DISCUSSION

4.0 DISCUSSION

4.1 IgA AND IgG SERUM ANTIBODIES TO RECOMBINANT EAs (p54 and p138) AND VCA (p23) PROTEINS IN ELISA

The association of EBV with NPC was first discovered in precipitating antibodies to EBV-related antigens found in the sera from patients having NPC (Old *et al.*, 1966). NPC patients were found to have high geometric mean titres (GMT) of antibodies to the Epstein-Barr viral capsid antigen (VCA) compared to patients with other head and neck cancers and normal subjects (Henle *et al.*, 1970; de Schryver *et al.*, 1974; Henderson *et al.*, 1974). Detection of elevated IgA to VCA by the indirect immunofluorescence method is widely used for the detection of NPC.

In any immunodetection methods, the accuracy and reliability of the test to detect antibodies or antigens in relation to presence of disease is of great importance. A part of this research objective is to find specific antigen(s) to complement the existing IgA-VCA IIF assay for NPC detection. Sensitivity is the ability of a test to detect a disease when present, calculated as the proportion of diseased subjects correctly classified. Specificity is the ability of the test to not detect the disease when it is absent and calculated as the

proportion of non-diseased subjects correctly classified (Crofts *et al.*, 1988). In this study, specificity was calculated as percentage of healthy subjects, and those with diseases other than NPC e.g. ENT diseases and RA, being unreactive to the antigen of concern.

In ELISA, p54 showed a higher reactivity with IgG than IgA where all 146 IgA-VCA, IIF positive (IIF-positive) NPC sera samples tested in IgG were positive whereas only 83.5% of the NPC sera reacted positively in p54-IgA ELISA (Table 3.4). p54-IgG ELISA was also able to detect 73.8% of NPC IgA-VCA IIF negative (IIF-negative) sera compared to 32.6% in p54-IgA-ELISA.

Tests of NPC sera done against p138-IgA ELISA detected 75.8% of those sera that are IIF-positive and 34.8% NPC sera that were IIF-negative. When 146 NPC, IIF-positive sera were tested in IgG-ELISA against p138, 107 (73.3%) were positive. In addition, p138-IgG-ELISA also identified 37.7% of IIF-negative NPC sera (Table 3.3). The proportion of serum IgA-p54, IgA-p138 and IgA-p23 detections are significantly higher in the IgA-VCA IIF-positive compared to the IgA-VCA IIF-negative NPC sera (χ^2 = 39.2, 21.4 and 33.0; $p<0.05$, for p54-, p138- and p23-IgA antibodies respectively). Similar observations were seen in the proportion of positivity in IgG antibody detections between the two NPC sera groups, in p54, p138 and p23 where χ^2 values are

41.7, 23.3 and 17.4 respectively ($p < 0.05$).

When tested with NHS controls, only 3.7% of the samples gave positive response in p54-IgA-ELISA compared to 19% positive response in p138-IgA-ELISA. Tests utilizing ENT disease sera other than NPC resulted in 13.7% and 11.8% positivity in p54-IgA and p138-IgA respectively. p138-IgA-ELISA did not react with any of the non-NPC cancers sera while IgA-p54 presented a 4.5% false positive reactions with 32 non-NPC sera tested. In p54- and p138-IgG-ELISA using the same EA proteins, tests with ENT disease sera showed undetectable antibody titers. Similar results were observed when 22 non-NPC cancer sera were tested for IgG antibodies in p138 whereas 5 (22.7%) sera showed significant IgG antibody titers elevation against p54.

The sensitivities of the two recombinant EA proteins (p54, p138) and p23, a VCA protein, was rather similar in NPC detection of both IIF-positive and negative sera. IgA-p23 gave false positive reactions with 9.1% of non-NPC cancer sera and 11.8% of ENT disease sera. IgA-p23 also reacted with 14.7% of NHS sera, giving it a specificity of 85.3%. Detection of IgG antibodies in various sera groups against p23 showed higher positivity in all groups including non-NPC cancers, compared to the EAs except in ENT disease sera where none were reactive.

We thus obtained sensitivities of 92% and 63% with all NPC samples tested for their IgG antibodies against p54 and p138 respectively. In contrast, Chen *et al.*, (1991) reported 75% sensitivity when working with an EA-D recombinant protein, pp58-IgG in ELISA. However they found a 100% specificity while our test yielded 93.9% and 97.2% specificity for IgG-p54 and IgG-p138 respectively. Chen and co-workers (1991) tested the same protein using immunofluorescence assay for serum IgG and reported a specificity of 96% and sensitivity of 66%. They also tested the protein using immunoblotting technique which increased the sensitivity to 88% while the speciificity remained at 96%.

Another group of researchers (Sigel *et al.*, 1994) used immunofluorescence method to test for serum IgA to EA in NPC samples. They reported 60.7% sensitivity of the test. IgA-p54 in our studies has a sensitivity of 68% with 96% specificity. p138-IgA has an overall sensitivity of 62% and was 81% specific. Sigel *et al.* (1994) detected 33% of non-NPC samples that were IgA-VCA- and IgA-EA-positive. The authors suggested the non-specificity of IgA-EA for NPC detection from their studies as the EA protein does not allow for unequivocal serological diagnosis of individual NPC cases and therefore is questionable for use in NPC screening. However, the authors believed that results presented in their report do not contradict the usefulness of utilizing IgA-

EA in monitoring of treated NPC patients and screening of populations in high-risk areas (Sigel *et al.*, 1994).

Hinderer *et al.* (1996) combined the two EA proteins p54 and p138 and tested it with four categories of NPC sera for IgA and IgG serum antibodies. They reported highest overall sensitivity of 87% in EAs-IgA and 82% in EAs-IgG. The four categories of patients included those who are presented with (i) no disease (ii) local relapse (iii) distant relapse and (iv) persistent disease. Group (iii) showed the highest reactivities while group (i) showed the lowest reactivities.

A few polypeptides of VCA have been identified to have immunogenic properties - a 160kD protein, a 125kD protein and a protein called p18. Immunofluorescence tests of serum immunoglobulin A against VCA has been used in extensive epidemiological studies in high risk areas of China. Researchers have suggested that the IgA-VCA antibody can be detected 8-60 months prior to clinical and histological diagnosis of NPC (Zeng *et al.*, 1985; Guo *et al.*, 1996; Liang *et al., 1996*).

The average immunofluorescence assay sensitivity of IgA-VCA is 95% and its average specificity is more than 94% (Gan *et al.*, 1996). A synthetic peptide featuring a combination of immunodominant epitopes in C-terminus of

VCA p18 was able to detect 97% of NPC serum IgG and 97% of 36 seropositive healthy individuals in IgG-ELISA (Uen *et al.*, 1988). The current p23 VCA recombinant protein has a specificity of 94% and sensitivity of 75% (combined average of 88% in IIF-positive and 62% in IIF-negative) in serum IgG detection via ELISA.

Hinderer *et al.*, (1996) reported difficulties in predicting relapse using IgG-p23 where non-specific increase or fluctuations of reactivities without subsequent relapse was observed. The authors suggested that the side effects of radiation was a possible cause of the phenomena. The presence of IgM antibody to p54 and p138 was earlier reported by Hinderer *et al.* (1990), working with IM sera. They associated high levels of IgM with acute primary infection of EBV or reactivation of the disease. Our search for markers in predicting recurrence or metastasis using EA-IgM was not significant (Table 3.6). p54-IgM was able to correctly detect the presence of disease in 14% of cases. Four of 12 cases which were subsequently confirmed to be NPC were p54-IgM-positive. In addition, only 27.5% confirmed NPC cases were IgM-p54 positive with 3 being recurrent cases. p138-IgM detected 16.7% of those with clinical symptoms of NPC and subsequently proven NPC. One of the IgM-p138 positive samples was a recurrent case. p23-IgM did not detect any recurrence and only 10% of the confirmed NPC sera samples were positive.

All three recombinant proteins we have tested may be useful diagnostic markers to complement the conventional IgA-VCA IIF assay. However, the recombinant p54 showed the highest sensitivity in detecting IgG antibodies. Furthermore, the protein p54 was able to detect 62% of NPC sera that were IgA-VCA IIF negative. The above reports showed that this EA-D is sensitive and specific marker for NPC.

4.2 IgA AND IgG REACTIVITIES IN PRE- AND POST-TREATMENT NPC SERA

Twenty one pre- and post-treatment NPC sera samples were tested for IgA and IgG reactivities against p54, p138 and p23 using indirect ELISA. The detection of serum antibodies is shown in Table 3.2 for IgA and Table 3.5 for IgG.

All 12 pre-treated NPC sera that were positive for IgA-p138 turned negative except in two cases where the IgA titers remained. Nine other NPC samples that were not reactive against p138 before treatment remained negative after treatment. p138-IgG was negative in all the 21 pre-treated NPC sera except in one. This IgG antibody positivity was negated after patient received treatment. No obvious correlation was observed between the disease

status of patients and the antibody levels detected by the three recombinant proteins; similar findings was reported by other researcher in detecting IgM antibodies using the same proteins (Hinderer *et al.*, 1996).

The other two proteins, p54 and p23, demonstrated both IgA and IgG antibodies fluctuations before and after treatment. Serum IgA reactivities against p54 fluctuated displaying 5 pre-treated sera without antibodies becoming positive after treatment. In p54-IgG, five positive pre-treated NPC sera were negative after treatment. Two other pre-treated sera sera that were IgG-p54-negative, had detectable level of IgG antibody after treatment. In p23, 4 pre-treated sera that were negative for IgA antibodies, turned positive after the patients were treated. Four samples that were p23-IgG-positive before treatment turned negative after treatment while two IgG-p23-negative pre-treated NPC sera became positive after treatment (Tables 3.2, 3.5).

The fluctuations may have been due to side effects after therapy (Hinderer *et al.*, 1996). IgA-VCA antibody fluctuations has also been reported previously by Sam and co-workers (Sam *et al.*, 1994) in their 5-10 years follow-up studies of NPC patients in Kuala Lumpur, Malaysia. From our studies, p138-IgA has a prognostic potential where sera reactivities showed predictable fluctuations.

4.3 IgA AND IgG REACTIVITIES IN MULTIPIN PEPTIDES FROM PEPSCAN

Recombinant EA-D p54 was selected for further studies to identify the reactive epitope regions due to its overall sensitivity for selecting NPC cases. p54-IgA was positive in 68% and p54-IgG has a sensitivity of 92%. p54 also has high specificity with 96% and 94% for IgA and IgG respectively. p54 was synthesized into 79 fragments consisting of 10-amino-acid residues per peptide according to methods of Geysen *et al.* (1984; 1987b). These non-cleavable peptides allow repititious testing of 30 sera samples for antibodies using ELISA system (Middeldorp and Meloen, 1988). The overall reactivities decreased abruptly after the 30th. sample although the same pattern of absorption remained e.g. peptides 52-54 showed higher degree of IgG binding compared to peptides 46-49 (Figure 3.13).

The initial IgG results of the 79 peptides (Figure 3.12) isolated peptides 1, 10, 52-54 and 77 as reactive. Figure 3.13 indicates the average level of antibody reactivity in 5 IgA-VCA IIF- and IgG-p54 ELISA-positive NPC sera to the 79 peptides. High absorbances can be seen in regions of peptides mentioned above. Since the clustering together of a few adjacent peptides with high specific reactivities and low background reactivities raised the possibilities of the region containing the antigenic determinants, only peptides 52-54 were

selected for further studies. This cluster studied was recognized by the majority (more than 70%) of seropositive individuals making it useful for serological purposes (Middeldorp and Meloen, 1988). Peptides 52, 53 and 54 showed more than 70% reactivities to NPC sera. Although other peptides (peptide 1, 10, 77) did show high reactivities with some sera, their adjacent peptides did not exhibit comparably high level of reactivities.

Preliminary IgG studies of these three peptides is shown in Table 3.7. Three categories of sera were tested with each of the peptides. Peptide 54 detected 100%, peptide 53 detected 92% and peptide 52 detected 83% of the IgA-VCA IIF-positive and IgG-p54 ELISA-positive NPC sera. Forty percent of NPC sera that were IgA-VCA IIF-negative but were positive in p54-IgG ELISA were being detected by peptides 54 and 52. Peptide 53 showed a 60% positivity with sera from the same group. Interestingly, certain percentages of IgA-VCA IIF-negative and p54-IgG ELISA negative sera also reacted with all three peptides. Peptide 54 showed positive reactivity with 86%, peptide 52 with 57% and peptide 53 with 43% of these sera. The reactivities observed were possibly due to differences of folding and reactive region presentations in antigens of various length. Recombinant p54 and peptides 52-54 undoubtedly have distinctly unique secondary structure presentations since p54 had 404-amino acid residues while the peptides were only 10-mers. Some antigenic

epitopes may have been folded inside into a hydrophobic core and are therefore inaccessible to antibodies. Reactivities of different sera may also be directed to different binding sites (Cheng *et al.*, 1995; Tedeschi *et al.*, 1995).

More copies of these three peptides especially peptide 54 were synthesized using procedures described previously (Geysen *et al.*, 1984; 1987b). In search of the most sensitive and specific epitope, NPC and NHS sera were tested for their serum IgA and IgG antibodies to these peptides. Peptide 54 was found to give the highest sensitivity and specificity to both IgG (Table 3.8) and IgA (Table 3.10) when compared with peptides 52 and 53. To further discriminate as to whether this epitope is more IgA or IgG specific, various other sera were tested (Table 3.10). Although peptide 54-IgA was more sensitive than its IgG counterpart, IgA antibodies was also present in 80% of ENT diseases, 84% of rheumatoid arthritis and 88% of non-NPC cancer serum samples. None of the non-NPC sera have detectable IgG antibody to peptide 54.

The lack of reactivity of peptide 54 for IgG antibody in sera of various diseases other than NPC was somewhat intriguing. The tests for IgA to peptide 54 and IgG to peptide 54 were run on consecutive days, utilizing the same technical procedures for both. Positive and negative controls, together

with a blank, were included in each test plate for ELISA tests. Tests were repeated only when controls were not reacting appropriately i.e. high absorbance for negative controls while positive control did not exhibit strong reactivities. This is due primarily to the limited amount of test peptides available for repeated testing. Each peptide synthesized on non-cleavable pins used in testing for polyclonal antibody reactivities in human sera gave reliable results with 30 or less repeated testing (Middeldorp and Meloen, 1988).

The cut-off points to determine the positivity of IgA and IgG antibody reactivities against peptide 54 differ. IgA anti-peptide 54 showed a very low level of elevation in NHS samples as compared to the reactivities of NHS samples for IgG anti-peptide 54. The average absorbance of IgG-peptide 54 when tested with the NHS sera was more than 3 times higher compared to the average absorbance detected for the same NHS samples tested for IgA anti-peptide 54. The high cut-off point (1.0) used to discriminate positive reactions for IgG-peptide 54 reactivities against test sera may have excluded some of the non-NPC sera samples from being picked-up as positives. Such use of high cut-off points for IgG were based on recommendations from the Biotest Diagnostic kit (Biotest Anti-EBV recombinant EA IgA/IgG, Dreieich, Germany) which had combinations of recombinant p54, p138 and p23 proteins as the antigens for ELISA tests.

Peptide 54 was also tested with 6 paired serum-saliva samples from patients presented with clinical symptoms of NPC. The presence of IgA was detected in one serum sample and two saliva samples. Presence of IgG was recorded in all six sera samples but none in their paired saliva samples (Table 3.9). The results indicated higher overall reactivities of peptide 54 to serum IgG. In 2 patients, IgA-p54 was present in saliva but not in the sera. this pattern of IgA reactivity was previously observed with IgA-EBNA1 antibodies in NPC (Foong *et al.*, 1990). The limited number of samples gathered here hinders a reliable statistical analysis of the results; at least 25 paired samples is required for a test to be statistically sound.

Our studies on various sera samples included those that were negative in recombinant p54-IgG-ELISA. NPC sera were found to contain IgG-peptide 54 antibodies although they were negative against the p54 recombinant protein. We identified EA peptide 54 as the reactive antigenic epitope. This reactivity may occur due to difference in binding residues in recombinant p54. Amino acids near to the reactive sequence of peptide 54 on the recombinant protein p54 backbone may bear charges that are attractive to the immunoplate of indirect ELISA. Binding of these amino acids may result in peptide 54 being bound to the bottom of the plate. Adsorption of the protein to plastic surface may distort its conformational structure formations or cause steric hinderence,

therefore causing changes in epitopes (Leinikki *et al.*, 1993), making the sequence of peptide 54 inaccessible to antibodies in ELISA. The protein concentration in indirect ELISA may also be lower than that of the pin-bound peptides (Savoca *et al.*, 1991) resulting in lower reactivities of recombinant p54. Peptide 54 has a specificity of 92% and sensitivity of 85%. It may prove a valuable peptide for screening of NPC in high risk areas.

4.4 ANTIGEN-ANTIBODY BINDING MECHANISMS

A protein antigen and its antibody typically repel each other at the macroscopic level. Specific attraction of the particular epitopic-paratopic sites enable local binding (van Oss, 1995). Subsequent to the first epitopic-paratopic encounter, much of the interstitial water becomes expelled (van Oss & Good, 1991; van Oss 1994a) giving rise to a bond that is principally caused by the Lifshitz-van der Waals forces. If the antigen-antibody binding involves a conformational change, then ***delta*** G_{net} (overall free energy) change must be less than the intrinsic, i.e. the maximum free energy change (delta G_{AgAb} - antigen-antibody) of binding (Geysen, 1985).

Hydrophobic compounds bind water less strongly (2-3.5 times) than hydrophilic compounds (van Oss & Good, 1991). Interactions between one hydrophilic and a hydrophobic molecule or particle can quite readily be attractive which explains why paratopes with hydrophobic sites tend to be found in concavities i.e. in paratopic clefts rather than on more exposed convex surfaces of immunoglobulins. When epitope and paratope approach each other closely, the neighboring chains of antigen and antibody also approach each other to approximately a few tenth of a nanometer. Hydrophobic attraction can occur even if just one of the neighboring chains has

hydrophobic site. Hydrophobic (i.e. Lewis acid-base) and electrostatic forces play an important role in the primary epitope-paratope interactions since they both act at a long range but not the Lifshizt-van der Waals.

Total macroscopic free energy of repulsion between two equal spheres and flat surface is directly proportional to the radius, R, of the spheres (van Oss *et al.*, 1988; van Oss 1994b). This indicates that protrusions, edges, curves, bends or "elbow" of particles, cells or biopolymers with the smallest R can easily pierce the repulsion fields. Thus epitopes that are located on prominent bends of an antigenic molecule or particle are most favoured to make contact with its corresponding paratope (Benjamin *et al.*, 1984; Thornton *et al.*, 1986; Getzoff *et al.*, 1988; Davies *et al.*, 1988; van Oss 1995).

Several factors are implicated for a successful peptide-antibody binding. Binding involves principally the complementarity between antigen combining site of antibody is maintained at least partially in both shape and charge (Geysen *et al.*, 1986; 1987b; Tribbick *et al.*, 1989). Binding requires molecular geometry. Previous tests with peptides having alternative geometry showed antibody binding at levels comparable to the parent peptide. Stereochemistry of residues in binding peptides, direction of the main chain and adequate peptide length are other contributing factors to substantial antigen-antibody

binding. Reversed direction of peptide's main chain seemed to attenuate its ability to bind antibody (Dryberg *et al.*, 1984; Geysen *et al.*, 1986). The immunological and disease status of patients may influence the responsiveness to different antigenic epitopes, resulting in variable antibody reactivity to different synthetic peptides (Dillner *et al.*, 1987).

Significant binding can occur to a minimum of three amino acid residues which have both correct identity (L or D isomers) and position in peptides, as determined via systematic replacement of individual amino acid experiments. In all the cases studied, at least two of the three residues were situated adjacent to each other (Geysen *et al.*, 1984; 1985; 1986). Adequate peptide length is critical as decreased peptide length leads to deletion of the specific sequence to which the antibody binds. Insufficient sequence length may result in less possibilities for firm binding.

The antibody binding sites generally lie on the solvent-accessible surface of protein structures. Most of B cell epitopes that have been mapped were on surface-accessible regions and these epitopes shared three important characteristics. B cell epitopes comprise of eight or fewer, usually non-contiguous amino acids, containing 5 ± 1.3 residues which make contact with

the antibody. A typical B cell epitope contains 4 ± 1.2 amino acid residues essential for binding to antibody (Saul and Geysen, 1990; Carter, 1994).

Peptide 54 comprising 10 amino acids was of adequate length and having a mixture of hydrophilic-hydrophobic amino acid residues to allow its conformation of sheet-turn-sheet structure, with proper stereochemistry. The importance of residue Y at position three in sequence RFYRSGIIAV may have contributed to the "turn" conformation (Chou and Fasman, 1977; 1978). The sheet-turn-sheet structure may have induced selectively specific IgG antibody binding to the 10-mer peptide. Several of the residues from this peptide are hydrophobic according to Hopp and Woods (1981; 1983) but initial binding can still occur since the forces involved in binding may work from a distance of one hundreth of a nanometer.

Initial hydrophilic interactions occur between surface-located amino acid of the peptide with the binding site of the antibody, leading to local denaturation of the antigen (Sheriff *et al.*, 1987). The local denaturation does not significantly change the global structure of the antigens. This denaturation process allows antibody to access buried regions of the antigens, the hydrophobic amino acids, to form stronger binding (Amit *et al.*, 1986).

Shorter peptides of 3- and 5-mers could have contained the essential amino acid for initial binding but not the overall charges and conformations to expose the structures adequately to the antibodies present. These factors may explain the decreased and non-specific reactivities of the shorter peptides.

4.5 ASSOCIATIONS OF HYDROPATHY PROFILES, SECONDARY STRUCTURES AND PEPSCAN ANALYSES

4.5.1 Hydropathy Profiles And Antigenicity

Available published methods of hydropathy (hydrophilicity and hydrophobicity) serve to help researchers in locating potential antigenic regions of proteins. Among the commonly known methods are that described by Hopp and Woods (1981; 1983), Kyte and Doolitle (1982) and Novotny *et al.*, (1986). However, none of the methods has been proven to be more superior than others in predicting the antigenic regions or epitopes (Doran, 1995).

Hydropathy plots facilitate among other things the identification of membrane-spanning segments of proteins, the differentiation of surface groups as opposed to those buried in the interior of a protein, the localization of turns in peptide chains and the determination of antigenic sites. Hydropathy profile is

dependent upon the choice of hydropathy scale i.e. hydropathy value assigned to each of the 20 amino acids, and the degree of smoothing utilized i.e. the window unit. In this study, the main interest is in locating the sites of antigenic determinants which could be correctly predicted from the peaks of hydrophilicity. These peaks would be associated with polar residues which are believed to be located on surface of proteins - the contact points with aqueous environment, the residues participating in antigen-antibody binding. Hydrophobic or non-polar residues are believed to be non-reactive and make up the internal core of proteins.

Various methods have been described for the predictions of antigenic determinants through the assigning of hydrophobic and hydrophilic values to each amino acid. The predictive methods rely on the correlation between structural properties of proteins and experimentally determined antigenic sites in proteins of known structure. Prediction is also correlated with the solvent accessible surface region of protein antigens of known structure or with local conformational flexibility in a polypeptide chains (Suhai, 1990). A few of the well known methods are that established by Hopp and Woods (1981), Kyte and Doolitle (1982), Rose and Roy (1980) and Levitt (1976). The method considered in this study is that of Hopp and Woods (1981; 1983).

In Hopp and Woods (1981), each amino acid in the protein sequence is assigned its hydrophilicity value. These values are then repetitively averaged down the length of the polypeptide chain, generating a series of local hydrophilicity values. An average of 6 hydrophilicity values were initially used, resulting in a 100% correct predictions. The percent correct predictions decreased as the number of hydrophilicity values averaged reached 8. The highest local upspike represents the maximum hydrophilicity, a point where an antigenic determinant has been consistently located. It was reported that neither the second nor the third highest points gave highly reliable prediction results (Hopp and Woods, 1981).

Hydropathy profiles usually reveal a lot of hydrophilic regions. In cases where a protein exhibited too many upspike peaks, considerations of the secondary structure provides additional help in locating the most likely region to be antigenic. Secondary structure consisting of turns represent protrusion points which may be the points of antigen-antibody binding initiation. There are no proofs however, that secondary structures are very useful in choosing the correct antigenic region. There have been reports on a sequence of pentapeptides giving different secondary structure in different proteins (Walter, 1986).

4.5.2 Recombinant EA p54

Hydrophilicity studies of recombinant p54 predicted four amino acid regions to be hydrophilic. Amino acid residues 310-315 (R3) with corresponding PEPSCAN peptides 61 and 62 were non-reactive for serum IgG. The highest reactivity to serum IgG among the 4 regions was against peptide 77 (46%), covering residues 378-394 (R4). A 100% specificity was recorded for residues 146-155 (R2) with corresponding PEPSCAN peptides 29 and 30 (Table 3.14). Predicted secondary structures of the four hydrophilic regions and the most reactive region from PEPSCAN were combinations of sheets, helices and turns (Table 4.1, Fig. 3.20). All of the regions predicted had at least 3 residues forming turns at various positions. The most reactive peptide from PEPSCAN had secondary structures consisting sheet-turn-sheet with 2, 5 and 3 residues in each structural position. Predicted hydrophilic regions did not exhibit the same combinations of secondary structures although two regions had 5 amino acids situated adjacent to each other and forming turns; these amino acids were different from YRSGI which formed a turn in peptide 54 (Appendix 3).

It is physically feasible for binding to occur in each region if one takes into account the number of amino acid residues present. Other factors such as charges, shape and molecular geometry could on the other hand, hinder binding to the proposed hydrophilic regions from hydropathy profile (Rhodes *et al.*, 1984; Geysen *et al.*, 1986; Savoca *et al.*, 1991; van Oss, 1995).

The non-correlation between PEPSCAN epitope analysis and predicted antigenic epitopes from hydropathy profile was also noted for two other EBV proteins studied below.

4.5.3 EBNA1

Hydropathy profile of EBNA1 showed 6 prominent upspike peaks with hydrophilicity values between 1.5-2.5 points (Figure 3.18). The region predicted to be antigenic comprised amino acid residues 50-53, 65-75, 355-380, 457-466, 570-585 and 625-640 (Table 3.15). The region most reactive in this protein by PEPSCAN ELISA analyses was not hydrophilic from hydropathy profile. Significant elevation of serum IgG antibody in NPC to the alanine-glycine-rich region or peptide 10 from PEPSCAN, was seen in 86% of samples. Serum IgA to the same region was elevated in 82% of samples. The serum IgA and IgG reactivities of identified hydrophilic regions by hydropathy profile are

shown in Table 3.15. The reactivities ranged from having maximum sensitivities of of 24% and 14% for serum IgA and IgG respectively, to having non-significant reactivities in both antibodies.

Two peaks at amino acid residues 355-380 (P1) and 625-640 (P2), were of approximately the same height, recognized here as highest peaks. P1 corresponded at least partially to peptides 14, 15 and 16 from PEPSCAN analyses for IgA and IgG antibodies. The highest reactivities recorded for the two Igs were to peptide 16 which was reactive to 24% and 2% of NPC serum IgA and IgG respectively. Peptide 16 was found to react with 92% specificity to both Igs. P2 corresponded to peptides 41 and 42 which were non-reactive for both serum IgA and IgG antibodies.

4.5.4 ZEBRA

Five hydrophilic regions were spotted by hydropathy plot of ZEBRA protein (Table 3.16, Figure 3.19). These regions corresponded at least partially to peptides 1, 13, 19, 22, 25 and 26 of PEPSCAN ELISA for IgG to ZEBRA peptide studies. The first region predicted covered amino acid residues 4-14 which corresponded to part of peptide 1. Although this region did not constitute the highest peak of the hydropathy profile (Figure 3.19), it proved to be the

most reactive region with more than 97% of NPC samples had IgG antibody to the sequence. Other predicted hydrophilic regions had IgG-peptide reactivities ranging from non-reactive to 35% sensitivity. The highest upspike peak in the region of amino acids 208-214, corresponding partially to peptides 25 and 26 in PEPSCAN, showed non-significant reactivities for IgG antibodies in NPC sera samples.

4.5.5 Three- and 5-mer Peptides from Peptides 52, 53 and 54 of EA p54

Shorter sequences of the antigenic region (PEPSCAN), synthesized as 5- and 3-mers peptides showed no profound changes to increased sensitivity and specificity of NPC detection. The pentapeptide RIPAV gave the the highest sensitivity for IgA antibodies among the 5, 5-mer peptides (Table 3.13). The overall reactivities of IgA in NHS increased from 9% in peptide 54 (RFYRSGIIAV) to more than 32% in RIP, RFY, IIA and VSVPI tri- and pentapeptides. Tests with the same panel of peptides for serum IgG antibodies in NPC and NHS sera resulted in moderately weak responses (Table 3.12). Peptide 54 had a sensitivity of 88% and specificity of 92%. IgG to the pentapeptide YRSGI conferred 70% sensitivity, the highest among all the 5-mer

peptides. The specificity however, has dropped to 62%. Tripeptide IIA was sensitive to 79% of NPC sera samples, also with a specificity of 62%. Peptide YRSGI which makes up part of the original peptide 54 (RFYRSGIIAV), has a "turn" or "bend" conformation according to secondary structure prediction, but found to be not as reactive and specific as its parent. The other residues may be required for the peptide chain to form a bend or possibly to impose certain steric or conformational restrains on the epitope (Radford *et al.*, 1990) thus rendering it more antigenic. The residue Y at the third place is predicted to form a bend, but not when it is situated in the first position (Chou and Fasman, 1977).

Further investigations of the peptide chains through systematic individual amino acid replacements (Valerio *et al.*, 1991) may result in increased sensitivity and specificity in monitoring for NPC cases as shown by Tedeschi *et al.* (1995). Radford *et al.*, (1990) detected certain dependencies on specific amino acids in peptide sequences in binding with monoclonal antibody to *Mycobacterium bovis*. Horal *et al.*, (1991) reported differences in binding activity of antibodies to peptides from HIV-1 virus when amino acids were replaced e.g. the change of leucine with histidine in sequence GKLICTT resulted in different serum samples showing binding.

Identical amino acid sequences in synthetic peptides and its native protein do not always indicate identical antigenic characteristics of the two antigens. Furthermore, reactivities of synthetic peptides may be limited due to the limited number of reactive amino acid residues within the sequence. Synthetic peptides can also develop conformation similar to epitopes of other microbial proteins, causing cross-reactivity and false-positive reactions (Leinikki *et al.*, 1993)

For diagnosis of NPC, it thus seemed that the original 10-mer immunodominant sequence of EA p54 RFYRSGIIAV is adequately useful and could be utilized as a novel immunological assay added to the battery of tests that are currently being employed.

Table 4.1: Predicted Hydrophilic Regions (Hopp and Woods, 1983) and Their Corresponding Predicted Secondary Structure (Chou and Fasman, 1978)

Hydrophilic Region, Amino Acid Residues No.	Corresponding Peptides from PEPSCAN	Secondary Structure
128-134	25, 26	sheet - helix - turn
146-155	29, 30	sheet - helix - turn
310-315	61, 62	turn
378-394	75, 76, 77, 78	helix - turn - helix - turn
➧266-275	54	sheet - turn - sheet

➧ most reactive region PEPSCAN, not hydrophilic by hydropathy plot