venous blood of normal subjects and SLE patients by fractionation over Ficoll - Hypaque

gradients (Boyum, 1968). Thirty to fourty milliters of venous blood from each person was collected in preservative free heparin (NOVO, Denmark, 5000 unit/ml) at the concentration of 20 unit/ml of blood under aseptic condition. A 30-40 ml of the heparinized blood was diluted with phosphate buffer saline (PBS), pH 7.2 (see appendix 1) to bring the total volume to 80 ml. Diluted blood was carefully over layered on the top of previously prepared 10 ml Ficoll - Hypaque mixtures (see appendix 2) in each of a 50 ml sterile conical tube with screw cap (Sarstedt, W. Germany), then the tube was centrifuged at 400 g for 40 minutes at room temperature. The interface layer consisting of PBMC was recovered with a sterile pasture pipette. These cells were washed three times with RPMI 1640 medium (see appendix 3) and centrifugation at 200 g for 10 minutes. Finally, the cell pellet was resuspended in complete RPMI 1640 (CRPMI) medium (see appendix 4). The viability of the final preparation was greater than 95% , as assessed using the nigrosine dye exclusion procedure.

2. Preparation of rested PBMC

A total of 0.5×10^6 or 1.0×10^6 freshly isolated PBMC in 1 ml CRPMI medium were cultured at 37°C in humidified atmosphere of 5 % CO_2 in air for 2 days in 13 x 100 mm screw cap tubes. At the end of the culture periods, 0.75 ml of the supernatants was discarded and the remainder cells were resuspended in fresh CRPMI medium before stimulated with PHA-P for IL-2 production.

3. Preparation of autologous red blood cells

Red blood cells of each individual person from the bottom of Ficoll-Hypaque centrifugation tube were washed two times with RPMI 1640 medium. Finally, the cell pellet was kept in Alsever's solution (see appendix 5) at 4°C for separating autorosetting T cells.

C. Assay for IL-2 activity

Con A induced lymphoblast obtained from mouse splenocytes were used to assess IL-2 activity in the culture supernatants (Dos Reis and Shevach, 1982). Normal spleen cells (1.0×10^6 cells/ml) from young (6 - to 8-week-old) inbred BALB/c mice were stimulated with 5 µg/ml Con A type IV (Sigma Chemical Co) in RPMI 1640 medium supplemented with 12.5 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol (2-ME), 1 mM sodium pyruvated and 10 % heat inactivated fetal calf serum for 3 days. The nonadherent cells were resuspended by gentle pipetting, collected and washed one times with RPMI 1640 medium. The cells suspension were separated the viable lymphoblast cells by fractionation over Ficoll - Hypaque gradient (see appendix 7 ; Davidson and Parish, 1975). The interface layer consisting of viable lymphoblast cells were collected, washed three times with RPMI 1640 medium and centrifugation at 400 g for 10 minutes. Finally, the cell pellet was resuspended in CRPMI medium supplemented with 0.1 M α-methyl-D-manoside (Sigma Chemical Co).

The viable lymphoblast cells were adjusted to 2 x 10^5 cells/ml, and a total of 2 x 10^4 cells/well in 100 µl were plated in each microplate well (flat-bottom culture plate). One hundreded microliters of either undiluted or two fold serial (log_2) diluted IL-2 containing supernatants (in triplicate) were added to each microplate well. After 24 hours, each microwell culture was pulsed with 0.2 µCi of ^{3}H-thymidine (Amersham, England) for an additional 18 hours. The culturs were harvested on the glass fiber filter strips using a semiautomatic cell harvester. The glass fiber filter strips were dried at room temperature overnight. Dried glass fiber filter strips were counted for Beta-radiation in a liquid scintillation counter (LKB, Finland). Results were expressed as unit of IL-2/ml (U/ml) as previously described by Gillis et al (1978a). Briefly, the incorporated radioactivity (counts per minute or CPM) of each sample was compared by probit analysis to that of the standard IL-2 preparation. The dilution of the IL-2 standard, which resulted in 50% of the maximum ^{3}H-thymidine incorporation, was arbitrarily defined as having an IL-2 concentration of 100 U/ml. Standard IL-2 was prepared by our laboratory (see below). Every assay was compared to our laboratory standard IL-2 supernatant.

D. Determination of optimum condition for IL-2 production

1. Determination of optimum PHA-P concentration

In order to obtain the appropriate concentration of PHA-P that stimulate maximum production of IL-2, PBMC from three normal subjects were used in this study. A total of 1.0 x 10^6 PBMC in 1 ml CRPMI medium were stimulated with PHA-P (Wellcome, North Carolina) at the final concentration of 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 µg/ml respectively and incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 24 hours. At the end of the culture periods, the culture supernatants were collected and stored at -40°C until assay for IL-2 activity.

2. Determination of optimum cells concentration

In order to obtain the appropriate concentration PBMC of which gave the maximum IL-2 production, PBMC from three normal subjects were used in this study. Various concentration of PBMC (0.25 x 10^6, 0.5 x 10^6, 1.0 x 10^6, 1.5 x 10^6, 2.0 x 10^6 and 4.0 x 10^6 cells/ml) were stimulated with PHA-P at the final concentration of 1.0 µg/ml and incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 24 hours. At the end of the culture periods, the culture supernatants were collected and stored at -40°C until assay for IL-2 activity.

3. Determination of optimum incubation time

In order to obtain the optimum incubation time for IL-2 production, PBMC from three normal subjects were used in this study. A total of 1.0 x 10^6 PBMC in 1 ml CRPMI medium were stimulated with PHA-P at the final concentration of 1.0 µg/ml and incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 4, 6, 8, 12, 18, 24, 48, and 72 hours respectively. At the end of the culture periods, the culture supernatants were collected and stored at -40°C until assay for IL-2 activity.

E. IL-2 production in vitro

A total of 1.0 x 10^6 PBMC of each subjects in 1 ml CRPMI medium were stimulated with PHA-P at the final concentration of 1.0 µg/ml and incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 18 hours. At the end of the culture periods, the culture supernatants were collected and stored at -40°C until assayed for IL-2 activity.

F. Standard IL-2 preparation

Normal rat spleen cells (1.0x10^6 cells/ml) were stimulated with 5 µg/ml Con A type IV in RPMI 1640 medium supplemented with 12.5 mM HEPES buffer, 5 x 10^{-5} M 2-ME, 1 mM sodium pyruvate and 10% heat inactivated fetal calf serum for 48 hours (Ayanlar - Batuman et al., 1987). At the end of the culture periods, the culture

supernatants were collected, aliquoted in several vials and stored at -40°C for used as standard IL-2.

G. Determination of optimum Con A concentration for preparation of AR^+ cells

1. Activation of PBMC with various concentration of Con A

Con A - activated PBMC of three normal subjects were established in 20 x 125 mm sterile screw cap tubes and consisted of 4 ml of CRPMI medium containing 4 x 10^6 PBMC. These cultures were incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 48 hours in the presence of Con A type IV (Sigma Chemical Co) at the final concentration 5, 10, 20, and 40 µg/ml. At the end of the culture periods, the cells were harvested and followed by successive washes in RPMI 1640 medium supplemented with 0.1 M α-methyl-D-mannoside (α-MM ; Sigma Chemical Co) to block the mitogenicity of residual Con A for 20 minutes at room temperature. The cells were harvested and gently resuspended in 2 ml of CRPMI medium supplemented with 0.1 M α-MM. The cells suspension were used for autorosetting T cells separation (see below).

2. Autorosette formation and separation of autorosetting T cells

Each 4 x 10^6 Con A - activated T cells in 2 ml of CRPMI medium supplemented with 0.1 M α-MM, 0.5 ml heat inactivated human AB serum and 1.0 ml of 1% autologous RBC in RPMI 1640 medium

(prewashed 3 times in RPMI 1640 medium) were mixed thoroughly and incubated at 37°C for 30 minutes. The mixture was then centrifuged at 150 g for 5 minutes and incubated on ice for 2 hours. The cells were gently resuspended for either counting the autorosettes or for their separation on Ficoll-Hypaque gradients. At least 200 lymphocytes were counted and T cells binding three or more autologous RBC were regarded as autorosette formation cells. For separation, the autologous RBC - T cell suspension was layered over Ficoll-Hypaque, centrifuged at 450 g for 20 minutes at 4°C, and autorosetting T cells and non-rosetting cells were collected separately. The autorosetting T cells from the bottom of Ficoll-Hypaque centrifugation tube were washed and the attached autologous red blood cells were lysed by treatment with 20 ml hypotonic ammonium chloride solution, pH 7.2 (see appendix 6) for 5 minutes at 4°C. These cells were washed one times with RPMI 1640 medium and resuspended in 2 ml of CRPMI medium. These cells were then treated with 100 µg/ml mitomycin C (MMC, Kyowa Hakko Kogyo Co) and 200 µg/ml cycloheximide (CY, Sigma Chemical Co) at 37°C in humidified atmosphere of 5% CO_2 in air for 45 minutes to inhibit DNA and RNA synthesis. Then, the cells were washed with RPMI 1640 medium 2 times to remove MMC and CY. These cells (MMC and CY treated autorosetting T cells ; AR^+ cells) were tested for their suppressive activity in the assay culture system (see below). The non-rosetting cells were treated with MMC and CY and washed. These cells were used for study crowding effect.

3. Assay AR^+ cells to suppress IL-2 secretion

Graded concentrations of viable cells of AR^+ cells (0.125×10^6, 0.25×10^6 and 0.5×10^6 cells/ml) in CRPMI medium were added to a constant concentration of rested PBMC (0.5×10^6 cells/ml) and stimulated with PHA-P at the final concentration of 0.5 µg/ml (suboptumal doses) and incubated at 37°C in humidefied atmosphere of 5% CO_2 in air for 18 hours. At the end of the culture periods, the culture supernatants were collected and stored at -40°C until assay for IL-2 activity. The percent of autorosetting T cells to suppress IL-2 secretion was calculated as follows :

$$\% \text{ Suppression} = \left\{1 - \frac{\text{IL-2 secreted by rested PBMC cultured with } AR^+ \text{ cells}}{\text{IL-2 secreted by rested PBMC cultured without } AR^+ \text{ cells}}\right\} \times 100$$

AR^+ cells = MMC and CY treated autorosetting T cells.

H. Preparation of AR^+ cells for assay system

A total of 4.0×10^6 PBMC in 4 ml CRPMI medium were stimulated with Con A at the final concentration of 5 µg/ml (minimum concentration) in 20 x 125 mm sterile screw cap tubes. These cultures were incubated at 37°C in humidified atmosphere of 5% CO_2 in air for 48 hours. Autorosetting T cells were separated as previously described. These cells were treated with 100 µg/ml MMC and 200 µg/ml CY at 37°C in humidified atmosphere of 5% CO_2 in air

for 45 minutes. Then the AR^+ cells were washed with RPMI 1640 medium 2 times to remove MMC and CY. These AR^+ cells were tested for their suppressive activity in the assay culture system as previously described.

I. To determine the inability of AR^+ cells to absorb IL-2 containing in culture supernatants

Autorosetting T cells from three normal subjects were prepared and treated with MMC and CY as previously described. These AR^+ cells were tested for their ability to absorb IL-2 activity in standard IL-2 preparation. Graded concentration of viable cells of AR^+ cells (0.125×10^6, 0.25×10^6, and 0.5×10^6 cells/ml) and control (no cells adding) were incubated with standard IL-2 at the final ratio 1:2 in the presence of PHA-P at the final concentration 0.5 µg/ml for 18 hours. At the end of the culture periods, the culture supernatants were collected by centrifugation at 200 g for 10 minutes and assayed as noted above for remaining (unabsorbed) IL-2.

J. To determine the inability of AR^+ cells to decrease PHA-P in culture supernatants

Autorosetting T cells from three normal subjects were prepared and treated with MMC and CY as previously described.

These AR^+ cells were tested for their ability to binding PHA-P in culture supernatants. If there were binding, the decreased PHA-P would stimulate less IL-2 production by rested PBMC. Graded concentration of viable cells AR^+ cells (0.125×10^6, 0.25×10^6, and 0.5×10^6 cells/ml) and control (no cells adding) were incubated with 0.5 ug/ml PHA-P at 37°C in humidified atmosphere of 5% CO_2 in air for 18 hours. At the end of the culture periods, each cells free culture supernatants (amount 0.8 ml) were incubated with rested PBMC (0.5×10^6 cells/ml) at 37°C in humidified atmosphere of 5% CO_2 in air for 18 hours. At the end of the culture periods, the culture supernatants were harvested and stored at -40°C until assayed for IL-2 activity.

K. Comparison between AR^+ cells and AR^- cells to suppress IL-2 secretion

Autorosetting T cells from three normal subjects were prepared and treated with MMC and CY as previously described. These AR^+ cells were tested for their ability to interfere the binding of PHA-P to rested PBMC and caused decrease IL-2 secretion (crowding effect). MMC and CY treated non-rosetting cells (AR^- cells) were used a control condition. A constant concentration of AR^+ cells or AR^- cells (0.5×10^6 cells/ml) were added to a constant concentration of autologous rested PBMC (0.5×10^6 cells/ml) and stimulated with PHA-P at the final concentration 0.5

µg/ml. These cultures were incubated at 37° C in humidified atmosphere of 5% CO_2 in air for 18 hours. At the end of the culture period, the culture supernatants were collected and stored at -40°C until assayed for IL-2 activity.

L. Viability test

A 0.1 ml of each cell suspension was mixed with 0.1 ml of 0.2 % nigrosine solution (see appendix 8) and incubated at room temperature for 5 minutes. The viable cells were not stained by nigrosine, whereas the dead cells were stained. Two hundreded cells were counted and the percentage of viable cells were calculated.

M. Statistical analysis

The two-tailed Student't test was used to determine the significance of differences between the mean of the two groups.