CHAPTER 3 : MATERIALS AND METHODS

3.1 EPIDEMIOLOGY OF GYNAECOLOGICAL NEOPLASMS

3.1.1 Collection of epidemiological data

The data for this study was collected from the General Hospital, Kuching, Sarawak, East Malaysia. The study population consisted of all gynaecological cancer patients admitted from January, 1985 to November, 1993 at the Radiotherapy Unit, General Hospital, Kuching. The registration number, name, address, ethnic group, age and type of cancer diagnosed were recorded. The total number of patients recorded during the nine year period was 789.

3.1.2 Analysis of data

The age, ethnic group and type of cancer (cervix, endometrium, ovary, vulva/vagina) prevalence and incidences in the total study population were analysed. A study of the distributions of cancer type in each major ethnic groups (Chinese, Malay, Iban, Other Indigenous Groups i.e. Melanau, Bidayuh, Kayan, Kenyah, Daro, Kelabit, Penan, Bisaya, Ukit, Kadazan, Dusun and Murut) was also carried out. Since the number of cases for the cancer of the endometrium, ovary and vulva/vagina were very small, the analysis of age and ethnic groups incidences were only done for cervical cancer. The 1985 - 1993 population year book was used to calculate the incidences
(Department of Statistics, Malaysia). The calculation of the age standardized rate (ASR) is based on the World Standard Population (modified by Doll et al., 1966, See Appendix) as reference population. The student’s t test was used for all statistical analysis.

3.2 SEROLOGY OF CERVICAL CARCINOMA

3.2.1 Collection of sera samples

Sixty-nine blood samples of patient with cervical carcinoma were collected at the Gynaecology Clinic of Hospital Kuala Lumpur (HKL), Malaysia. Forty-four blood samples from normal pregnant women were obtained from outpatients at the Antenatal Clinic of HKL. Ten millilitres of blood sample from each individual were spun down and the serum collected and aliquoted into Wheaton vials and stored at -20°C. All sera samples were inactivated by heating in a water bath at 56°C for 30 min.

3.2.2 Detection of IgA, IgG and IgM antibodies against HPV Type 16.

The IgA, IgG and IgM antibodies against HPV type 16 were detected using ELISA (Dillner, 1990). The synthetic peptide used to define the antibody response to HPV was HPV-16, E7 open reading frame (ORF) number 5, obtained from Dr. J. Dillner (Karolinska Institute, Stockholm).
i. Antigen coating

The synthetic peptide was diluted to 20 μg/ml in 10mM carbonate buffer, pH 9.6 (See Appendix) and added at a volume of 50 μl/well to half of the wells of a flat bottom microtitre plate (Costar) leaving the edge wells empty. The other half of the microtitre plate was filled with carbonate buffer as negative control. The plates were then kept at room temperature overnight.

ii. Blocking of plates

The peptide solution was discarded and after one wash with 150 μl/well phosphate buffer solution (PBS (See Appendix)) with 0.05% Tween 20 (PBS-T) the plates were blocked with 10% lamb serum (Gibco) in PBS (LS - PBS). The lamb serum was initially heat inactivated and filtered through Whatman paper and 0.45 μm membrane filter. The plates were then incubated for 60 minutes at 37°C. They were then frozen at -20°C until further use.

iii. Detection of antibodies

The plates were thawed at 37°C and the blocking solution was discarded. Human sera were diluted 1 : 30 in LS-PBS and added to the plates (50 μl/well) and incubated
for 2 h at 37°C. The plates were properly sealed with adhesive cover tape to avoid drying. After incubation the plates were washed five times with 150 μl/well PBS-T.

a) IgA detection

For the IgA detection, 50 μl/well of monoclonal anti-human IgA horseradish peroxidase conjugate (Janssen), diluted 1 : 500 in LS-PBS was incubated in the coated microtitre plates for 2 h at 37°C. The plates were then washed five times with 150 μl/well PBS-T and added with 50 μl/well of peroxidase substrate (Appendix). They were incubated for 1 h at room temperature and the absorbances were recorded at 415 nm using an ELISA reader (Titertek Multiskan MMC 340).

b) IgG detection

For the IgG detection, 50 μl/well of rabbit anti-human IgG-alkaline phosphatase conjugate (Dako), diluted 1 : 1,000 in LS-PBS was incubated in the coated microtitre plates for 2 hour at 37°C. The plates were washed five times with 150 μl/well PBS-T and once with 0.1 M diethanolamine buffer, pH 9.6 (Appendix). They were then added with 50 μl/well of phosphatase substrate (Appendix) and the plates were read at 405 nm after 90 minutes using an ELISA Reader (Titertek Multiskan MMC 340).
c) IgM detection

For the IgM detection, 50 μl/well of goat anti-human IgM-glucose oxidase conjugate (Seralab), diluted 1:800 in LS-PBS was incubated in the coated microtitre plate for 2 h at 37°C. The plates were washed five times with 150 μl/well PBS-T and added 50 μl/well of glucose oxidase substrate (Appendix) and were read at 415 nm after 1 hour using an ELISA Reader (Titertek Multiskan MMC 340).

Evaluation of ELISA Readings

An absorbance of 0.1 or more above background (same serum in uncoated wells) was considered as a positive reaction.

3.2.3 Detection of IgG antibody to CMV and HSV-II

Antibody detection of CMV and HSV-II were carried out using Enzygnost Anti-CMV Kit and Anti-HSV Kit (Behring).

Composition of Test kit:

The test plate consisted of 6 strips in a special holder. Each strip had 2 X 8 reaction wells which allowed 48 sera samples to be tested at one run. One row of the wells were coated with the antigen and the other row were coated with negative
control. For the detection of IgG antibody to CMV, the antigen were obtained from human cell cultures infected with CMV and inactivated before coating while the negative control were obtained from human cell cultures not infected with CMV. For the detection of IgG antibody to HSV-II, the antigen were obtained from HeLa cells infected with HSV and the negative control were obtained from HeLa cells which were not infected with HSV and inactivated before coating.

The positive control human serum (Enzygnost) to CMV and HSV antibody provided in the kit were used as a reference to validate the test results. The test results are valid if the positive control serum exhibits a positive reaction in the test dilution.

The anti-human alkaline phosphatase (AP) Conjugate (Enzygnost) provided in the kit, was prepared by coupling AP with highly avid antibodies from rabbits and diluted 1/40 in the dilution buffer provided.

The reagents provided in the kit include the AP Substrate buffer, AP substrate tablets, AP dilution buffer, AP washing solution (concentrate) and AP stopping solution (diluted in sodium hydroxide (NaOH)).
Procedure

The test kit were kept in 4°C before use. To begin using the kit, the test plate was allowed to stand for about 5 minutes at room temperature (RT). 0.15 ml of dilution buffer was introduced into each well. 0.05 ml of sera samples and the positive control serum (prediluted 1 : 11 in dilution buffer) were pipetted into the wells with antigen and the wells without antigen (negative control). The test plate was then incubated in a moist chamber at 37°C for 1 h.

After incubation, the sample dilutions were suctioned off. 0.2 ml of washing solution was pipetted into each well and suctioned off after about 1 to 2 min. The washing process was done twice. Each well was then added with 0.05 ml of the diluted anti-human IgG/AP conjugate solution and the plate was incubated in a moist chamber at 37°C for 1 h. Then the enzyme conjugate solution was suctioned off and the test plate was washed as described. The wells were then added with 0.1 ml of substrate solution and incubated for 30 min at 37°C in a moist chamber. At the end of the incubation the enzyme reaction was stopped by the addition of 0.05 ml of AP stopping solution (diluted with NaOH). The absorbance of the yellowish-green colour reaction was read at 405 nm using ELISA Reader (Titertek Multiskan MMC 340).
Evaluation of Absorbance Readings

The test results are valid if the positive control human serum exhibits a positive reaction in the test dilution. A positive reaction is indicated by a difference in absorbance whereby

$$A_{\text{antigen}} - A_{\text{control antigen}} \geq 0.2$$

($A = \text{Absorbance}$)

3.2.4 Cell Cultures and Maintenance

i. Cell lines

The cell lines used in the present study were EBV-DNA positive non-producer line Raji (Pulvertaft, 1965; Epstein et al., 1966) and producer cell line P3HR-1 (Hinuma et al., 1967) and were provided by Dr.D.V.Ablashi (NCI, Bethesda). The P3HR-1 cell line was used to prepare EBV-VCA and the Raji cell line for EBV-EA.

ii. Revival of Cells

The provial of cells was removed from liquid nitrogen and plunged into a beaker of ice. It was then transferred to a 37°C water bath for quick thawing. The cells were transferred into some supplemented RPMI 1640 (See Appendix) in a Falcon tube and then spun at 800 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 20% foetal calf serum (FCS) supplemented RPMI 1640 and incubated
in a tissue culture flask at 37°C in a CO₂ incubator. Excellent recovery of viable cells was obtained using this technique.

### iii. Maintenance of Cells

All cultures were maintained in supplemented RPMI 1640, in tissue culture flasks (Falcon) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air (Forma Scientific 3171 water jacketed incubator with automatic CO₂ controller). The cells were checked every day under an inverted microscope (CK Olympus, Tokyo), for any bacterial or fungal contamination. The cultures were fed twice a week by replacement of the supernatant with fresh supplemented RPMI 1640. The cultures were split when the concentration of cells exceed $2 \times 10^6$ per ml.

### iv. Cryopreservation of Cells

Six millilitres of exponentially growing cells (in sterile Falcon tubes) were spun down at 1000 rpm for 5 min using a bench centrifuge (MSE 20). The cells were resuspended in 3 ml of freezing solution (Appendix) and aliquoted into provials (swabbed with 75% alcohol), in 1 ml volumes. The provials were left to stand in ice and then kept in a styrene box with 1 inch thick walls. The box was placed in -70°C freezer overnight, before transferring the provials to liquid nitrogen.
3.2.5 Detection of IgA and IgG antibodies to EBV-VCA and IgG antibody to EBV-EA

i. Enhancement of EBV Antigen Expression

a) VCA Expression in P3HR-1

The P3HR-1 cell line was aged to produce VCA. This was done by transferring the cells growing exponentially at 37°C to a 34°C incubator and left there for 3 - 4 days without change of media. The cells were then harvested and slides were prepared with appropriate concentration of cells (3.2.5 ii).

b) EA Expression in Raji

One milligramme of 12-0-tetradecanoylphorbol-13-acetate (TPA) was dissolved in 10 ml dimethyl sulfoxide (DMSO, Grade 1, Sigma Chem. Co.) and aliquoted into provials in 1 ml volumes. The provials were gassed with nitrogen before sealing and then kept in liquid nitrogen.

Twenty nanogramme per millilitres of the TPA diluted in FCS free RPMI 1640 was added to a continuous culture of 1 - 5 X 10^5 cells/ml of Raji cells. The culture with the TPA was incubated for 72 h at 37°C in a CO₂ incubator. The cells were then ready for harvesting.
ii. Harvesting of Cells

The cells that were ready for harvesting were spun down at 800 rpm for 5 min using an MSE 20 bench centrifuge. The supernatant was discarded and the cells were washed 2X with PBS (Appendix). Finally, the cells were resuspended in a small volume of PBS.

Using a pasteur pipette, a drop of suspended cells was placed onto a well of the 12-well teflon-coated slide (Cooke) and the distribution of the cells was checked under an inverted microscope (Olympus, Tokyo). The well should be filled with a confluent layer of cells. The dilution of the cells was altered if the spread of cells was not satisfactory.

The slides with the drops of cells were dried under cold air. They were then fixed in cold acetone (-20°C, Analar R) in a covered Coplin jar for 10 min. After drying, the slides were kept in slide boxes at -20°C for short periods. For long periods storage, -70°C freezer was used. The slides were then ready for use in the immunofluorescence test.
iii. Indirect Immunofluorescence Assay

A modification of the indirect immunofluorescence test described by the Henles (Henle et al., 1970; Henle et al., 1977) was used for titration of IgG and IgA antibodies to VCA and EA.

The serum were diluted in a series of double dilutions of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640 and 1/1280 in PBS (Appendix) in a microtitre plate (Dako). 20 μl of diluted serum was dispensed onto the well of the multi-test slide (with the antigen). This was done for all 8 dilutions for each serum sample. A positive NPC sera (high antibody titre) and negative NPC sera (titre value less than 10) were used as controls. The slides were incubated in a humidity chamber at 37°C for 45 min. After incubation the slides were rinsed off with PBS and placed in slide holder filled with PBS and placed on a rocker to circulate the buffer for 10 min. The washing procedure was done twice.

The sides of the slides and in between the wells were dried using absorbent cotton buds while the wells were not allowed to dry. The wells were then added with 20 μl/well of fluorescein-conjugates. The conjugates used were fluorescein-conjugated rabbit anti-human IgA (diluted 1/20) and rabbit anti-human IgG (diluted 1/15). The slides were then incubated in a humidity chamber at 37°C for 45 minutes and were washed with PBS twice. They were then dried under cold air and mounted with
glycerol buffer (Appendix) and covered with 22 X 50 mm coverslips (Mitsunami). The slides were ready for reading.

Reading of slides

The fluorescence of the wells were observed using a UV-microscope (Olympus). At each end point, fluorescent cells were absent. Hence, the antibody titre was taken as the reciprocal value of the highest serum dilution giving specific fluorescence. Titre values of 10 and above are considered as positive. The geometric mean titre (GMT) value was also calculated (See Appendix).

Analysis of data

All data were analyzed statistically using the student’s t test.