Chapter 4

Native Early Antigen of Epstein-Barr Virus, A Promising Antigen for Diagnosis of Nasopharyngeal Carcinoma

Dewi K Paramita¹, Jajah Fachiroh¹, Wayan T Artama¹, Eric van Benthem¹, Sofia M Haryana³, and Jaap M Middeldorp³

¹Department of Histology and Cell Biology, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia
²Faculty of Veterinary, Gadjah Mada University, Yogyakarta, Indonesia
³Department of Pathology, VU University Medical Center, Amsterdam, the Netherlands

Journal of Medical Virology 2007; 79: 1710-1721
Early sero-epidemiological studies indicated a close relation between EBV infection and NPC as revealed by elevated IgG and IgA antibody titers to viral capsid antigen (VCA), early antigen diffuse (EAd) and nuclear antigen (EBNA) complexes (18). NPC patients are most specifically characterized by elevated levels of EBV-reactive IgA responses, reflecting the tumor’s origin in the mucosa of the nasopharynx (18, 19, 50). Although EBV gene expression in NPC tumor cells is largely confined to the latency-II program, small nests of differentiating tumor cells may provide a local source of EA and VCA antigens driving the characteristic IgA responses (14, 56). The outstanding relevance of EA-IgA antibodies for the diagnosis and prognosis of NPC was first claimed by the Honj’s (18), but somewhat disputed in more recent publications (12, 36, 51). EBV-specific IgA antibodies, in particular EA-IgA increase with stage of diseases and tumor burden at presentation and decrease with successful therapy. Re-appearance or increase in EA-IgA is indicative of tumor relapse and development of distant metastasis (8, 20, 22, 32, 38).

For NPC-related serodiagnosis, indirect immunofluorescence assay (IFA) methods are still used widely as golden standard, but these are time consuming and poorly suitable for large scale automated handling (15, 41, 51). EBV ELISA is increasingly used, with sensitivities and specificities comparable to IFA (7, 11, 16, 22, 43). Development of ELISA requires the availability of high quality purified EBV antigens and in recent years many studies addressed this topic using either defined extracts from EBV producer cells (11, 12, 43, 49), purified recombinant proteins (1, 3, 5, 7, 24, 39) or synthetic peptides as antigen (2, 13, 45). Recently, we described an EBV-IgA screening assay based on a combination of VCA-p18 and EBNA1 derived synthetic peptides that might fulfill the criteria for a cheap first line NPC screening test [13]. However, an independent serological test based on EA-only might be useful for confirmation as well as for post-treatment prognostic monitoring in NPC (22, 38).

Multiple studies have addressed the value of individual EA marker proteins for NPC diagnosis, but no consensus has been obtained thus far (3, 5, 7, 15, 21, 24, 39). Reported sensitivity and specificity values show considerable variation from laboratory-to-laboratory. Recently the molecular diversity of EBV-specific IgG and IgA responses in NPC patients from different geographical origins were characterized and noticed that IgG and IgA antibodies in individual NPC patients have frequently different antigen specificity, reflecting distinct B-cell triggering events (14). This, in part, may explain the diverse results in previous studies. Comparing the molecular diversity for EA-IgG responses in NPC patients, with acute mononucleosis and chronic EBV infection, a clear difference in antigen-recognition pattern is seen (26, 31, 44). Such underlying diversity in antibody reactivity is not revealed by IF-serology. Considering the diagnostic relevance of EA-specific antibody responses in NPC patients and the need to improve further the sensitivity and specificity but also affordable serological methods in high incidence regions of the developing world, we explored further the diagnostic value of defined EBV-EA markers, produced as recombinant proteins or synthetic peptides, using EA-specific extracts prepared by differential salt extraction from induced HHSiS16c16 cells as reference. We used ELISA, immunofluorescence and
method of Dolken et al. [1986] (10) who used high concentration of salt (1-4 M NaCl) to extract the EAd p52 from the EBV producer cells. In initial experiments, purified EA nuclei were centrifuged at 13,000g for 5 min and the pellet was resuspended in 675 ml PBS with 75 ml 1.5 M NaCl (0.15 M NaCl) and incubated on a rotary shaker at ambient temperature for 1 hr. The mixture was centrifuged at 13,000g for 5 min. The supernatant was collected as 0.15 M NaCl and the pellet was resuspended in 600 ml PBS plus 150 ml 1.5 M NaCl and incubated on a shaker at RT for 1 hr. The supernatant collected from the second step is the extract of 0.3 M NaCl. This process was repeated by increasing the concentration of NaCl until 1.05 M in the extract. For routine use in DNA-ELISA the solubile 0.3 M NaCl EA-nuclear extract was used.

Recombinant EA Proteins. Constructs expressing full-length TK (BGLF5), DNase (BGLF5), EA-p47/S4 (BMRF1) and ZEBRA (BZLF1) were made by cDNA cloning of intact open reading frames (ORFs) from EA-induced B95-8 (EBV type-1) mRNA into pcScript plasmids using the pcScript-II cloning kit (Stratagene, Cedar Creek, TX) followed by sequencing (ABI310, Applied Biosystems, Foster City, CA) using standard procedures. Oligos containing the correct sequences were either used for expression in E. coli or reckoned into Fastbac vectors (Invitrogen, Venk, The Netherlands). In addition to home made E. coli recombinant proteins, commercial purified EAd-p47/S4-MRP fusion protein was obtained from Biosource (Nivelles, Belgium) and Ross Southern labs (Salt Lake City, UT). Furthermore purified E. coli derived EAd-p47/S4 and EAd-p138 autolysin fusion protein (BALF2), as used in the EBV line assay, were kindly provided by Milcheck AG (Neuried, Germany). Purified Baculovirus-derived DNase from P3HR1 background (EBV type-2) was kindly donated by T. Ooka (Lyon, France). Insect cells were cultured in serum-free SF900-II medium at 28°C. Baculovirus cDNA stocks were prepared by plaque purification on SF9 insect cells and propagation at low mode of infection (MOI) of 0.1 plaque forming units (PFU) per cell. For recombinant proteins production, a high MOI was used of 1-5 PFU/cell and cells were harvested at 48 hr post infection (pi).

EBV Synthetic Peptides. Immunodominant epitopes on Ebv proteins EAd-p47/S4 and -p138 were defined as described before (24, 27, 43). Briefly, from the predicted amino acid sequence of the BRLF1 and BFLF2 open reading frames on the EBV genome all possible 12-mer peptides with an overlap of 11 were synthesized on polypropylene pins and tested by PeptideScan. Individual EBV-EA reactive Mabs were mapped for their specific epitope reactivity and served as positive control. Unique clusters of human IgG, IgM, and IgA reactive immunodominant epitopes were defined for the individual EA proteins using sera from patients with different EBV disease syndromes. Individual 30-mer polyepitope peptides were synthesized by FMOC chemistry and purified by HPLC (Neosystem, Strassbourg, France). Occasionally, separate 30-mer peptides were covalently linked by S-S linkage (43). The S-S linked combi-peptides were once more purified by HPLC to achieve >90% purity and all peptides were stored as powder at -20°C until use.
Immunofluorescent (IF) Staining. For IF experiments, SP9 cells were infected with either wt baculovirus or recombinant baculovirus at 1 PFU/cell for 48 hr, leaving about 55% of uninfected cells in the preparation which served as internal specificity control. IF slides were fixed in cold acetone and preincubated in PBS/2% FCS for 10 min. All washings were done three times in PBS/0.05% Tween-20 (PBS-T) and primary Ab dilutions were made in PBS supplemented with 1% FCS and 1% normal rabbit serum and all incubations performed at RT. Monoclonal antibodies were diluted in 1:1000 and incubated for 1 hr. After washing the slides were incubated with rabbit anti-mouse IgG/FITC (Dako) diluted 1:100 for 30 min, followed by washing and counterstained with DAPI (Parteck, Enthuizen, The Netherlands) in 0.5% Evans blue, sealed in IF mounting fluid and evaluated with Leica DMIRB fluorescence microscope (Leica, Cambridge, England).

Determination of Protein Concentration. Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL) using BSA as a standard according to the manufacturer’s instructions.

SDS - PAGE and Western Blot Analysis. Protein extracts were solubilized and separated under standard reducing conditions in 10% gels using the Mini Protean II system (BioRad, Hercules, CA). Proteins were transferred onto 0.2-μm nitrocellulose (Schleicher & Schuell’s, Hertogenbosch, The Netherlands) by Western blotting (Mini-Trans bkt cells, BioRad). After transfer, the nitrocellulose sheets were washed with H2O, dried between filter paper and stored at 4°C until use. 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 monoclonal or polyclonal antibody or sera in blocking buffer. Specific antibody binding was detected with horseradish peroxidase (HRP)-conjugated second antibody (Dako, Glostrup, Denmark) in blocking buffer as described before (43).

Recombinant EBV Line Immunoassay. The Mikrogen EBV RecombLine immunoassay (Mikrogen) was used to validate human IgG and IgA antibody responses to purified EBV antigens expressed recombinant EBV proteins according to the manufacturer’s instructions.

ELISA to EA Extract. Individual wells of standard ELISA plates (Greiner, Frickenhausen, Germany) were coated at 4°C overnight with 150 μl EA extract at 1 μg/ml in 50 mM sodium carbonate buffer, pH 9.6. Non-specific binding was blocked for 1 hr with PBS/3% BSA at 37°C. Serum and conjugate incubations were performed for 1 hr at 37°C followed by four washes with PBS. Human sera were diluted 1:100 in dilution buffer (PBS, 0.1%Tween-X, 1% BSA and 1% normal rabbit serum). HRP-labeled rabbit anti-human IgG and IgA (Dako, Copenhagen, Denmark) were used at 1:6,000 and 1:4,000 in dilution buffer, respectively. HRP activity was detected by 3,3’,5,5’-tetramethylbenzidine (bioMerieux, Biotel, The Netherlands) and stopped by adding 1 H2SO. Optical density was determined at 450 nm (OD450) and cutoff values were determined by the mean OD450 of 4 EBV-negative sera plus 2 S.D.

Epitope Linearization and Test for Avidity. EA epitopes were denatured by boiling the EA extract in coating buffer Na2CO3, prior to coating. The test for avidity was done in two ways; first by washing after the serum incubation with washing buffer containing 8 M urea (17), and secondly by diluting and incubating the sera in dilution buffer containing 8 M urea.

Data Analysis. All statistical analysis was done by GraphPad Prism Ver. 6.00 program (San Diego, CA). Cut off values were determined by the ROC analysis based on the OD value of sera from healthy donors and NPC patients (6).

RESULTS

Antibody Responses to Individual Recombinant EA Proteins. Our prior studies indicated that for serological studies expression of EBV proteins in the baculovirus system was preferred by E. coli due to the low levels of antibody reactive with insect cells and baculovirus proteins in human sera (26). Individual full-length recombinant EBV proteins with predicted diagnostic value, that is, TK (HXL1-F), DNase (BGLF5), and ZEBRA (BZLF1) were expressed in Sf9 insect cells and harvested at 48 hr postinfection. Recombinant baculovirus deleted of the gly-la repeat, was used as control (28). Protein expression was analyzed by immunofluorescence (IF) and immunoblot (IB) techniques to assess antibody binding to native and denatured epitopes, respectively.

In a first series of IF staining, sera were selected for strong reactivity and high diversity IgG and IgA antibody pattern (14). Overall, IF results showed rather low (~<1:100) IgG reactivity with DNase and rTK in only 33% and 50% of sera tested, respectively, whereas IgG to rZEBRA was observed at somewhat elevated titer (<1:200) in 90% of the sera tested. IgG to rEBNA1 was present at higher titers (~>1:200) in 100% of the sera. In simultaneous IF analysis, IgA reactivity to rDNase, rTK, and rZEBRA was observed at lower titer (~<1:50) and at lower frequency in only 10%, 10% and 30% of the sera, respectively. IgA to rEBNA1 was observed at slightly higher titer (1:100) in about 60% of the sera (data not shown). Subsequently, a set of NPC sera (n = 101) was tested for IgG and IgA by IB analysis using individual baculovirus-expressed EA proteins: rTK, rDNase, and rZEBRA and E. coli expressed purified recombinant EAD-p47/F4 and EAD-p138 proteins (Fig. 1). Monospecific antibodies were used to define antigen-specific bands on IB as reference for specificity with human sera (Fig. 1A). Reactivity with the “golden standard” HHF35-F/P5 extract, used as positive control in all experiments (Fig. 1B,C, strips E), has been described before (14). Healthy donors showed significant different responses compared in NPC patients (Fig. 1B,C).
Chapter 4

Figure 1

Immunoblot analysis of HH514 VCA antigen and individual recombinant EA proteins using monospecific antibodies and representative sera from NPC patients and healthy controls. Standardized HH514 VCA EA nuclear extract and individual rEA proteins (T, D, E1, E2, and Zebra) were separated by reducing gel electrophoresis and transferred onto nitrocellulose filters. These were subsequently cut into 3-mm strips and used for incubation with antibodies and human sera [Fachiroh et al., 2004]. Panel A shows the staining pattern of using monospecific antibodies to reveal the location and banding pattern for each rEA protein on the blot. Panel B shows the characteristic diverse IgG staining patterns for 4 representative NPC sera on the standard HH514 VCA reactivity is shown for the individual rEA proteins, identified as T, D, E1, E2, and Zebra, respectively. Interestingly, whereas most NPC sera showed diverse reactivity on HH514 antigen, reactivity to EA was mostly limited to T, E1, and Z proteins. Panel C shows the analysis for IgG antibodies in sera from healthy EBV carriers (HD1-4) revealing a much more limited reactivity pattern, mainly restricted to VCA-p18 and EBNA1 proteins, without reactivity to the EA proteins, as described before [Mukkheap and Hertsinsk, 1998; van Grunsven et al., 1999b].

| Table 1. IgG responses of NPC patients (NPC) and healthy blood donors (normal) to individual EBV-EA recombinant proteins tested by IB using recombinant baculovirus-infected SF9 insect cells |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                               | rTK             | rDNase          | rEA(D)-p47      | rEA(D)p138      | ZEBRA           |
| NPC (n=101)                   | 52.9            | 17.3            | 90.7            | 76.9            | 84.6            |
| Normal (n=37)                 | 0               | 8.6             | 5.7             | 1.7             | 11.4            |

None of healthy donor sera (n=37) showed IgG reactivity to rTK and only few gave a generally weak positive response to other EA markers; that is, 8.6%, 5.7%, and 11.4% to rDNase, rEA-p47 and ZEBRA, respectively (Table 1). NPC sera showed significant more IgG reactivity to recombinant EA proteins, that is, 53% to rTK, 17% to rDNase, 90.7% to rEA-p47, 76.9% to rEA-p138 and 84% to ZEBRA. NPC sera produced more intense bands than healthy EBV positive controls. NPC sera were also tested for IgA reactivity revealing different responses to the individual rEA proteins as compared to IgG. Ninety percent of NPC sera gave both IgG and IgA responses to rTK and 68.5% gave both IgG and IgA response to rEA-p138 and 53.7% to rEA-p138, whereas fewer NPC sera with IgG to rDNase and rZEBRA showed IgA reactivity to these proteins, that is, 45.5% and 27.1% respectively (data not shown). rEA-p47 and -p138 reactivity were confirmed in separate experiments using a commercial line immunoblot based on E. coli expressed purified proteins [Paramita et al., 2004, manuscript in preparation]. Interestingly, for all rEA proteins analyzed there was no correlation between IgG and IgA reactivity proteins in individual sera, in line with our prior observations in HHS14 cell extracts (14).

Antibody Response of NPC Sera to EA Derived Peptides. A significant number of NPC sera showed reactivity with denatured EAD proteins p47/p54 and p130, thus potentially interacting with linear epitopes. Therefore, NPC sera were also tested against a selected set of synthetic peptides comprising combined epitopes of these immunodominant EA proteins. These epitopes were previously mapped by PEPSAN for reactivity with IgG and IgM antibodies in sera from patients with acute IM or chronic EBV syndromes (27). In previous studies these peptides were shown to react with IgG (40% for both p47 and p138; n=40) and IgA (p47+55%; p138+62%, n=38) antibodies in sera from a limited number of NPC patients (24). Most of the 101 NPC sera tested in this study contained IgG antibodies reactive with one or multiple synthetic EA peptides, similar to IM and chronic EBV sera and thus being non-discriminatory for NPC. Contrary, only about 50% showed significant IgA reactivity in these peptides (data not shown). Because of the restricted and usually low IgG responses to EA peptides we did not pursue further analysis and concluded that IgA responses may only involve marginally interaction with linear (peptide) epitopes on EBV EA proteins.

Native EA Extract Production. Prior studies showed that EAD complex antigens for diagnostic use could be made by high salt extraction from virus producer cells like P3HR1 and B95-8, requiring further purification by ion-exchange or affinity chromatography (10, 42, 43). We pursued this approach by extracting the nuclear fraction of EA induced HHS14.c16, a cell line previously shown to yield very high levels of EA antigen (31). Using stepwise increases of 0.15-1.05 M NaCl, native EA proteins were prepared in a simple 2-step procedure removing virtually all cellular and EBNA1 proteins. Cellular (histone) proteins were predominantly extracted at higher salt concentration as shown in Figure 2A. The EBV proteins in the EA extract were revealed by IB analysis using a poly-reactive serum from a NPC patient (Fig. 2B) and defined monospecific antibodies (Fig. 2C). Most relevant EAD reactivity...
Chapter 4

was present in the 0.15 - 0.3 M extracts. The 0.3 M NaCl extract contained all relevant EA marker proteins, that is, TK, DNAse, EA-p47/S4, EA-p130 and ZEBRA (Fig. 2C, lanes 1-4). Interestingly, the 0.3 M NaCl extract did not contain any EBNA1 (Fig. 2C, lane 5), which eluted partly at 0.15 M NaCl and mostly at >0.75 M NaCl concentrations, as demonstrated by EBNA1-specific MoAb staining (data not shown). No VCA proteins were detectable, indicating effective blocking of viral DNA-polymerase by using PAA (Fig. 2C, lane 6). Therefore, 0.3 M NaCl extraction of purified EA nuclei yielded a fraction predominantly consisting of relevant EA marker proteins, which was further evaluated for use in ELISA.

Native EA ELISA in NPC and Regional Controls. Conditions for EA-extract coating, non-specific background blocking, serum and conjugate dilution were optimized yielding the procedure described in Materials and Methods. Since IgG-EA present in NPC sera might interact with identical epitopes as IgG-EA, serum reactivity was compared with and without prior IgG removal using GuHCl, which previously was effective for enhancing EBV-IgM detection in ELISA (45). IgG removal had no effect on IgG-EA reactivity in NPC sera and occasionally no effect on IgG-EA reactivity (data not shown), confirming previous findings for IgG to VCA and EBNA1 (13) and indicating that IgG and IgA are not interacting competitively with similar epitopes on EA. To validate the diagnostic performance of the EA-extract ELISA, optimal cutoff values (CoV) were defined by ROC analysis using a large panel of sera of regional health care workers and NPC patients (6). Table II shows the CoVs and associated sensitivity and specificity values for IgG and IgA to native EA extract in a panel of 155 NPC sera and 259 health controls, the latter being all positive for IgG against VCA and/or EBNA1 (13). Overall, the results are shown in figure 3 and table III. The IgG-EA and IgA-EA ELISAs yielded positive predictive value (PPV) and negative predictive value (NPV) of 86.6%, 97.1% and 79.4%, 94.9% respectively (table II).

Native EA-ELISA and Non-NPC Patient Groups. An extract of EA was analyzed further for IgG and IgA reactivity in sera from non-NPC patients (n=40), including patients with carcinomas of breast, larynx, oral and nasal cavity and tongue, Burkitt lymphoma (n=39) and Hodgkin lymphoma (n=54), and infectious mononucleosis (n=70). IgG antibodies specific to the native EA extract were detected in 92.9% of NPC sera, while only 6.9% of healthy donors, 10% of non-NPC, and 5.6% of HD, whereas 72.9% of IM sera and 84.6% of BL sera were IgG-EA positive at low level (Fig. 3), confirming previous findings (26).

Seven of 155 (4.5%) NPC sera had no detectable IgG-EA. On the other hand, IgA-EA was detected in 143/155 (92.3%) of NPC sera, but only 14.5%, 5%, 3.7%, and 28.2% of healthy donors, non-NPC, HD, and BL sera, respectively, the latter giving mainly low OD450 values. Some IM sera (41.4%) contained IgA-EA, but all with distinct lower reactivity compared to IgG-EA, as shown in Figure 3. Twelve of 155 (7.7%) NPC sera were negative for IgA-EA. Five of these 12 samples were also negative for IgG to EBNA1+VCA-p18 in ELISA (13) and negative or weakly reactive in the EAU-p47 and -p138 by IB and Recomb-Line assay, reflecting a lack of EBV responsiveness overall.
There was no correlation between IgG and IgA reactivity to EA extract in any of the patient groups, with r² value of 0.2297, 0.00589, 0.2095, 0.04932, 0.002595, and 0.3708 for NPC, normal healthy, IM, non NPC, HD, and BL, respectively (Fig. 4). This confirms our previous suggestion that IgG and IgA responses are directed to different antigens or epitopes, triggered by distinct B-cell responses in NPC patients (14).

<table>
<thead>
<tr>
<th>Cutoff value (OD450)</th>
<th>Value for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-EA</td>
<td>0.2999</td>
</tr>
<tr>
<td>IgA-EA</td>
<td>0.2162</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensitivity (%)</th>
<th>95.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity (%)</td>
<td>95.5</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>95.6</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>97.1</td>
</tr>
</tbody>
</table>

*Cutoff value was determined by ROC analysis (Crowther, 2001).

Native EA ELISA and NPC Stage. To further evaluate the value of native EA ELISA in NPC diagnosis we studied IgG-EA and IgA-EA in Indonesian NPC patients in relation to sex, age and stage. The male/female ratio was 2.31, with an age distribution of 11-80 (mean 46.8 years). No significant age-related differences could be observed and no significant difference in mean or median values for either IgG-EA or IgA-EA was found in the 155 NPC patients analyzed (P > 0.10 for all). Most patients presented with advanced stage (87.9%; stage III or higher). Patients with stage I and IIA showed the lowest IgG and IgA responses (Fig. 5), being significantly different compared to stage III and above.

One patient with stage I NPC however had a significant IgG-EA response, with associated high diversity in IB, whereas IgG-EA was rather low. However, the numbers of patients at early stage is too low to allow statistical analysis between early and late stage NPC. At higher stage both IgG-EA and IgA-EA reactivity are high, but show a wide range. No correlation was found between IgG-EA and IgA-EA OD450 values in the same individual with NPC (data not shown).

Native EA Epitope Interaction. It was determined whether antibody reactivity to EA antigen involves interaction with conformational rather than linear epitopes. Denaturing the EA-extract by boiling prior to coating, reduced significantly both IgG and IgA reactivity, but more dramatically for IgG, and to different extents for

<table>
<thead>
<tr>
<th>Panel of sera</th>
<th>n</th>
<th>No. of positives (%)</th>
<th>No. of positives (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC</td>
<td>155</td>
<td>148 (96.0)</td>
<td>143 (93.2)</td>
</tr>
<tr>
<td>Non-NPC cancer</td>
<td>40</td>
<td>4 (10.0)</td>
<td>2 (5.0)</td>
</tr>
<tr>
<td>Healthy</td>
<td>259</td>
<td>23 (8.8)</td>
<td>37 (14.3)</td>
</tr>
<tr>
<td>HD</td>
<td>54</td>
<td>3 (5.6)</td>
<td>3 (5.9)</td>
</tr>
<tr>
<td>IM</td>
<td>70</td>
<td>9 (28.6)</td>
<td>11 (39.1)</td>
</tr>
</tbody>
</table>

*Cutoff values were determined by ROC analysis using NPC patients and the blood donor population, all being EBV carrier as defined by IgG to VCA (IgG > 100), Yu et al. 2004. Both IgG and IgA ELISA responses to EBV EA extract are significantly higher in NPC sera compared to other patient groups, which was significant (P < 0.01) for NPC versus all groups.
IgG and IgA in the same serum specimen. The mean OD450 values were 44.5% and 52.5% of the original IgG and IgA reactivity to native EA-extract, respectively. The avidity of EA antigen-antibody interaction was also evaluated by urea treatment (17). The presence of 8 M urea in the washing buffer decreased OD450 values for IgG to 50.9%, whereas IgA reactivity was reduced to 37.6%. The presence of 8 M urea during serum incubation more significantly reduced both IgG and IgA reactivities to 25.2% and 11.1% of original value, respectively, indicating a reduced avidity for IgA-EA responses. These results show that IgG and IgA responses in NPC sera involve recognition of both linear and native epitopes, the latter contributing significantly to the diagnostic performance of the EA-extract.

DISCUSSION

EBV-specific antibodies for NPC diagnosis are detected traditionally by immunofluorescence or immunoenzymatic methods using EBV transformed lymphoblastoid cell lines as antigen (18, 52). These methods are still used widely in routine practice for NPC screening and diagnosis (4, 41, 51). Particularly IgA antibodies to EBV EA have been shown to provide a valuable marker for (early) NPC diagnosis (18, 23, 54, 55), for monitoring treatment efficacy and for prediction of relapse and late distant metastasis (20, 22, 23, 32, 38). ELISA methods are becoming increasingly available for the diagnosis of NPC with similar sensitivity and specificity compared to the traditional IF method (7, 13, 16, 22). However, ELISA requires high quality purified EBV antigens and, in particular for use in developing countries, these antigens should be cheap and readily available. In recent years recombinant proteins and synthetic peptide antigens have shown potential to replace infected cell-derived antigens, but the results may be biased by irreproducible loss of natural native (conformational) epitopes. Thus far little consensus exists regarding the choice of individual EBV EA components for NPC serodiagnostic use, and initially promising markers may not be consistent (5, 7, 21, 24, 39). In this study, using different methodological approaches, disappointing results were noted initially for a range of individual recombinant and synthetic EA antigens, possibly due to lack of appropriate epitopes for interaction with IgA in NPC sera (Table I; unpublished data). For instance, we found that EBV-DNAse (BGLF5), when expressed in insect cell and purified to homogeneity, proved an excellent antigen in DNase enzyme-inhibition experiments (39) [Middeldorp and Ooka, unpublished work]. However, this same protein yielded only low antibody titers when evaluated by IFA, immunoblot or ELISA (Table I). For other EBV EA proteins less dramatic differences were found, but overall recombinant proteins and/or synthetic peptides proved inferior to EA proteins extracted from natural EBV infected cell lines. Similar results were described recently by Liu et al. (2004) (24). These findings reflect most likely the importance of native epitopes or post-translational modifications for interaction with human antibodies, especially in NPC sera. The present data also indicate that a single EA marker protein as antigen might not be adequate to comprise the diverse anti-EBV antibody responses for NPC diagnosis (14). Multiple EA protein species may be required, as indicated by Dardari et al. (2000), Cheng et al. (2002), Liu et al. (2004), and Fachiroh et al. (2004) (3, 7, 14, 24).
Chapter 4

The EA-complex consists of various virus-encoded enzymes essential for viral replication. The diagnostically relevant EA antigens consist mainly of proteins associated with viral DNA replication complex, accumulated in the nucleus as revealed by previous studies (10, 31, 54) [Lu and Chen, 2006]. To provide a specific EA antigen, EBV proteins should be separated from DNA and host cell components. Extraction at high salt has been used to solubilize the EA-related DNA binding proteins but also EBNA1 (10, 25). Tsurumi et al. [1996] (42) showed the EAd-p138 (the major DNA-binding protein encoded in BALF2) could be eluted in 0.15 M, and completely separated from the contaminated DNase activity in 0.3 M NaCl. We modified these prior EA extraction procedures to yield a reproducible low-salt extract containing the main EA reactive components EAd-p47/54, EAd-p138, TK, DNase and ZEBRA, whereas VCA and EBNA1 were absent. This low-salt extraction also preserved the native epitope structure of the EA complex, yielding an excellent antigen for ELISA, showing a specificity of 90.4% and 95.5% for IgG and 85.7% and 94% for IgA, respectively (Table II).

In line with previous observations, the (epitope) specificity of EA reactive IgG and IgA antibodies in NPC sera may be different. This is indicated by the absence of competition between IgG and IgA as revealed by the lack of improved IgA reactivity after removal of IgG using GullSorb pre-treatment (data not shown), and as suggested by the different response to EA heat-denaturation and 8 M Urea treatment. This is supported further by the complete lack of correlation between IgG and IgA responses in EA ELISA as determined for a larger series of sera from patients with different EBV-related disease syndromes (Fig. 4).

In line with observations by others, EA-IgG and EA-IgA can be found occasionally in other patient groups, but these responses are generally low when compared to NPC patients (Fig. 3). The data, summarized in Figure 3 and Tables II and III, show that the low-salt EA extract provides an antigen that is highly sensitive and specific for diagnosing NPC, either when using IgG or IgA antibodies. This is particularly true in populations with low incidence of IM, including most developing countries. Only a few prior studies determined the diagnostic value of EA-IgA in NPC and multiple non-NPC patient populations, as most studies compare NPC only to healthy controls. Sigel et al. [1994] (36) showed by using IFA that 63.7% of NPC patients exhibited positive EA-IgA, but other groups (i.e., non-NPC tumor patients, immunosuppressed patients, patients with inflammatory diseases or mononucleosis-like symptoms, HIV-positive patients) exhibited significant percentages of EA-IgA positive cases as well (33% for the total population of non-NPC patients; ranging between 26% and 42% for individual groups). The difference in percentile EA-IgA positive sera between NPC and non-NPC patients was statistically significant, which is confirmed in the present study (P < 0.001). Recent studies by Wong [2005], also using IFA to EA as first line screening antigen showed that 81.2% NPC sera were positive EA-IgA. The EA extract ELISA method described here provides an improvement over these IFA studies in terms of sensitivity and specificity in large patient groups and healthy EBV carriers as controls. The results confirm data from a prior collaborative study in a relatively small population of juvenile and adult NPC cases (n=30 each) from North-African origin, showing better sensitivity for ELISA compared to IFA in detecting IgA-VCA and IgA-IgA antibodies (22).

In conclusion, the results show that ELISA using native EBV-EA proteins provides a promising tool for NPC-specific serodiagnosis. The simple low-salt extraction yields an EA-specific antigen mixture devoid of host cell proteins, and not containing VCA and EBNA1. The native EA-extract provides high sensitivity and specificity due to virtual absence of EA-reactive antibodies in healthy EBV carriers and non-NPC cancer patients in an NPC endemic region. Native EBV-EA proved to be a superior antigen compared to synthetic peptides and recombinant EAd proteins. This method may complement (as confirmation test) the synthetic peptide-based ELISA measuring IgA to VCA + EBNA1 as first-line screening test in high-risk populations (13).

Acknowledgments

We thank the NPC team of Dr. Sardjito Hospital, Faculty of Medicine, Gadjah Mada University, Indonesia for support in collecting patient samples and Dr. Bambang Hariwijanto (ENT specialist) and Dr. Harijadi (pathologist) for providing clinical and pathological data. We also thank the EBV team in Department of Pathology, Vrije Universiteit Medical Centre, Amsterdam, the Netherlands, for providing facilities and assistance.
REFERENCES


Chapter 4

Native EA - EBV for NPC diagnosis


