CHAPTER III

MATERIALS AND METHODS

1. Sites of study

1.1 The Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society : All laboratory activities which included the determination of the percentage of NK cells in PBMC using immunoperoxidase technique and the study of the function of cells with natural killing activity were performed at the Research and Development unit of QSMI.

1.2 Bumrasanaradura Communicable Disease Control Hospital: Blood samples were collected from human rabies patients admitted there.

1.3 Chula Hospital : Blood samples were collected from patients with non-fatal encephalitis other than rabies.

2. Materials

2.1 Rabies patients

A total of 13 patients with rabies, admitted to Bumrasanaradura hospital from November 1989 to December 1990, were included (Table 1). Seven were males and six were females.

Their ages ranged from 6 to 65 with a mean age of 34.3 (SD=20.5). Ten patients presented as encephalitis and the remaining 3 with paralysis. Only patients who were alert and coherent were enrolled into this study. The clinical diagnosis was based on a reliable history of a recent dog or cat bite, and by typical clinical manifestations including aerophobia, hydrophobia and/or inspiratory spasms. Blood was collected on the day of admission, usually 2 to 3 days after the onset of disease.

2.2 Patients with non-rabies encephalitis

The 6 encephalitic patients consisted of 2 with tuberculous meningoencephalitis , 2 with herpes simplex encephalitis and 2 with unidentified viral encephalitis were included. Two were males and four were females. Their ages ranged from 22 to 51 with a mean age of 35.8 (SD=9.9). These patients recovered but with mild to moderate neurological sequele. Blood samples were collected at the time of admission; usually 2-3 days after the clinical onset of the disease.

2.3 Normal healthy control

Thirty-one healthy blood donors at National Blood Center, Thai Red Cross Society and volunteers, with age and sex matched, had their blood collected and served as controls. Seventeen were males and fourteen were females. Their ages ranged from 17 to 58 with a mean age of 35.9 (SD=13.4).

3. Preparation of peripheral blood mononuclear cells

3.1 Collection of blood specimens

Only heparinized blood was used in this study (Heparin; Leo Pharmaceutical products, Ballerup, Denmark, 20 units per 1 ml of blood). A total of 30 ml of blood was drawn from each patient and control.

3.2 Mononuclear cell seperation

Mononuclear cells were separated from heparinized peripheral blood samples using Ficoll Hypaque density gradient centrifugation. Each six aliquots of five millilitres of heparinized blood was gently over - layered on 3 ml of the Ficoll Hypaque mixture (density of 1.077 g/ml). They were then centrifuged at 1,800 rpm for 30 minutes at room temperature (184). The mononuclear cells at the interface between plasma and Ficoll Hypaque was collected and washed 3 times with RPMI 1640 pH 7.4 by centrifugation at 1500 rpm for 10 minutes at room temperature. PBMCs were used for quantitate of NK cells and NK function study.

4. Immunoperoxidase staining

4.1 Cytocentrifugation step

4.1.1 10^4 PBMCs were spun onto slides by cytospin (Shandon Southern Instruments, Sewickley, PA, USA) at 1200 cycles per minute for 3 minutes.

4.1.2 After air drying, cells were fixed in periodate - lysine - paraformaldehyde - glutaraldehyde (PLPG) (see appendix II) for 20 minutes.

4.1.3 Slides were washed in PBS pH 7.4, 4 times and kept in PBS with 0.02 % Sodium Azide until use.

4.2 Staining

Mouse monoclonal antibodies to CD 56 and CD 57 were used to determine the number of NK cells in PBMC. Immunoperoxidase technique, using avidin - biotin as an amplification step was used as described by Hsu and co-workers (185) and Johnson et al (186) with some minor modifications.

The following steps were carried out at room temperature.

4.2.1 Slides were rinsed very gently with PBS, pH 7.4, for 2 minutes.

4.2.2 Nonspecific reaction were blocked by incubating with 2 % normal horse serum in PBS, pH 7.4, for 20 minutes, and washing once with PBS, pH 7.4, for 1 minute.

4.2.3 Cells were incubated with 100 ul of primary antibodies ; mouse anti human CD 56 or CD 57 monoclonal antibody (Becton Dickinson & Co., Cockeysville, MD, USA.) at dilution of 1:100 and 1:50 respectively in 2 % normal horse serum in PBS, pH 7.4, 45 minutes.(Normal mouse serum at a dilution of 1:100 was

used as control) After incubation with primary antibodies (and normal mouse serum as control) , cells were washed three times with PBS, pH 7.4 (1 min once and 5 mins twice).

4.2.4 Cells were then incubated with 100 ul of secondary antibody ; biotinylated horse anti mouse IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:100 in 2 % normal horse serum in PBS, pH 7.4, for 30 minutes. The washing step was done as described above (4.2.3).

4.2.5 Endogeneous peroxidase activity was blocked by 1 % hydrogen peroxide in Methanol for 30 minutes followed by washing with PBS, pH 7.4.

4.2.6 Preformed avidin - biotin - horseradish peroxidase complex (ABC)(Dako, Als, Denmark) (see appendix II) were applied on the cells, incubateed for 30 minutes and then washed as described in step 4.2.3.

4.2.7 Substrate reaction : Slides were incubated with freshly prepared substrate, 3-3'-diaminobenzidine (DAB) 0.5 mg/ml and 0.1 % hydrogen peroxide in PBS, pH 7.4, for 20 minutes or until the color became light brown. Drained off DAB into diaper and rinsed immediately in double distilled deionized water (DDDW) two times, 2 minutes each.

4.2.8 Intensification of the color by soaking in 0.5% copper sulphate in 0.15 M NaCl for 5 minutes and rinsed in DDDW for 5 minutes.

4.2.9 Counterstaining with Gill's hematoxylin # 1 (Sigma, St.Louis, USA) for 1-2 minutes, rinsed with DDDW 2 minutes.

4.2.10 Incubating with DDDW substitute (see appendix II) for 2-3 minutes followed by rinsing with DDDW.

4.2.11 Dehydrating cells twice in graded ethyl alcohol (70 %, 95 % and 100 %) 30 seconds to 1 minute each.

4.2.12 Clearing in xylene for 1 minute.

4.2.13 Mounting the cells in permount and covering with cover glasses.

4.3 Method of quantitation

Cells were examined under a high power objective (40X) light microscope. The antigen-positive cells contained either dark brown granules or a diffuse staining reaction in the cytoplasm (Figure 3).

Number of cells with CD 56 or CD 57 phenotype was assessed from 200 mononuclear cells and the percentage was calculated.

5. Determination of factors affecting the immunoperoxidase reaction

To achieve meaningful results in obtaining the greatest intensity of specific staining with the least amount of background interference and in avoiding false negative and false positive, the staining technique was tested in different conditions as following :

Affect on antibodies

Checkerboard titration for the determination of optimal dilution of primary antiboby and secondary antidody

The primary antibody : mouse anti human CD 56 and CD 57 monoclonal antibodies was diluted 1:25 , 1:50 , 1:100 and 1:200 with 2 % normal horse serum in PBS , pH 7.4 and 100 ul of each dilution was applied onto the slides and further steps were the same as previously mentioned in protocol. Slides with different concentrations of primary antibody were incubated with varying concentrations of secondary antibody at 1:100 and 1:200 with 2 % normal horse serum in PBS , pH 7.4.

Time course for incubation with primary antibodies

Incubation time for primary antibody varied from 45 minutes , 1 hour and 1.5 hours at room temperature.

6. Specificity of reaction in immunoperoxidase staining ,ABC technique

Slides of mononuclear cells of normal healthy donor, non-rabies encephalitic and rabies patients were incubated with 100 ul of normal mouse serum at dilution 1:50 , 1:100, 1:500 and 1:1000 in 2 % normal horse serum for 45 minutes.

7. 4-hr microcytotoxicity assay

7.1 Preparation of effector cells

PBMCs obtained from Ficoll hypaque centrifugation were used as effector cells.

7.2 Preparation of target cells

K 562 cells (187) a continuous cell line from a pleural effusion of a patient with chronic myelocytic leukemia at blast crisis, obtained from Prof. Diane E. Griffin, Department of Neurology, The Johns Hopkins Hospital, Maryland, USA. were used as target cells.

7.2.1 Target cell cultures

K 562 was cultivated in sterile plastic disposable tissue culture flasks (Costar, Cambridge, USA). They were grown in RPMI 1640 supplement with 100 U/ml penicillin, 100 ug/ml streptomycin, 10 mM HEPES and fetal calf serum (FCS) to 10% final concentration. Subculture was done every three days with a

splitting ratio of 1:2 to 1:3 and passed one day before used. Cells were maintained in a humidified 5 % CO_2 incubator at 37 C.

7.2.2 Target cell labelling

Cells were transfered from the culture flasks and were washed 3 times with RPMI 1640 pH 7.4. Their viability was checked by 0.1 % trypan blue staining with a count of more than 90 %. A suspension of 1×10^6 target cells in 0.5 ml RPMI 1640 medium was labelled for 1.5 hrs with 100 uCi of $Na_2{}^{51}CrO_4$ (Amersham, International plc., Buckinghamshire, England) in a 15 ml conical tube and then washed thrice with RPMI 1640 . These cells were suspended in 10 ml RPMI 1640 with 10 % FCS and left in 4 C for 1 hr.to minimize spontaneous release of radioactivity. They were then centrifuged once at 500 rpm for 10 minutes with supernatant fluid discarded and cells adjusted to a concentration of 5×10^4 cells/ml with 10 % FCS in RPMI 1640.

7.3 assay

7.3.1 100 ul of 5×10^4 labelled K 562 target cells/ml were added into each well of round bottomed microtiter plate.

7.3.2 100 ul of different amounts of effector cells were added into each well resulting in an effector : target (E:T) ratio of 100:1, 30:1, 10:1, 3:1 and 1:1 (triplicate assays were done). Triplicates of spontaneous release as well as maximum lysis were also prepared for each experiment.

Determination of spontaneous release activity was performed by incubating 5×10^3 labelled target cells with medium without effector cells . The maximum release activity was achieved by incubating 5×10^3 labelled target cells with 1 % sodium dodecyl sulphate (SDS) in RPMI 1640.

7.3.3 Plates were incubated in a humidified 37 C incubator containing 5 % CO_2 for 4 hrs.

7.3.4 After centrifugation at 500 rpm for 10 minutes , 100 ul of supernatant were carefully collected into plastic tubes.

7.3.5 Radioisotope activity was counted for 1 minute in a gamma counter.

7.3.6 The average count per minute (CPM) of each triplicate and percent specific lysis were calculated.

$$\% \text{ specific lysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximum lysis - spontaneous release}} \times 100$$

NK cell activity was expressed as lytic units (LU)/10^7 PBMC as determined by least squares analysis. One LU was defined as the number of effector cells required for 20% specific lysis of 5×10^3 target cells (Figure 4).

^{51}Cr released from the spontaneous well was usually less than 15% of the maximum cytotoxicity. Data were rejected in any test where spontaneous release was greater than 15% .

8. In vitro activation of cytotoxic cells

PBMC in RPMI 1640 with 5% FCS were cultivated at 37 C overnight at a concentration of 10^6 cells/ml in the presence of 100 units/ml of recombimant human α -IFN (Schering Cooperation, USA.) or recombinant human IL-2 (Genzyme,USA.)and then assays for lytic activity against ^{51}Cr-labelled K 562 cells were done as described above.

9. Statistical analysis

Data were analysed using the nonparametric Mann-Whitney U test for NK number and function, and Wilcoxon signed-rank test for in vitro activation.