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CHAPTER 7

Humoral Immune Responses to Epstein-Barr virus encoded Tumor Associated Proteins and their Putative Extracellular Domains In Nasopharyngeal Carcinoma Patients and Regional Controls

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Humoral Immune Responses to EBV tumor associated protein in NPC

ABSTRACT

Nasopharyngeal carcinoma (NPC) displays an Epstein-Barr virus (EBV) latency type II gene expression profile in the tumor cells, characterized by formation of viral proteins EBNA1, LMP1, LMP-2A/-2B and BARF1. IgG and IgA humoral immune responses to these tumor-associated non-self antigens were analysed in a large panel of NPC patients (n=125) and regional controls (n=100) by three different approaches, i.e. indirect immunofluorescence assay (IFA) and immunoblot (IB) using baculovirus-expressed recombinant protein and Enzyme Linked Immunosorbent Assay (ELISA) using distinct synthetic peptide epitopes of these proteins, in particular focusing on the putative extracellular domains.

Compared to the abundant IgG and IgA antibody responses to multiple lytic antigens and EBNA1 in NPC sera (15) in the same patients only low levels of antibodies to LMP1, LMP2A and BARF1 could be detected. Using IFA, NPC patients had low titer (1:25 - 1:100) IgG to LMP1 (81.2%), LMP2A (95.6%) and BARF1 (84.8%) respectively, while IB showed such reactivity in 24.2%, 12.5% and 12.5% at 1:50 serum dilution, respectively. As positive control, IgG antibody responses to EBNA1 were found at high titer (>1:200) in 100% and 94.9% of NPC patients using IFA and IB, respectively. Few IgA responses were detected in NPC sera against the examined proteins, except for EBNA1 (81.8%) (IFA) and 56.5% (IB)). Healthy regional EBV carriers from the same region were virtually devoid of any antibody response to these EBV tumor-associated antigens, except for IgG to EBNA1. ELISA using synthetic peptides derived from different intracellular and putative extracellular domains of LMP1, LMP2A and BARF1 also yielded low and mostly negative IgG and IgA antibody responses in NPC patients. Fine mapping revealed that, when existing, most positive responses consist of IgG to intracellular C-terminus of LMP1 (62.9%).

Immunization of rabbits with synthetic peptides representing extracellular domain yielded specific antibodies serving as positive controls. Importantly, rabbit antibodies against putative LMP1 and LMP2 extracellular domains were shown to specifically stain extracellular domains of LMP1 and LMP2 on viable EBV transformed cells. These anti-peptide antibodies were able to mediate complement-driven cytolyis on 50.4% and 59.3% of X50/7 and 35.0% and 35.9% of RAJI cell lines by anti-LMP1 loop-1 and -3 antibodies respectively, and nearly 22% of both cell lines either by anti-LMP2 loop-2 or -5 antibodies.

Data generated in this study demonstrate that EBV-encoded tumor-associated antigens are at best marginally immunogenic for humoral immune responses in NPC patients. However, specific stimulation using exogenous peptide constructs may generate such antibodies, which can mediate killing activity through antibody dependent cytotoxicity. This opens options for peptide-based tumor vaccination in patients carrying EBV latency-II type tumors such as NPC.

INTRODUCTION

Epstein-Barr virus (EBV) is a human γ-herpesvirus, that infects more than 90% of the world population, and is associated with a spectrum of diseases, including infectious mononucleosis (IM) (19), Burkitt’s lymphoma (BL) (13), Hodgkin’s disease (22, 53), extranodal T/NK cell lymphoma (9, 51), immunoblastic B-cell lymphomas in immunocompromised individuals (44), gastric carcinoma (50) and nasopharyngeal carcinoma (NPC) (55).

EBV persists in life in the human host after the primary infection and is well controlled by the host’s immune system. Life-long immunosurveillance is reflected by the persistence of antiviral antibodies and virus reactive (cytotoxic) T cells (41). Different sets of proteins expressed during EBV’s lytic and latent life cycle induce qualitatively and quantitatively different immune responses (14, 21). Similar to other herpes viruses, EBV reactivation can occur in patients with immune defects or immune suppression reflected by aberrant IgG/M/A antibody responses (30). Importantly, EBV may cause a number of malignancies of lymphoid and epithelial origin in both immunosuppressed and immunocompetent individuals, which are also reflected by aberrant antibody responses to EBV.

In the neoplastic cells of these malignancies, several EBV latent gene products are expressed corresponding to the latency type. NPC is one of the latency type II tumors and is characterized by expression of EBNA1, LMP1, LMP-2A/-2B proteins (3, 20, 24) with co-expression of the epithelial oncogene BARF1 (2, 11, 43). In view of potential immunogenicity of virus-encoded “nonself” proteins, it is surprising that LMP1, LMP2 expressing tumors occur in immunocompetent individuals, who are considered to have the capacity of mounting an effective immune response to these “non-self” proteins.

CD8+ T cell responses to EBV latent antigens are skewed towards immunodominant epitopes derived from the EBNA3A, 3B, and 3C protein family. Accompanying subdominant responses map to additional epitopes from the same EBNA3 family or from LMP2, and much less often to epitopes from EBNA2, EBNA-1p, or LMP1 (21, 25, 37). Only limited data are available for T cell responses to BARF1 (29). Early work on EBNA1 as CD8 T cell target showed that the internal 250 amino acid glycine-alanine repeat (GAr) protects the endogenously expressed EBNA1 from CD8 T recognition (27), as consequence from GAr-mediated interference with proteasomal degradation (8). EBV has multiple evasion strategies in establishing and maintaining latency in the face of a CD8 T cell response by switching-off antigen expression in those cells constituting the latent reservoir (48), by inducing T-cell anergy (12) or Treg’s (28) or by active interference with antigen processing and presentation during lytic replication (52, 54).

EBNA1 is well recognized as a major target for humoral immune responses. However, only few studies addressed the role of LMP1 and LMP2 proteins as targets for humoral immune responses in detail. Antibody reactivity to LMP1 has been described in different EBV-related patient populations, including
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NPC, Hodgkin Disease, mononucleosis, and Burkitt Lymphoma patients, using different techniques, such as ELISA, immunoblot, and migration inhibition assays (4, 26, 30, 31, 36, 42, 45). Previous studies indicated that LMP1 is a protein with a low immunogenicity for the humoral immune response in humans. In NPC only 7.5% (3/40) patients had low serum levels of LMP1 directed antibodies, whereas antibodies to LMP2A/2B were detected at low titer in about 40-60% of NPC sera from different ethnicity (26, 30). Structurally, LMP1 and LMP2A/2B are suggested to protrude from the cell surface via several conserved small kopl domains connecting the transmembrane helices (36). However these kopl-domains have not been studied as target for humoral immune response to date. Importantly, such anti-kopl antibodies may have potentially important function in targeting complement and/or FcR-bearing killer cells to LMP1, 2 expressing tumor cells. A prior study of antibody to BARF1 in sera with EBV-associated diseases including NPC suggested that the BARF1 protein may serve as target on EBV-infected cells for antibody dependent cytoxicity (ADCC) (47). However, this study has not been confirmed and recent data indicate that BARF1 is rapidly and completely secreted from the EBV positive cells, making it a disputable target for ADCC (10, 43).

In this study, we evaluate in detail antibody responses to EBV-tumor associated antigens LMP1, LMP2 and BARF1 in NPC patients compared to healthy EBV carriers. We further developed specific antibodies to the putative LMP1 and LMP2 extracellular kopl domains and evaluate whether such antibodies can mediate complement killing of the LMP1 and LMP2 expressed tumor cells.

MATERIAL AND METHODS

Sera from NPC patients and Healthy EBV Carriers. Serum panels from histologically confirmed NPC patients (overall n=125) were collected from department of Ear, Nose and Throat (ENT), Dr. Sardjito General Hospital, Yogyakarta. NPC sera were taken on the first visit of patients to the clinic, prior to treatment. NPC staging was done by ENT examination and CT-scan and classified according to the 1996 criteria established by UICC (Union International Cancer Control). Sera from healthy EBV carriers (overall n=100) were obtained from the local red cross blood bank. All sera were extensively analysed for reactivity to multiple EBV-encoded lytic cycle proteins in prior studies (14, 15, 39, 40). NPC tissues from available formalin fixed paraffin embedded tumor NPC biopsies were examined the EBV status by EBER in situ staining (DAKO, PNA) and analysed the expression of LMP1 using S12 or OT21C MoAbs based immunohistochemistry (31).

Cell culture. The EBV positive RAJI Burkitt Lymphoma cell line, the in vitro EBV transformed B cell line X50/7, BJAB-LMP1 (kind gift of M. Rowe) and Daudi-LMP1 (kind gift of P Busson) were cultured in RPMI-1640 medium comprising 25mM Hepes and glutamin (Sigma, St.Louis, USA), 10% fetal calf serum (FCS, Hyclone, Perbio, Sweden), 100 II/ml penicillin and 50 g/ml streptomycin (p/s) at 37 °C in a humidified 5% CO₂ atmosphere. Both cell lines express relatively high levels of LMP1 and LMP2 (1, 33). Insect cells were cultured as described below.

The BJAB-LMP1 cell is originally from EBV negative cell line BJAB transfected with LMP1 expression vectors. The LMP1-transfected clones of BJAB were established using a tetracycline-regulated vector system and were maintained in culture medium containing 1.5 mg/ml G418, 0.5 mg/ml hygromycin B, and 1 g/ml tetracycline. Tetracycline withdrawal induced LMP1 expression as previously described (16).

Recombinant proteins. The Baculovirus constructs expressing full-length LMP1, LMP2A, BARF1 and EBNA1 without the GAr domain were made under control of the polyhedrin promoter (32, 33). Sf9 cells were cultured to the log phase (1 x 10⁶ cells/ml) and infected with one of the Baculovirus constructs. A high dose of 1-5 PFU/cell was used for recombinant protein production and cells were harvested at 48 hours post infection (pi). For immunofluorescence experiments infection at 1 PFU/cell for 48 hours was used leaving about 50% uninfected cells in the preparation, which were used as specificity control. Insect cells were cultured in serum-free SF900-II medium at 28°C.

EBV synthetic peptides. Immunodominant epitopes on EBV proteins were derived by computer prediction techniques, as described by Modrow and Wolf (36), using high scores for hydrophicity, flexibility, and β-turn probability. Peptides mimicking different domains of LMP1, LMP2 and BARF1 proteins were synthesized with a peptide synthesizer (433 A; Applied Biosystem, Foster City, CA). Peptides representing putative extracellular kopl domains of LMP1 and LMP2 were also synthesized as circular peptides by inserting two cystine residues at the ends forming a S-S bridges upon oxidation (49). Most peptides were extended at the N-terminus with additional lysine residues for improving solubility and coupling options. All peptides were purified in reverse phase high performance liquid chromatography (Beckman System Gold Mijdrecht, The Netherlands). Peptide coupling to carrier proteins KLH or TTD was performed by standard techniques using commercial reagents (Sigma, St.Louis, USA). Peptide denomination and amino acid sequences are listed in table 1.

Monoclonal and polyclonal antibodies. Monoclonal (MoAb) and polyclonal (PoAb) antibodies were obtained by immunization of mice and rabbits with synthetic peptides or purified recombinant EBNA1, LMP1, LMP2 and BARF1 proteins expressed in insect cells. Female Chinchilla rabbits were immunized with either keyhole limpet hemocyanine (KLH) or tetacon toxoid (TTG) conjugated synthetic peptides or istochrophesin isolated recombinant proteins (30). Before immunization pre-serum of each rabbit was drained from the ear. For primary immunization 1 mg antigen was mixed well with 1 ml Freund’s Complete Adjuvant.
Immunofluorescent (IF) staining on fixed recombinant antigen-expressing cells.

Cytospins were made with SiP cells either infected with wild type (wt) baculovirus or recombinant baculovirus. Slides were fixed in cold (-20°C) acetone and pre-incubated in PBS containing 2% fetal calf serum (PBS/2%FCS) for 10 min. All washings were done three times in PBS/0.05% Tween-20 (PBSt). Antibody dilutions were made in PBS/2% FCS and incubated at RT. MoAbs were diluted in 100 - 1000 times human sera and used in a 1:25, 1:50, 1:100 and 1:200 and incubated for 1 h unless stated otherwise. After washing, the slides were incubated for 30 min with FITC-labeled rabbit anti-mouse Ig or anti human IgG secondary antibodies (DAKO-PATTS, Denmark). Finally slides were counterstained for 5 min. with a 1:1 mix of DAPI and Evans blue or 1:500 ToPro 3 (Partec, The Netherlands) washed, dipped with mounting fluid Vectashield sealed with a coverslip and evaluated with a Leica DMRB fluorescence microscope (Leica, Cambridge, England).

**Table 1. Amino acid sequence of the LMP1, LMP2 and BARF-derived synthetic peptides**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
</tr>
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<tbody>
<tr>
<td>OTF1</td>
<td>HKKCTAMVMFTGQALWYCLT</td>
</tr>
<tr>
<td>OTF2</td>
<td>HNKSCASQDVWRVPHLLC</td>
</tr>
<tr>
<td>OTF3</td>
<td>HSKHLEVPQPKRMPWPPVIF</td>
</tr>
<tr>
<td>OTF4</td>
<td>HCSMCDQDDTQKPOQSVYD</td>
</tr>
<tr>
<td>OTF5</td>
<td>HACLWIEDQPMQNLQAL</td>
</tr>
<tr>
<td>OTF6</td>
<td>HAKLAVDWQPMQNLQAL</td>
</tr>
<tr>
<td>OTF7</td>
<td>HHCNQTVVCTDEEHRPFPACYQDC</td>
</tr>
<tr>
<td>OTF8</td>
<td>HSCSGDQPMQNLQAL</td>
</tr>
<tr>
<td>MAFv</td>
<td>HCGVESEKFLKGDGSQGDAKHMNHEQFRAFKC</td>
</tr>
<tr>
<td>MAFv</td>
<td>HSSLQPKPMQPLKQPQKDEKAYCVSVSCQQL</td>
</tr>
</tbody>
</table>

**SDS-PAGE and western blot analysis.** Recombinant proteins were solubilized in standard Laemmli sample and boiled for 5 min. and separated in 10% acrylamide gels using the Mini Protean II system (BioRad, Hercules, USA) under reducing condition. Polypeptides were transferred from the gel onto 0.2 μm nitrocellulose (Schleicher & Schuell, Hertogenbosch, the Netherlands) by Western blotting (Mini-Trans blot cells, BioRad). After transfer, nitrocellulose sheets were washed with H2O and dried between filter paper and stored at 4°C until use. Marker proteins (Bio-Rad Low MW marker) were run on the side to indicate the molecular weight of polypeptides. Non-specific binding sites were saturated with blocking buffer (5% horse serum and 5% non-fat dry milk (Campina, Eindhoven, the Netherlands) in PBS pH 7.2) followed by incubation with Moab or PoAb at appropriate dilutions or sera at different dilutions made in blocking buffer. After washing with PBSt, specific bound IgG and IgA were detected with horseradish-peroxidase (HRP) conjugated secondary antibody (Dako, Gohstrup, Denmark) in blocking buffer and HRP-activity was visualized by using 4-chloro-1-naphthol (15).

**Synthetic Peptide ELISA.** Standard microtiter plates (Biobasic, Canada) were coated overnight at 4°C with 135 μl of one of the peptides in a concentration of 1 g/ml in 0.05M carbonate buffer pH 9.6. Excess coating fluid was removed and non-specific binding sites were blocked subsequently for 1 h with 200ul/well of PBS/3% BSA at 37°C. Further incubations were performed for 1 h at 37°C followed by four washes with PBS. Human sera were diluted 1:50 in ELISA sample buffer (PBS; 0.1% Triton-X100, 1% BSA), followed by washing and incubation with HRP-labeled rabbit anti-human IgG (1:3000) and IgA (1:2000) (DAKO, Copenhagen) diluted in conjugate buffer (PBS; 0.1% (v/v) Triton X-100, 1% BSA and 2% normal rabbit serum). Peptide-specific Moab or PoAb were diluted in ELISA sample buffer and detected with rabbit anti-mouse or swine anti-rabbit HRP conjugates (Dako, Gohstrup, Denmark) in blocking buffer and HRP-activity was detected using 3,3’,5,5’-tetramethylbenzidine (TMB) (BioMérieux, Boxtel, The Netherlands) and the reaction was stopped by adding 1M H2SO4. The optical density was determined at 450 nm (Anthus 2001 reader, Anthus Labtec, Austria).
Humoral immune responses in NPC patients and healthy EBV carriers to recombinant EBV-encoded Tumor Associated Protein. In this study, we explore the antibody responses of NPC patients to individual recombinant proteins LMP1, LMP2 and BARF1. Antibody responses to the individual proteins were analyzed by indirect immunofluorescence (IF) and immunoblot (IB) techniques. Sf9 insect cells infected with recombinant Baculovirus expressing full-length LMP1, LMP2A and BARF1 were used as antigen (rLMP1, rLMP2A, rBARF1, respectively), mainly as described previously (30, 32, 33). A low MOI was chosen to leave 40-60% Sf9 cells uninfected, serving as internal specificity control in each experiment. Recombinant EBNA1 deleted of the GAr (rEBNA1) was used as positive control and all sera and MoAbs were analyzed in parallel on Sf9-cells infected with wild type Baculovirus (wtBac). Expression of LMP1, LMP2A, BARF1 and EBNA1 in the infected Sf9 cells was confirmed by staining with specific MoAbs to the individual EBV proteins (Figure 1). Human antibody staining was interpreted with the MoAb staining pattern as reference.

MTT assay. To evaluate the cytolytic capacity of anti-LMP1 and -LMP2 ksp-specific antibodies, complement cytotoxicity studies were performed with MTT read-out (Cell Proliferation Kit I, Roche, Germany) using EBV, LMP1, 2 positive RAJ and X50/7 cell lines and appropriate controls. All incubations were performed at 37°C and 5% CO₂. Prior to the experiment, lymphoprep purification was performed to remove dead cells. Cells were placed on a 96 well plate at 10⁴ cells /25 µl per well. Antibody anti-loop-1 and -3 LMP1 and anti-loop-2 and -5 LMP2 (1:3, 1:10, 1:50 and 1:250) were added, followed by the addition of 50 µl 30 times diluted rabbit complement (Innovative Research, USA) and incubated for 2 hrs. As controls, cells were incubated with rabbit preserum or beta-2 microglobuline. Subsequently 5 µl MTT labeling reagent was added. After 4 hrs, 50 µL solubilization reagent was added and after overnight incubation, the optical density was determined at 550-600 nm. Percentage of dead cell was calculated by using the formula below.

\[
\text{Percentage (\%)} \text{cell death:} \quad \frac{\text{OD of untreated cells (blank) - OD of treated cells}}{\text{OD of untreated cells (blank)}} \times 100\%
\]

Overall, IF results with NPC sera showed rather low (most 1:25 - 1:100) IgG reactivity to acetone-fixed rLMP1, rLMP2A and rBARF1 being detectable in 81.2%, 95.6% and 84.8% of tested sera, respectively, whereas IgG to rEBNA1 was present at higher titers (> 1:200) in 100% of the sera (n=32) (Figure 1).
general, observed background reactivity with uninfected Sf9 cells and Sf9-wtBac was minimal and, when present, wt-Bac staining pattern could be discriminated from EBV antigen-specific staining. In simultaneous IF analysis, IgA reactivity to rLMP1, LMP2A and BARF1 was observed at even lower titer (< 1:25) and at lower frequency in 40.9%, 54.5%, and 59.0% of NPC sera respectively. IgA to rEBNA1 was observed at slightly higher titer (1:100) in 81.8% of the sera (n=22) (data not shown).

Subsequently, to reveal potential immune responses to possible linear epitopes in fully denatured EBV tumor proteins, a set of NPC sera (n=123) was tested for IgG and IgA reactivity by IB analysis at dilutions of 1:50 using lysates of Sf9 cells expressing either rLMP1, rLMP2A, rBARF1 or rEBNA1. Figure 2A-C show that control MoAbs OT21C, 14B7 and 4A6 recognize clear bands at 63kD (LMP1), 54kD (LMP2a) and 30kD (BARF1). In contrast to IF, IB analysis revealed very low IgG responses to LMP1, LMP2A and BARF1 indicated by weak intensity of the specific protein band in 24.2, 12.5% and 12.5% NPC patients, respectively. In general EBV-protein specific staining by IB was only detectable using the lowest dilution (1:50), if detectable at all. IgG reactivity to EBNA1 was observed at 94.9% of NPC patients (Figure 2D), and showed similar clear band at 55kD as revealed by OT1X Ab (figure not shown). None of NPC patients had detectable IgA response to the LMP1, LMP2A and BARF1 by IB analysis, but a weak IgA response to EBNA1 was observed at 56.5% NPC patients. These data indicated that NPC patients, who have high-level antibody reactivity to multiple lytic cycle antigens and EBNA1 [14, 15], are largely lacking potent antibody responses to tumor associated membrane antigens LMP1 and LMP2A, as well as BARF1, as examined with intact full length recombinant proteins.

**LMP1 expression and antibody reactivity in NPC cases.** No relation was found between LMP1, LMP2 and BARF1 responses (when present) with TNM stage of the tumor. In cases analyzed for serological responses to LMP1 by IFA (n=32) or IB (n=125) we also detect the presence of LMP1 at the tumor level using MoAb base immunohistochemistry. Results are shown in table 3A and 3B. Overall 80% of the NPC were found to LMP1 expression using immunohistochemistry. In cases having antibody reactive with LMP1 by IFA has positive correlation with LMP expression on the tumor (68.8% concordance), but by IB has negative correlation (33.6% concordance) (table 3). IFA and IB may detect different epitopes, which is related to the level of denaturation of the antigen used, being minimal in IFA aceton fixation, and maximal in IB SDS boiling. Therefore we decided to analyse this option in more detail. The functional importance of detecting Ab-responses to LMP1 and LMP2 conformational domain will be of particular interest when expressed on the tumor cell surface.

**Antibody Responses to defined extracellular peptide-epitopes of and LMPs, LMP2s and BARFs.** To more precisely study the epitope specificity in the sera of NPC patients, defined synthetic peptides representing putative extracellular domains of LMP1, LMP2 and BARF1 were created and used as antigens in ELISA. Cytoplasmic peptide epitopes of LMP1 and LMP2 and extracellular domain of BARF1 were selected for having high scores for hydrophilicity, flexibility and -turn probability as...
Described before (30, 35, 36). In addition, for LMP1 and LMP2 synthetic peptides were also created representing the extracellular loop 1 and 3 (connecting the 1st to 2nd and 5th to 6th transmembrane helix, respectively) and loop 2 and 5 (connecting the 3rd to 4th and 9th to 10th helix, respectively) (Figure 3A & 3B). Synthesis of cytoplasmic peptide domains of LMP1 have been described previously (30). For LMP1 we used peptide domain in circular conformation to more closely mimic the in vivo structure. Circular peptides were created by oxidation of the sulfide bridge in peptides OTP 405 and OTP 407 (Table 1) (49). These peptides were used as antigens in indirect ELISA. Epitope-specific antibodies were generated by rabbit immunization using carrier proteins conjugated to the peptides. These newly developed antibodies were used as positive control in the ELISA (Figure 3). All human sera used were strongly responsive to VCA-p18 and EBNA1 synthetic peptides as described before (14).

Analysis of LMP1, 2 and BARF1 peptide-epitope specific antibody response by ELISA did not show major differences between NPC patients and healthy EBV carriers. When detectable, positive responses were marginal in most cases and the most significant response (62.9% positive) in NPC patients is confined to IgG against the intracellular C-terminus of LMP1 (Figure 3C). Overall analysis is depicted in table 2.

Table 2A. Positive IgG responses (%) in NPC and healthy EBV carriers to LMP1, LMP2 and BARF1 peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Loop</th>
<th>C-terminus</th>
<th>N-terminus</th>
<th>Loop</th>
<th>C-terminus</th>
<th>N-terminus</th>
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<tbody>
<tr>
<td>LMP1</td>
<td></td>
<td></td>
<td></td>
<td>LMP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>5.0</td>
<td>5.0</td>
<td>10.0</td>
<td>5.0</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>NPC</td>
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<td>21.8</td>
<td>13.2</td>
<td>13.2</td>
<td>21.8</td>
</tr>
</tbody>
</table>

Table 2B. Positive IgA responses (%) of NPC and healthy EBV carriers to LMP1, LMP2 and BARF1 peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Loop</th>
<th>C-terminus</th>
<th>N-terminus</th>
<th>Loop</th>
<th>C-terminus</th>
<th>N-terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMP1</td>
<td></td>
<td></td>
<td></td>
<td>LMP2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>5.0</td>
<td>5.0</td>
<td>10.0</td>
<td>5.0</td>
<td>5.0</td>
<td>10.0</td>
</tr>
<tr>
<td>NPC</td>
<td>1.8</td>
<td>2.4</td>
<td>3.2</td>
<td>1.8</td>
<td>2.4</td>
<td>3.2</td>
</tr>
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Analysis of LMP1, 2 and BARF1 peptide-epitope specific antibody response by ELISA did not show major differences between NPC patients and healthy EBV carriers. When detectable, positive responses were marginal in most cases and the most significant response (62.9% positive) in NPC patients is confined to IgG against the intracellular C-terminus of LMP1 (Figure 3C). Overall analysis is depicted in table 2. Table 2A shows the number of donors and patients having IgG responses to the individual peptides of tumor-associated EBV proteins. IgG responses to LMP2 in healthy EBV carriers were lower compared to responses to LMP1 and BARF1. There was no difference in LMP1 loop peptide responses when using circular (created by S-S bridge oxidation) or linear peptides (data not shown). None of healthy EBV carriers had IgG responses to LMP2 loop peptides. Responses to C-terminus and N-terminus LMP2 are found only in 2.0% and 1.8% of donors and patients.
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Humoral Immune Responses to EBV tumor associated protein in NPC

of healthy EBV carriers, respectively. About 5% of healthy EBV carriers had IgG response to subfragments of LMP1 and BARF1. Table 2B shows the number of NPC patients and healthy EBV carriers with IgA responses to peptides of LMP1, LMP2 and BARF1. IgA responses are lower as compared to IgG responses, and most of the IgA responses in NPC patients can also be addressed the C-terminus of LMP1 (27.4%).

Accessibility of LMP1 and LMP2 loop domains on viable EBV transformed cells. LMP1 and LMP2 are transmembrane proteins, with six or twelve membrane-spanning domains, respectively, connected by intracellular and extracellular loops. The extracellular loop domains are potential targets for functional immune responses, and may mediate killing of EBV transformed cells via complement dependent cytotoxicity (CDC) or killer cell (ADCC) dependent cytotoxic pathways. To study the accessibility of the extracellular loops of LMP1 and LMP2 on viable cells, we evaluated specific antibody recognition of these proteins expressed on viable RAJI, X50/7, Daudi-LMP1, BJAB-LMP1 and BJAB cell lines by FACs analysis and confocal microscopy. All cell lines except BJAB cells were positive for LMP1 and LMP2A mRNA as determined by reverse transcription PCR and by intracellular protein staining. For the latter, permeabilized cells were treated with monoclonal antibodies GT21C and 14B7, recognizing the intracellular epitopes of LMP1 and LMP2A respectively (data not shown). Both LMP1 and LMP2A revealed a heterogeneous intracellular staining pattern between individual cells of a cell population as described before (26, 42). The presence of LMP1 and LMP2A loop domains on the surface of those cell lines were detected by FACs analysis using anti-loop specific antibodies for LMP1 loop 1 and 3 and LMP2A loop 2 and 5. LMP1 clearly expressed on RAJI and X50/7 (5-15% of the cells), but clearly negative with Namalwa and BJAB. Figure 4 shows a fine patch-like staining observed on RAJI cells with soluble loop 1 and 3 LMP1 and BJAB as negative control and FACs analysis of RAJI cells using similar antibodies. On cells artificially expressing LMP1 (Daudi-LMP1 and BJAB-LMP1) by vector transfection much higher staining was seen (20-50%). LMP2 best expression was seen on loop 2 on X50/7 cells (data not shown). Rabbit antibody against J2M reacted with >88% of all cell lines. Staining pattern of individual viable cells was determined by confocal microscopy, revealing a heterogeneous staining pattern similar to the cytoplasmic staining patterns, with some cells being negative, and others being positive and showing a patch-wise distribution of LMP1 and LMP2 related epitopes. This is the first demonstration that extracellular LMP1 and LMP2 related loop domains, can potentially function as targets for antibody-based therapy.

Complement lysis by anti-LMP1 and LMP2 loop-specific antibodies. Since LMP1 and LMP2 are expressed in multiple EBV tumors, including NPC, targeting of the extracellular domains may have therapeutic potential. We demonstrated that immunization of rabbits using synthetic peptides mimicking the extracellular loop domains of LMP1 and LMP2 could generate specific anti-loop antibodies. This approach might be applicable to humans as well aiming for therapeutic

Vaccination. Considering this option, we evaluated the functional activity of the anti-loop antibodies and analysed complement mediated lysis using RAJI and X50/7 cell lines. Figure 5 shows that by 4 hr complement lysis 35%, 35.3%, 22% and 22.3% of RAJI cells were killed by anti-LMP1 loop 1 and 3 and anti-LMP2 loop 2 and 5 antibodies respectively, and 50.4%, 59.4%, 22% and 22.7% of X50/7 as measured by MTT assay. Killing potential of each antibody depend upon dilution (Figure 5). No cell lysis was observed by pre-serum obtained from these rabbits and no lysis was observed with Namalwa or EBV negative Ramos or BJAB cell lines. Using J2M as target closely to 80% cells were lysed by rabbit anti-J2M in this assay. These results demonstrate that newly developed anti-loop antibodies can functionally target extracellular domains of LMP1 and LMP2, which may have important therapeutic implications.
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Heussinger. In addition, recent studies revealed the expression and secretion of latent EBV proteins such as LMP1 and LMP2 are regularly detected in NPC (20, 24). However, informations on humoral immune responses to LMP1, LMP2A and BARF1 antibodies is rather limited (17, 26, 30, 47). In our study using IF-analysis on acetone-fixed recombinant proteins expressed in insect cells, IgG antibodies to LMP1 and LMP2 were found in a significant number of NPC patients (81.2%) and 95.6%, respectively, albeit in low titers, but hardly in controls. This confirms and extends previous studies that used smaller numbers of patients and controls (17, 26, 30). In all samples tested, the responses were low compared to IgG-EBNA1. By using a similar method we found that 84.8% NPC patients have a detectable but low titered IgG response to BARF1. Antibody responses against BARF1 protein have been studied before using sera from chronic and acute IM and NPC patients (47). Using transduced RAJI cells they demonstrated significant ADCC reactivity to BARF1-expressing RAJI cells in sera from NPC patients. However no study has yet confirmed antibody responses to BARF1 to strengthen these findings. In fact, BARF1 seems to be rapidly and completely secreted by BARF1-expressing cells, leaving little protein in or on the cell for detection (10, 43). The role of anti-BARF1 immune responses remains to be further established.

Immunoblottting confirmed the low-level antibody responses, being detectable at 24.2%, 12.5%, and 12.5% of NPC patients for LMP1, LMP2 and BARF1, respectively. The lower response rates compared to IF-analysis may be due to the fact that antigens prepared by SDS-PAGE may have lost certain conformational epitopes. Again anti-EBNA1 antibodies were clearly detected, confirming the immunodominance of EBNA1. IgA-specific analysis showed similar low responses to LMP1, LMP2A and BARF1, but again clearly detectable responses to EBNA1. This demonstrates a lack of local mucosa-specific responses to the tumor-associated latent EBV membrane antigens, hinting at specific defects in their presentation to the immune system. These observations are clearly in contrast to the responses to the marginally expressed but highly immunogenic lytic antigens, to which abundant IgG and IgA antibody responses are detectable in the same NPC patients (14, 15, 39). Importantly, most (80%) NPC cases analysed showed LMP1 expression. We found a positive correlation between LMP1 expression and Ab-responses using IFA analysis, but a negative correlation when using IIB (table 3). This may suggest that conformational epitopes, which are more reactive by IFA may triggered in LMP1 positive tumor cases, whereas antibodies to linear (denatured) LMP1 are triggered differently (i.e. by cross presentation). Our finding in NPC differ from previous observation in HD, where LMP1 antibodies were most prevalent in EBV negative cases (31).

The data from this study using EBV-recombinant proteins showed that NPC patients only have weak humoral immune responses to LMP1, LMP2 and BARF1. However the potential importance of LMP1 and LMP2 as targets for immunotherapy, prompted us to further analyse the presence of antibodies in NPC.

Figure 5. Complement mediated cell lysis. RAJI cells were incubated with a dilution series of anti-loop antibodies (3x, 10x, 30x, and 125x) and 30 times diluted complement solution. Percentage cell death is plotted against antibody dose. In dilution of anti-loop antibodies, approximately 49% RAJI cells were killed by anti-loop-1 and -3 LMP1 and 35% by anti-loop-2 and anti-loop-5 LMP2. Similar results or even higher killed cells were obtained in other EBV-carrying LCL lines but not in Namalwa, an EBV positive cell line with LMP1 and LMP2 negative. Anti-B2M antibody was taken as a positive control, and used at larger dilution (1:5000) and therefore is not reaching >80% lysis. Preserum was used as negative control and to distinguish with specific binding of specific antibodies.

Discussion

Individuals with EBV infection develop antiviral immune responses to a wide variety of EBV proteins and epitopes. Monitoring of anti-EBV antibody responses has yielded useful applications for diagnosis in various EBV-associated diseases, such as NPC. Elevated antibody titers to EBV antigen e.g. early antigens (EA), viral capsid antigens (VCA) and the EBNA1 protein are frequently found in NPC patients and are relevant as diagnostic and prognostic markers (5, 14, 15, 23, 30). However most of the EBV antigens used for diagnosis are not expressed in NPC patients and are relevant as diagnostic and prognostic markers (5, 14, 15, 23, 30). In all samples tested, the responses were low compared to IgG-EBNA1. By using a similar method we found that 84.8% NPC patients have a detectable but low titered IgG response to BARF1. Antibody responses against BARF1 protein have been studied before using sera from chronic and acute IM and NPC patients (47). Using transduced RAJI cells they demonstrated significant ADCC reactivity to BARF1-expressing RAJI cells in sera from NPC patients. However no study has yet confirmed antibody responses to BARF1 to strengthen these findings. In fact, BARF1 seems to be rapidly and completely secreted by BARF1-expressing cells, leaving little protein in or on the cell for detection (10, 43). The role of anti-BARF1 immune responses remains to be further established.

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patients directed to defined extracellular epitopes of LMP1, LMP2 and BARF1 in the form of synthetic peptides. No such information was available yet, and, in fact, the extracellular accessibility of domains of LMP1 and LMP2 has not been clearly demonstrated before. Therefore we extended our previous studies and explored responses to defined peptide epitopes mimicking these domain (30). In rabbits, polyclonal epitope-specific antibodies were developed directed against distinct domains of LMP1, LMP2 and BARF1. These antibodies, having a high affinity for their epitopes in denaturated as well as in the native conformation on viable cells, were used as positive controls. Using these anti-looop antibody reagents we were the first to demonstrate the presence and functional accessibility of extracellular loop domains of LMP1 and LMP2, opening option as targets for therapeutic applications (34). However, in naturally EBV infected NPC patients and healthy EBV carriers these LMP1 and LMP2 loop domains seem to evade from immune recognition, as anti-loop antibody responses are mostly negative (Table 2). The results of peptide-specific analysis confirm the presence of some antibody responses to the intracellular C- and N-terminal domains of LMP1 and LMP2, although only at a low levels (Figure 4B). Intrinsic properties of LMP1 and LMP2 and their short existence in the plasma membrane may be responsible for the low immunogenicity. On the other hand, this study shows that LMP1 and LMP2 antibodies specifically directed against the extracellular loop domains can be generated by immunization of rabbits using related peptides and these antibodies can activate the complement system to kill LMP1 and LMP2 expressing cells. X50/7 cells can be killed by complement (50.4% and 59.4%) in higher percentage compared to RAJI cells (35% and 35.9%) most likely reflecting different level of LMP1 and LMP2 expression or differences in loop-accessibility. This requires further analysis but is in line with known LMP1 expression levels in different cell lines (33). Detection of extracellular domains requires viable cells and low temperature incubation to inhibit aggregation and internalization activity. A heterogeneous staining pattern of small patches of FITC-labeled anti-loop antibodies was demonstrated in the cell membrane. Also individual cells among the cell population showed a clear distribution (Figure 5). This corresponds with the known heterogeneous expression of LMP1, being abundant in some cells and barely detectable in others in the same culture (42). The relation between intracellular situated and membrane-associated LMP1 and LMP2 remains to be analysed in detail (studies in progress). Conclusion from this study suggests that limited humoral immune responses to EBV-encoded tumor antigens LMP1, LMP2 and BARF1 allow malignant cells to escape from control. Augmentation of immune reactivity to EBV-tumor associated antigens especially LMP1 and LMP2, by active or passive immunization, may be important to the prevention and treatment of NPC as a member of latency type II tumors. Our finding that immunization of rabbits using these peptides can generate highly reactive epitope-specific antibodies opens new prospects for immunotherapy and vaccination of patients suffering from EBV associated tumors (34).

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