CHAPTER 8

General Discussion
EBV infection is ubiquitous in mankind. The great majority of adult humans are lifelong virus carriers from childhood age onwards (72). EBV has various biological behaviours. EBV initially infects submucosal resting naive B lymphocytes and drives the activation and proliferation of these cells (“growth program”). On the other hand, the virus can reside quietly in circulating resting memory B cells (latency program), and occasionally, the infected memory B cell can be activated to Ig-secreting plasma cells and then becomes a virus producer cell (lytic program). These behaviours correlate with the location of the infected cell. Lytic virus production is active in the nasopharyngeal lymphoid system, including the tonsils, whereas the virus is dormant in the peripheral circulation (44, 123, 138). When B cells with dormant EBV pass through lymph nodes, the virus can be partially activated to promote host cell growth and survival (default program). In addition, EBV can infect epithelial cells in latent and productive cycle (44, 108). EBV in latently infected B cells can turn-off all latent gene expression (4), and exit the tonsil into the circulation as resting memory cells, where the virus can persist for life. Since the growth-promoting latent genes are not expressed (11, 113, 140), the cells are maintained in the periphery by normal memory B cell homeostasis mechanisms. Moreover these cells are non-pathogenic and the virus persists in a benign state (123). Furthermore, due to the lack of gene expression, (11, 113, 140), the virus inside circulating memory B-cells is also hidden from the immune response and cannot be eliminated. EBV persistence is an example of a dynamic equilibrium between the immune response and the various states of infection. The numbers of virus-infected cells are increased by new infection and by expansion of cells expressing the growth and latent (default) programmes. This is counterbalanced by viral-neutralizing antibodies and infected-cell death induced by cytotoxic T-cells (CTLs) directed against cells expressing latent or lytic proteins (55, 138).

The different biological activities (latency, default, growth program) are also seen in various EBV-associated malignancies. These can be of lymphoid or epithelial origin, reflecting the dual cell tropism of EBV. EBV is officially recognized as a human class I carcinogenic agent, with a proven role in Burkitt’s lymphoma, Hodgkin disease, extranodal T/NK cell lymphoma, immunoblastic B-cell lymphomas in immunocompromised individuals, gastric carcinoma and nasopharyngeal carcinoma (NPC). Nasopharyngeal carcinoma (NPC), one of the most prevalent malignancies in certain regions, is an EBV-linked epithelial tumor of the nasopharynx. It is a rare tumor in most parts of the world, but common in certain geographic areas such as China, South East Asia and Northern Africa. The etiology of NPC is multi-factorial, including genetic susceptibility, exposure to carcinogens, and prior infection EBV. The virus is considered to give growth advantage and apoptosis resistance to premalignant or (epi)genetically modified cells in the nasopharynx (107). Molecular analysis identified that undifferentiated NPC biopsies are 100% positive for EBV by immunohistochemical staining for EBNA1, LMP1, LMP2, EBER and BamHI A RNAs within all tumour cells. EBV genome is clonal in NPC tumor cells, suggesting early onset involvement in the carcinogenic process. The association is confirmed serologically by the elevated antibody titer to capsid antigen (VCA) and a group of antigen (EA) synthesized early in the viral replication process (114).

Host immune responses are of central importance both in limiting primary infection and in controlling lifelong virus carrier state. Under normal circumstances in healthy individuals, EBV infections are not life-threatening and are generally effectively controlled by the immune system through the action of antigen-specific T lymphocytes (55, 120). EBV infection in human triggers both humoral and cellular immunity (75). Immune control in viral infection is predominantly mediated by CD8+ CTLs (120), essentially supported by helper and regulatory T-cells (55). T cell responses to several peptide epitopes of immediate early, early and late EBV antigens have been detected (53-55, 112, 129), but due to difficulty of isolating cells in lytic cycle, these effectors have never been demonstrated to specifically eliminate productively infected EBV target cells. To date, there is no report on a protective role of antibody specific to lytic antigens EA and VCA in eliminating EBV cells, except neutralizing antibodies to gp350/220 and gp155 which may also mediate killing of lytically infected cells via complement or killer cells and protecting the virus-carrying host from subsequent infection with a new exogenously transmitted virus strain (139, 146).

Control of virus replication and proliferation of transformed EBV-infected B cells by virus specific-CTL is crucial for the host-virus relationship (immunosurveillance). Virus production in the lytic cycle would benefit from effective evasion of CTL responses (2, 47, 109, 117). As part of their normal lifestyle, herpesviruses use general immune evasion approaches, such as blocking the induction of programmed cell death and shutting down host protein synthesis (121). In addition, herpesviruses specifically perturb recognition by virus specific T cells via several mechanisms to thwart the MHC class I and II processing and presentation pathway. Down regulation of class surface MHC class I is a common strategy to escape from class I-restricted immunity (22, 117, 118). With regards to EBV, the entry into lytic cycle is paralleled by reduced expressed of surface HLA class I and II up to 5-fold. The BNLF2a protein inhibit TAP function, thus preventing peptide-MHC association (22). The viral G-protein-coupled receptor (GPCR) encoded by BILF1 was shown to associate with MHC I in plasma membrane, leading to its internalization and degradation (153). An early lytic gene, BZLF1, inhibits upregulation of surface MHC class I expression (66). LMP1, one of latent type proteins, also expressed in the lytic cycle, is known to induce expression of MHC class II molecules, and numerous surface proteins in B cells relevant for cell-cell contact (124, 144, 152). However, the viral pp65 directly binds to MHC II, preventing proper maturation and blocking TCR recognition. The BGLF5, viral DNAse serves as host shut-off protein, sufficient for reducing of all these cellular MHC proteins (22, 119).

The detection of antibodies directed against viral structural proteins and the EBV nuclear antigens is important for the diagnosis of EBV infection (75). However, the simple detection of anti-EBV antibodies is not sufficient to discriminate between healthy EBV carriers and patients with EBV associated diseases. Analysis of the dynamics and diversity of anti-EBV responses are more informative for diagnosis, since this better reflects the status of the virus-host interplay (142).
Previously, measuring humoral immune responses to EBV was done by using indirect immunofluorescence assay (IFA) tests for antibody titers to four serologically defined antigenic complexes of the virus life cycle, such as VCA, MA including the viral envelope gp350/220 and gp85, EA and EBV nuclear antigen (EBNA). Primary and persistent phases of infection are associated with different combination of antibody reactivities. Primary infection is characterized by detectable IgM and IgG antibodies to VCA and EA, a detectable IgM but relatively weak IgG response to membrane antigen (MA), and the absence IgG reactivity to EBNA1. The long term virus carrier stage is characterized by stable IgG reactivities to VCA, MA, and EBNA, and weak or undetectable IgG anti-EA (48, 50, 51).

NPC is an aggressive tumor, with a high predisposition to metastasis, following 85% of patients rapidly die of the disease and virtually all succumb within 3 years (137). In Indonesia, close to 80% of the patients are diagnosed at late stage (II & IV) [Tobing, unpublished] with poor prognosis which requires combined chemo-radiotherapy, whereas early stage NPC may reach complete remission by radiotherapy only (83). Therefore, better tumor markers are needed, since the improvement of treatment and patients survival depends, at least in part, on rapid diagnosis and early detection of relapse (34). Early studies showed that NPC patients frequently have elevated serum antibodies against two lytic cycle antigens, namely viral capsid antigen (VCA) and early antigen (EA) (49, 56). Elevated IgG antibodies to EBV antigens have been used in NPC diagnosis (65), but this response is also found in other EBV-associated diseases such as EBV-related lymphoma as well as normal healthy carriers (26, 65). EBV-IgA is an outstanding marker for NPC rather than -IgG (49, 56), reflecting the tumor’s origin in the nasopharyngeal mucosal surface. Therefore, IgA-based ELISA is more suitable for NPC serodiagnosis. Recent studies have further elucidated the role of EBV in the pathogenesis of NPC and have demonstrated the utility of EBV markers in diagnosis, prognosis, and post-therapeutic monitoring (136).

In this chapter, EBV-based diagnostic aspects of NPC will be discussed first. Subsequently we address immunological aspects of EBV tumor associated antigens in NPC, and discuss options for inducing therapeutic antibodies in NPC patients targeting tumor-associated antigens expressed on the tumor cells.

1. EBV-based diagnostic aspects of NPC

From the first identification in 1960s (73, 74), there has been significant interest in using antibody responses to various EBV proteins in the diagnosis and prognosis of NPC. Quantitative analyses of EBV antibodies and EBV DNA have been shown to be clinically useful (17, 103). In NPC diagnosis, indirect IFA for measuring EBV-specific antibody has proven very useful, but is also labor-intensive and subjective (151). In recent years, based on progressing insights in the molecular basis of anti-EBV antibody responses, more economical and objective tests have been introduced using recombinant EBV proteins and enzyme-linked immunosorbent assay (ELISA) technology, multiplexed microparticle based-immunoassay (MMIA) and related methods (37, 151). Most recently, rapid filter test have been introduced (EBV-TRU and Immunoquick) (61). IFA does not provide insight into the molecular basis of anti-EBV responses, because EBV-infected cells each contain multiple different EBV proteins that can serve as the target for antibody (96, 97, 143).

In order to find detailed information about predominant markers for NPC, we analyzed the molecular basis of IgG and IgA antibody responses in patients with NPC from different ethnic origin such as Javanese (Indonesia), Chinese (Hongkong), and white (Europe), compared with non-NPC control subjects and healthy EBV carriers as described in chapter 2. Regardless of the ethnic background of patients, immunoblot analysis on extract of HH514.c16 cells expressing lytic EBV antigens revealed the underlying complexity of anti-EBV responses in NPC patients. Anti-EBV IgG diversity shows a positive correlation with stage of malignancy, while IgA reactivity is more variable among patients. Our results indicate that IgG and IgA-producing B cells are triggered differently, and react to different EBV proteins and epitopes. This is probably related to the location of EBV reactivation, paralleling NPC development. In normal EBV carriers, EBV-IgG reactivity is characterized by dominant responses to EBNA1 (BRRF1; 72 kD) and VCA-p18 (BFRF3; 18 kD) with occasional weak responses to VCA-p40 (BdRF1; 40–45 kD) and Zebra (BZLF1; 36–38 kD). In higher stages of malignancy, additional IgG responses to EBV proteins, including EA polypeptides p138 (BALF2), TK (BZLF1), DNAse (BGLF5), EAd-p47/54 (BMRF1) and Zebra (BZLF1) were detected. The antibody diversity pattern in NPC differs from healthy EBV carriers and other EBV associated diseases allowing this diversity profiles to be used reliably as marker in NPC diagnosis.

Regarding the clinical use of different EBV lytic markers, IgA-EA is the best marker for diagnosis of NPC, by showing the highest specificity compared to VCA (141). Chapter 3 of this thesis confirms that EAd proteins, particularly EAd-p47 and -p138 together with EBNA1 are best discriminator for NPC compared to other proteins such as VCA-p18 and -p23. The results confirm previous findings that the combination of IgG and IgA to EA and EBNA1 can be used to discriminate NPC from healthy carriers and other EBV-related diseases (18, 60).

Most NPC patients have significantly higher antibody titers to EBV lytic proteins, such as viral capsid antigen (VCA), ZEBRA, DNAse, DNA polymerase, and thymidine kinase (TK) (3, 16, 33, 64, 85, 86). Some of these were considered to be good indicators for prognosis of NPC because their levels fell after clinical treatment and rose again upon recurrence (16, 27, 87). IgG ZEBRA has been proposed to be a prognostic marker for NPC patients after radiotherapy. Patients with elevated IgG ZEBRA are predicted to develop distant metastasis or cranial extension (25, 148). IgG ZEBRA is also a more sensitive marker for NPC diagnosis in children (24). The combination of IgG ZEBRA with IgG EAd-p47+p138 improved the detection of NPC to 95% in overall of NPC population (23). However, these ZEBRA findings could not be confirmed in our population. Neutralizing IgA antibodies against DNAse is highly specific marker for NPC (12, 13), and it was shown to be a valuable marker using recombinant antibody and anti-DNase have high risk to develop NPC in the future (12, 14). None of these individual markers proved very useful in our hands, possibly related to the differences in expression system and purity. Most recently, in parallel with this
Chapter 8

In our previous study, a further development was made by the construction of synthetic peptide-based ELISA test combining VCA- and EBNA1-IgA detection, with promising diagnostic performance (30, 31). Considering the diagnostic relevance of EA-specific antibody responses in NPC, we developed a new way to isolate native EA protein from nuclei of induced HH514 c16 cells by using a low salt extraction (Chapter 4). Native EA protein extracted in a low salt yield an EA protein complex consisting of EAd-p138, TK, DNase, EAd-p47/54 and ZEBRA. This native EA extract served as an excellent antigen for NPC diagnosis compared to individual recombinant and synthetic EA peptides, by showing sensitivity and specificity of 90.6% and 95.5% for IgA and 85.7% and 94% for IgA, respectively. These findings reflect most likely the importance of native epitopes or post-translational modifications for interaction with human antibodies, especially in NPC sera. Furthermore, it indicates that a single EA marker might not be adequate to comprise the diverse anti-EBV antibody responses for NPC diagnosis (33, 37). Therefore multiple EA proteins may be required (15, 24, 33, 88). The use of multiple tests in combination is preferable, since they provide better sensitivity and specificity (10, 18, 23, 31, 32, 60).

In order to promote improved treatment outcome and patients’ survival, screening of at risk population for having early stage NPC is important. The IgA-EA ELISA system developed in chapter 4 is a promising method for NPC screening in Indonesia. Besides NPC, IgA-EA is found significantly in IM patients (41.1%), but low in other EBV related malignancies. For that reason, initial exclusion of IM is proposed to be done prior the screening program to minimize the recruitment of IM cases using this method. However, IM is hardly found in Indonesia. Therefore IgA antibody detection using native EA antigen provides a highly sensitive and specific for screening early stage NPC in population with low incidence of IM, such as Indonesia. A disadvantage is that production of the native EA extract still depends on culture cell, which is expensive and complicated. For that reason, the IgA-EA ELISA is proposed only as confirmation test in a screening program, using a cheap peptide EBNA1-VCA-p18 IgA ELISA as first step.

Fachiroh et al [2006], developed an ELISA test was detecting IgA against two synthetic peptides derived from immunodominant epitopes of EBNA1 and VCA-p18 (IgA [EBNA1-VCA-p18]), which showed good sensitivity and specificity of 85.4% and 90.1%. This test may be suited as first step in an affordable (cheap) mass-screening program. Therefore, we proposed to apply the peptide based IgA-ELISA as first line screening test and IgA(native) EA extract as confirmation test. In Chapter 5 we show that by using this two-step ELISA approach the sensitivity & specificity increases from 85.4% to 96.7% and 90.1% to 98%, respectively. Previous studies (149, 150) have clearly demonstrated the value of EBV-IgA serology in NPC screening. This was recently further highlighted by the demonstration that increased IgA-VCA levels precede NPC presentation by an average of 2 years (63). These studies still used IFA-based methods, which should be replaced by more standardized and objective methods such as ELISA. The two-step ELISA approach developed in this thesis is currently under evaluation in prospective studies in several “high risk goups” in the Yogyakarta region [Hutajulu, in prep].

Chapter 5

Based on good expertise in the (post-transplant) EBV+ lymphoma field (130, 131), other biological markers have also been suggested in NPC diagnosis/screening, such as EBV-DNA detection in serum or plasma (59, 102, 111, 126, 127). Recent developments in molecular technology and instruments will provide new (affordable) tools for detecting tumor-derived nucleic acids in the blood. EBV DNA serum or plasma has been hypothesized to be due to the leaking of DNA from dead cells (102). This was proven by demonstrating that circulating EBV DNA in NPC patients (but also in HD patients) largely consist of small fragmented DNA of about 150 bp in length derived from apoptotic cells (9). Circulating EBV DNA in plasma and serum may aid in the diagnosis of NPC (91, 102), prognosisstication (78, 82, 89), monitoring the progress of NPC patients (59, 90). Furthermore, it might serve as a screening tool for individuals at high risk for developing NPC (147). Serum EBV DNA represents released tumor DNA, not originating from other latently infected cells such as epithelium or B lymphocytes, because active NPC specific BARF1 RNA expression could not be detected in the circulation (Chapter 6). Circulating EBV DNA is potentially useful for confirmation of sero-diagnostic risk stratification in mass screenings. However, large variations in peripheral blood EBV DNA positivity in NPC patients is reported by using different DNA target size in PCR (Chapter 6). The percentage of NPC patients positive for EBV DNA in peripheral blood varies from 30-98%, most having very low EBV DNA loads (10, 57, 59, 102, 127). In this thesis, initially a 213-bp region of EBNA1 was targeted by PCR for EBV DNA quantification in blood of NPC patients, 72.5% positivity and only 29.5% had EBV DNA above CoV level. In addition, a 99-bp PCR was developed showing 85.9% positivity, with 60.4% having EBV DNA loads above CoV. In contrast to the initial study of Lo et al, we conclude that circulating EBV DNA load quantitation is not sensitive enough for NPC diagnosis, confirming numerous other studies from regions where NPC is endemic (79, 102, 111, 127). Therefore, we find that EBV DNA load measurement may have limited value for the primary diagnosis of NPC in population screening in high incidence area (chapter 6).

However, the disappearance of EBV DNA after radiotherapy in NPC patients and its reappearance during recurrence may reflect the tumor burden in selected patients. A transient increasing EBV DNA concentration occurs in patients after receiving radiotherapy, within 3 days of the start of radiotherapy and reaches the peak at 2 weeks after the start of radiotherapy. Following this initial surge in circulating EBV DNA concentration, the decrease in EBV DNA concentration occurred. The median half-life of EBV DNA decay was determined to be 3.8 days (92). These data are confirmed in the recent study from Jakarta, Indonesia [Adham and Middelhoop, pers. comm]. Therefore, monitoring EBV DNA concentration in blood over a time during treatment may enable direct monitoring the in vivo effect of treatment (7, 8, 57).
Chapter 8

2. Antibody against EBV-encoded tumor-associated antigens in NPC

Studies on EBV-specific antibody responses in different EBV-related disorders have concentrated mainly on analyzing antibody reactivity to components of the EBNA, EA, and VCA complexes, reflecting local (mucosal) lytic virus replication which in some (direct or indirect) way is associated with the oncogenic process. However in most EBV-related tumors that arise in immunocompetent individuals, such as BL, HD, T-NHL, and NPC, only latent gene products are expressed in the tumor cells.

NPC tumor cells consistently express EBNA1, LMP1 and LMP2, and BARF1 with CD4 and CD8 T cell memory production to these antigens at levels equivalent to those seen in healthy carriers (84). These "non-self" protein expressed in the tumor are now becoming prime targets for immunologic intervention (70).

HLA class I-restricted CTLs play an important role in controlling EBV infection, and also the pathogenesis of EBV-associated tumors including NPC. Adoptive transfer of EBV-specific CTL has been used for treatment of EBV-positive tumors, such as PTLDs (69, 106, 122). The effector cells in these treatments predominantly consist of CTLs targeting the dominant EBNA3 family of latent proteins. NPC tumor cells only express LMP1, LMP2 and EBNA1. Although LMP1 and LMP2 are subdominant antigens, they provide targets for immunotherapeutic approaches (134). However, immunotherapy targeting EBV in NPC might not work as well as in PTLDs (136). Several investigations using EBV-specific CTLs indicate that immune intervention in NPC is feasible. The first trial in NPC patients with advanced diseases showed that infusion with autologous EBV CTLs safe and led to increase the number of CTL and reduced plasma EBV DNA levels (19). A trial by Comoli (20, 21) using LMP2 specific CTLs also observing the LMP2-specific immune response in NPC patients. Another trial by Straathoff (133) indicated that treatment using autologous CTLs showed remarkable anti-tumor effect. However, there are some obstacles in the development of CTL based strategy: T cell responses to viral proteins are restricted through HLA alleles, which hamper a generic approach in the patient population. Furthermore, only weak CTL responses to tumour-associated EBV antigens can be detected in the blood of a minority of NPC patients, but remain undetectable at the tumour site (81). Moreover, there is the possibility of CTL resistance and immune evasion by the tumour cells, and there is no guarantee whether the effector T cells will home to the tumour site. A clear understanding of important molecular mechanisms in this process is limited (135).

LMP1 is expressed in most NPC cases (68). Therefore, LMP1 has become a major target for vaccination against EBV-induced malignancies. Study by Duraiswamy (29) propose an LMP1 epitope-based vaccination strategy to enhance EBV-specific CD8+ CTLs as a preferred approach for the treatment of EBV-associated relapse HD and NPC. However, LMP1 protects against apoptosis by increasing bcl-2 activity (46), thus increasing resistance to CTL therapy and LMP1 may have multiple additional roles in tumor immune evasion (98). Therefore, LMP1 expressing tumors may resist CTL attack. Therefore, more knowledge is needed to provide a rationale for using anti-LMP1 T-cell responses as a therapy for NPC.

NPC is characterized by a large cellular infiltrate comprised mainly of CD4+ and CD8+ T lymphocytes that is intimately associated with the tumour cells. These contains few, if any EBV-specific reactivities (77), but may be include significant number of T regulatory cells (76), indicating immune evasion to occur. The effort to target NPC should involve means to overcome the potentially suppressive effects of the local tumour environment and deliver virus-specific T cells to the tumor site. In addition, a study from our population show that close to 50% (38/72) patients with more than 25% of granzyme B positive TILs did not reach complete remission. The poor prognosis in cases with many granzyme B-positive TILs can be explained by assuming that under the pressure of a strong CTL-mediated immune response, selection for apoptosis-resistant tumour cells occurs, resulting in resistance to both CTL and radio-therapy-induced apoptosis (105). More detailed study of these cases indicated that apoptosis resistance may indeed be a fundamental mechanisms underlying CTL resistance (104). Despite this, many studies on cell-mediated immunity are ongoing using either vaccination or CTL infusion treatment, although the efficacy is still in debate.

However, humoral immune responses to EBV tumour associated protein, when existing, may provide a protective mechanisms against tumor lysis effectuated via complement or Fc-R bearing natural killer cells. This has not been studied in detail before. Therefore, chapter 7 of this thesis explores the potential presence and relevance in NPC patients of antibodies targeting tumor-associated antigen expressed in or on the NPC tumor cells. In previous chapters it was shown that NPC patients have high IgG and IgA antibody responses to a range of EA and VCA antibodies, plus EBNA1. In contrast, analysis of natural humoral immune responses (IgG and IgA) to individual EBV encoded tumor associated proteins (i.e. EBNA1, LMP1, LMP2A and BARF1) revealed the presence of very low or mostly undetectable natural antibody responses to these proteins. In particular, the potentially extracellular "bop" domains of LMP1 and LMP2 seem rather non-immunogenic, as well as secreted BARF1 protein. However, immunization of rabbit using synthetic peptides of selected epitopes on "putative extracellular domains" of those proteins result in the generation of specific antibodies, capable binding to the surface of viable latent EBV infected cells. Furthermore, we show that antibodies against these putative LMP1 and LMP2 extracellular domains can mediate complement-driven cytolysis of LMP1 and LMP2 expressing cell lines. This opens possibilities for inducing therapeutic antibodies in NPC patients targeting tumor associated antigen expressed on the NPC tumor cells, either vaccination or infusion of specific antibodies. This hypothesis recently proven in a mouse model (28).

Antiviral drugs have not had a great impact on the treatment of EBV related diseases (38). Therefore, a therapeutic EBV vaccine is worth considering (99). A therapeutic vaccine would be much less expensive and more practical than adoptive transfer of EBV-reactive T-cells (6). A peptide-based vaccine has recently been designed to induce specific cellular responses (71). To overcome the problem
of HLA specificity of viral CTL responses, the extensive allelic variation of human HLA genes, and variation in epitopes between EBV strains, a "polytype" vaccine containing multiple epitopes to which over 94% of the population should respond has been formulated (29, 94). Again, this vaccine is not designed to induce "sterile immunity" but in a hope that it will be effective in the prevention of disease states. The circular synthetic peptides developed in chapter 7 may provide a new means for inducing anti tumor immune responses, which needs to be explored in future studies. A recent study using so-called "long peptide" of HPV E6 and E7 have provided a strong supportive evidence for the ability of inducing effective immune responses against weakly immunogenic intracellular proteins (67). This is the first proof that therapeutic vaccination with peptides is feasible for treatment of cancer.

Another option for EBV vaccination is the induction of antibody responses. So far, the investigations aimed to explore the use of gp150 vaccination for preventing primary infection in young children (42) and infectious mononucleosis (101, 128), and recently to children with end stage renal failure awaiting transplantation (116). The data supports the clinical feasibility of using EBV vaccine to prevent acute primary infection and infectious mononucleosis in teenagers and young adults. This vaccine induces specific antibody responses and reduced the proportion of symptomatic primary EBV infection in teenagers and young adults (101, 128). Additionally, the vaccine is able to induce the production of a neutralizing antibody response, which inhibit the EBV transformation of B lymphocyte (62, 100, 101).

Neutralizing antibody provides a humoral defense against cell entry of EBV. Antibodies may penetrate mucosa and cell compartments, not hindered by (local) immunosuppressive influences. This is an attractive feature for a preventive vaccine. A preventive vaccine in PTLD has also been explored. EBV-naive candidates for haematopoietic stem cell or solid organ transplantation who are at the high risk developing PTLD were identified and immunized before transplantation. These individuals could be monitored after transplantation, not only for the development of PTLD but also to evaluate the effect of vaccination on the quantity of EBV viral load (116). Unfortunately, the vaccine formulations used so far do not provide long lasting immunity nor from exogenous EBV infection. Endemic BL and NPC are also worthy targets for preventive trials, because of the seriousness of these malignancies (115). Therefore, EBV-specific vaccination for inducing antibody response against EBV tumor associated protein should be considered. The recent initiation of prophylactic HPV vaccination, using structural HPV proteins to prevent cervical cancer expressing only non-structural E6 and E7 is a promising example of the potential antiviral vaccination to prevent cancer (1, 49).

Alternatively, new treatment modalities using monoclonal antibody targeting antigens on the surface of tumors cells are used for several types of malignancies, including tumors associated with anti-CD20 (rituximab), -CD22 (epratuzumab), -CD80 (galiximab) (35) and EGFR (Erbitux, Nimotuzumab) (5, 41). The action of rituximab appears to be mediated via three potential humoral and cell-mediated effector mechanisms, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and direct apoptosis signaling. CDC appears to be the predominant mechanism by which rituximab exerts its therapeutic effect (40, 45). When CD22 binds to a natural ligand or antibody will be rapidly internalized and provide pro-apoptotic signal within B NHL cells (125). A preclinical study demonstrated that anti-CD80 antibodies inhibit lymphoma cell proliferation by inducing ADCC (110). Inhibition of EGFR signaling with the monoclonal antibody cetuximab (Erbitux), or the tyrosine kinase inhibitor gefitinib (Iressa), has been shown to retard cell growth and induce apoptosis in NPC cells. The knowledge on kinase-mediated cell signaling, which is commonly deregulated in epithelial cancers, and the ability to pharmacologically inhibit such kinases, has led to the development of protein kinase inhibitors in oncology. The rationale of targeting the EGFR-mediated signaling in NPC is based on both preclinical and clinical groundwork. EGFR gene amplifications can be found in 40% of NPC tissues, and EGFR overexpression is associated with poor prognosis following chemoradiation in patients with advanced NPC (93). The recent introduction of Nimotuzumab, which has less side effect compare to Erbitux and is less expensive may be an important step forward in treating NPC. However the price of this treatment is still prohibitory for many developing countries.

The development of (monoclonal) antibody-based therapy for cancer and our result with immunization of rabbits using LMP1, LMP2 synthetic peptides to induce extracellular domain-specific antibodies as discussed in this thesis (Chapter 7), creates the option of using peptide-based vaccination for inducing antibody responses against external domains of LMP1, LMP2 proteins in NPC patients. During natural infection or even in patients with LMP1 and LMP2 expressing tumors barely any anti-LMP1, 2 antibody responses is formed, in particular not against the evolutionary well conserved extracellular domains (Chapter 7). This provides an open window for vaccination. Induced antibodies against the putative LMP1 and LMP2 extracellular domains, when having sufficient avidity, can mediate complement-driven cytolysis of LMP1 and LMP2-expressing cell lines. This may allow inducing therapeutic antibodies targeting virus-encoded antigen expressed on the NPC tumor cells, either using active or passive immunization. For passive immunization, anti-LMP1 and -LMP2 kopi reactive human antibodies may be selected from phage libraries using the peptides described here in this thesis. Phage library technology has shown strong potential in recent years (58). Further technological developments led to advances in the generation of chimeric antibodies (65-90% human), partially humanized antibodies (95% human), and most recently fully humanized antibodies (58, 80, 145). An obstacle in treating cancer: cells with targeted therapy is the ability of cancer cells to become resistant to a targeted therapy by activating alternative pathway to evade apoptosis. It suggests the use of several antibodies or combination of antibodies with chemotheraphy. The study of using combination targeted therapy in NPC has been done by combining cetuximab and platinum in NPC cell lines, cetuximab and carboplatin in patients with metastatic NPC (93).

Antibody based therapy targeting EBV tumor associated antigen is considered to be the advantageous option and can be used as a component of an
immunotherapeutic for NPC. However the considerable cost of (human) antibody therapy, precludes its widespread use in developing countries (95). Therefore, more work aiming for less costly "small molecule" therapeutic agent is still needed.

On the other hand, recent studies have indicated the option using "common" drugs for epigenetic modification of latent viral promoters to trigger EBV into lytic cycle production, thus becoming sensitive to antiviral drugs and more potent immune responses. This so-called "lytic" induction therapy was proven successful in mouse models and awaits further evaluation in humans (36, 132).

In conclusion, the aberrant humoral immune responses of NPC patients against EBV, mainly to components of the EBNA, EA, and VCA complexes, is important for diagnosis and prognosis and may find application in screening for early-stage NPC. However, IgG antibodies to EBNA1 and VCA frequently present in healthy carriers. Therefore, IgG and particularly IgA antibodies to EA are proposed to be specific marker for NPC. The latter appear to be directed to conformational rather than linear epitopes (this thesis). Together with detection of IgA specific for VCA and EBNA1 peptide, IgG/IgA-EA can be used as NPC discriminator from healthy carrier and other EBV-related diseases. The EBV-encoded tumor associated antigens are marginally immunogenic for humoral immune responses in NPC patients. However, this may be stimulated specifically using exogenous LMP peptide constructs to generate such responses. These "anti-loop" antibodies can mediate killing activity through antibody dependent cytotoxicity and open options for peptide-based tumor vaccination in patients carrying EBV latency-II type tumors such as NPC.

Reference


Chapter 8


Chapter 8

Leuk Lymphoma 43:831-40.


