Chapter 2

Molecular Diversity of Epstein-Barr Virus IgG and IgA Antibody Responses in Nasopharyngeal Carcinoma: A Comparison of Indonesian, Chinese, and European Subjects

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Abstract

Epstein-Barr virus (EBV), a γ-herpesvirus, is well established in the human population and is efficiently transmitted by mucosal secretions. EBV infection usually occurs silently in life, but it may be symptomatic when infection is delayed until adolescence [13]. EBV is also a human carcinogen that has been implicated in the development of malignancies of lymphoid and epithelial origin, including Burkitt lymphoma, Hodgkin disease (HD), immunodeficiency-related B cell lymphoma, extranodal T/NK cell lymphomas, gastric carcinoma, and nasopharyngeal carcinoma (NPC) [2, 46]. Most EBV-associated malignancies are of different genetic background has not been described. The study described here provides insight into the molecular basis of EBV-specific IgG and IgA antibody responses in patients with NPC of defined tumor stage from Javanese (Indonesia), Chinese (Hong Kong), and white (Europe) origin, compared with those in regional non-NPC control subjects and healthy EBV carriers. Our parallel analysis of IgG and IgA antibody responses revealed differences in EBV antigen recognition profiles, which suggest independent B cell triggering events.

INTRODUCTION

Epstein-Barr virus (EBV), a γ-herpesvirus, is well established in the human population and is efficiently transmitted by mucosal secretions. EBV infection usually occurs silently in life, but it may be symptomatic when infection is delayed until adolescence [13]. EBV is also a human carcinogen that has been implicated in the development of malignancies of lymphoid and epithelial origin, including Burkitt lymphoma, Hodgkin disease (HD), immunodeficiency-related B cell lymphoma, extranodal T/NK cell lymphomas, gastric carcinoma, and nasopharyngeal carcinoma (NPC) [2, 46]. Most EBV-associated malignancies are of different genetic background has not been described. The study described here provides insight into the molecular basis of EBV-specific IgG and IgA antibody responses in patients with NPC of defined tumor stage from Javanese (Indonesia), Chinese (Hong Kong), and white (Europe) origin, compared with those in regional non-NPC control subjects and healthy EBV carriers. Our parallel analysis of IgG and IgA antibody responses revealed differences in EBV antigen recognition profiles, which suggest independent B cell triggering events.
SUBJECTS, MATERIAL, AND METHODS

Serum samples and antibodies. Serum samples from Indonesian Javanese (non Chinese) subjects consisted of samples from a panel of 135 patients with histologically confirmed NPC, 5 patients with non-NPC head and neck cancer (all of which were collected at the Department of Ear, Nose, and Throat (ENT), Dr. Sardjito General Hospital, Yogyakarta), and 70 healthy donors obtained from the local Red Cross blood bank. The NPC serum samples were obtained on the first visit of patients to ENT during 2001-2003. From all patients with NPC, nasopharyngeal and/or lymph-node biopsy samples were obtained and confirmed histologically for the presence of undifferentiated carcinoma cells and the presence of EBV, by EBER1,2 in situ hybridization, by use of the Dako PNAkit (Dako) and by immunohistochemistry (Labvision) with EBNA1- and latent membrane protein (LMP) - specific monoclonal antibodies OT1X [32] and OT21C [23, 33], respectively. NPC staging was done by ENT examination and computed tomography scan and was classified according to the 1997 Union International Cancer Control (UICC) classification.

Serum samples from persons of Chinese ethnicity living in Hong Kong were provided as a blind panel and included samples from 40 healthy donors, 35 patients with head and neck-related non-NPC tumors, and 40 patients with histologically confirmed NPC (obtained by M.H.N.). The EBV serological profile of the Chinese panel was analyzed without knowledge of the clinical diagnosis. VCA and EA IgG and IgA antibody titers were determined by standard IFA techniques, the Chinese panel was analyzed without knowledge of the clinical diagnosis. VCA and EA IgG and IgA antibody titers were determined by standard IFA techniques, and this information was revealed only after breaking the code.

Serum samples from 7 white patients with NPC were obtained from hospital in Germany, the United Kingdom, and The Netherlands. One series (n<5) of follow-up samples from a white Dutch patient with NPC was obtained from the Vrije Universiteit medical center, Amsterdam, The Netherlands. All serum samples were stored at 20°C until use.

Monoclonal and polyclonal monospecific antisera samples were produced by the immunization of animals with synthetic peptides or purified recombinant proteins, as described elsewhere. Antibodies to defined EBV proteins consisted of OT13B (anti-EA-p138; BALF2) [34], rabbit anti-EBNA1 (BKR1F1) [35], rabbit anti-DNAase (BGLF5) [27], OT14F (anti-EA-p47; BMRF1) [36], BZ-1 (anti-Zebra; BZLF1) [37], OT41A (anti-VCA-p40; BDF1) [38], and OT12E (anti-VCA-p18; BFRF3) [39].

Cell culture and antigen preparation. The supernadichable P3HR1-derived cell line HH514.c16 was kindly provided by Dr. G. Miller (Yale University, New Haven, CT). Cells were cultured and induced for EBV lytic cycle antigen expression (EA only or EA plus VCA), and the nuclear fraction was prepared by hypotonic detergent treatment and Ficoll separation, exactly as described elsewhere [21-23]. The EBV-negative cell line BJAB was used as a control.

RESULTS

The P3HR1-derived HH514.c16 cell line can be induced to express high levels of lytic-phase EBV antigen (i.e., Zebra, EA, or VCA) on treatment with 12-O-tetradecanoylphorbol 13-acetate and sodium butyrate. The use of phosphonoacetic acid during induction effectively blocked the synthesis of EBV-DNA and the concomitant expression of latent antigen (VCA), as revealed by the absence of the VCA-p40 (BDF1F1) and VCA-p18 (BFRF3) marker proteins (figure 1A). The results of previous cell-fractionation studies have shown that diagnostically relevant antigens mainly reside in the nuclear fraction of both EA- and EA-plus-VCA-induced cells [21, 22].

Reference antibody staining. The position of EBV marker proteins on the blot strips was defined by use of a panel of antibodies of defined specificity. Figure 1A shows the position of EA(d)-p138 (BALF2; 138 kD), EBNA1 (BKR1F1; 72 kD), EA-DNAase (BGLF5; 55 + 57-kD doublet), major EA(d) (BMRF1 47/54-kD diffuse smear), VCA-p40 (BDF2+BD1F1; 50 + 40-kD sharp bands), Zebra (BZLF1; 36 + 38-kD fine doublet), and VCA-p18 (BFRF1; 20 kD) on strips that contained EA and VCA. The position of EA-TK just below EBNA1 was revealed in a previous study.
EBV-antigen recognition pattern in healthy EBV carriers. Healthy blood donors from Indonesia (np 70) and control subjects (np 5) showed a highly restricted IgG reactivity pattern, as was previously found in white persons from Europe and the United States [21, 22] (figure 1B, lanes 3 and 4), that were characterized by dominant responses to EBNA1 (BFRF1; 72 kD) and VCA-p18 (BFRF3; 18 kD), with occasional weaker responses to VCA-p40 (BdRF1, 40 + 45 kD) and Zebra (BZLF1; 3638 kD). IgA-EBV reactivity was absent in all samples except in 1 Indonesian control subject with non-NPC cancer, who showed weak IgG and IgA recognition of the EA-p47/54 (BFRF1) and VCA-p40 (BFRF3) proteins (data not shown). This individual had World Health Organization class 1 squamous carcinoma, and the tumor tested negative for all EBV markers. Healthy Chinese individuals (np 40) and control subjects with non-NPC cancer (np 35) were included in the “blinded” Hong Kong panel and were identified after breaking the code. Comparable to healthy Indonesians and white EBV carriers, the healthy Chinese blood donors and control subjects revealed a similar restricted antigen-recognition spectrum for IgG that involved the immunodominant EBV proteins (specified above; see table 1). No IgA antibody reactivity was detected, except in Chinese donor 22 and control subject 17, who showed IgA reactivity to VCA-p18 (BFRF3) and weak IgAEBNA1 responses (data not shown). The restricted recognition pattern in control subjects confirms the absence of EBV activation in non-NPC head and neck cancer.

EBV-antigen recognition patterns in patients with NPC. The Indonesian NPC panel consisted of 3 patients with stage 1 NPC, 15 with stage 2 NPC, 47 with stage 3 NPC, and 105 with stage 4 NPC cancer, according to the 1997 UICC classification. The high number of patients with stage 4 NPC reflects the delayed hospitalization of patients. A limited number of representative samples are shown in figure 2 and demonstrate the results for parallel analysis of IgG and IgA reactivity on blot strips that contained both EA and VCA antigens. Additional samples from Indonesian patients were tested in parallel for IgG and IgA reactivity on blot strips that contained either EA or VCA only antigens, as shown in figure 3. The absence of immunodominant VCA-p18 and VCA-p40 bands on the EA only strips provided a dominant reactivity pattern, as was previously found in white persons from Europe and the United States [21, 22] (figure 1B, lanes 3 and 4), that were characterized by dominant responses to EBNA1 (BFRF1; 72 kD) and VCA-p18 (BFRF3; 18 kD), with occasional weaker responses to VCA-p40 (BdRF1, 40 + 45 kD) and Zebra (BZLF1; 3638 kD). IgA-EBV reactivity was absent in all samples except in 1 Indonesian control subject with non-NPC cancer, who showed weak IgG and IgA recognition of the EA-p47/54 (BFRF1) and VCA-p40 (BFRF3) proteins (data not shown). This individual had World Health Organization class 1 squamous carcinoma, and the tumor tested negative for all EBV markers. Healthy Chinese individuals (np 40) and control subjects with non-NPC cancer (np 35) were included in the “blinded” Hong Kong panel and were identified after breaking the code. Comparable to healthy Indonesians and white EBV carriers, the healthy Chinese blood donors and control subjects revealed a similar restricted antigen-recognition spectrum for IgG that involved the immunodominant EBV proteins (specified above; see table 1). No IgA antibody reactivity was detected, except in Chinese donor 22 and control subject 17, who showed IgA reactivity to VCA-p18 (BFRF3) and weak IgAEBNA1 responses (data not shown). The restricted recognition pattern in control subjects confirms the absence of EBV activation in non-NPC head and neck cancer.

Classic immunofluorescence serological data were available for the Hong Kong panel and were compared with the blot staining pattern shown in table 1. Seven white patients with NPC were at stage 3-4, and their IgG patterns are shown in figure 4A.
Virtually all patients with NPC, irrespective of their ethnic background, had an aberrant IgG antibody recognition pattern, compared with regional control subjects (figures 2 and 3). In stage 1 NPC, the IgG diversity pattern is still largely similar to that of healthy subjects and control subjects, although weak staining for both IgG and IgA were seen at EAd-p47/54 (BMRF1) in patients 10 and 15 of the Hong Kong panel (table 1). In contrast, at higher stages of malignancy, IgG antibodies showed reactivity to an increasing number of EBV proteins, including EA polypeptides p138 (BALF2), TK (BXLF1), DNAse (BGLF5), p47/54 (BMRF1), and Zebra (BZLF1). It is noteworthy that only a few patients with NPC produced IgG antibodies to the EA-R characteristic BHRF1 protein located at 17 kD, just below the VCA-p18 marker. Similarly, few samples from patients in Hong Kong with NPC were found to recognize this protein (data not shown), but none of those from the white patients with NPC did (figure 4).

Table 1. Comparison of Epstein-Barr virus (EBV) early antigen (EA) and viral capsid antigen (VCA) IgG and IgA antibody responses measured in immunoblotting for EA and dominant IgG and IgA reactive EBV EA and VCA polypeptides defined as non-overlapping, by use of samples from Chinese patients with nasopharyngeal carcinoma (NPC) and control subjects from Hong Kong.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Stage</th>
<th>EA IgG</th>
<th>VCA IgG</th>
<th>EA IgA</th>
<th>VCA IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC 1</td>
<td>1</td>
<td>-</td>
<td>640</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>NPC 2</td>
<td>2</td>
<td>40</td>
<td>640</td>
<td>-</td>
<td>640</td>
</tr>
<tr>
<td>NPC 3</td>
<td>3</td>
<td>10</td>
<td>640</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>NPC 4</td>
<td>4</td>
<td>640</td>
<td>640</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>Control subjects</td>
<td>5</td>
<td>10</td>
<td>640</td>
<td>-</td>
<td>10</td>
</tr>
</tbody>
</table>

NOTE: EA and VCA were tested at 1:100 dilutions. Immunoblots were scored (+ = +) for reactivity with the polypeptide identified by name (for EA: Zebra, p138, p54, p80, and p138; and for VCA: p18, p40, and p160).
NPC in white patients showed more-abundant IgG reactivity to EA-TK than that in Indonesian or Chinese patients. Our data reveal elevated IgG responses to the lytic switch protein Zebra (36-38-kD doublet) in most patients with NPC, irrespective of the genetic background. This was much less evident for IgA-Zebra. At higher NPC stages, the IgG reactivity for VCA-p18 and EBNA1 showed a relative increase; however, it is apparent that different individuals have different spectra of anti-EBV reactivities for both IgG and IgA antibody classes. For all NPC serum samples at NPC stages 2, the most distinctive IgG reaction, compared with control samples, was directed against the early antigens EA(d)-p47/54 (BRLF1), DNAse (BGLF5), and TK (BRLF1) protein (figures 2 and 3). The increasing diversity of antibody responses against EBV proteins at higher stages of malignancy reflects viral replication that is associated with NPC tumor growth.

**Comparison of IgG and IgA reactivity patterns in patients with NPC.** Parallel analysis of antigen-recognition patterns for IgG and IgA antibodies in serum samples from the Hong Kong and Indonesian patients with NPC were done. Direct comparison of the individual NPC serum samples revealed a clear overall dissimilarity of EBV antigens recognized between IgG and IgA antibodies, as shown in figure 2. IgG reactivity does not seem to increase significantly with NPC stage, as was observed for IgG, but is, rather, more variable among individuals. Although IgA reactivity to EBNA1 and VCA-p18 was most frequently observed, the distribution and intensity of additional IgA reactive bands varied considerably among patients. For instance, some serum samples with strong IgG responses to multiple EBV proteins showed hardly any IgA response, as revealed by NPC 27 and 31 from the Indonesian panel (figure 2). The reverse situation, with dominant IgA reactivity, was found in patients 3 and 8 with NPC in the Indonesian panel (figure 2). Although IgG against Zebra was frequently detectable, IgA reactivity to Zebra was not predominant in the Hong Kong and Indonesian NPC groups. Overall, IgA responses frequently displayed a different pattern than IgG in the same sample. This implies that IgG- and IgA-producing B cells are triggered by different antigens or antigen fragments (epitopes), possibly at different locations in the body.

**Comparison of IFA antibody titer and immunoblot detection.** Table 1 shows an overview for the serum samples from patients with NPC from Hong Kong of IgG and IgA antibody titers to EA and VCA, as determined by routine IFA testing, in combination with a listing of the major EBV-specific antigen bands for each serum, defined by IgG and IgA immunoblot. The overall data showed a lack of correlation between IFA titer and immunoblot reactivity pattern to individual EBV proteins for either an IgG or IgA response. In some serum samples, high titers in IFA (e.g., NPC 25) were related to antibody recognition of only a limited number of EBV proteins, whereas, in other serum samples with similar titers, the results of immunoblot revealed the recognition of multiple EBV polyproteins (e.g., NPC 9). In reverse, some serum samples with low IFA titers (e.g., NPC 31) bound to multiple EBV proteins, as revealed by immunoblot analysis. This result is in agreement with those of recent studies that compared EBV recombinant line-blot and IFA results [40] and clearly reflects that IFA titers provide only limited information about the true diversity of anti-EBV responses in patients with NPC.

**Follow-up case.** In figure 4R, the follow-up analysis of a white patient with stage 4 NPC is presented. Sampling started at the end of combined chemoradiation therapy and continued at 3-month intervals for 15 months. In this patient, dominant IgG responses were directed against the TK and Zebra proteins and relatively minor, but diagnostically significant, responses to EA-p138, DNAse, and EAd-p47-54 proteins. The overall IgG diversity pattern in this patient remained stable over time but showed a gradual reduction in staining intensity, which reflects a waning antibody response to EBV lytic proteins (VCA and EA). This was paralleled clinically by complete clinical remission after 15 months of follow-up.
DISCUSSION

EBV-associated diseases are characterized by distinct antibody patterns to various EBV-determined antigen specificities, as defined by IFA serological testing [8, 41]. Aberrant levels of EA and VCA-reactive IgG and IgA antibodies can be detected in the serum and saliva of patients with NPC at early stages of the disease [8, 12, 19, 29, 42]. The precise location of the NPC-related EBV lytic gene expression that triggers antibody responses remains undefined. Zhang et al. [36] showed that EA and VCA expression associates with sporadic epithelial cell differentiation within the NPC tumor, which might trigger characteristic IgA antibody responses, but additional sites of lytic EBV replication may exist. Murphy [43] proposed that serum IgA results from spillover at mucosal sites, but IgA antibody responses are triggered differently. Multiple examples in figures 2 and 3 reveal that IgA and IgG responses are triggered differently. At present, the overall molecular diversity of systemic EBV-specific IgG responses is rather unexplored, and a direct comparison of the molecular fine specificity of NPC-related IgG and IgA responses in patients of different geographical and ethnic origin is lacking. Previous studies have addressed molecular aspects of EBV serology for the diagnosis of NPC, by use of single purified proteins or related peptides, such as DNase [25-27], EA-D-p138 [44], EAd-p7/54 [17, 30], TK [20], Zebra [16, 30, 31, 45], EBNA1 [17, 19, 28, 31], and VCA-p18 [19, 29]. In the present study, we used the immunoblot technique [21, 22], which allows side-by-side analysis of IgG and IgA reactivity against nearly the full spectrum of EBV proteins. Our results in Southeast Asian blood donors extend previous findings in whites from Europe and the United States, which have shown that healthy EBV carriers and patients without EBV-linked diseases have a highly restricted IgG antibody diversity, regardless of their geographic origin [2123, 37]. EBV-reactive IgA was not detected in most EBV healthy carriers, except for an occasional response to either VCA-p18 or EBNA1. This uniform response to a limited set of EBV proteins reflects the well-balanced virus-host relationship [22]. Compared with those in healthy carriers, significantly different diversity patterns are found in patients with acute and chronic EBV syndromes, including infectious mononucleosis [2123, 37], HD [46], and NPC (present study). The antibody-recognition pattern in patients with NPC differs from that in patients with other EBV syndromes and reflects the distinct underlying viral activity in NPC. Of importance, similar IFA antibody titers in different EBV-associated diseases may represent different antibody diversity patterns, the latter of which more directly reflect different EBV involvement. Thus, the immunoblot system provides a more detailed insight into virus-host interaction in different disease syndromes. The overall EBV-specific IgG antibody reactivity tends to increase with NPC stage, in line with the results of previous serological studies that have used IFA testing [6, 8], and the IFA titer does not reflect the underlying antibody diversity. Patients with stage 1 NPC showed restricted responses, largely without IgA, that were comparable to those of healthy EBV carriers and patients with non-EBV-related malignancies. Although EBV is involved in the early and premalignant stages of NPC [6], this may proceed without keratinization and lytic gene expression and, thus, not trigger IgA EA and VCA antibody responses [36]. The lack of reactivity to EA-R-p17 (a BHRF1-encoded bcl-2 homologue) is in agreement with the results of previous IFA studies [8] and with recent data on the expression of BHRF1 in NPC [47]. Patients with NPC have rather limited antibody responses to the tumor-associated latent membrane proteins LMP1 and LMP2 [23, 46, 48], as has also been found in HD [46]. However, EBNA1 protein released from lysed NPC tumor cells induces strong anti-EBNA1 responses. The biological and immunological basis underlying different immune responses to individual EBV proteins remains a subject for further study.

It is generally assumed that IgG and IgA responses to EBV proteins have similar antigen reactivity. However, our results indicate clearly that IgG and IgA responses are triggered differently. Multiple examples in figures 2 and 3 reveal that IgG and IgA in the same serum bind to different sets of EBV proteins. In addition, no correlation was found between IFA titer and the number of EBV protein bands.
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identified by immunoblot (Table 1), which indicates that both techniques detect different sets of antibodies binding to different EBV-encoded proteins or epitopes. Immunoblot reactivity more directly reflects the biological activity of EBV in patients with EBV-linked diseases. As is shown in Figure 4, a white patient with a complete clinical response after combined chemoradiation therapy showed decreasing antibody reactivity over period of 15 months. Thus, antibody profiling by immunoblot may be used as a prognostic marker.

In conclusion, our results show that the molecular complexity underlying anti-EBV antibody responses in patients with NPC differs significantly from that of healthy EBV carriers and patients with non-NPC cancer. The antigen-recognition patterns of both IgG and IgA increases with NPC stage, most significantly stage "2. The EBV antigen diversity of IgG and IgA varies considerably between individual patients with NPC and seems to be driven by different antigen-triggering events. The EBV immunoblot diversity pattern has significant value for discriminating between NPC and non-NPC tumors and provides valuable information for the development of molecularly defined EBV serology.

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References

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