MATERIALS & METHODS

2.0 MATERIALS AND METHODS

2.1 PROTEINS AND PEPTIDES

The proteins/peptides tested were derived from various regions of the Epstein-Barr virus antigens. They are:

2.1.1 Recombinant Proteins:

- p54 - 404-amino-acids long, 52/47kD recombinant protein from EA-D / accessory protein of DNA polymerase, encoded by BMRF1 reading frame
- p138 - 376 amino-acids long, 39kD fusion of two protein segments from EA-D within the BALF2 reading frame
- p23 - recombinant protein from a part of VCA

p54, p138 and p23 were cloned in *Escherichia coli* bacteria JM109 strain, via a pUC plasmid as carrier. The proteins were isolated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. Isolated fragments were then tested in immunoblots to determine the presence of their antigenicity (Hinderer *et al.*, 1990). They were tested positive with control sera therefore concluded to be antigenically viable for antigen coating in enzyme-linked immunosorbent assay (ELISA) tests. The recombinant proteins were supplied by Dr. Walter Hinderer of the Research Division of Biotest AG, Germany.

2.1.2 Synthetic Peptides:

Peptides 1 to 83 of p54 recombinant EA, including controls as specified by kit supplier. The peptides were synthesized using the Chiron's non-cleavable multipins mimotope mapping technique as 10-residue with 5-amino acid overlap peptide sequences (Geysen *et al.*, 1984; 1987b; Houghten, 1985; Houghten *et al.*, 1986) (see Appendix 1)

2.2 SERUM SAMPLES

2.2.1 NPC Sera:

Serum from histologically confirmed NPC patients were collected at the ENT Clinic, University Hospital, Kuala Lumpur (UHKL). Alll of the sera were of histologically confirmed NPC patients who have been treated with radiotherapy and/or chemotherapy.

2.2.2 Control Sera:

Control sera included those from normal healthy subjects (NHS), patients with diseases other than NPC e.g. ENT diseases and patients with cancers other than NPC i.e. gynaecological, stomach and colon cancers. NHS sera were collected from various places around the country during blood donation campaigns. The samples collected were mainly from Chinese males between the ages of 35-50 years old.

2.2.3 Suspect NPC Sera:

Serum from patients presented with clinical symptoms indicative of NPC e.g. nose bleed, double vision and neck node, were collected at the ENT Clinic, UHKL. These patients were biopsied and their specimens studied

histologically. Thirty four of those biopsied were histologically confirmed to have NPC while 10 were histologically normal. Twenty one of those confirmed NPC patients were followed up.

2.2.4 Immunoglobulin A anti VCA (IgA-VCA) and IgG anti EA (IgG-EA) Titers:

The titers of IgA against VCA and IgG against EA were determined by indirect immunofluorescence method as in Sam *et al.*, 1989. P_3HR-1 and Raji cells were grown in RPMI culture medium at 37°C. The EBV lytic cycle was induced on day seven with addition of phorbolester and sodium butyrate for 60 and 48 hours for P_3HR1 and Raji respectively. Cells with viability of 80% and above were harvested and seeded onto multiwell slides. Cells were fixed onto the slide wells using acetone (at 0°C - 4°C) for 10 minutes. Slides were kept at -20 °C until use.

Sera were tested using these slides with positive and negative controls being included in each run. Sera were diluted serially 1:5, 1:10, 1:20, 1:40 and 1:80 in phosphate buffered saline (PBS), pH 7.2. Slides with diluted sera were incubated for 45 minutes at 37°C in a humid chamber. Three washes followed and slides were then allowed to air dry. 20µl of fluorescein conjugated immunoglobulin A (IgA) or G (IgG) (Dako, Germany) were then added onto

each well and a second incubation was carried out. Slides were washed 3 times as was done previously. After they were air dried, slides were mounted with 1:10 glycerol:PBS and read under an ultraviolet light microscope (Olympus BH2, Japan). The highest serum dilution showing distinct cell fluorescence was taken as the specific antibody titer. A titer of >1:5 was considered positive. Each test run was accompanied by an EBV negative serum as negative control and a high-titered NPC serum as a positive control.

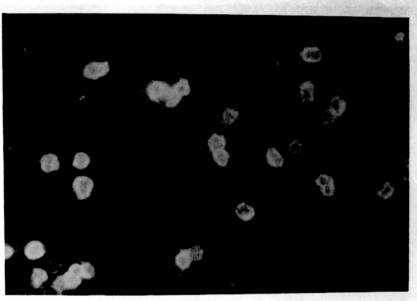

Figure 2.1: Positive staining of IgA antibody of Epstein-Barr virus viral capsid antigen (VCA) detected by Indirect Immunofluorescence (IIF)

Figure 2.2: Negative staining of IgA antibody to Epstein-Barr virus viral capsid antigen (VCA) detected by Indirect Immunofluorescence (IIF)

2.3 PROTEIN HYDROPATHY PLOTS AND SECONDARY STRUCTURE PREDICTION

Hydropathy plots of p54 were done using MacDNASIS and an internet Protscale (http:\\expasy.hcuge.ch/cgi-bin/protscale.pl) analysis, based on method of Hopp and Woods (1981; 1983) while the protein secondary structure prediction was based on the Chou and Fasman's (1978) technique. Both MacDNASIS and Protscale programs require the use of single letter amino acid codes of the protein sequence for analyses. Hydropathy profiling and secondary structure prediction analyses were done using a 7-amino-acid window.

Hydrophobicity and hydrophilicity plots of other proteins i.e. EBNA1 and ZEBRA that have been studied in our laboratory were also determined using the same computer programs. Hydropathy and hydrophilicity plots provide the first basis of antigenicity prediction for a region of interest in a protein, before shorter peptide fragments were synthesized to determine protein's specific immunodominant epitopes.

2.4 MULTIPIN PEPTIDE SYNTHESIS

To further analyse the specific epitopes particularly on p54, short 10 amino acid peptides with a 5-amino-acids overlap were synthesized onto non-cleavable polyethylene pins (Chiron Mimotopes, Australia).

2.4.1 Storage of the Multipin Peptide Synthesis Kit:

The kits included polyethylene pins attached to pin blocks for amino acid synthesis, positive and negative control pins with their corresponding monoclonal antibody which are packed in polyethylene sandwich boxes. These boxes are kept at -20°C until use. The 96-wells polyethylene reaction trays of 8 x 12 configuration were also supplied, together with pin blocks (holders). The trays and empty holders were stored at room temperature, away from direct heat.

2.4.2 Reaction Tray Numbering System and Pins Holder:

Although the reaction trays and the holder are in 8 x 12 configuration similar to those of standard microtiter plates, their numbering systems are different.

Figure 2.3 illustrates the tray's numbering system:

In this figure, reference number A1 (1,2) refers to block A, row 1, columns 1 and 2. A1 (1,2) corresponds to wells H1 and G1 on a standard microtiter plate. Peptides were sequenced from A1 (1) to A12 (1), up to A2 (1) to A2 (12) and so on. A1 (1) would normally be the synthesized positive control PLAQ, followed by GLAQ. The first sequence of interest would follow at A1 (3).

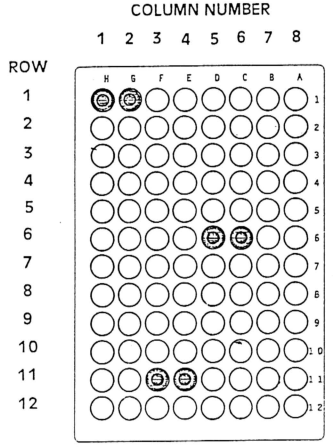

Figure 2.3: Numbering system of tray in Mimotope as used by the software supplied with the synthesis kit. Wells are identified by a letter that represents the block, followed by the column number(s) in parenthesis. Assuming that this is block A, filled wells (dark) are identified as A1(1,2), A6(5,6) and A11(3,4); from top to bottom

2.4.3 Synthesis Procedures:

The synthesis procedures involved:

1) Synthesis Schedule Generation

The entire amino acid sequence of the 404-amino-acids p54 was entered into a 486-DX2 computer and saved in an ASCII format. Synthesis schedules generation was accomplished by using Pepmaker program (Chiron, Australia), utilizing a general net (Gnet). Amino acid concentration was chosen at 60mM with 150µl/well and a maximum of 15% overfill. Positive and negative controls were PLAQ and GLAQ respectively. AASET1 amino acid sequence containing 20 different amino acids of fluerenyl-methyloxycarbonyl (F-moc) chemistry was selected as the amino acid reference/base.

Synthesis schedules generated provided complete information on the number of pins to be used, amount of amino acids and its solvents necessary, also the well positions of each amino acid to be coupled each time. The synthesis schedule generated was checked to ensure correct information (e.g. amino acid sequence, number of segments and control peptides) has been entered before the actual synthesis was carried out.

2) Amino Acids Couplings

Before starting any coupling cycles, the required number of pins were fastened onto their appropriate places on a pin block. The amino acids required for each coupling, were weighed and stored away from light at 0°C - 4°C in air-tight universal bottles. Care was taken to avoid cross-contamination by rinsing spatula with 70% alcohol in between weighings and securing caps onto appropriate bottles after each weighing. Amino acids for a coupling reaction were taken out of storage and allowed to reach room temperature before use. The amount of N-hydroxybenzotriazole (HoBt) (Chiron, Australia) and diisopropylcarbodiimide (DIC) needed were measured and dissolved in purified N,N-dimethylformamide (DMF) (Sigma, St. Louis, USA) (Appendix 1) according to the amount stated in the synthesis schedule. Bromophenol blue solution (Appendix 1) was added to HoBt to a final dilution of 1:200. Addition of bromophenol blue (Sigma) was an optional step; it helped to indicate filled wells during amino acids dispensing. A specific amount of DMF was added into DIC. The solution was shaken to ensure homogeneity. Homogenous HoBt solution was then added to individual amino acids and shaken until the amino acids were fully dissolved. DIC, the amino acid activator was added last. The sequence of amino acids activation and dispensing is as follows:

i) Alanine (A)
ii) Aspartic acid (D)
iii) Glutamic acid (E)
iv) Phenylalanine (F)
v) Glycine (G)
vi) Isoleucine (I)
vii) Leucine (L)
viii) Methionine (M)
ix) Proline (P)
x) Serine (S)
xi) Threonine (T)
xii) Valine (V)
xiii) Tyrosine (Y)
xiv) Tryptophan (W)
xv) Glutamine (Q)
xvi) Asparagine (N)
xvii) Lysine (K)
xviii) Cystein (C)
xix) Histidine (H)
xx) Arginine (R)

Couplings were done at a 4-hour to overnight incubation period.

3) Washing and Deprotection of Pins

i) coupling reaction was stopped by removing pins from reaction tray.

Pins were then washed according to the steps below:

- in methanol covering half pins height for 5 min, with agitation
- air drying for 2 min
- in DMF bath covering half pins height for another 5 min, with agitation

ii) deprotection step was carried out immediately after the washing procedures

- place block of pins in piperidine:DMF solution (20%; v/v) (Appendix 1) covering half pins height i.e. the solution must cover the whole area of the gears, for 20 minutes with agitation
- block of pins was removed from the piperidine:DMF solution and transferred into DMF for 5 minutes with frequent agitation
- pins were soaked in 3 methanol baths for 5 minutes each. Each soaking uses a fresh methanol preparation
- pins were left to dry at room temperature for at least 30 minutes.

While the block of pins was drying, amino acids required for the next coupling reaction were prepared and dispensed into their appropriate wells as was described in 2a(2) above.

4) Acetylation of Terminal Groups

Acetylation was done after the last amino acid had been coupled. The block of pins was washed and Fmoc deprotected before acetylation was carried out. Pins were placed into a reaction tray containing 150µl/well of the following mixture:

DMF:acetic anhydride:N-ethyldiisopropylamine

193:6:1 (v/v/v).

The reaction was allowed to proceed for 90 minutes at RT. The block of pins was then removed from the reaction tray and washed in methanol bath for 15 minutes, followed by air drying for another 15 minutes.

5) Side-chain Deprotection

The block of pins was ready for side-chain deprotection after the acetylation of terminal groups was completed. Deprotection was accomplished by using a mixture of:

trifluoroacetic acid:ethandithiol:anisole (v/v/v)

38:1:1

Pins were left in the bath for 150 minutes at room temperature. The mixture used covered up to half pins height, ensuring complete immersion of the gears.

After removal from the deprotection bath, the block of pins was completely soaked in ethanol for 10 minutes. Excess methanol was flicked off and the block was soaked in a mixture of 0.5% acetic acid in methanol:purified water (1:1 v/v) for an hour. This was followed by two immersion washes in fresh methanol, 2 minutes each. Excess solvent were flicked off and block of pins was ready for ELISA testing.

Note: A block of pins was placed in an empty, new microtiter plate to minimize the risk of damage on the gears and stored in a sealed container packed with silica gel to ensure dryness. The package was stored at 4°C.

2.5 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA tests were carried out on recombinant proteins and synthetic peptides to determine their reactivity and specificity in NPC detection.

2.5.1 Recombinant Proteins as Antigens in ELISA:

p54, p23 and p138 were coated 50µl/well onto their respective flat bottomed microtiter plates (Nunc Immuno Plates F96 Maxisorp) at 1µg/ml in 10mM carbonate buffer pH 9.8 (Oshima and Atassi, 1989) (Appendix 1). Buffer alone was applied to serve as antigen negative controls. Coated plates were incubated overnight at 4°C. The next day, the plates were washed once in PBS/0.05% Tween-20 (PBS-Tw). Blocking steps followed, with the addition of 100µl/well PBS-1% bovine serum albumin (PBS-BSA) (Sigma) for two hours at RT. Washings (three times) in PBS-Tw were carried out after which 50µl/well of sera diluted 1:50 in PBS-BSA-0.01% Tw (PBS-Tw-BSA) were dispensed and further incubated for two hours at RT. Plates were washed four times in

PBS-Tw. Goat anti human IgA- or IgG-peroxidase conjugated, diluted in PBS-Tw-BSA at 1:1000 and 1:3000 respectively were added at 50µl/well and left to react at RT for an hour. Plates were then washed five times in PBS-Tw and colorimetric reaction was conducted. 50µl/well, 0.5mg/ml of enzyme substrate o-phenylenediamine (OPD), dissolved in citrate-phosphate buffer with 0.01% H_2O_2 were dispensed into microtiter plate wells and reaction was allowed to proceed at room temperature (RT) until a dark orange color develops. Reactions was stopped using 4M H_2SO_4. The optical absorbance was measured using Titertek Multiskan ELISA reader (MCC 340, Flow, Australia) at wavelengths of 492nm/540nm.

Reactions was considered positive when the delta absorbance (dOD) or the average difference between the antigen and buffer coated well is greater than the average absorbance for all NHS tested against a particular protein, plus 1-2 standard deviation.

2.5.2 PEPSCAN Using Enzyme-linked Immunosorbent Assay:

The PEPSCAN testing was conducted using a modified ELISA technique. A conjugate (antibody) test was carried out before any ELISA analysis of NPC and control samples to check the background reactions. This reaction may be mistaken as the reactivity of the pin bound peptide with test

samples. The actual concentration of the conjugate was optimized. The conjugate test utilizes the same procedure as ELISA testing excluding the addition of serum/first antibody. Conjugate testing was also done after several ELISA tests to ensure maximum antibody removal has taken place after the disruption procedures.

1) Precoating of pins (blocking step)

Precoating was done to reduce non-specific binding so as to decrease the background-to-signal ratio. The block of pins was incubated for 60 minutes at room temperature on a shaker table, with the gears fully immersed in 200µl/well of precoating buffer which included:

2% w/v bovine serum albumin (BSA)

0.1% v/v Tween 20

0.1% w/v sodium azide, in 0.01M PBS, pH 7.2. The pins were then washed once with 0.01M PBS bath for 10 minutes.

2) First antibody (primary antibody)

Human sera diluted 1:1000 in precoating buffer were dispensed 170µl/well into microtiter plates. Pins were incubated with the sera overnight at 4°C. The following day, the blocks of pins were washed four times in 0.01M

PBS, pH 7.2; fresh PBS was used each time. The washings were done at room temperature, with agitation.

3) Conjugate reaction

Conjugate diluent was prepared, making a mixture of:

1% v/v sheep serum

0.1% v/v Tween 20

0.1% w/v sodium caseinate, into 0.01M PBS, pH 7.2

The solution was dispensed at 170µl/well into the microtiter plates and pins were allowed to incubate for 1 hour on a shaker table, at room temperature. Four washings of 10 min each followed.

4) Chromogenic development

Substrate solution for the chromogenic development was prepared using: 35.6g Na_2HPO_4 and 33.6g citric acid monohydrate, making up a 2L solution with distilled water. The pH was adjusted to 4.0 using either 1M Na_2HPO_4 or 0.8M citric acid monohydrate. 10mg/ml of 2,2'-azino-bis[3-ethylbenz-thiazoline-6-sulfonate] (ABTS) (Sigma) was dissolved in the substrate solution and 0.01% H_2O_2 was added. 200µl/well of the solution was

dispensed into Nunc microtiter plates and incubated on a shaker table (Belly Dancer, USA) for 45 min at room temperature. The reaction was stopped by removing the blocks of pins away from the microtiter plate wells. The result was read on a Titertek Multiskan ELISA reader at 405nm. An average absorbance of 1.0 and above were considered positive reactions.

5) Antibody removal

The block of pins was subjected to sonication for 10 min at 65°C. The disruption buffer contained 1% sodium dodecyl sulphate (SDS) (Sigma) in PBS. 2-mercaptoethanol was addded to a final concentration of 0.1% v/v. The buffer was preheated to 60°C. Pins were placed downward into the disruption buffer placed in a sonication bath after the temperature has equilibrated at 60-65 °C. The blocks of pins was rinsed twice in hot distilled water (60°C) for 30 sec. This was followed by washing in water bath with starting temperature of 60°C for 30 min. Excess water was shaken off the blocks and immersion in hot methanol for 15 sec was carried out. The blocks of pins were left to dry at room temperature for at least 15 min, after which it would be ready for the next ELISA testing.