RESULTS

3.0 RESULTS

3.1 DETECTION OF IgA ANTIBODIES TO EBV RECOMBINANT PROTEINS

The IgA response to the two recombinant EA proteins p138 and p54, and a recombinant VCA protein p23 were evaluated by reacting them with sera from:

i) 136 NPC patients

ii) 163 NHS

iii) 51 ENT diseases patients

iv) 22 non-NPC cancers patients

v) 44 patients with clinical symptoms of NPC; 34 of whom were subsequently histologically proven to be NPC while 10 were histologically normal

Table 3.1 shows the percentage of positive serum IgA responses to the three recombinant proteins.

3.1.a.(i) IgA-p54 (Early Antigen)

In the IgA-VCA IIF positive NPC sera, 85.3% (78/91) had IgA antibodies to p54. In the control sera, 3.7% of NHS, 4.5% of non-NPC cancers and 13% of ENT disease samples showed positive IgA-p54. Interestingly, in the IgA-VCA IIF-negative NPC sera, 32.6% had IgA antibodies to p54. The distribution of IgA anti-p54 antibody binding to NPC and control sera are shown in Figures 3.1 and 3.2.

3.1.a.(ii) IgA-p138 (Early Antigen)

p138 reacted with 75.8% (69/91) of NPC, IgA-VCA IIF positive sera. IgA-p138 was also present in 34.8% of NPC that were IgA-VCA IIF-negative. In the control sera, 19% of NHS and 11.8% of ENT disease samples had elevated IgA antibodies. None of the non-NPC cancers sera showed any significant serum IgA antibody elevations against p138. The distribution of IgA anti-p138 antibody binding to NPC and control sera are shown in Figures 3.3 and 3.4.

3.1.a.(iii) IgA-p23 (Viral Capsid Antigen)

79.1% (72/91) of IgA-VCA IIF-positive NPC sera had IgA antibodies to p23. IgA-p23 was also detected in 28.3% of NPC, IgA-VCA IIF-negative sera.

In the control sera, 14.7% of NHS, 12.8% of ENT samples and 9.1% of non-NPC cancer samples had IgA antibodies to p23. The distribution of IgA anti-p23 antibody binding to NPC and control sera are shown in Figures 3.5 and 3.6.

3.1.b.(i) IgA Analysis in Suspect NPC Serum Samples

Forty four sera from patients with clinical symptoms of NPC were tested for the presence of serum IgA antibodies against the recombinant proteins. Thirty two of these suspect cases were subsequently histologically confirmed to have NPC. IgA-p54 was detected in 38.6% (17/44) of the sera. Two of the 17 sera (4.5%) with IgA antibodies to p54 were later found to be histologically normal. 50% (22/44) of the suspect NPC sera had IgA antibodies to p138 with 1 serum (2%) later confirmed to be histologically normal. p23 identified 31.8% (14/44) of the suspect NPC sera. 2% of these were later confirmed to be histologically normal.

χ^2 test revealed non-significant difference in the reactivity of the two EA proteins, p54 and p23 for IgA antibody in differentiating NPC cases among patients suspected of suffering from NPC ($\chi^2 = 3.9$, $p > 0.05$; $\chi^2 = 4.0$, $p > 0.05$ for p54 and p23 respectively) . These two proteins showed non-discriminatory IgA reactivities in sera of patients subsequently proven to have NPC (n=32)

and those who were normal (n=12), after their biopsies were analysed. p138 on the other hand showed a significant difference in IgA antibody reactivity in NPC patients compared to those who do not suffer from NPC ($\chi^2 = 11.8$, $p < 0.05$). The same samples were used for the IgA antibody level detection against the three recombinant proteins. Statistical analysis of the above results showed that the IgA antibody levels detected by the three proteins could not be used as discriminating tests of NPC detection.

3.1.b.(ii) IgA Anlysis in Follow-up NPC Sera, Pre- and Post-therapy

Twenty one patients in remission were followed up after treatment (Table 3.2). Blood samples were collected from these patients during their first visit to UHKL ENT clinic, 3 months post-therapy. Eight cases had a significant level of IgA-p54 antibodies before treatment. Their IgA-p54 were negative after treatment. Five cases had detectable level of IgA-p54 antibodies only after treatment. The presence of IgA-p23 in was also found in 4 patients. In the p138 ELISA, 12 samples had specific IgA antibodies before treatment. Ten of the 12 sera were IgA-p138 negative after treatment. Although all patients were categorized as "in remission" during the visit, fluctuations can still be seen due

Table 3.1: Percent positivity of IgA responses to the recombinant EAs and VCA proteins in various sera groups

Sera Group	p54	p138	p23
NPC, IgA-VCA IIF Positive (91)	85.3	75.8	79.1
NPC, IgA-VCA IIF Negative (46)	32.6	34.8	28.3
NHS (163)	3.7	19.0	14.7
ENT Diseases (51)	13.7	11.8	11.8
Non-NPC Cancers (22)	4.5	0.0	9.1

The number in parenthesis indicate the sample size of each sera group

Table 3.2: Presence of serum IgA anti-EBV proteins in pre- and post-treatment NPC cases

Serum No.	p54 Pre-	p54 Post-	p138 Pre-	p138 Post-	p23 Pre-	p23 Post-
1	+	-	+	-	-	+
2	-	-	-	-	-	-
3	-	-	-	-	-	+
4	-	+	-	-	-	-
5	-	-	-	-	-	+
6	+	-	+	-	-	-
7	+	+	+	-	+	-
8	+	-	-	-	-	-
9	+	+	+	-	+	-
10	-	-	+	-	-	-
11	-	+	-	-	-	-
12	+	-	+	-	-	-
13	-	+	+	-	+	+
14	-	+	+	-	-	+
15	-	-	-	-	-	-
16	-	+	-	-	+	+
17	+	+	+	+	+	+
18	-	-	-	-	-	-
19	+	+	+	+	+	+
20	-	-	+	-	+	+
21	-	-	+	-	+	+

(+) indicates presence of antibody, (-) indicates absence of antibody

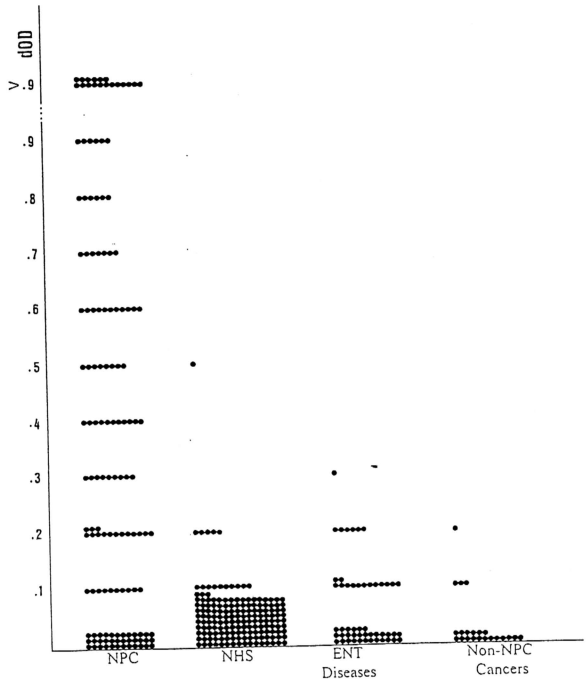


Figure 3.1: IgA-p54 reactivities in NPC, NHS, ENT diseases and non-NPC cancers sera

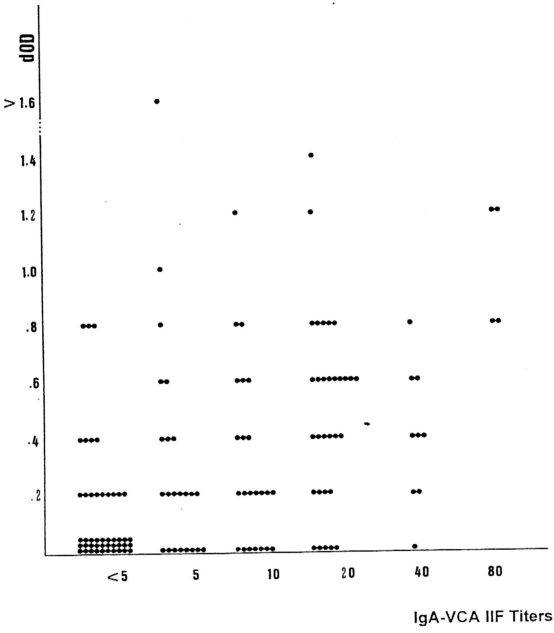


Figure 3.2: IgA anti-p54 antibody binding and IgA-VCA IIF titers in NPC sera

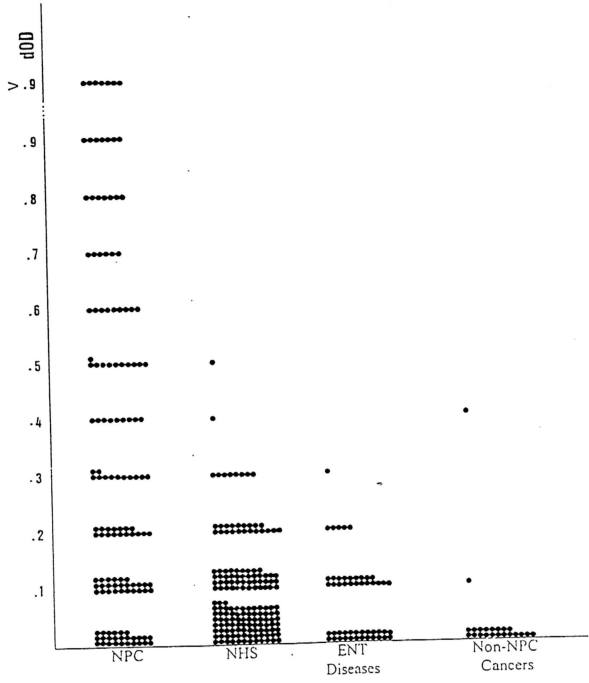


Figure 3.3: IgA anti-p138 reactivities in NPC, NHS, ENT diseases and non-NPC cancers sera

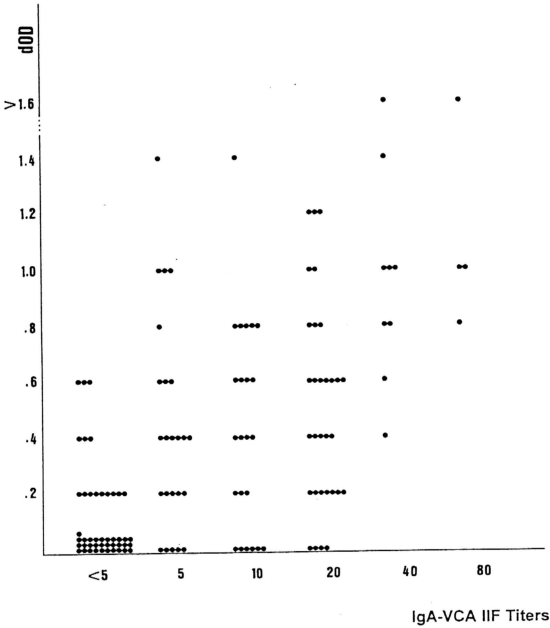


Figure 3.4: IgA anti-p138 antibody binding and IgA-VCA IIF titers in NPC cancers sera

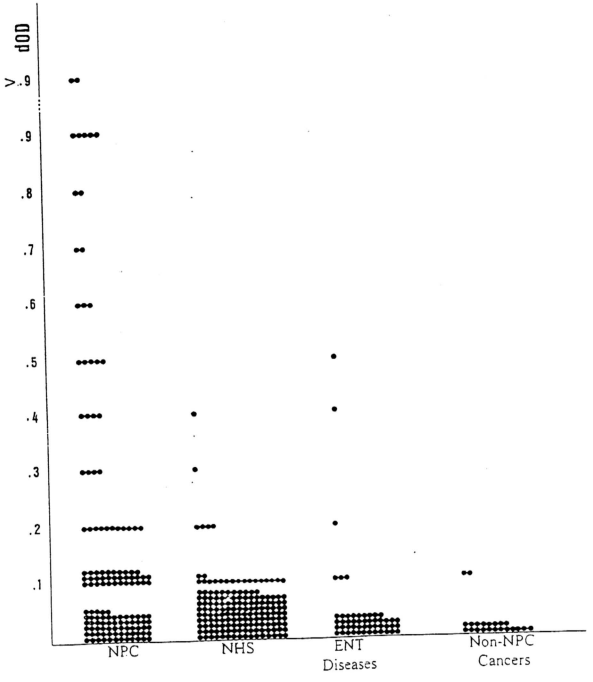


Figure 3.5: IgA-p23 reactivities in NPC, NHS, ENT diseases and non-NPC cancers sera

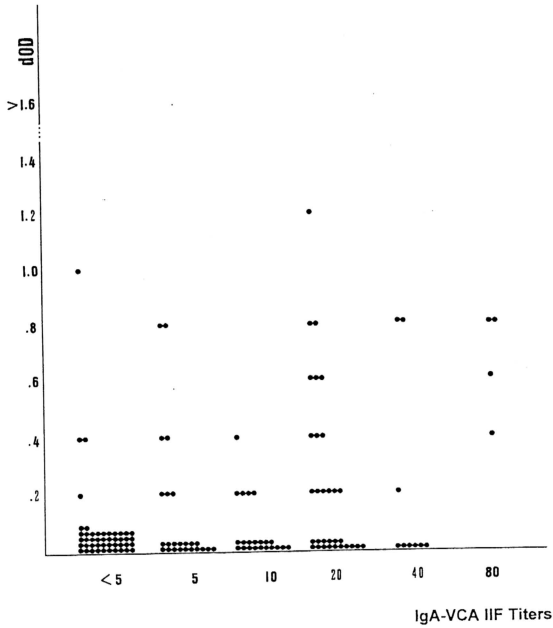
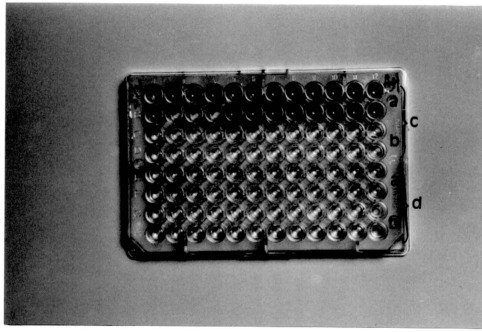


Figure 3.6: IgA anti-p23 antibody binding and IgA-VCA IIF titers in NPC sera



a: **antigen positive wells**

b: **antigen negative wells**

c: **positive reaction**

d: **negative reaction**

Figure 3.7: IgA antibody to EBV recombinant EA p54 detected by ELISA

possibly to the difference in binding of different sera to different regions of a protein/peptides (Cheng *et al.*, 1995; Tedeschi *et al.*, 1995).

3.2 DETECTION OF IgG ANTIBODIES TO EBV RECOMBINANT PROTEINS

IgG responses to the three recombinant proteins were determined by reacting them with 207 NPC sera (61 were IgA-VCA IIF negatives), 108 NHS sera, 51 non-NPC ENT disease sera and 22 non-NPC cancer sera (Table 3.3).

3.2.a.(i) IgG-p54 (Early Antigen)

All of the IgA-VCA IIF positive NPC sera tested (n=146) had IgG antibodies to p54. in the control sera, 5.6% of NHS, 22.7% of non-NPC cancers and 0% of ENT diseases samples showed positive IgG-p54. Interestingly, in the IgA-VCA IIF negative NPC sera, 73.8% had IgG antibodies to p54. The distribution of IgG anti-p54 antibody binding to NPC and control sera are shown in Figure 3.8.

3.2.a.(ii) IgG-p138 (Early Antigen)

Seventy three percent of the NPC, IgA-VCA IIF positive sera had IgG antibodies to p138. In the IgA-VCA IIF negative samples, 37.7% also had positive IgG-p138. IgG-p138 was detected in 4.6% of NHS samples. In the controls, no IgG antibodies to p138 was detected in ENT diseases and non-NPC cancer sera. The distribution of IgG anti-p138 antibody binding to NPC and control sera are shown in Figure 3.9.

3.2.a.(iii) IgG-p23 (Viral Capsid Antigen)

In the IgA-VCA IIF positive NPC sera, 87.7% had IgG antibodies to p23. In the control sera, 10.2% of NHS, none of ENT diseases and 40.9% of non-NPC cancer samples had elevated IgG antibodies to p23. In the IgA-VCA IIF negative NPC sera, 62.3% also had IgG antibodies to p23. The distribution of IgG anti-p23 antibody binding to NPC and control sera are shown in Figure 3.10.

The sensitivities and specificities of serum IgG antibody reactivities to the three EBV proteins, p54, p138 and p23 are summarized in Table 3.4.

Table 3.3: IgG Antibodies to p54, p138 and p23 EBV Recombinant Proteins in Several Sera Groups

Sera Group	p54	p138	p23
NPC, IgA-VCA IIF Positive (146)	100.0	73.3	87.7
NPC, IgA-VCA IIF Negative (61)	73.8	37.7	62.3
NHS (108)	5.6	4.6	10.2
ENT Diseases (51)	0.0	0.0	0.0
Non-NPC Cancers (22)	22.7	0.0	40.9

The number in parenthesis indicate the sample size of each sera group

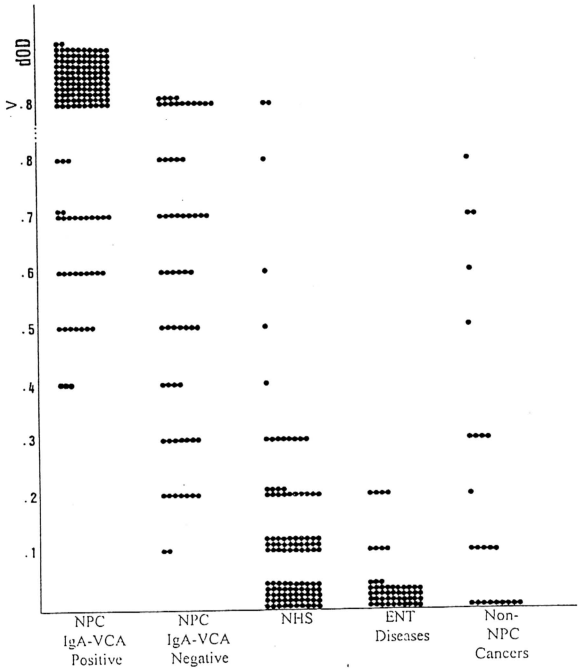


Figure 3.8: IgG anti-p54 antibody binding by ELISA in NPC and control sera

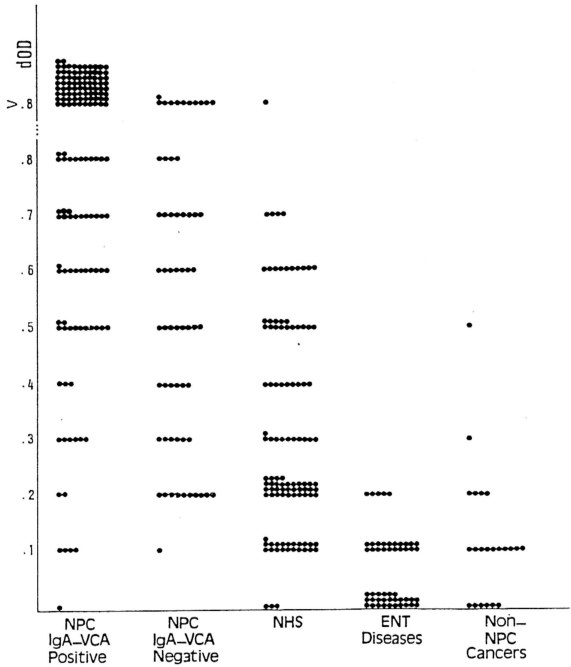


Figure 3.9: IgG anti-p138 antibody binding by ELISA in NPC and control sera

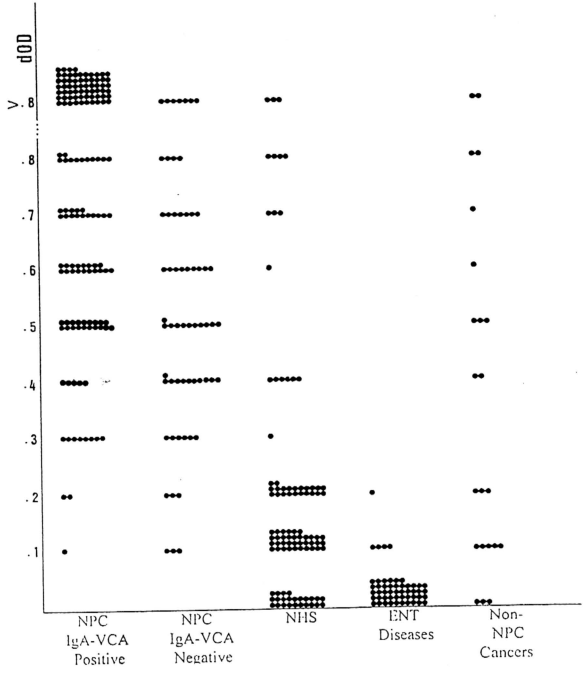


Figure 3.10: IgG anti-p23 antibody binding by ELISA in NPC and control sera

Table 3.4: Percent Sensitivity and Specificity of IgA and IgG to EBV Recombinant Proteins p54, p138 and p23

Antigens	IgA Sensitivity (136)	IgA Specificity (163)	IgG Sensitivity (207)	IgG Specificity (108)
p54	67.6	94.1	92.3	93.9
p138	61.8	84.3	62.8	97.2
p23	61.8	86.4	80.2	89.0

The numbers in parenthesis indicate the sample size tested

3.2.b.(i) IgG Analysis in Suspect NPC Serum Samples

The proteins were also tested with 44 sera from patients suspected of suffering from NPC. IgG-p54 was detected in 70% (31/44), IgG-p23 in 36% (16/44) and IgG-p138 in none of the NPC sera. Four sera (12.9%) with IgG-p54 antibodies and 3 sera (18.8%) with IgG-p23 antibodies were later found to be histologically normal. p23 showed non-significant difference in reactivities against NPC and NHS sera samples for IgG antibody ($\chi^2 = 1.03$; $p > 0.05$). There is however, a significant difference in the reactivities of p54 against NPC and NHS sera samples for IgG antibody ($\chi^2 = 8.63$; $p < 0.05$).

3.2.b.(ii) IgG Analysis In Follow-up NPC Sera, Pre- and Post-therapy

Twenty one of those 44 confirmed to have NPC were followed up (Table 3.5). Blood samples were collected from these 21 patients in remission from NPC during their first visit to UHKL ENT clinic, 3 months post-therapy. In the 16 NPC cases, IgG-p54 were positive before treatment. After receiving treatment, six of the sera became IgG-p54-negative. In 4 patients, the IgG-p23 positivity was negated after treatment. However, in both IgG-p54 and IgG-p23 detection, 2 NPC samples had positive ELISA reactions only after treatment.

The IgG to p54 and IgG to p23 before and after treatment of NPC, did not correspond to the IgA titres of the two peptides. Such fluctuations in the antibody levels are not surprising since antibody levels to EBV proteins in remission and in recurrence of NPC have been reported to fluctuate (Sam *et al.*, 1994; Hinderer *et al.*, 1996). The fluctuations suggest a lack of correspondence between the state of remission/recurrenceof the NPC and the expression of VCA and EA proteins of EBV.

3.3 DETECTION OF IgM ANTIBODIES TO EBV RECOMBINANT PROTEINS

IgM responses to the three recombinant proteins were determined by reacting them with 40 NPC sera, 8 NHS sera, 12 sera from patients presented with clinical symptoms of NPC and later confirmed to have NPC (Table 3.6). IgM-p54 was present in 27.5% of NPC sera, 12.5% of NHS and 33.3% of sera from patients with clinical symptoms indicative of NPC. IgM-p138 was detected in 20% of NPC sera, 16.7% of sera from suspect NPC and none in NHS samples. IgM-p23 was present in 10% of the NPC sera tested. NHS samples and suspect NPC sera showed no reactivity for IgM-p23. IgM responses in NPC sera were generally low for all the three recombinant proteins.

Table 3.5: Presence of Serum IgG Anti-EBV Proteins in 21 Pre- and Post-treatment NPC Cases

Serum No.	p54 Pre-	p54 Post-	p138 Pre-	p138 Post-	p23 Pre-	p23 Post-
1	+	-	-	-	-	-
2	-	-	-	-	-	-
3	+	-	-	-	-	-
4	-	+	-	-	-	-
5	+	-	-	-	-	-
6	+	-	-	-	-	-
7	+	+	-	-	-	-
8	+	+	-	-	-	-
9	+	+	-	-	-	+
10	-	-	-	-	-	-
11	-	+	-	-	-	+
12	+	+	-	-	-	-
13	+	+	-	-	+	-
14	+	+	-	-	+	-
15	+	+	-	-	-	-
16	+	+	-	-	-	-
17	+	+	-	-	-	-
18	-	-	-	-	-	-
19	+	+	+	-	-	-
20	+	-	-	-	+	-
21	+	-	-	-	+	-

(+) indicates presence of antibody, (-) indicates absence of antibody

Table 3.6: Percent Positivity of IgM Antibody to Recombinant Proteins p54, p138 and p23 in NPC, NHS and sera of patients with clinical symptoms of NPC

Sera Status	**p54**	**p138**	**p23**
NPC (40)	27.5	20.0	10.0
NHS (8)	12.5	0.0	0.0
Clinical Symptoms of and Subsequently Proven NPC (12)	33.3	16.7	0.0

The number in parenthesis indicate the sample size of each sera group

3.4 MULTIPIN PEPTIDE SYNTHESIS

Due to the high sensitivity and specificity of the recombinant protein p54 compared to the other proteins, further research was conducted to identify its specific antigenic epitope(s).

Seventy nine, 10 residue peptides with 5 overlapping amino acids were synthesized onto non-cleavable pins according to Geysen *et al.* (1984; 1987). Each peptide was synthesized in duplicate so that an average absorbance from each test serum can be obtained (Appendix 2). The 79 peptides were tested in ELISA with 24 NPC sera, one EBV-negative serum and 6 NHS sera. An example of the reactivity of one NPC serum to peptides 1-46 in IgG-ELISA is shown in Figure 3.11. Peptides 52, 53 and 54 gave the three highest sensitivity and positive titrers when tested with NPC sera (Figures 3.12 and 3.13).

Considering the high percentage of overall positivity in peptides 52, 53 and 54 from preliminary IgG studies of the 79, 10-residue peptides (Table 3.7), more copies of these three peptide sequences, RIPAVSVPIL, SVPILRFYRS and RFYRSGIIAV were synthesized for larger serum samples testing (Geysen *et al.*, 1987a; Maeji *et al.*, 1995).

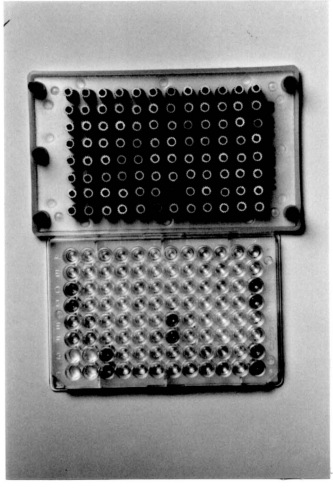


Figure 3.11: IgG antibody to 10-residue, non-cleavable-pin peptides 1-46 of recombinant EA.

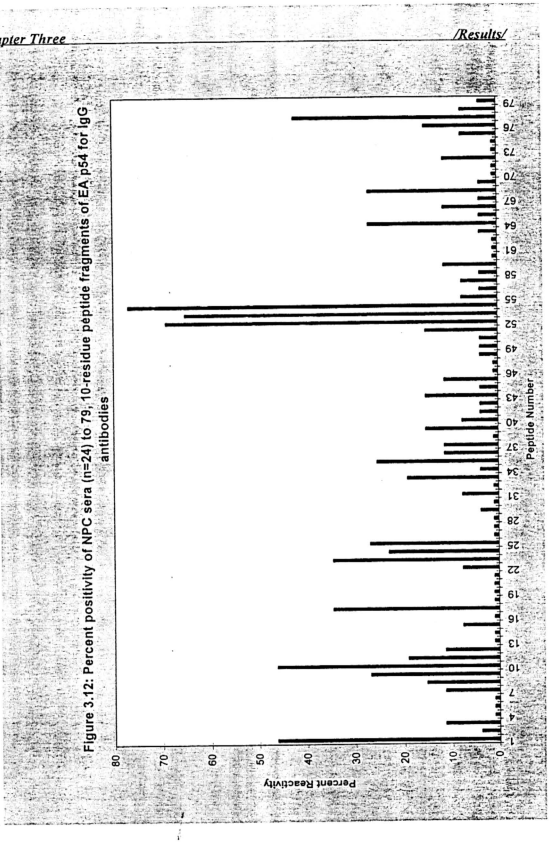


Figure 3.12: Percent positivity of NPC sera (n=24) to 79, 10-residue peptide fragments of EA, p54 for IgG antibodies

Figure 3.13: IgG antibody reactivities in 5 NPC sera to 79, 10-residue peptides of EA p54, showing strength of absorbance to each peptide

Table 3.7: Percent Positive IgG Reactivities to EBV Early Antigen Peptides 52, 53 and 54 in 24 NPC sera (Initial Results)

Sera Type	Peptide 52	Peptide 53	Peptide 54
NPC IgA-VCA IIF Positive & IgG-p54 ELISA Positive (12)	83.3	91.7	100.0
NPC IgA-VCA IIF Negative & IgG-p54 ELISA Positive (5)	40.0	60.0	40.0
NPC IgA-VCA IIF Negative & IgG-p54 ELISA Negative (7)	57.1	42.8	85.7

The numbers in parenthesis indicate the sample size of each sera group

Peptides 52 and 53 were tested with 64 NPC and 16 NHS for the same antibodies. Peptides 52 and 53 have reactivities of 54.7% and 68.8% to serum IgG respectively. Peptide 53 showed a higher specificity at 62.5% than peptide 52 at 50% (Table 3.8).

Peptide 54 was tested with a set of 149 NPC sera, 106 NHS sera, 32 non-NPC cancer sera, 44 ENT disease sera and 44 rheumatoid arthritis sera for IgG antibodies. The proportion of IgG anti-peptide 54 positive sera was significantly higher in NPC than in NHS ($\chi^2 = 133.1$, $p<0.001$). In addition, 6 paired serum-saliva samples from suspect NPC patients were also tested for their serum IgA and IgG antibodies against peptide 54. One serum and two saliva samples were positive for IgA-peptide 54 antibodies. All 6 sera samples had IgG antibodies to peptide 54 while none of the saliva samples showed any reactivities for IgG antibody (Table 3.9).

The three peptides were also tested with NPC and NHS to detect the presence of their serum IgA antibodies for a comparison of IgA-IgG sensitivity and specificity. The results are presented in Table 3.10. Tests for serum IgA against peptides 52 and 53 resulted in 12.5% and 25% sensitivity respectively. Both peptides exhibited 68.8% specificity for serum IgA antibody. Peptide 54-IgA gave a higher overall sensitivity than peptide 54-IgG. Peptide 54-IgG also

Table 3.8: Percent Positive IgG Reactivities to EBV Early Antigen, Peptides 52, 53 and 54 in NPC and NHS

Sera Type	Peptide 52	Peptide 53	Peptide 54
NPC	54.7 (35/64)	68.8 (44/64)	85.2 (127/149)
NHS	50.0 (8/16)	37.5 (6/16)	8.5 (9/106)

Table 3.9: Presence of IgA and IgG Antibodies in Paired Serum-Saliva to Early Antigen Peptide 54

Serum-Saliva	Presence of IgA Serum / Saliva	Presence of IgG Serum / Saliva
S1	- / -	+ / -
S2	+ / -	+ / -
S3	- / -	+ / -
S4	- / -	+ / -
S5	- / +	+ / -
S6	- / +	+ / -

(+) indicates presence of antibody; (-) indicates absence of antibody

Table 3.10: Percent Positive IgA Reactivities to EBV Early Antigen, Peptides 52, 53 and 54 in NPC and NHS

Sera Type	Peptide 52	Peptide 53	Peptide 54
NPC	12.5 (4/32)	25.0 (8/32)	93.4 (141/151)
NHS	31.2 (5/16)	31.2 (5/16)	9.2 (8/87)

detected 91.5% of NPC IgA-VCA IIF-negative sera which suggested a diagnostic potential. Peptide 54-IgG was more specific (91.5%) compared to that of IgA (90.8%). None of the other sera from ENT diseases, rheumatoid arthritis and non-NPC cancers tested had IgG-peptide 54 antibodies. The frequency of positive reactivities for serum IgA to peptide 54 in NPC sera is significantly higher than that of NHS samples ($\chi^2 = 167.4$, $p<0.001$). However, serum IgA antibodies were also detected in 80% of ENT disease samples, in 84% of rheumatoid arthritis samples and in 87.5% of non-NPC cancer samples (Table 3.12).

Among the three 10-mer peptides tested, peptide 54 appeared to be the most sensitive and specific for both the IgA and IgG antibodies in NPC and NHS samples.

Figures 3.14 and 3.15 show the δ absorbance of NPC sera with positive and negative IgA-VCA IIF and IgG-EA IIF titers respectively, to peptide 54. There was no obvious correlation of the IgG anti-peptide 54 binding with both the IgA-VCA and IgG-EA IIF titers in NPC sera observed. Figure 3.16 shows the correlation of serum IgG anti-recombinant p54 absorbances with the absorbances of serum IgG anti-peptide 54. Statistical analysis showed a

Table 3.11: IgG and IgA Reactivities of Various Sera Against Peptide 54

Sera Group	IgA % Reactive	IgG % Reactive
NPC (Overall)	93.4 (141/151)	85.2 (127149)
IgA-VCA Positive	-	79.5 (62/78)
IgA-VCA Negative	-	91.5 (65/71)
NHS	9.2 (8/87)	8.5 (9/106)
ENT Diseases	80.0 (40/50)	0.0 (0/44)
Rheumatoid Arthritis	84.0 (42/50)	0.0 (0/44)
Non-NPC Cancers	87.5 (28/32)	0.0 (0/32)

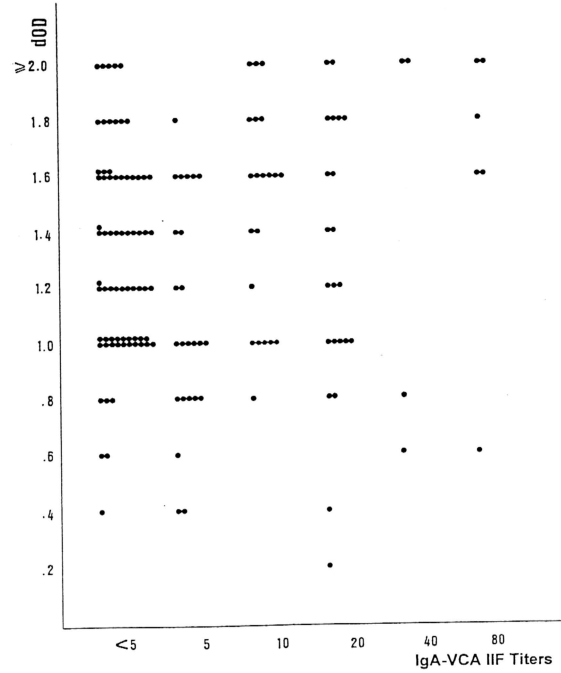


Figure 3.14: IgG anti-peptide 54 antibody binding and IgA-VCA IIF titers in NPC sera

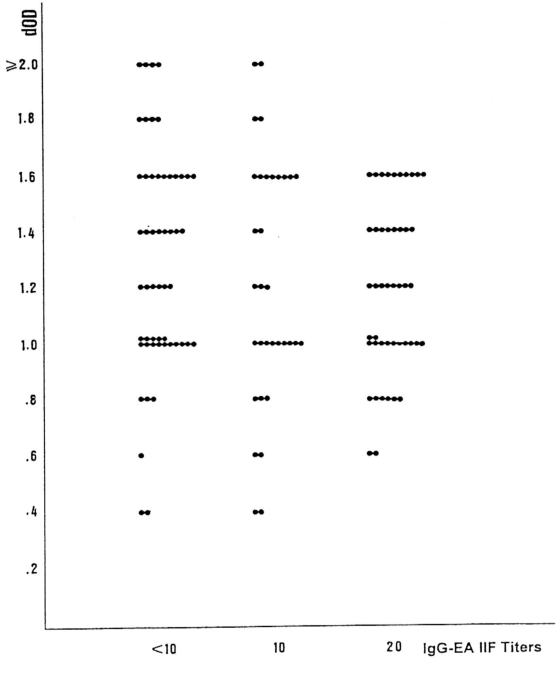


Figure 3.15: IgG anti-peptide 54 antibody binding and IgG-EA IIF titers in NPC sera

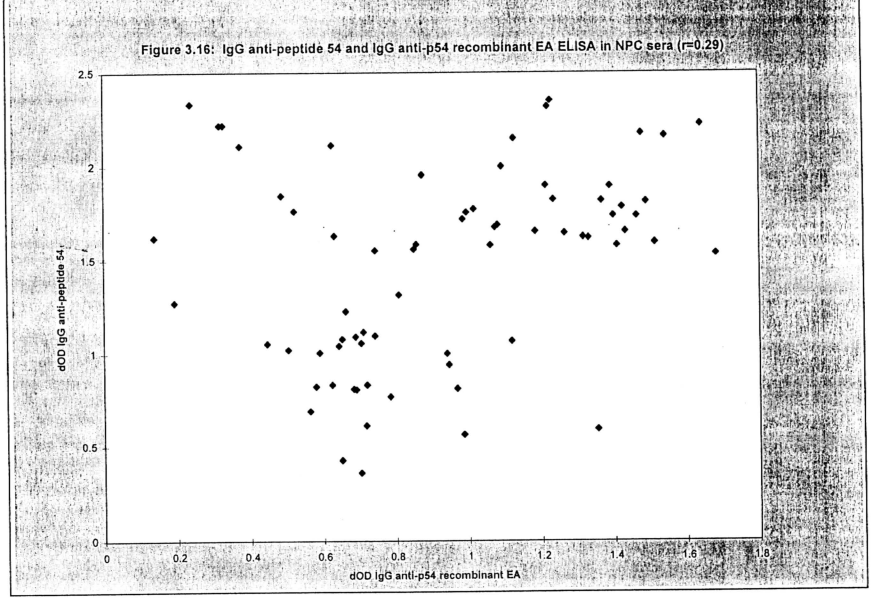


Figure 3.16: IgG anti-peptide 54 and IgG anti-p54 recombinant EA ELISA in NPC sera (r=0.29)
dOD IgG anti-peptide 54
dOD IgG anti-p54 recombinant EA
0
0.5
1
1.5
2
2.5
0.2
0.4
0.6
0.8
1.2
1.4
1.6
1.8

moderately low correlation between the two antigens for serum IgG reactivities (r=0.29).

High reactivities in NPC sera against the 3 overlapping peptides 52, 53 and 54 have prompted the synthesis of shorter sequences of these peptides as peptides of 5- and 3-amino-acid residues. The aim is to locate the shortest, most reactive residues against NPC sera. On average 4-5 amino-acids in an epitope are required to determine the specificity and provide binding energy (Geysen *et al.*, 1988).

The reactivities of serum IgG (Table 3.12) against all the 10-, 5- and 3-mer peptides is higher than that of serum IgA (Table 3.13) in NPC sera. An exception was seen in the peptide RFYRSGIIAV (peptide 54) where serum IgA detection (93.4%) was higher compared to 85.2% for serum IgG. IgG to the 10-mer peptide 54 appears to have the highest specificity in NPC detection compared to all other 10-, 5- and 3-mer peptides tested.

Shorter EA peptides of 5- and 3-mers did not help to improve the sensitivity and specificity of serum IgG antibody detection in NPC samples. False positive binding was also observed in tests for serum IgG to all the pin-bound peptides, excluding peptide 54, with non-specific reactivities ranging from 25% to 50%. Among the 5-mer peptides, peptide YRSGI showed the

highest sensitivity in NPC detection (70%), with a specificity of 62.5%. Three of the 3-mer peptides detected IgG anti-peptide antibodies in more than 70% of NPC samples. Peptides IIA and PAV had specificities of 62.5% while peptide VPI showed a 50% specificity in NPC detection (Table 3.12).

Among the 5-mer peptides, peptide RIPAV showed the highest sensitivity for IgA antibodies, whereas peptides RIP, VPI and IIA showed similar sensitivity in serum IgA detections (31.2%). All of the non-cleavable peptides except peptide 54 exhibited non-specific reactions when tested for serum IgA antibody with percentage of false positive reactions ranging from 18.8% to 50% (Table 3.13).

Table 3.12: IgG Reactivities in NPC and NHS Sera Against Mimotopes

Mimotope	Percent Positivity in IgG-NPC	Percent Positivity in IgG-NHS	χ^2 (d.f =1)
RIPAVSVPIL (peptide 52)	54.7 (53/64)	50.0 (8/16)	0.11 n.s
SVPILRFYRS (peptide 53)	68.8 (44/64)	37.5 (6/16)	5.34*
RFYRSGIIAV (peptide 54)	85.2 (127/149)	8.5 (9/106)	133.1***
RIPAV	65.0 (26/40)	50.0 (8/16)	1.08 n.s
VSVPI	40.0 (16/40)	25.0 (4/16)	1.91 n.s
ILRFY	67.5 (27/40)	37.5 (6/16)	2.25 n.s
YRSGI	70.0 (28/40)	37.5 (6/16)	5.07*
IIAVM	52.5 (21/40)	43.8 (7/16)	0.35 n.s
RIP	62.5 (15/24)	50.0 (4/8)	0.30 n.s
PAV	70.8 (17/24)	37.5 (3/8)	2.84 n.s
VSV	41.7 (10/24)	37.5 (3/8)	0.02 n.s
VPI	70.8 (17/24)	50.0 (4/8)	1.06 n.s
ILR	58.3 (14/24)	25.0 (2/8)	2.67 n.s
RFY	58.3 (14/24)	25.0 (2/8)	2.67 n.s
YRS	45.8 (11/24)	37.5 (3/8)	0.17 n.s
SGI	33.3 (8/24)	25.0 (3/8)	0.34 n.s
IIA	79.2 (19/24)	37.5 (3/8)	2.60 n.s
AVM	54.2 (13/24)	25.0 (2/8)	2.08 n.s

d.f = degree of freedom; χ^2 = chi square test; n.s = non-significant; * = significant at p<0.05; ** = significant at p<0.01; *** = significant at p<0.001

Table 3.13: IgA Reactivities in NPC and NHS Sera Against Mimotopes

Mimotope	Percent Positivity in IgA-NPC	Percent Positivity in IgA-NHS	χ^2 (df=1)
RIPAVSVPIL (peptide 52)	12.5 (4/32)	31.2 (5/16)	2.46 n.s
SVPILRFYRS (peptide 53)	25.0 (8/32)	31.2 (5/16)	0.38 n.s
RFYRSGIIAV (peptide 54)	93.4 (99/104)	9.2 (8/87)	167.4***
RIPAV	68.8 (22/32)	31.2 (5/16)	7.40**
VSVPI	28.1 (9/32)	37.5 (6/16)	0.87 n.s
ILRFY	0.0 (0/32)	18.8 (3/16)	6.40*
YRSGI	31.2 (10/32)	18.8 (3/16)	0.79 n.s
IIAVM	31.2 (10/32)	31.2 (5/16)	0.00 n.s
RIP	31.2 (10/32)	37.5 (3/8)	0.11 n.s
PAV	12.5 (2/16)	25.0 (2/8)	1.13 n.s
VSV	12.5 (2/16)	25.0 (2/8)	1.13 n.s
VPI	31.2 (10/32)	25.0 (2/8)	0.12 n.s
ILR	6.2 (1/16)	25.0 (2/8)	1.49 n.s
RFY	18.8 (3/16)	37.5 (3/8)	1.24 n.s
YRS	6.2 (1/16)	25.0 (2/8)	1.71 n.s
SGI	6.2 (1/16)	25.0 (2/8)	1.71 n.s
IIA	31.2 (10/32)	37.5 (3/8)	0.11 n.s
AVM	12.5 (2/16)	25.0 (2/8)	0.31 n.s

d.f = degree of freedom; χ^2 = chi square test; n.s = non-significant; * = significant at p<0.05; ** = significant at p<0.01; *** = significant at p<0.001

3.5 HYDROPHATY PLOTS AND SECONDARY STRUCTURE PREDICTIONS

Hydropathy plots were done on three EBV proteins namely p54 (EA), EBNA1 and ZEBRA. Figure 3.17, 3.18 and 3.19 show the hydropathy plots of p54, EBNA1 and ZEBRA respectively, according to the Hopp and Woods (1983) method. Figure 3.20 shows the secondary structure prediction of p54 based on the Chou and Fasman (1978) calculations. Numerical values of secondary structure calculations of the recombinant protein are shown in Appendix 3.

Based upon studies done on myoglobins and other proteins, antigenic determinants were consistently located at the point of maximum hydrophilicity; the highest upspike points on the hydrophaty profiles plotted. However, not all high points were associated with antigenic determinants and not all antigenic determinants can be associated with high points (Hopp & Woods, 1981). In the case of p54, antigenic determinants are predicted at locations of amino acid residues 128-134, 146-155, 310-315 and 378-394 (Table 3.14). The first region (amino acids 128-134) is predicted to have a combination of sheet-helix-turn secondary structure. Residues 146-155 has the same combinations of sheet-helix-turn as the first predicted region by Chou and Fasman (1978) secondary

structure prediction method. The third antigenic region consists of only a turn while the last region (a.a. 378-394) has a repetition of helix-turn-helix-turn predicted structure. The antigenic regions identified from PEPSCAN analysis however falls in the region of residues 266-275 with secondary structure of sheet (amino acids 266-267), turn (amino acids 268-272) and sheet (amino acids 273-274) (Figure 3.17, Appendix 3).

Comparison studies using hydrophilicity plots (Hopp and Wood, 1983) were done on recombinant proteins EBNA1 and ZEBRA. PEPSCAN analyses to determine the reactive epitopes of these two proteins have been done previously (Foong *et al.*, 1990; Cheng *et al.*, 1995).

EBNA1 has three rather prominent hydrophilic regions at amino acid residues 355-380, 357-463 and 625-640 (Table 3.15, Figure 3.18). However from IgA- and IgG-ELISA studies done on synthetic peptides of EBNA1, highly antigenic region is located between amino acids 85-325, a region rich with alanine-glycine repeat residues. This region was found to be antigenic with IgA and IgG of NPC sera with positive reactivities reported at 82% and 86% respectively (Foong *et al.*, 1990). The alanine-glycine rich region was calculated as mildly hydrophobic under the Hopp and Woods (1981; 1983) methods. Amino acid residues 65-75 and 570-585 cover the residues of the

20-amino-acid peptides 6,7 and 35,36,37 from synthetic peptide studies. The peptides reactivities against IgA in NPC sera ranges from 2-24% while reactivities in IgG ranges from 2-14%.

Studies on ZEBRA synthetic peptides using IgG ELISA technique has described antigenic regions to be between amino acids 1-22 with 97.3% NPC sera reactive to the sequence (Cheng *et al.*, 1995). Hydrophilicity studies show the antigenic ZEBRA region to be located at amino acids 4-14, 112-118, 160-162, 186-190 and 208-214 (Table 3.16, Figure 3.19). The first antigenic region identified by the Hopp and Woods (1983) method thus corresponded with the ZEBRA synthetic peptide studies (Cheng *et al.*, 1995).

Table 3.14: Predicted Antigenic Regions of p54 Early Antigen and Their Corresponding Peptide(s) Reactivities in IgG from PEPSCAN Studies

Predicted Hydrophilicity Regions (Amino Acid Residue Numbers)	Corresponding Peptide Numbers in PEPSCAN Studies	Highest Sensitivity / Specificity of Peptides in IgG PEPSCAN Studies (%)	
		Sensitivity	Specificity
128-134	25*, 26	29	50
146-155	29*, 30	4	100
➨310-315	61, 62	N.R.	
378-394	75, 76, 77*, 78	46	67

➨highest upspike from hydropathy plot; * sensitivity and specificity calculated from antibody reactivities to this peptide; N.R. = non-reactive

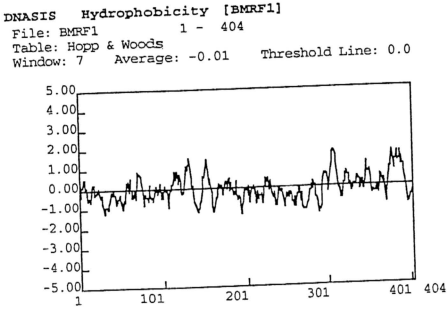


Figure 3.17: Hyrophilicity plot of recombinant EA p54 protein using method of Hopp and Woods (1983)

Table 3.15: Predicted Antigenic Regions of EBNA1 and Their Corresponding Peptide(s) Reactivities in IgA and IgG from PEPSCAN Studies

Predicted Hydrophilicity Regions (Residue Numbers)	Corresponding Peptide Numbers in PEPSCAN Studies #	Percent Sensitivity / Specificity of Peptides in IgA-PEPSCAN Studies		Percent Sensitivity / Specificity of Peptides in IgG-PEPSCAN Studies	
		Sensitivity	Specificity	Sensitivity	Specificity
50-53	6*	4	97	14	100
65-75	6, 7*	20	100	10	92
←355-380	14, 15, 16*	24	92	2	92
457-466	24*, 25	10	100	6	94
570-585	35, 36, 37*	2	100	4	100
←625-640	41, 42	N.R.		N.R.	

← highest upspike from hydropathy plot
N.R. non-reactive

* sensitivity and specificity calculated from antibody reactivities to this peptide

Cheng *et al.*, 1991

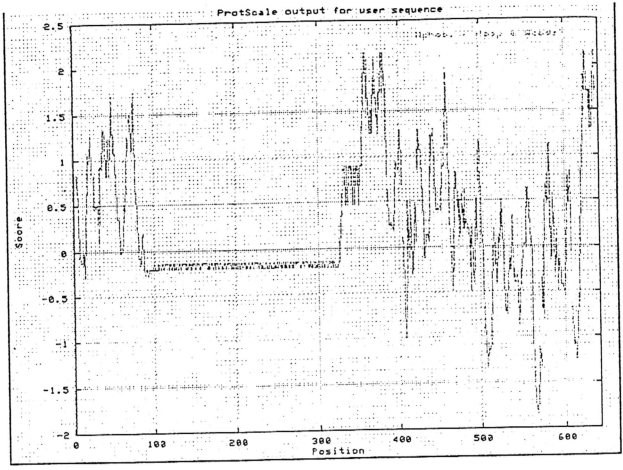


Figure 3.18: Hyrophilicity plot of EBNA1 using method of Hopp and Woods (1983)

Table 3.16: Predicted Antigenic Regions of ZEBRA and Their Corresponding Peptide(s) Reactivities from PEPSCAN Studies

Predicted Hydrophilicity Regions (Amino Acid Residue Numbers)	Corresponding Peptide Numbers in PEPSCAN Studies #	Sensitivity of IgG-Peptides in PEPSCAN Studies (%)
4-14	1	97.3
112-118	13	5.4
160-162	19	35.1
186-190	22	18.9
☛ 208-214	25, 26	N.R.

☛ highest upspike from hydropathy plot; N.R. = non-reactive

Cheng *et al.*, 1995

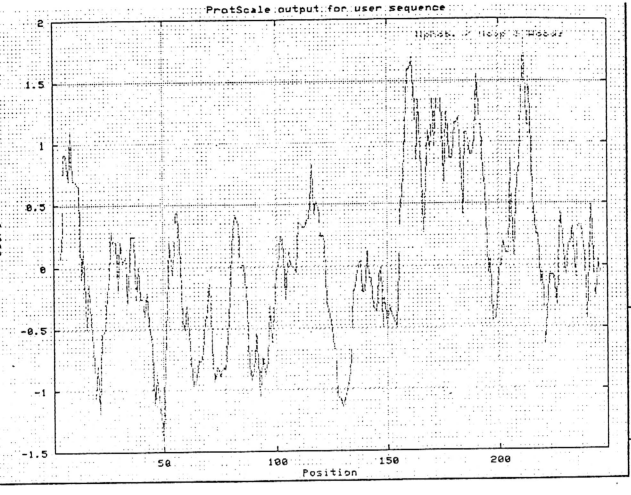


Figure 3.19: Hyrophilicity plot of ZEBRA protein using method of Hopp and Woods (1983)

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DNASIS   Protein 2D Structure Map [BMRF1]
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 Seq:        1 -      404
 Function: Chou and Fasman
▬▬▬ : HELIX  ▪▪▪▪▪▪▪▪▪▪ : SHEET  ▒▒▒▒▒▒ : TURN  ▒▒▒▒▒▒ : COIL
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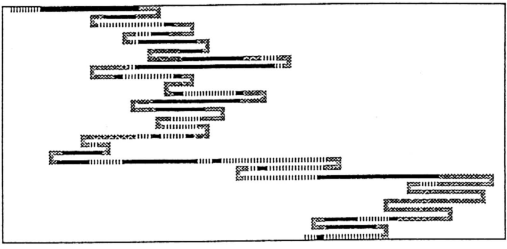

Figure 3.20: Secondary structure of recombinant EA p54 protein as predicted by Chou and Fasman (1978)