MATERIALS AND METHODS

2.1. Sample Collection

Nine hundred sixty nine sera were collected from 15 October 1997 to 30 June 1998. Out of the 969, 867 sera were from the Institute For Medical Research (IMR), where the sera were previously collected from different parts of Malaysia for a dengue fever survey. The remaining 102 sera were random pediatrics samples from the Clinical Diagnosis Laboratory (CDL) of the University Hospital Kuala Lumpur (UHKL).

Nine hundred sera were diluted and the endpoint titre of IgG antibodies against EBV and HHV-6 were determined by indirect immunofluorescence assay (IFA). These 900 sera consisted of 867 sera from IMR and 33 sera from CDL, UHKL. The sera were divided into 12 age groups (0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8, 8-9, 9-10, 10-15, 15-20 years) with 75 sera in each age group. 862 from these 900 were also tested for EBV seroreactivity by enzyme-linked immunosorbent assay (ELISA).

The remaining 69 samples were sera from newborn babies (60) and from babies aged 5 to 11 months (9). Since only microlitre quantities were obtained, the sera were only used to screen for EBV and HHV-6 seroreactivity by using IFA at 1:10 serum dilution.

All the sera were inactivated for 30 minutes at 56^0 C in a water bath. The sera were then stored at -20^0 C until use.

2.2. Epstein-Barr Virus Indirect Immunofluorescence Assay

2.2.1. Cell Line

EBV-positive cell line, P3HR-1 (Hinuma *et al.*, 1967) was used as the source of EBV capsid antigen (EBV-VCA) in this study. EBV-DNA negative BJAB cell line (Menezes *et al.*, 1975) was used as the negative cell control to check for non-specific binding in IFA.

2.2.2. Culture Medium

10.38 g (1 packet) of RPMI-1640 (Flow Lab, Australia) containing L-glutamine and without HEPES (N-2-Hydroxyethyl-piperazine-N^1 -2-ethane-sulfonic acid) was dissolved in 980 ml freshly deionised distilled water. 2.0 g of sodium bicarbonate (May & Baker LTD, England) was added into the medium. The pH of the medium was adjusted to pH 7.2 with sodium hydroxide (Merck, Germany) or hydrochloric acid (J.T.Baker, USA) and the volume made up to 1 L. The medium was then filter sterilised through a 0.22 μm Millipore filter. The filtered RPMI-1640 medium was checked for sterility and kept at 4^0 C.

The culture medium was made up of RPMI-1640 medium containing 10% of heat-inactivated fetal calf serum (Flow Lab), 100 IU/ml penicillin and 100 μg/ml streptomycin (Flow Lab). This 10% fetal calf serum supplemented culture medium is referred to as RPMI-10.

2.2.3. Reviving of Cells

A vial of cells was removed from the liquid nitrogen tank and plunged into a 37^0 C water bath for quick thawing. The cells were then transferred into 5 ml RPMI-10 and centrifuge at 800 rpm (400 x g) for 3 minutes. The supernatant was discarded and the pellet resuspended in 5 ml RPMI-10 and dispensed in a 50 ml tissue culture flask (Nunclon, Denmark). Incubation was at 37^0 C in a 5% CO_2 incubator (Jouan, France).

2.2.4. Culturing of Cells

Cultured cells were grown in RPMI-10 in 50 ml tissue culture flasks and incubated at 37^0 C in 5% CO_2 incubator. The cultures were closely monitored to ensure there was no contamination by microorganisms and for the maintenance of pH. The lymphoblastoid cells were passaged (1:3) twice weekly.

2.2.5. Solutions

2.2.5a. *Phosphate Buffered Saline (PBS)*

80.0 g sodium chloride (Univar, Australia), 2.0 g potassium chloride (Merk), 11.5g di-sodium hydrogen orthophosphate anhydrous (BDH, England) and 2.0 g potassium dihydrogen orthophosphate (Merck) were dissolved in 1 litre of distilled water. The pH of the buffer was adjusted to 7.2, and the volume made up to 10 litres with distilled water.

2.2.5b. ***Sodium Butyrate (NBA)***

0.33 g NBA (Sigma, USA) was dissolved in 10 ml of PBS to make a solution of 300 mM NBA. The solution was filter sterilised using 0.20 μm single use filter unit (Sartorius, Germany). This sterilised solution was then aliquoted into microcentrifuge tubes and stored at -20^0 C.

2.2.5c. ***12-0-tetradecanoyl phorbol 13-acetate (TPA)***

1 mg TPA (Sigma) was dissolved in 1 ml of Dimethyl sulfoxide, (DMSO; Sigma) to make a stock solution of 1 mg/ml. The working solution was 20 μg/ml in RPMI-1640 medium (100 μl of stock of 1 mg/ml to 4.9 ml of RPMI-1640). To avoid oxidation, solution of TPA was gassed with nitrogen before sealing and storage at -20^0 C.

2.2.6. Cell Count and Viability Test

Cell count and viability test was done to evaluate the quantity and quality of cells in the cell culture. 0.1 ml of cell culture was added to 0.9 ml of 0.1% trypan blue solution (BDH) before they were counted in a haemocytometer (Weber, England). Dead cells stained blue. Only cultures with at least 95% viability were used for cell induction.

2.2.7. Induction of P3HR-1 Cells

TPA and NBA were used as inducers of EBV cycle. P3HR-1 cells were made to 5 x 10^5 cell /ml with fresh culture medium. 10 μl NBA /ml culture medium and 1μl TPA /ml culture medium were added into the flask. Incubation was continued at 37^0 C with 5% CO_2 for 3 days before the cells were harvested.

2.2.8. Harvesting of Induced P3HR-1

Cells were centrifuged at 800 rpm (400 x g) for 5 minutes. The supernatant was discarded and the cells washed 3 times with PBS before being resuspended in a small volume of PBS. A drop of this cell suspension was placed onto wells of a 12-well multi-test teflon-coated slide (Sigma) to obtain monolayers of cells on the wells. The slides were air dried and then fixed in cold acetone (-20^0 C) for 10 minutes. Fixed slides can be kept at -70^0 C before use.

2.2.9. IFA for EBV Serosurvey

Serial dilutions of sera with PBS were carried out using a microtitre plate (Flow Lab). 20 µl serum was used per-well of the P3HR-1 slide, and incubation was in a humid chamber at 37^0 C for 45 minutes. At the end of the incubation, the sera were rinsed off with PBS and washed 3 times with PBS, 5 minutes each time. The slides were then blotted dry and 20 µl of diluted fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human IgG (Dako. Diluted 1:40 with PBS and store at 4^0 C in the dark) was added. The slide was reincubated in the humid chamber at 37^0 C for 45 minutes.

After the incubation, the slides were washed in PBS as was done after the incubation with the primary antibodies. The slides were then blotted dry and mounted with glycerol-buffer mountant (1:1 glyserol : PBS). They were examined under an ultraviolet light microscope using 200 x magnification (Olympus BH2, Japan). The samples were marked as VCA-EBV seropositive when more than 10% of the cells in the well showed specific fluorescence. Any non-specific fluorescence was cross-checked with

EBV negative BJAB cells. An EBV negative serum (negative control) and a high-titred NPC serum (positive control) were included in every assay.

After the seroreactivity screening at 1:10 dilution, all the positive sera were further diluted in a series of double dilutions of 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280 in the assay to find the endpoint titre. The titre of the serum was taken as the highest serum dilution giving specific fluorescence.

2.3. EBV Viral Capsid Antigen IgG ELISA Test

The PanBio (Australia) IgG-VCA enzyme-linked immunosorbent assay (ELISA) kit was used. The kit contains a 12 x 8 microwell-plate coated with recombinant VCA, wash buffer, serum diluent, horseradish peroxidase (HRP) conjugated sheep anti-human IgG, tetramethylbenzidine (TMB) reagent, stop solution, one positive control serum, one negative control serum and one cut-off calibrator serum.

All reagents and sera were brought to room temperature (25^0 C) before use. Sera, positive control, negative control and cut-off calibrator were diluted 1:100 in a microtitre plate. 100 µl of the diluted samples were pippetted into their respective microwells. The plate was then covered and incubated at 37^0 C for 20 minutes. At the end of incubation, the plate was washed 4 times with diluted wash buffer. 100 µl of HRP conjugated sheep anti-human IgG was then pippetted into each well. The plate was again covered and incubated at 37^0 C for 20 minutes. After the second incubation, the plate was washed 6 times with wash buffer. Next, 100 µl of TMB reagent was pippetted into each well, after which the plate was left at room temperature for 10 minutes before 100 µl stop solution

was pippetted into each well. The absorbance of each well was read at a wavelength of 450 nm in an MRX microplate reader (Dynex, Germany).

The results were calculated as PanBio Units using the formula shown below :

$$\text{PanBio Unit} = 10 \times \frac{\text{Absorbance of sample}}{\text{Mean absorbance of cut-off calibrator}}$$

A unit of >10 was considered positive for specific IgG antibodies against EBV-VCA.

2.4. Human Herpesvirus 6 (HHV-6) Indirect Immunofluorescence Assay

2.4.1. Solutions

2.4.1a. *Human Interleukin-2 (IL-2)*

5 µg of Human IL-2 (GIBCO) was reconstituted in 50 µl of 100 mM acetic acid (Merck) supplemented with 0.5% bovine serum albumin (Sigma). This mixture was then dispensed into 50 polypropylene tubes (Sarstedt, Germany) with 1µl in each tube, and stored at -20^0 C.

2.4.1b. *Phytohaemagglutinin (PHA)*

One tube of lyophilized PHA (Gibco) was rehydrated with 10 ml of distilled water. It was then dispensed into 5 microcentrifuge tubes (ISC, Utah) with 2 ml in each tube and stored at -20^0 C.

2.4.2. Isolation and Culture of Human Cord Blood Mononuclear Cells (HCBMC)

Fresh cord blood was collected aseptically from umbilical cords of newborns in the labour ward of UHKL by using 5-ml syringes. 10 µl of 1000 unit/ml heparin (Gibco, USA) was added to each 1 ml of cord blood to prevent clot formation. 5 ml of cord blood was mixed with the same volume of RPMI-10 at 37^0 C in the laboratory. This mixture was gently layered onto 3 ml of Ficoll-paque, density = 1.077 (Flow Lab) and centifuged at 1500 rpm (500 x g) for 30 minutes. The HCBMC in the interface was then harvested carefully by pasteur pipette and washed twice, each time in 5 ml of PBS.

Freshly isolated HCBMC was suspended into 5 ml pre-warm (37^0 C) HCBMC growth medium in a 50 ml tissue culture flask (HCBMC Growth Medium consists of RPMI-1640 containing 10% FCS, 0.001 µg/ml IL-2, 20 µl/ml PHA, 100 IU/ml penicillin and 100 µg/ml streptomycin). The pH of the medium was adjusted to 7.6 and the flask incubated at 37^0 C in a 5% CO_2 incubator. The culture was checked daily.

2.4.3. HHV-6 Infection of HCBMC

At the end of 3 days, actively dividing and aggregating HCBMC should be seen. These activated HCBMC were centrifuged at 1500 rpm for 10 minutes. The supernatant was discarded and the cell pellet was washed twice, and resuspended in 1 ml of HHV-6 stock containing a minimum of 2000 HHV-6 infected cells (HCBMC infected by HHV-6 variant B, strain Hashimoto (Japan) was a kind gift from Dr. Chua Kaw Beng from Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur). Cell suspension was centrifuged at 1500 rpm for 30 minutes to enhance infection. At the end of infection, the supernatant was discarded and the pellet was

resuspended in 5 ml of RPMI 1640 maintenance medium (RPMI 1640 with 2% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin) in a 50 ml tissue culture flask. The flask was incubated at 37^0 C in 5% CO_2 incubator. On the fourth day of incubation, about 50% of the cells should be refractile and significantly enlarged. Whenever the number of cells showing the cytopathic effect (CPE) was low, the virus titres were enhanced by passaging: 0.6 ml of 4-day old infected cells were added to freshly isolated HCBMC from 5 ml of cord blood. 80% of the cells should be showing CPE at 4 days post-infection.

2.4.4. Harvesting of HHV-6 Infected Cells

HHV-6 infected cells were harvested on the 4th day of post-infection when at least 50% of the cells were showing CPE. The harvested cells were washed 3 time in PBS and dropped onto teflon-coated 10-well slides and then fixed in cold acetone.

2.4.5. Confirmation of HHV-6 Infection

A mouse monoclonal antibody (OHV-3) against a specific HHV-6 variant B (Hashimoto strain) antigen (a gift from Professor Koichi Yamanishi, Japan) was used to confirm HHV-6 infection in HCBMC. FITC-conjugated goat anti-mouse IgG (Dako) was used as the second antibody in the IFA. All the steps used in HHV-6 IFA were the same as used in EBV IFA.

2.4.6. Electron Microscopy

5 ml of 4th day HHV-6 infected HCBMC culture was centrifuged at 1500 rpm for 10 minutes. The infected cells were washed 3 times with PBS before being resuspended in 1 ml of PBS. A drop of the suspension was placed on a formvar (Sigma) coated copper grid (Agar Scientific LTD.). Particles in the suspension were allowed to set for 5 minutes before the excessive fluid was dripped off. The copper grid with a layer of infected cells was blotted dry and negatively stained with 1% uranyl acetate (Agar Scientific LTD.). After 5 minutes, it was rinsed with water and allowed to air dry. The copper grid was viewed under a CM12 Transmission Electron Microscope (Philips, Holland).

2.5. Assessment of Results

Seroprevalence of EBV and HHV-6 were calculated according to age group, sex group, race group and geographic distribution. χ^2 test was then employed to determine the differences in seroprevalence of EBV and HHV-6 in different groups.

The geometric mean titre and Z-test were then performed to determine the differences between mean titres of antibodies (calculated based on $\log_{10}$ GMT values) in different groups.

All the statistical analysis were two-tailed with significance level at $\alpha = 0.05$ or $\alpha = 0.01$. The formulae used in the tests are shown in appendix.