INTRODUCTION

1.0 INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a malignant tumour affecting various populations worldwide. It is claimed to be the most common form of nasopharyngeal malignancy in all human populations (Shanmugaratnam, 1984). NPC is documented to arise in the post nasal space, namely the fossa of Rosenmuller (Prasad, 1979; Prasad *et al.,* 1983). It is common in South China, Hong Kong and the Greenland Eskimos (de The *et al.,* 1982). It is also not uncommon in other Southeast Asian countries and some African states. The encyclopedia of Chinese Medical Terms edited by Wu in 1921 has mentioned a disease termed "shih ying" which was most likely to be caused by primary carcinoma of the nasopharynx (Ho, 1972).

Incidence rate in most populations is less than 1:100 000 per year (de The *et al.,* 1982). However in certain Chinese population, the age-adjusted incidence rate per 100 000 per annum varies between 10 to 20 for males and 5 to 10 for females (Muir, 1971). A rate of 15 to 30 per 100 000 per annum was also cited (Simon and Shanmugaratnam, 1982). The age incidence curve for NPC high risk population tends to increase in the late twenties and reaches its

plateau between the ages of forty to sixty. The curve does not increase again with age (Simon and Shanmugaratnam, 1982).

1.1 CLINICAL SYMPTOMS OF NPC

The clinical symptoms of NPC vary considerably. According to Neel and Taylor (1983) the most common and obvious symptom is the neck lump, seen in 80% of the patients. Other symptoms include plugging or fullness of ears (41%), hearing loss (37%), nasal bleeding (30%) and nasal obstruction (29%). NPC patients also complain of headaches, ear pain and deep neck pain. Unfortunately those who present with these symptoms are usually already having a later stage of the disease. This is so since the supply of nerves in the nasopharynx is not abundant, making pain not an early characteristics of this carcinoma.

NPC is one of the most misdiagnosed malignancies due to its wide range of symptoms (Neel and Taylor, 1983). 80% of those with NPC have a characteristic elevation of antibody titers against an antigen from a virus closely linked with the disease. Diagnosis is confirmed from histological examination of biopsies taken primarily from the post nasal space or from a secondary site such as the cervical lymph node. False negative results although rare, may

occur due to inadequate specimens and/or the cells' histological features is obscured by traumatic distortion or tumour necrosis (Shanmugaratnam, 1984).

1.2 CLASSIFICATIONS OF NPC:

The histological typing and classification of NPC is based on light microscopy. There are several classifications available and one of them is the WHO classification as described by Shanmugaratnam and Sobin in 1978. WHO classifies NPC into three categories:

i) WHO 1 or squamous cell carcinoma (SCC)

Evidence of squamous differentiation with intercellular bridges and/or keratinization over most of its extent is distinct characteristics of this class. WHO 1 may be graded into three subdivisions:

a) well differentiated,

b) moderately differentiated

c) poorly differentiated

ii) WHO II or non-keratinizing carcinoma (NKC)

This category shows well-defined cell margins with stratified or pavemented arrangement. However, no evidence of squamous differentiation can be seen in the tumour cells.

iii) WHO III or undifferentiated carcinoma (UC)

Tumour cells categorized as WHO III generally have pale or vesicular nuclei with prominent nucleoli. They have syncytial appearance. Spindle-shaped tumour cells exhibiting hyperchromatic nuclei may also be present. Cells arrangement may be irregular with moderately well defined masses in strands of loosely connected cells in a lymphoid stroma.

WHO I accounts for 20% of NPC cases in lower risk populations in Europe and United States of America (Weiland, 1978). It is uncommon in Malaysia. 55% of Malaysian cases are of the WHO II (Yadav, 1987), while about 44% of Malaysian patients presented the WHO III (Prathab *et al.*, 1983). Undifferentiated carcinoma comprises significantly higher proportion of tumors occurring among children and adolescence whereas SCC is more common among older patients.

Classifications are done with the objective of assisting histological diagnosis of tumors by categorizing and describing morphological variants. Subdividing the tumors into histo-morphological types that share important biological characteristics in clinical behavior, response to therapy, epidemiology and etiology is a more significant objective.

The prognosis of NPC is dependent upon its histological identification and clinical stage of the tumor at time of detection. WHO II and WHO III respond better to therapy; they also have a longer disease-free period (Scanlon *et al.,* 1967; Dickson 1981). Patients with WHO I have lower survival rate compared to the other two WHO types, although in general survival rate is higher with earlier detection. Ho *et al.* (1981) reported the survival rate to have a positive correlation with tumor burden - recurrence-free rate is 63% for stage I, 50% for stage II, 28.8% for stage III, and 15.8% for stage IV. The overall 5-year survival rate for NPC was approximately 20-30%. In cases where tumors are confined to the nasopharynx, 60-70% of patients may survive for five years.

1.3 GEOGRAPHICAL AND RACE DISTRIBUTION OF NPC:

NPC is a rare malignancy in most parts of the world with the exception of China and certain regions of the Southeast Asian countries. The frequency of cases is highest among Chinese both within and outside of China. However there is a drop in frequency as one travels from south to the north of China (Ho, 1972; Shanmugaratnam *et al.,* 1979; Chen-Chuan *et al.*, 1985). The frequency is intermediate among North Africans (Camoun *et al.*, 1974) and low in Europeans and Americans (Bailar 1967; Waterhouse *et al.*, 1982). The incidence rate is higher in emigrant Chinese population than the native populations. In the United States for instance, NPC occurs more commonly among the Chinese Americans than the whites and blacks (Levine *et al.*, 1983). However the rate is lower in American-born Chinese if compared to those who emigrated (Zippin *et al.*, 1962; Buell, 1965). On a similar note, the African- and Asian-born Jews showed significantly higher frequency of developing NPC than those born elsewhere (Waterhouse *et al.*, 1976; Waterhouse *et al.*, 1982).

In Malaysia, NPC is listed among five most common cancers affecting male population. Armstrong *et al.* (1979), from his 10-year studies on Malaysian NPC patients concluded that the Chinese hold the highest relative risk; the

Cantonese were most affected followed by the Kheks (Hakka), the Hokkiens and the Teochews. The Kadazans in Sabah, East Malaysia were also found to have a higher relative risks compared to the Malays or Indians. 71.5% of NPC patients were Chinese, 15.3% were Malays, 3.3% were Ibans, 2.8% were Kadazans, 2.2% were Dayaks and Indians made up 0.9% of total patients (Yadav, 1987). The Cantonese have a frequency of 28.9%, the Hakka 23.2% and the Hokkien 19.7% of developing NPC (Yadav *et al.*, 1985).

In an eleven years epidemiological studies on NPC incidence per 100,000 population in West and East Malaysia, the Chinese was reported to have the highest incidence rate at 6.83. Malays and Indians have incidence rates of 0.73 and 0.21 respectively. In the East Malaysia, 3.41 and 1.06 incidence per 100,000 per year were recorded for the Ibans and the Kadazans. Age group studies reported having a patient below 4 years of age. The incidence curve for the Chinese, Malays and Ibans according to age groups were of continuous nature with peaks at the ages of 40-44 years for the Chinese and Ibans, and 50-54 years for the Malays. The incidence curve representing the Kadazans showed bimodality with peaks at 10-24 years and 45-49 years of age. A higher frequency of cases in subethnic Chinese was also reported where Cantonese were at 34.3%, Hokkiens at 37% and Hakka/Kheks at 23.1% (Norhanom, 1989).

Singapore Cancer Registry in its preliminary statistics of cancer incidences, revised in 1990, stated nasopharyngeal cancer to be the fifth killer among males (7.3%) after lung (23.7%), colo-rectum (12.5%), stomach (11.5%) and liver cancer (17.5%). NPC was reported to be the eighth most common cancer among the females with an age-standardized rate of 6.1 per 100 000 per annum. About 95% of the affected males were Chinese of various dialect with the Cantonese being the largest number of patients (9.8), followed by the Hakka (7.9), Hokkien (6.4), Teochew (6.1) and Hainanese (6.1). On the other hand, the Hainanese women were the largest group of female NPC patients (5.9), followed by the Cantonese (4.8), the Hakka (4.7), the Hokkien and Teochew, both at a 4.0 age-standardized incidence rate per 100 000 per annum.

Singapore-born Chinese studied have a higher risk of developing NPC compared to those born in China and elsewhere. There were 1852 reported cases in males over the years of 1978-1982; 986 of the patients were Singapore-born whereas 515 were born in China and the rest were born elsewhere. Similar observation was seen in females where 410 out of 793 patients were Singapore-born as opposed to 208 who were China-born (Lees *et al.*, 1988).

1.4 ETIOLOGICAL FACTORS OF NPC:

Genetic and environmental cofactors play important roles in the development of NPC.

1.4.1 Genetic Factor

NPC is very common among the Southern Chinese. The incidence rate for this population ranged from 15-30 per 100 000 per year. The high NPC frequency was also observed in Hong Kong, among emigrant Southern Chinese populations in South East Asia and California (Shanmugaratnam 1982; Waterhouse *et al.*, 1976). The relative risk of NPC in Singapore Chinese (immigrants from Southern China) was highest at 20.2 compared to Malays and Indians whose relative risks were 5.8 and 0.2 respectively (Shanmugaratnam, 1971).

In a study by Prasad and Rampal (1992), the age-adjusted incidence rate per 100 000 populations for Chinese in Malaysia was 11.3 for males and 4.1 for females. The Malays were reported to have incidence rates of 1.3 for males and 0.3 for females. The Malaysian Indians had the lowest incidence

rates per 100 000 population with 0.3 for males and 0.2 for females. The natives of Alaska (Lanier *et al.,* 1980), Canada (Mallen and Sandro, 1974) and Greenland (Neilson *et al.,* 1977) develop NPC at more than 15 times the rate of white Caucasians in the United States. The rates of 13.5 per 100 000 per year in males and 3.7 in females were observed in the Alaskans (Lanier *et al.,* 1980).

There are evidences from familial clustering in NPC cases among people of Alaska (Lanier *et al.,* 1980), Hong Kong (Ho, 1978), China (Degos *et al.,* 1984), and Malaysian Chinese (Prasad *et al.*, 1983).The significance of familial clustering however, only applies to those in the high risk groups e.g. the Cantonese (Ho, 1972; Fisher *et al.*, 1984; Degos *et al.*, 1984; Tarone *et al.*, 1990).

The involvement of genetic factor is further suggested by observations of increased incidence rates of NPC among offsprings of a Southern Chinese parent (Muir, 1971; Ho, 1972). Studies on HLA markers provided rather strong indications of genetic involvement in the onset of NPC. The risk of developing NPC has been reported to be significantly associated with HLA-BW46, HLA-

BW17 and HLA-BW19 genes in Chinese but not in other populations. HLA-BW46 was associated with an increased risk in older onset patients (> 30 years), HLA-BW17 was associated with both older and younger onset patients but particularly in younger patients while HLA-B13 was associated with decreased risk of NPC in older patients. (Simon *et al.*, 1974; 1977; Chan *et al.*, 1983; 1985; Lu *et al.*, 1990). HLA-A11 on the other hand is associated with a decreased risk. An increased risk is also associated with the presence of HLA-A2 and HLA-Bsin2 major histocompatibility complex (MHC) alleles in Chinese individuals (Ren *et al.*, 1995). Chan *et al.* (1983) indicated that the existence of HLA locus A2 and BW46 rather than BW46 alone as the determinant of risk. The presence of BW46 without A2 resulted in no excess risk. In Malays, the relative risk of developing NPC is associated HLA-B58 rather then A2 or BW46 (Chan *et al.*, 1985).

Gm allotypes are a highly polymorphic system of markers associated with antigenic differences in the constant region of IgG heavy chains and are inherited in fixed combinations known as haplotypes (Propert, 1984; van Loghem, 1984). A consistent pattern of change in haplotypes frequency in Asian populations can be seen (Propert, 1984) suggesting an association of certain Gm haplotypes with susceptibility of NPC in South China, and the

association would have persisted in Southeast Asia (Tarone *et al.*, 1990). However, the haplotype differences were not observed in NPC cases and controls of Malaysian Chinese and Malays (Tarone *et al.*, 1990). The similarity of haplotype patterns indicate the availability of other genetic and environmental cofactors in determining NPC susceptibility.

Burt *et al.* (1994) reported consistency of relative risk of NPC in Chinese populations to be associated with HL-A2. However, the same antigen was found to have a protective effect in Caucasians whereby HL-A2.1 (A* 0201) molecule was present in 96% of the Caucasians studied.

A study by Ren *et al.* (1995) on HL-A2 allelic microvariants contrasted the findings of Burt *et al.* (1994) above where A* 0201 was associated with increased relative risk. These contrasting findings suggest a more complex, interactive role of EBV proteins and HLA susceptibility alleles in causing NPC.

Another genetic aspect that has been looked into recently was the T-cell receptor (TCR) genes. Many studies have been conducted on the associations of TCR and HLA to autoimmune diseases (Ito *et al.*, 1988; Funkhouser *et al.*, 1992). Some researchers reported the association of HLA types in autoimmune diseases e.g. Graves diseases and rheumatoid arthritis patients to

have the same HLA associations with NPC (Yeo *et al.*, 1989; Tan *et al.*, 1988; Boey *et al.*, 1992). Studies done in Singapore (Chen and Chan, 1994) suggested that NPC susceptibility is strongly linked to a 20kb protein fragment from restriction fragment length polymorphism (RFLP) analysis using BamH1/TCR Vβ11 combination cut sites.

1.4.2 Environmental Cofactors

NPC carcinogenesis has been associated mainly with nitrosamines, a chemical substance known to have tissue-specific oncogenic effect. Ho *et al.* (1971) first suggested the Cantonese-style salted fish ingested may be a contributing factor to increased risk of NPC among Southern Chinese. Cantonese-style salted fish extracts were proven to contain volatile nitrosamines which were known mutagens (Fong and Walsh, 1971; Ho *et al.*, 1978; Huang *et al.*, 1978; Shao *et al.*, 1988). Two groups of researchers studied the effect of Cantonese-style salted fish on Wistar albino rats. Mutagenic urine was passed by rats fed with salted fish. Four of 20 rats given salted fish diet subsequently developed carcinoma in the nasal and paranasal regions while none of the 6 controls did (Huang *et al.*, 1978; Fong *et al.*, 1979). Results from the studies strongly supported the notion that Cantonese-style

salted fish consumption is an important etiological factor in NPC carcinogenesis.

Salted fish was also reported to be a common food item in the intermediate and high risk NPC populations in Southeast Asia, e.g. the Kadazans in East Malaysia (Rothwell, 1978). Lanier *et al.* (1980) reported similar associations among the Eskimos, Aleut and Indian natives of Alaska, where salted fish was among childhood diet. However, the report stated no significant difference in salted fish intake between NPC and control groups.

A dose-response effect was observed in both childhood and adulthood consumption of Cantonese-style salted fish in China. The geographical incidence distribution of NPC in China is also consistent with the intake of Cantonese-style salted fish, which is the favourite food among Southern Chinese of Guangdong Province but rarely eaten by the Northerners (Yu *et al.*, 1986). The occurrence of NPC among those ages 15-34 years in China suggested an early exposure to etiological agent(s). The high NPC rates among immigrants to low NPC risk areas e.g. North America, have also been attributed to Cantonese-style salted fish (Yu *et al.*, 1981).

Yu *et al.* (1985; 1988) also looked into the consumption of other foods and their relative risks of causing NPC in young Hong Kong Chinese subjects. 2-7% of the NPC patients interviewed in the study reported eating salted pork, pork sausage, dried shrimps, dried red date, cooling soup and dried fruits at least once a week at the age of 10. 18% and 25% of NPC cases reported consumption of at least once weekly, the moldy bean curd and salted mustard green respectively, also at the age of 10 years old. Interestingly, 46% of the cases studied had consumed Cantonese-style salted fish at least once per week when they were 10 years old. However, all the other foods mentioned above were found to have non-significant correlation to NPC after adjustments were made in the calculations, taking salted fish into account.

NPC is also linked to volatile nitrosamines, smoke and dust inhalation, and the use of Chinese herbal medication for nasal illnesses (Lin *et al.*, 1973; Henderson and Louie, 1978; Shanmugaratnam *et al.*, 1978; Ito, *et al.*, 1982; Armstrong *et al.*, 1983; Poirier *et al.*, 1987; Yu *et al.*, 1988; 1990;). The only etiological factor associated with NPC in all populations worldwide is the Epstein-Barr virus.

1.5 EPSTEIN-BARR VIRUS (EBV):

The Epstein-Barr virus is a gamma-herpes virus, classified into the family Herpesviridae. It is an ubiquitous human virus. EBV was first observed by Epstein in a culture of endemic African Burkitt's lymphoma (BL) (Epstein *et al.*, 1964). Typically more than 95% of the adult population carries the virus which was acquired in early childhood (Henle and Henle 1966a; 1967). Delayed infection results in clinical illness known as infectious mononucleosis (IM) (Henle *et al.*, 1968; Epstein and Achong 1979). Once acquired, EBV persists in host throughout life and is carried asymptomatically in latent form, in B lymphocytes (Yao *et al.*, 1985). Its existence can be detected as elevated serum antibodies to specific antigens. The viral capsid antigen (VCA) for instance, was noticed from observation of Henle and Henle (1966a) where a small percentage of EBV producer cultures e.g. P3HR-1 reacted against various human sera and were brilliantly stained using indirect immunofluorescence technique (Henle and Henle, 1966b; 1979; Henle *et al.*, 1967; 1974).

EBV is believed to have an association with BL although its precise pathogenicity remains unclear (Klein, 1983; Magrath, 1990). It is also associated with the development of high-grade B cell non-Hodgkin's

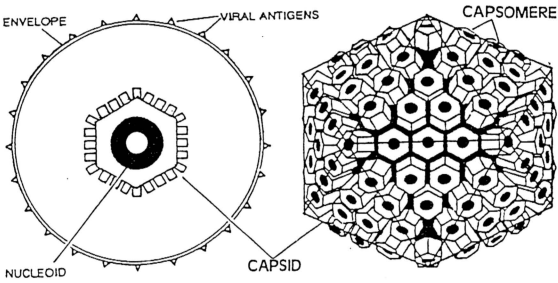

Figure 1.1: The Epstein-Barr Virus (EBV)

lymphomas (NHLs) in patients with both congenital and acquired immunodeficiency states (Pallesen *et al.*, 1993). Recent studies have shown that EBV nucleic acids and antigens were present in about 50% of Hodgkin's disease cases and in substantial number of peripheral T cell lymphomas (PTLs) and related lesions (Pallesen *et al.*, 1993).

EBV is strongly linked to NPC as results from various studies showed the existence of EBV in NPC patients. The first association can be seen in the elevation of antibody titers against EBV antigens in a majority of NPC patients (Henle and Henle, 1976). Another link is the presence of EBV DNA or protein (latent membrane protein) in NPC biopsies studied by *in situ* hybridization (Wolf *et al.*, 1984; Permeen *et al.*, 1990; Pathmanathan *et al.*, 1995a). Researchers working with NPC biopsies have observed the presence of EBV associated RNAs, EBERs and EBNAs indicating another plausible influence of EBV to NPC (Huang *et al.*, 1974; Chen *et al.*, 1992; Pathmanathan *et al.*, 1995a; 1995b).

1.6 EPSTEIN-BARR VIRUS AND GENOME STRUCTURE:

EBV has a linear, double stranded DNA genome of 172kb in length, encoding approximately 100 genes (Farrel, 1989). EBV has a protein core

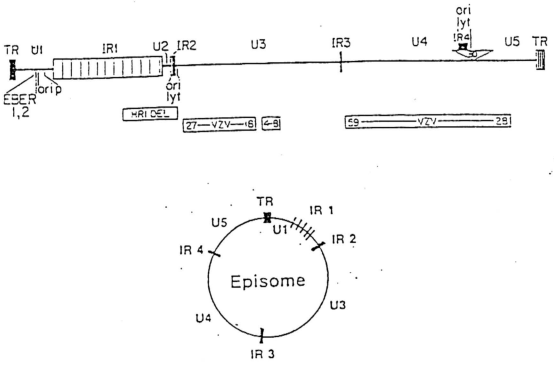

Figure 1.2: Linear EBV DNA and cicular EBV episome showing the general organization of unique (U) and major internal repeats (IR) DNA domains (From Tosato, 1987; Fields, 1996)

which is wrapped with DNA, a nucleocapsid with 162 capsomeres, a protein tegument between the nucleocapsid and an outer envelop with external glycoprotein spikes typical of other herpesviruses (Epstein *et al.*, 1964; 1965; Pope *et al.*, 1968; Dolyniuk *et al.*, 1976). The EBV genome has been completely sequenced (Baer *et al.*, 1984) and mapped for promoters, open reading frames, RNAs and other structural elements (Baer *et al.*, 1984; Farrel, 1989).

The presence of EBV DNA was detected in Burkitt's lymphoma and anaplastic NPC (zur Hausen *et al.*, 1970). Nucleic acid hybridization studies showed that certain BL lines and cells transformed *in vitro* that were not actively producing mature virus particles, contained EBV genome. The genome was present as latent extrachromosomal circular DNA molecules in these cell lines (Adams and Lindahl, 1975).

The genome structure of EBV includes 0.5kbp terminal direct repeats and 3kbp internal direct repeats; these repeats separated the genome into short and long unique sequence domains (Davison and Wilkie, 1981; Davison and Scott; 1986; Davison and Taylor, 1987; Baer *et al.*, 1984; Cameron *et al.*, 1987; Pallesen *et al.*, 1993). EBV isolates differ in their tandem repeat reiteration frequency (Heller *et al.*, 1982; Katz *et al.*, 1989). EBV genome has a

short unique sequence (US) of about 10kb long, followed by an internal repeat sequence (IR) with length of about 40kb and an approximately 120kb long, long unique region. Four direct tandem internal repeats (IR1 to IR4) were identified; IR1 are multiple repeats of a 3072bp sequence, IR2 and IR4 are tandem repeats of 125 and 103bp sequence respectively (Cheung and Kieff, 1982; Dambaugh and Kieff, 1982). IR3 has a unique characteristic of having 3 nucleotide triplets, GGG, GCA and GGA in a sequence of 708bp repeat array (Heller *et al.*, 1982). The IR sequence separates the EBV genome into five unique (U) region known as U1, U2, U3, U4 and U5. At each end of the EBV DNA, there are 4-12 copies of a 500bp terminal repeats (TR). TR is believed to play a role in facilitating EBV DNA circularization following host infection (Lindahl *et al.*, 1976). Viral DNA becomes covalently linked inside infected cells and the genome persists as multiple covalently closed circular episomes.

There are two types of EBV identified, designated type A and B (or 1 and 2) (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985; Rickinson *et al.*, 1987; Rowe *et al.*, 1989; Sixbey *et al.*, 1989; Sculley *et al.*, 1989; Arrand *et al.*, 1989). Studies conducted showed that EBV-1 is more commonly detected in the Western population while virus isolated from Africans have an even distribution between types 1 and 2 (Zimber *et al.*, 1986). Later studies using

biopsies from Southern China NPC patients showed the predominance of EBV type A in the area (Chen *et al.*, 1992). EBV types 1 and 2 share extensive homology with one another. Variations documented in latently infected cells, occur in genes coding for EB virus nuclear antigens (EBNAs) and small polyadenylated RNAs (EBERs) (Allday *et al.*, 1989), showing 16-47% differences (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985; Sample *et al.*, 1990).

1.7 EBV TRANSMISSION AND STAGES OF INFECTION:

1.7.1 Transmission:

Healthy carriers shed EBV intermittently into their saliva, indicating presence of productive viral infection in the epithelial, oropharynx and/or salivary glands (Gerber *et al.*, 1972; Morgan *et al.*, 1979; Sixbey *et al.*, 1983; Wolf *et al.*, 1984). EBV is transmitted via saliva to non-infected individuals. EBV has also been detected in genital secretion but no reports have suggested that EBV is transmissable via the secretions (Sixbey *et al.*, 1986; Se Thoe, 1991).

1.7.2 Infection:

EBV infection occurs in two stages:

a) Latent Infection

Acquired EBV will infect and immortalize B lymphocytes. This is accomplished through interaction and binding of viral envelop glycoprotein gp350 and 140kDa B cells-associated C3d complement receptor, CR2, also designated CD21. The viral DNA circularises upon entering host cells via fusion of terminal repeats. The episome is then maintained as a multicopy episomal plasmid (Kieff and Liebowitz, 1990). EBV-transformed B lymphocytes are controlled by HLA-restricted cytotoxic T cell response, *in vivo* (Rickinson, 1986; Murray *et al.*, 1992). *In vitro*, infected B lymphocytes enter the cell cycle and continue to proliferate indefinitely. Peripheral blood EBV-infected B lymphocytes give rise to permanent clones of transformed, lymphoblastoid cell lines (LCLs) spontaneously in culture (Pallesen *et al.*, 1993). EBV infection also stimulated RNA and DNA synthesis, cell division and expression of B-cell activation markers (Kintner *et al.*, 1981; Thorley-Lawson *et al.*, 1982). The latently infected LCLs support predominantly latent viral life cycle in which about 11 of the 100 viral genes are expressed (Rogers *et al.*, 1992; Khanna *et al.*, 1995). Among the genes expressed are EBER1, EBER2, six nuclear antigens (EBNA1 through EBNA6), and three integral membrane proteins (LMP1, LMP 2A and LMP 2B) (Murray *et al.*, 1990;

1992) with EBNA5 being the first nuclear antigens to be expressed (Alfieri *et al.*, 1991). All EBNA proteins would have reached the level that is maintained consistently during latent infection within 48hrs of primary B-cell infection, *in vitro* (Moss *et al.*, 1986; Allday *et al.*, 1989; Khanna *et al.*, 1995), whereas EBERs will only reach a substantial level after 70hrs of infection (Alfieri *et al.*, 1991).

In general less than 1% of cells in any given LCL will enter lytic cycle whereby most or all the viral genes are expressed, producing virions and resulting in host cell death. The background triggering of untreated LCL is not understood although a number of natural and artificial stimuli help to increase the number of cells entering lytic cycle (Rogers *et al.*, 1992).

b) Lytic/Productive Infection

Lytic infection can be induced in vitro utilizing phorbol esters or cross-linking of surface Igs or treatment with calcium ionophore (zur Hausen *et al.*, 1978; Lin *et al.*, 1979; Luka *et al.*, 1979). Induction can also be accomplished through superinfecting of Raji cell line with a latent antigen-defective strain of EBV from P3HR-1 cell line (Biggin *et al.*, 1987). Induced cells will exhibit changes such as margination of nuclear chromatin, synthesis of viral DNA,

synthesis of viral DNA, assembly of nucleocapsids, virus envelopment and inhibition of host macromolecular synthesis (Gergely *et al.*, 1971; Takagi *et al.*, 1991). About 80 virus-specific RNA are produced during this phase (Takada and Ono, 1989). The switch from latency to lytic infection is activated by the expression of BZLF1 gene, producing BZLF1 (Z) or ZEBRA protein (Countryman and Miller, 1985; Kenny *et al.*, 1989; Taylor *et al.*, 1989). Cytohybridization studies showed that EBV replication occurs in the oropharyngeal epithelial cells (Sixbey *et al.*, 1984). Replicated virus is released into the oral cavity from epithelial cells of the upper respiratory tract and salivary glands and become available for infection of susceptible individuals (zur Hausen *et al.*, 1970). Uptake of immunoglobulin A (IgA)-virus complexes by the epithelium or the fusion of circulating EBV-positive B cells with oropharyngeal epithelium are alternative explanations of EBV oral epithelium infection mechanism (Sixbey and Yao, 1992).

1.8 EPSTEIN-BARR VIRUS ANTIGENS

EBV produces various antigens during its latent and lytic cycles. Several of the antigens are described below.

1.8.1 Epstein-Barr Virus Nuclear Antigens (EBNAs):

EBNAs can be found in latently infected cells; detection may be done by complement immunofluorescence (ACIF) (Reedman and Klein, 1973). There are 6 types of EBNAs identified to date namely EBNA1, EBNA 2A and 2B, EBNA 3A, 3B (EBNA 5/L*eader* protein) and EBNA3C/6.

a) EBNA1:

EBNA1 was identified by immunoblotting and radioimmunoelectrophoresis (Strnad *et al.*, 1981). The EBV positive cell lines utilized in EBNA detection yielded antigens of different molecular weights, ranging from 65 kDa to 75 kDa. The size variations of EBNA1 was found to be determined by viral genome (Gergely *et al.*, 1984; Sculley *et al.*, 1984). It is particularly dependent on the length of the third internal repeat array (IR3) in the BamK fragment (Heller *et al.*, 1982; Hennessy *et al.*, 1983). It was later found that EBNA1 is mainly responsible for the EBNA ACIF reaction, due to its 22-residue copolymer of glycine and alanine repeats (Dillner *et al.*, 1987) termed peptide 107 (p107). EBNA1 is encoded by BKRF1 open reading frame in the BamK fragment (Dillner *et al.*, 1984). EBNA1 protein structure can be described as having a short N-terminal sequence, followed by a 20-45kDa glycine-alanine copolymer, flanked by basic arginine-rich sequence and a

highly acidic carboxy terminal sequence (Dillner and Kallin, 1988). The acidic carboxy terminal of EBNA1 corresponded with synthesized 28kDa protein in a bacteria. The protein was shown to have three sequence-specific binding sites on EBV genome (Rawlins *et al.*, 1985). One of the regions lies in the oriP which requires the presence of EBNA1 gene for the maintenance of EBV plasmids (Yates *et al.*, 1985).

b) EBNA2:

EBNA2 is an 81kDa protein encoded by BYRF1 reading frame (Dillner *et al.*, 1985) in the BamWYH region (Sculley *et al.*, 1984; Dambaugh *et al.*, 1984). EBNA2 amino acid structure contains a 26-amino-acid long proline polymer, a 12-amino-acid glycine-arginine repeat and a highly charged, acidic carboxy terminal (Dillner and Kallin, 1988). EBV is subtyped into EBV A and EBV B due to the variation of hybridizability of BYRF1 region (Zimber *et al.*, 1986). EBNA2 coding region BamWYH is necessary for EBV transformation, but not in its maintenance (Jones *et al.*, 1984; Ernberg *et al.*, 1986). EBNA 2A is 85kDa in B95-8 virus with about 491 amino acids whereas EBNA 2B is 75kDa in AG876 Burkitt's lymphoma-associated strains with about 443 amino acids (Dambaugh *et al.*, 1984; Rymo *et al.*, 1985; Hennessy and Kieff, 1985). EBNA 2A virus-transformed cells grow quicker than EBNA 2B (Rickinson *et al.*,

1987). EBNA 2A is found mainly among Europeans and the North Americans while EBNA 2B is predominant in Africa (Zimber *et al.*, 1986). EBNA 2 induces cellular antigens CD21 and CD23 in EBV-negative cell lines, therefore influencing host cells' gene expressions (Wang et al., 1987). EBNA 2 also transactivates LMP promoter (Wang *et al.*, 1990; Fahraeus *et al.*, 1990) and the promoters of viral terminal proteins. Lees *et al.* (1993) studied sequence homology among membrane proteins derived from three cell lines namely AG876, P_3HR1 and B95-8 in the process of developing an EBV candidate vaccine from antigen gp340/220. The data obtained indicated that the EBV membrane antigen is highly conserved (>97% identity) between type A and B strains.

c) EBNA 3:

EBNA 3 is found in all EBV infected cell lines (Hennessy *et al.*, 1985). EBNA 3 is divided into 3 families designated EBNA 3A, 3B and 3C. EBNA 3A is encoded by Bam H1L open reading frame (BLRF3) exon and a neighboring long exon BERF1 of the Bam H1E reading frame (Hennessy *et al.*, 1986; Joab *et al.*, 1987). EBNA 3B is encoded by BERF 2A and BERF 2B (Petti and Keiff, 1988). EBNA 3C or EBNA 6 (Ricksten *et al.*, 1988) is encoded by BERF 3 and BERF 4 (Petti *et al.*, 1988; Ricksten *et al.*, 1988). The EBNAs are

suggested to have roles in regulating mRNA processing or the copy number of EBV episome in the maintenance of EBV latency (Petti *et al.*, 1988). The presence of EBNA 3C in Raji cells altered the expression of LMP1. EBNA 3C appears to either relieve the apparent repression of LMP1 in cells progressing through early G_1 or possibly alter the stage at which the cells growth arrest to one where they are permissive for LMP1 expression.

d) EBNA 5 or Leader Protein:

EBNA 5 is a 41-70kDa protein encoded by exons from Bam H1 WYH restriction fragments. Its size is dependent on the length of IR1 repeat numbers of each EBV isolates (Dillner *et al.*, 1986; Fields *et al.*, 1996). EBNA 5 protein is inadequate to restore the immortalizing function of P3HR-1 viral strain but may contribute to the B lymphocyte growth rate (Hammerschmidt and Sugden, 1989). EBNAs 1, 3A or 3C have EBNA 5 coding sequence at their 5' ends. These cDNAs either lack the necessary splice-generated ATG start codon for EBNA 5 or have an incomplete 5' ends, which preclude the translatability of EBNA 5 from corresponding messages.

Alternative splicing appears to be an important mechanism of ensuring translation of all genes in the transcriptional unit. EBNA 5 was reported to be

associated with the survival of Group 1 BL cells from apoptoxis *in vitro*. EBNA was suggested to be of a requirement for the transformation of B cells at a particular stage of differentiation and/or for the continued survival of infected cells against selective pressure *in vivo* (Sillins and Sculley 1995). A combination of EBNA LP and EBNA2 were shown to induce G_0 to G_1 transition of primaary B lymphocytes, co-stimulated with gp350 (Sinclair *et al.*, 1994)

1.8.2 Viral Capsid Antigen (VCA):

VCA is a lytic infection antigen appearing late in permissively infected cells (Pearson, 1980). The number of detectable positive cells are small after superinfection of non-producer lines such as Raji with P3HR-1 (Henle *et al.*, 1970; Pearson *et al.*, 1971). VCA expression is dependent upon viral DNA synthesis (Summers and Klein, 1976) where inhibition of DNA synthesis also inhibits the synthesis of VCA. A few major VCA protein have been detected, having molecular weights of 125K, 152K and 160K. The 125K glycoprotein was isolated from purified nucleocapsids and virus-producing cell lines.

This protein is encoded by Bam H1A; present in the nuclei and cytoplasm of virus producing cells (Pearson and Luka, 1986). The 160K protein on the other hand, is found primarily in nuclei of infected cells (Dolyniuk

et al., 1976). Vroman *et al.* (1985) reported from their immunofluorescence microscopy studies, the monoclonal antibody directed against this 160K protein labelled both viral particles and soluble proteins of the nucleus. VCA was noticed from observation of Henle and Henle (1966b) where a small percentage of EBV producer cultures e.g. P3HR-1 reacted against various human sera and were brilliantly stained using indirect immunofluorescence technique. Immunoelectron microscopy revealed only naked but not the enveloped virus particles reacted with anti-VCA antibodies, an indication of VCA being a structural component of the virus capsid (Silvertre *et al.*, 1971).

Recently VCA p18 has been shown to be highly immunogenic to humans. Immunodominant epitope clusters were also identified. These clusters were combined together in a synthetic protein which reacted positively with 95% of sera that were IIF positive in IgG-VCA (van Grunsven *et al.*, 1994).

1.8.3 BZLF1 Protein, ZEBRA:

ZEBRA is a 40kDa nuclear protein (Countryman *et al.*, 1987) encoded by the BZLF1 gene in the Bam H1Z fragment of EBV (Countryman and Miller, 1985; Chevallier-Greco *et al.*, 1986). BZLF1 gene is believed to be responsible for the switch of EBV latency to productive cycle. EBV latency can

be disrupted into producing lytic cycle antigens by chemical inducement i.e. using phorbol esters or 12-o-tetradecanoylphorbol-13-acetate (TPA) or anti-Igs (IgM) or superinfection of Raji cell lines with P3HR-1 cells (zur Hausen *et al.*, 1978; Lin *et al.*, 1979; Luka *et al.*, 1979; Flemington and Speck, 1990a). ZEBRA is expressed only in a small percentage of NPC cells (Cochet *et al.*, 1993). ZEBRA has been shown to downregulate the BCR2 promoter (Kenny *et al.*, 1989; Saint-Clair *et al.*, 1992) and upregulate the Fp promoter which is responsible for EBNA 1 expression in NPC tumors (Lear *et al.*, 1992). ZEBRA may be at least partially responsible in shutting off the expression of all EBNA proteins, except EBNA 1 (Cochet *et al.*, 1993). ZEBRA has been reported to induce abortive productive cycle in LCLs (Gradoville *et al.*, 1990).

Clinical studies showing the direct relationship between the disease onset and serological antibody titers suggested the association of EBV lytic cycle expression and tumor presence (Martel-Renoir *et al.*, 1995). BZLF1 gene encoding the ZEBRA antigen was demonstrated to be expressed in all NPC specimens studied (Cochet *et al.*, 1993; Martel-Renoir *et al.*, 1995). Other researchers (Lau *et al.*, 1992) however, found no evidence for Z- expression in oral hairy leukoplakia (OHL) biopsies or B95-8 cells induced into lytic cycle or Akata cells treated with anti immunoglobulin. Three BZLF1 promoter (Zp) regions were identified - two regions were responsive to either TPA or the

BZLF1-encoded protein itself (Flemington and Speck, 1990a; 1990b), and another region negatively regulated the BZLF1 expression (Montalvo *et al.*, 1991; Shimizu and Takada, 1993).

Zhang *et al.* (1994) showed that BZLF1 interacts with p53, a tumor suppressor protein where overexpression of wild-type p53 inhibits the ability of ZEBRA to disrupt viral latency. Likewise, BZLF1 protein inhibits p53-dependent transactivation in lymphoid cells. In general ZEBRA is not expressed in EBV-positive Burkitt's lymphoma *in vivo* or in latently infected, immortalized B-cell lines *in vitro* (Keiff and Leibowitz, 1990; Miller, 1990). However, up to 60% of EBV-positive lymphomas from immunocompromised patients (e.g. AIDS patients and organ transplant recipients) contained cells expressing BZLF1 protein (Pallesen *et al.*, 1990).

1.8.4 Early Antigen (EA):

Early antigens are synthesized early in abortive or permissive infection (Pearson, 1980). EA production can be induced in latently infected cell lines through superinfection with P3HR-1 virus or by the use of chemical inducers (Gerber, 1972; Hampar *et al.*, 1973; Bayliss and Nonoyama, 1978; Kallin *et al.*, 1979). Unlike VCA, no synthesis inhibition was seen when cultures were

treated with inhibitors such as phosphonoacetic acid (Nyormi *et al.*, 1976; Summers and Klein, 1976; Feighny *et al.*, 1981). EA has been divided into two distinct components, EA-R (restricted) and EA-D (diffused), based on their immunofluorescence staining patterns with different human sera (Henle *et al.*, 1971). EA-D showed a diffused staining of the nucleus and cytoplasm whereas EA-R stained only the cytoplasm. EA-D is expressed in both methanol- and acetone-fixed cells unlike EA-R which is methanol sensitive (Henle *et al.*, 1971).

Antibodies to EA complex are frequently elevated in patients with EBV-associated diseases but not in the latently infected populations (Pearson, 1980). Sera from patients with infectious mononucleosis and nasopharyngeal carcinoma react primarily with the diffused component of EA while those of African BL react mainly with the EA-R component (Henle *et al.*, 1971). Studies by Pearson *et al.* (1983) and Lin *et al.* (1985) utilizing monoclonal antibodies concluded that EA-R is an 85K protein and EA-D has a molecular weight between 50K to 55K. These proteins were identified to be EA-D and EA-R after testing their sensitivity/resistance to methanol, and also their intracellular locations. The 85K protein was methanol sensitive and restricted to the cytoplasm. In contrast, the 50-55K protein was resistant to methanol and largely intranuclear. The smaller protein appeared in the nuclei within hours of

largely intranuclear. The smaller protein appeared in the nuclei within hours of induction of permissive EBV infection whereas the 85K protein appeared later in the cytoplasm (Pearson *et al.*, 1983). These findings are consistent with those found by Henle *et al.* (1971). EA-D is encoded by Bam H1M in the regions of BMLF1 and BMRF1. Being nuclear, EA-D may play a role in an early stage of viral DNA or RNA synthesis (Pearson *et al.*, 1983a).

1.9 EBV SEROLOGY

EBV is believed to be an etiological factor of NPC. Presence of antibodies against EBV antigens was shown by immunoprecipitation and immunofluorescence techniques (Old *et al.*, 1966; Henle and Henle, 1970). Antibody (IgA) to VCA was frequently found to be elevated in NPC compared to those of other cancers including the head and neck cancers (Henle *et al.*, 1970). Detection of IgA antibodies to EBV antigens can serve as a useful diagnostic markers for NPC and occult tumors of the head and neck area (Henle and Henle, 1976; Ho *et al.*, 1976; Pearson *et al.*, 1983b; Foong *et al.*, 1990; Cheng *et al.*, 1993).

Serology has been used to screen and identify individuals of high NPC risks in areas of high incidence rates (Zeng *et al.*, 1983a;b; Zeng, 1985). NPC

patients usually present with high titers against EA-D component (Henle *et al.*, 1971; de Schryver *et al.*, 1974; de-The *et al.*, 1974).

Antibodies to EA and VCA had been documented to correspond to tumor burden; the geometric mean titer (GMT) increased as the tumor stage progressed (de-The *et al.*, 1974). Henle *et al.* (1977) reported the fluctuations of antibody titers in NPC patients during recurrence, presence of metastasis and in remission after radiotherapy, corresponding to their clinical status.

Patients who had undergone radiotherapy treatment would have a decreased level of IgA against VCA titers (Henle *et al.*, 1973). A study in our laboratory on the fluctuations of IgA serological markers in Malaysia found that although the majority of IgA-VCA titers decreased after radiotherapy, the titers fluctuated without correlation to their clinical status. Local recurrence and presence of metastatic lesions were not indicated by increased IgA-VCA titers (Sam *et al.*, 1994).

Studies on North American NPC patients demonstrated 84-90% of the subjects having a moderate- to highly elevated titers of IgA-VCA (Pearson *et al.*, 1983). Serological studies on NPC patients in China revealed that 80-90%

of sera from individuals with less differentiated NPC were IgA positive to EBV VCA antigen, in contrast with 5-15% of control populations.

Antibodies against EBNA were detected in more than 90% of NPC patients (Foong *et al.*, 1990; Cheng *et al.*, 1993). 7-14% of normal healthy donors reacted positively to EBNA and 11-14% of sera from other malignancies or cancers showed an elevated titers to this antigen. Antibodies against ZEBRA was also detected in 75-87% of NPC patients (Joab *et al.*, 1991; Mathew *et al.*, 1994) with only 2-4% of healthy seropositive donors demonstrated the presence of elevated ZEBRA antibodies (Joab *et al.*, 1991; Mathew *et al.*, 1994).

Researchers working with Chinese patients in Southern China suggested the importance of performing IgA-VCA tests for the purpose of early detection of NPC. Data collected indicated the presence of antibodies against VCA 3-5 years prior to NPC diagnosis (Ho *et al.*, 1976; 1978; Zeng, 1985). Other antigens such as LMP, EA-R, EBV thymidine kinase and membrane antigen may also be used to complement the standard IgA-VCA test (Rowe *et al.*, 1988; Littler *et al.*, 1990; Gan *et al.*, 1996). LMP was reported to detect 67% of NPC cases (Rowe *et al.*, 1988). NPC patients may have

antibodies to EA-R but its presence was obscured due to the abundance of EA-D.

Serological tests using various EBV antigens may be utilized to complement each other in hope for earliest detection hence better prognosis of NPC.

Disease specificity of antibody response can vary for different epitopes even within the same protein (Cheng *et al.*, 1991; Tedeschi *et al.*, 1995). In order to identify the epitopes, a relatively simple, flexible and inexpensive technique of producing short peptides and peptide analogs i.e. the mimotope synthesis technique is utilized.

1.10 IMMUNOGENIC EPITOPE ANALYSIS BY PEPTIDE SYNTHESIS

Investigations of the sites and nature of epitopes of protein antigens proceeded slowly until the solid-phase methods of peptide synthesis was introduced (Merrifield, 1963). New synthetic peptides are being extensively used in fields of immunology, hormone-receptor interactions and vaccine development research (Leinikki *et al.*, 1993). Generally, peptide homologs or

analogs are synthesized and their relevant biological activities evaluated (Houghten *et al.*, 1985; 1986; Geysen *et al.*, 1987a).

Synthetic peptides can mimic intact proteins. Epitope mapping technique allows the synthesis of overlapping amino acid sequences that may be easily tested. This relatively simple technique therefore allows the study of proteins at their amino acid level and this in turn allows the identification of epitope(s) or epitope clusters. Epitope in an operationally satisfactory definition can be described as an entity able to react with a paratope in a specific way irrespective of its relationship to the original protein. Identical amino acid sequences do not always correspond with identical antigenic characteristics of the synthetic peptides and the intact proteins. Mimotopes or mimic of the epitopes may well be constructed using different amino acids by testing all possible amino acid combinations in order to eventually find a structure that reacts best with the antibody (Geysen *et al.*, 1987a,b; 1988).

It was initially believed that two necessary conditions must exist for a synthetic peptide to be usable:

1) the antigenic determinants on the particular protein identified (Atassi 1975; 1980; 1984; Atassi and Lee, 1978)

2) presence of similar conformational structure of both the native protein and the peptides (Arnon *et al.*, 1971).

Later studies have shown that prior knowledge of the determinants was not essential and the lack of similar conformation may not necessarily be a limiting factor (Walter *et al.*, 1980; Audibet *et al.*, 1981).

Cross reactivity between proteins and their linear peptides have allowed synthetic peptides to become standard biological tools in researching immune response (van Regenmortel, 1987). An example is synthetic peptides derived from a fragment of EBNA1, the p107, which is a copolymer of alanine and glycine. Antibodies to the fragment was detected by anti-complement immunofluorescence (ACIF) (Dillner *et al.*, 1984) indicating the peptide as antigenic determinant of the native EBNA protein. Due to limitations on the number of peptides which can be readily synthesized and tested, the choice of peptides has often been made on the basis of predictive algorithms which were published in scientific literature (Hopp and Woods, 1981; Fraga 1982; Westhof *et al.*, 1984; Novotny *et al.*, 1986; Thornton *et al.*, 1986). Other limitations include costs, facilities requirement and peptide presentations as affected by coupling procedures during synthesis (Geysen *et al.*, 1987a).

Antigenic determinants or epitopes are classified into two categories (Benjamin *et al.*, 1984; Geysen *et al.*, 1987a)

1) sequential or continuous epitopes consisting of linear sequence of amino acids homologous with the inducing antigens

2) assembled epitopes whereby tertiary structure/protein folding/conformational determinant is essential for antibody binding

Identification of continuous epitopes can be accomplished by making complete set of all possible overlapping synthetic peptides of a given length, homologous to the sequence of antigens of interest. The relationship between antibody-binding peptides and continuous epitopes can then be established by various methods such as competitive inhibition of binding and use of monospecific antibody preparations. Presently, the length of sequential epitopes is documented to be from 5-8 residues (Kabat, 1970; Schechter, 1971). The extent of specificity in binding of each amino acid residue in the identified epitopes can be assessed by replacing the residue with another e.g. alanine with glycine or serine (Geysen *et al.*, 1987a; 1988; Tedeschi *et al.*, 1995).

A newly developed method for epitope scanning is the synthesis of mimotopes which induced the antibody to bind (Geysen 1985; Geysen *et al.*, 1986; Geysen *et al.*, 1987a,b). Mimotope is based on the believe that the only essential criterion for effective binding of antibody to a peptide is that complementarity between the antigen-combining site of the antibody and the molecular surface of the binding peptide is maintained in regard to both shape and charge (Geysen *et al.*, 1987a).

Synthetic peptides made using the mimotope technique have been used in research involving the mapping of antigenic epitopes of several important virus such as the human immunodeficiency virus (HIV), human T-cell leukemia virus types 1 and II, picornaviruses and Epstein-Barr virus.

Gnann *et al.* (1987) had located the immunodominant region of HIV utilising overlapping sequences of immunodominant regions from HIV transmembrane glycoprotein gp41. Horal *et al.* (1991) defined five epitopes within the identified areas of immunodominant regions of HIV type-1 virus by using a 6-amino-acid overlapping peptides. Studies on poliovirus type 3/sabin capsid protein were done to identify immunogenic portions of protein antigens, using 14-mer peptides (Roivainen *et al.*, 1991). Synthetic peptides have been used in a simple hemagglutination test in HIV-1 detection. It is a rapid and

easy to use test, needing only fingertip blood sample and the results can be read in less than a minute (Kemp *et al.*, 1987).

Two other studies that were published with methods of pin-coupled peptides included the investigations of continuous epitopes of allergens derived from *Dermatophagoides pteronyssinus* (*Der p* 1) and *Dermatophagoides farinae* (*Der f* 1), and the epitope mapping of *Mycobacterium bovis*. Investigation on *Der p* 1 and *Der f* 1 concluded that the specific IgE binding epitopes to be discontinuous (Collins *et al.*, 1996). Researchers dealing with *Mycobacterium bovis* identified an epitope where strong reactivity was seen between the amino acids 32 and 39 of MPB70 antigen. This research can possibly set a basis for serological testing of bovine tuberculosis (Radford *et al.*, 1990).

Identification of immunodominant epitotope (s) of EBV using the mimotope procedures may result in a more reliable and easy to use test kits.

1.11 ANTIGENIC EPITOPES AND PROTEIN SECONDARY STRUCTURE PREDICTIONS

B cells detect conformationally restrained antigenic sites while T cells detect proteolytically processed sites. Two rather prominent views of B cell epitopes are that the epitopes are discrete and are located in highly accessible surface locations while the rest of the protein molecule devoid of antigenicity. The entire accessible surface of a protein is a continuum of overlapping epitopes that are potentially able to combine with appropppriate paratopes. It is generally accepted that regions of high hydrophilicity are regions that are antigenic since it is exposed on the surface of proteins therefore accessible for antigen-antibody binding (Doran, 1990).

A lot of methods have been devised in view of predicting the antigenic sites of proteins such as those of Chou and Fasman (1974), Levitt (1976), Maxfield and Scheraga (1976), Rose and Roy (1980), Hopp and Woods (1981), Kyte and Doolittle (1982) and Novotny *et al.* (1986).

Hopp and Woods (1981; 1983) formulated methods of hydrophilicity calculations based upon running average of 6 amino acid residues. The hydrophilicity values for each of the 20 amino acids were derived from solvent

parameters. Values of acidic residues and that of proline were subjectively adjusted to make them more antigenic. Regions with high index values will be considered B-cell epitopes. Kyte and Doolittle (1982) used hydropathicity coefficients derived from both water vapor transfer free energies and the interior versus exterior distribution of amino acid side chains. Arbitrary value adjustments were also done. Calculations using 9 residue windows reported to be the best for distinguishing the interior from exterior residues. Methods of Novotny *et al.* (1986) requires x-ray structure of proteins to determine the most protruding surface areas. A 1nm radius probe is rolled over the protein surface to identify the most protruding atoms. Antibodies are known to contact up to 6 amino acid residues surrounding the most protruding points, therefore locating the antigenic epitopes.

One of the most well known method for secondary structure predictions is that produced by Chou and Fasman (1974). The method claims to be able to predict known amino acid sequences of their helix, β and coil regions with 80% accuracy. The method is based on statistical analysis of a database of crystallographic protein structures. The database allows the calculations of coefficients of each amino acid in each of their three states i.e. helix, β sheet and random coil and the secondary structure is then predicted. β turns are

often found on surface of proteins hence more likely to be candidate of B cell epitopes, whereas a significant portion of most helices and sheets are inaccessible or buried.

1.12 OBJECTIVE OF STUDY

The objectives of this study are:

1) to evaluate the sensitivity and specificity of 3 recombinant EBV proteins namely p54, p138 and p23 using Enzyme-linked Immunosorbent Assay (ELISA) technique in the detection of NPC

2) to determine the immunodominant epitope(s) of the most sensitive and specific antigens evaluated above, by synthesizing 10-residue with 5-amino-acid overlapping peptides using mimotope strategy. The reactivities of these non-cleavable, pin-bound peptides will be evaluated in ELISA system

3) to compare the correlation of antigenic epitopes as predicted by hydropathy profiling utilizing the methods described by Hopp and Woods (1981; 1983) with the epitopes identified through PEPSCAN analysis