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

Jajah Fachiroh, Tabitha Schouten, Bambang Hariwiyanto, Dewi K Paramita, Ahmad Harijadi, Sofia M Haryana, Mun H Ng, and Jaap M Middeldorp

1 Gadjah Mada University, Yogyakarta, Indonesia
2 Department of Microbiology, Queen Mary's Hospital, Hongkong, China
3 Department of Pathology, Vrije Universiteit Medical Center, Amsterdam, The Netherlands

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Abstract

Epstein-Barr virus (EBV)-specific immunoblot analysis was used to reveal the molecular diversity of immunoglobulin (Ig) G and IgA antibody responses against Epstein-Barr nuclear antigen (EBNA), early antigen (EA), and viral capsid antigen (VCA) in serum samples from patients with nasopharyngeal carcinoma (NPC) and control subjects, by use of immunofluorescence assay (IFA). Control donors (n=150) showed IgG responses to few EBV proteins VCA-p18, VCA-p40, EBNA1, Zebra - and sporadically weak IgA reactivity to EBNA1 and VCA-p18. Patients with NPC stage 1 (n=6) had similar response patterns. Patients with NPC stage 24 (n=132) showed significantly more diverse IgG and IgA responses to EA and VCA proteins-VCA-p18/-p40, EBNA1, Z-encoded broadly reactive activator; and EAd-p47/54,-DNAse, thymidine kinase, and -p138. No correlation was found between IFA titers and the number of EBV proteins recognized by IgG or IgA. Our results reveal dissimilarity between EBV polypeptides recognized by IgG and IgA Antibodies, which suggests 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 early 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 reveal the molecular diversity of immunoglobin (Ig) G and IgA antibody responses against various EBV proteins and antigen complexes [8]. Undifferentiated NPC is 100% associated with EBV and forms an unusual tumor with intriguing epidemiological and biological characteristics [6,10]. The highest incidence is found in persons of Chinese ethnicity living in southern China, Hong Kong, Taiwan, and Singapore. Intermediate incidence occurs in certain African and Mediterranean populations, in Inuits from Greenland and Alaska, and in Malays from Singapore and Malaysia. A low incidence is found in American and European whites, Hispanics, and Japanese. In Indonesia, NPC has an overall intermediate incidence (3.9 cases/100,000 population) similar to that in Malaysia. However, in the Yogyakarta area, NPC constitutes 21.8% of tumors in men and 7.9% of tumors in women, which ranks NPC as the most frequent tumor in men and the fourth most frequent tumor in women [11]. The results of seroepidemiological studies have indicated a close relationship between EBV infection and NPC, as revealed by elevated IgG and especially, IgA responses to EBV viral capsid antigen (VCA), early antigen (EA), and Epstein-Barr nuclear antigen (EBNA) complexes [8, 12]. Elevated total and EBV-specific serum IgA levels are indicative of NPC stage [8, 13, 14] and can precede tumor development by 15 years, which suggests that a reactivation of EBV infection plays a role in tumor development [14, 15]. In addition, a decline in anti-EBV antibody responses after radiotherapy may have prognostic value [16]. At present, the indirect immunofluorescence assay (IFA) is still used as the reference standard for the serodiagnosis of EBV in NPC [2, 8, 12, 14]. This method, however, is difficult to standardize and is not suitable for large-scale testing in developing countries; it is gradually being replaced by more-defined EIAs [17-20]. Of importance, IFA does not provide insight into the molecular basis of anti-EBV responses, because EBV-infected cells each contain a multitude of different EBV proteins that can serve as the target for antibody interaction [21-23].

Recent molecular serological testing approaches in the diagnosis of NPC have focused on the use of defined recombinant EBV proteins. Tedeschi et al. [24] showed that antibodies against the Z-encoded broadly reactive activator (Zebra) protein are regularly found among patients with NPC. Others have proposed the anti-Zebra IgG antibody titer to be a prognostic marker for NPC [16]. However, anti-Zebra IgG antibodies are detectable in 74% of healthy EBV carriers, according to the results of a sensitive immunoblot assay [22]; this has been confirmed by exchanging blind serum samples (I. Joab and J.M.M., unpublished data). EBV DNAse neutralizing antibodies have been found in 83%-94% of patients with NPC [25], and they appear to be a good marker for NPC screening and prognosis. However, there is no correlation between the level of anti-DNAse antibodies and antibody titer to VCA or EA-D, according to the results of IFA serological testing [26]. Stolzenberg et al. [27] detected IgA antibody against recombinant DNAse in patients with NPC but rarely in patients with other EBV-related malignancies. Shimakage et al. [28] suggested the use of the EBNA1-IgA serum level as a prognostic marker for monitoring patients with NPC after radiation therapy, and Fooong et al. [29] suggested the use of serum and salivary IgA levels against an EBNA1 Gly-Ala repeat peptide as a suitable NPC marker. Connolly et al. [30] suggested the use of thymidine kinase (TK) as the antigen in IgA-ELISA for the diagnosis and screening for NPC. Dardari et al. [31] indicated that the combination of IgG-Zebra and IgA-EB [p54]+(p138) should be used. Most recently, Chan et al. [31] proposed the combined use of EBNA1-IgA and Zebra-IgG as the best predictor for NPC. It may be obvious from the above that there is no consensus on the use of defined EBV proteins in serological testing for the diagnosis and prognosis of NPC.

Still very little detail is known about the overall molecular diversity (complexity) of anti-EBV IgG and IgA antibody responses in patients with NPC [23]. Moreover, a comparison of antibody profiles among patients with NPC who 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 Japanese (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 responses revealed differences in EBV antigen recognition profiles, which suggests independent B cell triggering.
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. Sardijsjo General Hospital, Yogakarta), 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) 1-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, and concomitant expression of late antigen (VCA), as revealed by the absence of the LMP 1-specific monoclonal antibodies OT1X [32] and OT21C [23, 33], respectively.

Serum samples from 7 white patients with NPC were obtained from hospitak 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 OT138 (anti-EA-p138; BALF2) [34], rabbit anti-EBNA1 (BKRFI) [35], rabbit anti-DNAase (BGLF5) [27], OT146 (anti-EA-p47; BMRF1) [36], BZ-1 (anti-Zebra; BZLF1) [37], OT41A (anti-VCA-p40; BDRF1) [38], and OT15E (anti-VCA-p18; BBRF3) [39].

**Cell culture and antigen preparation.** The superinducible 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.

**SDS-PAGE and immunoblot analysis.** The nuclear fractions were sonicated and boiled for 5 min in standard Laemmli buffer and clarified by centrifugation at 14,000 g. Polypeptides were separated by SDS-PAGE in 10% acrylamide gels by use of the Bio-Rad mini-gel system (Bio-Rad) and transferred onto 0.2-μm nitrocellulose membranes (Schleicher & Schuell) that were subsequently cut into 3-mm strips. Marker proteins (Bio-Rad Low MW Marker) were run on the side of the gel. Blot strips were immersed for 1 h in blocking buffer (5% [vol/vol] horse serum [Gibco BRL] and 5% [wt/vol] nonfat dry milk in PBS), to prevent nonspecific binding. For IgA detection, serum samples were treated with GelSorb (Meridian Diagnostic) to remove IgA antibodies, as described by the manufacturer.

In all experiments, human serum samples were tested at 1:100 dilution in blocking buffer and incubated with the strips for 1 h at room temperature. Subsequently, the strips were washed 3 times with PBS that contained 0.05% Tween-20 (PBS-T), and horseradish peroxidase (HRP)-conjugated anti-IgG or anti-IgA antibody (DAKO) was added in appropriate dilutions and incubated for 1 h at room temperature. After washing 3 times with PBS-T and 2 times with PBS, bound HRP was visualized by 0.07% 4-chloro-1-naphtol and 0.01% (vol/vol) H2O2 in PBS. Stained strips were washed overnight with 10 ml of H2O, dried, and stored in the dark until photography.

The position of characteristic EBV antigens was defined by monoclonal or polyclonal antibodies of known specificity (figure 1), which were detected with HRP-labeled anti-mouse or anti-rabbit antibodies (DAKO). In addition, from every batch of blot strips, 3 random strips were stained with reference human serum samples from a healthy seronegative and seropositive donor and serum from a patient with severe, chronic EBV infection [22, 23].

**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 late antigen (VCA), as revealed by the absence of the VCA-p40 (BDRF1) and VCA-p18 (BBRF3) 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 (BKRFI; 72 kD), EA-DNAase (BGLF5; 55 + 57-kD doublet), major EA(d) (BMRFI 47/54-kD diffuse smear), VCA-p40 (BDRF2+BDRF1; 50 + 40-kD sharp bands), Zebra (BZLF1; 36 + 38-kD fine doublet), and VCA-p18 (BBRF3; 20 kD) on strips that contained EA and VCA. The position of EA-TK just below EBNA1 was revealed in a previous study.
[22]. On strips that contained EA only, the VCA-p18 and VCA-p40 bands were absent, but EA and EBNA1 bands were detectable. EBV marker proteins were defined on the basis of size, staining pattern (doublet, diffuse, or sharp band), and EBNA, EA, and VCA characteristics. Strips that contained BJAB nuclear extract did not show any bands. In all experiments, human reference serum samples were used as controls (figure 1B). These included 2 EBV-negative serum samples as specificity controls (samples 1 and 2); 2 serum samples from healthy seropositive donors (samples 3 and 4), to represent “normal” staining patterns observed in most healthy carriers worldwide [22, 23]; an infectious mononucleosis serum sample with the characteristic dominant EA-D p47/54 band [21] (sample 5); a serum sample from a patient with chronic, severe EBV infection (sample 6); and a sample from a patient with NPC (sample 7), to represent “strong” positive staining control. These serum samples reproducibly gave identical banding patterns and staining intensities on different batches of EA and EA-plus-VCA blot strips.

**Fig. 1.** Immunoblot reactivity patterns with reference antibodies. (A), Antigen applied to the blot: polypeptides from the nuclear fraction of HH514.c16 cells induced to express early antigen (EA) and Epstein-Barr virus (EBV) viral capsid antigen (VCA) (V, VCA induced), EA only (E, EA induced), and an EBV-negative cell line (C, EBV negative). The monoclonal antibodies used to panel A are specific for EA-p138, Epstein-Barr virus nuclear antigen (EBNA1), DNAse, major EA-D p47/54, VCA-p40, Z-encoded broadly reactive activator (ZEBRA), and VCA-p18, as detailed in Subjects, Materials, and Methods. (B), Characteristic IgG (G) and IgA (A) reactivities for a set of human reference serum samples from EBV-negative donors (1 and 2), healthy EBV carriers (3 and 4), a patient with mononucleosis (5), a patient with severe chronic EBV infection (6), and a patient with stage 4 nasopharyngeal carcinoma (7). MW, molecular weight.
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 EΔd-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 (BRLF1), 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 4A).
Follow-up case. In figure 4, 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.

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, IgA 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 IgG 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 polypeptides (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.

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 (30–38 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 (BMLF1), DNAse (BGLF5), and TK (BBLF1) 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.

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At present, the overall molecular diversity of systemic EBV-specific IgA 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-p47/54 [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], but 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 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 lyed 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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