The disease associations of the antibody response against the Epstein–Barr virus transactivator protein ZEBRA can be separated into different epitopes

R. Tedeschi,1,2 Y. T. Foong,1,3 H. M. Cheng,3 P. dePaoli,2 T. Lehtinen,4 T. Elfborg5 and J. Dillner1*

1 The Microbiology and Tumor Biology Center, Karolinska Institute, Box 280, S-17177 Stockholm, Sweden, 2 Centro Regionale Di Riferimento Oncologico, Aviano, Italy, 3 Nasopharyngeal Carcinoma Research Laboratory, University of Malaya, Kuala Lumpur, Malaysia, 4 Department of Oncology, Tampere University, Finland and 5 Euro-diagnostica, Ideon, Malmö, Sweden

The BamHI-Z-encoded Epstein–Barr virus (EBV) replication activator (ZEBRA) is a key mediator of the switch from latency to productive cycle in EBV virus. Antibodies against ZEBRA are a marker of EBV reactivation and are regularly found among patients with infectious mononucleosis (IM) or nasopharyngeal carcinoma (NPC), but are only rarely found among healthy EBV-seropositive donors. In order to define the serologically reactive epitopes in the ZEBRA protein, we synthesized a set of overlapping peptides and tested them for reactivity with serum samples from EBV-seronegative persons, patients with NPC, IM, chronic fatigue syndrome, lymphoma or from healthy donors.

Three major EBV-specific epitopes were found. These epitopes were further defined and optimized using substitution or truncation analogues of the peptides. Reactivity with epitope number 22 was found in 63% of NPC patients' sera, with < 2% of healthy donors' sera being positive. Serological reactivity with epitope number 19 was associated with IM (57% positive, 5% healthy donors positive). Serum antibodies against epitope 1 were found among healthy donors, but were significantly elevated among patients with NPC, IM or lymphomas. In conclusion, different serologically reactive epitopes in the ZEBRA protein associate with different EBV-associated diseases.

Introduction

Epstein–Barr virus (EBV) is the causative agent of infectious mononucleosis (IM) and is linked with several human malignancies: Burkitt’s lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin’s lymphoma and lymphoproliferative diseases in the immunosuppressed. More than 90% of the healthy adult population carry EBV in a stable, latent form. Signs of EBV reactivation are seen among some patients with AIDS, lymphomas or chronic fatigue syndrome, although the significance of this reactivation is unclear (Dillner, 1991).

The switch between latency and replication of EBV is mediated by the BZLF1 gene in the BamHI Z fragment of EBV (Countryman & Miller, 1985). The BZLF1-encoded protein (ZEBRA) is a 40 kDa nuclear protein (Countryman et al., 1987). Antibodies against ZEBRA have been detected among 75–87% of NPC patients (Joab et al., 1991 a; Mathew et al., 1994), among 85% of IM patients and among 32% of sera from human immunodeficiency virus (HIV)-infected subjects (Joab et al., 1991 b). In contrast, only 2–4% of healthy EBV-seropositive donors have ZEBRA antibodies (Joab et al., 1991 a, b; Mathew et al., 1994), implying that the presence of antibodies to ZEBRA is a serological marker of EBV-associated disease rather than of EBV infection.

Each EBV-associated disease has its own typical pattern of antibody response against the various EBV-determined antigens. For example, NPC is characterized by strongly elevated IgA responses as well as elevated IgG responses against the viral capsid antigen (VCA), the diffuse early antigen (EA-D) and against the EBV nuclear antigen (EBNA), whereas BL is characterized by elevated IgG against VCA and the restricted early antigen (EA-R) (Henle & Henle, 1979). We have also described that the disease specificity of the antibody response can vary for different epitopes even within the same protein (Cheng et al., 1991). Therefore, in order to define reactive epitopes and optimize the disease specificity of the ZEBRA serology, we mapped serologically
reactive epitopes in the ZEBRA protein using overlapping synthetic peptides.

Methods

Peptide synthesis. Peptides were synthesized by the multiple simultaneous peptide synthesis method (Houghten, 1985), as detailed by Dillner et al. (1990). Homogeneity of peptides in each synthesis was assayed by reversed-phase HPLC on a C8 column.

ELISA. Peptides at 20 μg/ml were coated in 0.1 M-Tris–HCl, pH 8.8, onto half-area ELISA plates (Costar) overnight at room temperature. After two washes with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T), the plates were blocked with 10% horse serum (HS-PBS) for 30 min at 37 °C. Sera were diluted at 1:30 in HS-PBS and incubated on the plates for 2 h at 37 °C. Before each subsequent step, five washes with PBS-T were made. Monoclonal antibodies against IgG, IgA or IgM (Euro-dagnostica, Malmö, Sweden) were diluted in HS-PBS at 1:1000 for IgG or 1:800 for IgA and IgM and incubated on the plates for 90 min at 37 °C. A horseradish

Fig. 1. Screening for serologically reactive epitopes within the ZEBRA protein. The primary amino acid sequence of the ZEBRA protein was synthesized as a set of 29 22-amino-acid peptides, overlapping each other by 14 amino acids. Peptide 1 is situated at the ZEBRA N terminus, peptide 29 at the C terminus. The results of IgG ELISAs (a) or IgA ELISAs (b) obtained with 16 nasopharyngeal carcinoma patients’ sera are displayed as box plots. The line in the box represents the median value, the box boundaries represent the values for 75% and 25% of the sera, the horizontal lines connected to the boxes with vertical lines represent 10% and 90% of the sera and the remaining outliers are plotted individually.
Epitopes in EBV ZEBRA protein

Reactivity was expressed as difference in absorbances at 405 nm (ΔA) by subtracting the absorbances obtained with antigen-coated wells from the values obtained when the same serum was incubated on wells coated only with buffer. Cut-off level for determination of positivity was calculated for each antigen as the mean plus four standard deviations of the reactivity of EBV VCA-negative sera, or in the case of the ZEBRA-22 antibody kit using a cut-off determination standard serum provided in the kit. Differences in reactivity between patient groups were analysed both in dichotomous analysis using exact methods from contingency tables and in continuous analysis using the Mann–Whitney non-parametric ranking test.

Serum samples. Sera from 55 EBV VCA-negative donors were obtained from Dr A. Linde, Swedish Institute for Infectious Disease Control, Stockholm. These sera had been gathered from serum samples that had been submitted for EBV VCA immunofluorescence analysis as part of a clinical routine.

Samples from 96 untreated patients with malignant lymphomas were collected at admission for treatment in the Department of Oncology, Tampere University Hospital, Finland. Serum samples from 61 patients with untreated nasopharyngeal carcinoma were collected at the University Hospital, Kuala Lumpur, Malaysia. Serum samples from 44 patients with infectious mononucleosis (diagnosis verified by immunofluorescence) were collected at the Division of Microbiology and Immunology, Aviano, Italy. Serum samples were also obtained from 65 patients attending the Division of AIDS & Medical Oncology at the Regional Oncologic Center in Aviano with the diagnosis chronic fatigue syndrome. The diagnostic criteria were those formulated by Holmes et al., (1988). As a reference group for comparisons of disease specificity, serum samples from healthy blood donors (not selected according to EBV VCA IgG status) were obtained from the Karolinska Blood Donor Centre, Stockholm (n = 79), the Tampere University Hospital Blood Donor Centre (n = 76), the Blood Donor Laboratory in the Regional Oncologic Center in Aviano (n = 106) and from healthy Malaysian chinese (n = 10). The donors from Tampere were age- and sex-matched with the lymphoma patients, who attended the same hospital. Similarly, the healthy donors from Aviano were matched with the IM sera.

Results

The primary amino acid sequence of the EBV transactivator protein ZEBRA was synthesized as a set of 29 22-amino-acid peptides overlapping each other by 14 residues. The peptides were first screened for reactivity with IgG or IgA antibodies present in the sera of 16 patients with nasopharyngeal carcinoma. IgG reactivity present in most patients' sera could be found for 12 peptides. Only two peptides were regularly reactive with IgA antibodies in these patients' sera and at much lower titres (Fig. 1). In contrast, the EBNA-1-derived peptides EBNA-1/p107 and EBNA-1/p19 (Cheng et al., 1991; Foong et al., 1990) that were assayed in parallel showed high levels of IgA reactivity in most NPC patients (not shown).

The EBV-specificity of the response was investigated for five of the most reactive peptides (peptides 1, 4, 17, 19 and 22) containing unique epitopes (some overlapping peptides, e.g. 1 and 2, showed a closely related reactivity among the different individual sera and were therefore likely to contain the same epitope in the overlapping part of the peptide). Serum samples from 50 EBV VCA-
Table 1. Optimization of NPC disease specificity/sensitivity by synthesis and testing of modified peptides*

<table>
<thead>
<tr>
<th>Peptide synthesis number</th>
<th>Modifications made</th>
<th>Rationale for modification/position of original peptide</th>
<th>Reactivity with healthy donor sera (mean ΔA × 10³)</th>
<th>Reactivity with NPC patient sera (mean ΔA × 10³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4801</td>
<td>Original peptide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZEBRA-1</td>
<td>Modifications</td>
<td>Amino acids 1–22 in protein</td>
<td>390</td>
<td>495</td>
</tr>
<tr>
<td>6508</td>
<td>N-terminal C addition</td>
<td>Allow dimerization</td>
<td>352</td>
<td>475</td>
</tr>
<tr>
<td>6509</td>
<td>F to A replacement</td>
<td>Reduce hydrophobicity</td>
<td>512</td>
<td>674</td>
</tr>
<tr>
<td>6510</td>
<td>Extension with 3 aa at C terminus Removal of 3 aa at N terminus</td>
<td>Do additional C-terminal amino acids constitute part of epitope? Maintaining easy-to-make length</td>
<td>159</td>
<td>249</td>
</tr>
<tr>
<td>6511†</td>
<td>Removal of 3 aa at C terminus Removal of 3 aa at N terminus</td>
<td>Reduce hydrophobicity Facilitate synthesis</td>
<td>222</td>
<td>372</td>
</tr>
<tr>
<td>6512</td>
<td>Removal of 3 aa at C terminus</td>
<td>Reduce hydrophobicity</td>
<td>277</td>
<td>427</td>
</tr>
<tr>
<td>4819</td>
<td>Original peptide</td>
<td>Modifications</td>
<td>Amino acids 145–166 in protein</td>
<td>58</td>
</tr>
<tr>
<td>6515†</td>
<td>V to A replacement</td>
<td>Reduce hydrophobicity</td>
<td>101</td>
<td>422</td>
</tr>
<tr>
<td>4822</td>
<td>Original peptide</td>
<td>Modifications</td>
<td>Amino acids 169–190 in protein</td>
<td>127</td>
</tr>
<tr>
<td>6506</td>
<td>C-terminal C to S replacement</td>
<td>Limit disulphide bond formation Facilitate synthesis</td>
<td>306</td>
<td>357</td>
</tr>
<tr>
<td>6505</td>
<td>N-terminal C to S replacement Omission of 1 aa at both termini</td>
<td>Limit disulphide bond formation Facilitate synthesis</td>
<td>54</td>
<td>264</td>
</tr>
<tr>
<td>6505†</td>
<td>As above, but measured using ELISA kit</td>
<td>Increase sensitivity Easy and quick (4 h) assay</td>
<td>72</td>
<td>835</td>
</tr>
</tbody>
</table>

* The peptide analogues were tested with a panel of 29 healthy donors and 29 NPC patients. In the case of the ZEBRA-1 analogues, 29 healthy donors who had tested positive with the original version of ZEBRA-1 were chosen, whereas the other analogues were tested with sera chosen at random.
† Peptide analogues that were considered optimal.

negative donors were tested concomitantly with samples from 10 NPC patients in an ELISA assay for IgG and/or IgA. Whereas the serological reactivity of all five peptides was higher among the NPC patients (Fig. 2), reactivity against peptides 4 and 17 was also present among several of the EBV-negative persons’ sera and these peptides were not studied further.

For peptides 1, 19 and 22, we tried to optimize the sensitivity and specificity for NPC as compared to healthy donors by synthesizing and testing modified analogues of the original peptides. The changes made and the resulting effects on serological reactivity are detailed in Table 1.

For peptide 1, a truncated 16-mer was considered to have the best disease specificity, although the amplitude of the reactivity was somewhat lower. For peptide 19, a valine to alanine replacement analogue had about the same disease specificity as the original peptide, but was reactive at higher levels. The original peptide 22 contained two cysteine residues and we reasoned that extensive formation of disulphide bonds could have resulted in the formation of cyclizations or multimers containing non-specific reactivity. If the N-terminal cysteine was replaced with a serine the serological reactivity was virtually restricted to NPC patients. In contrast, the analogue where the C-terminal cysteine was replaced with serine had a greatly increased reactivity with healthy donor sera (Table 1), suggesting that the background reactivity among healthy donors seen with the original peptide had been caused by dimerization of the peptide formed by disulphide bonds between the N-terminal cysteines. For the optimized version of peptide 22, we also developed a standardized antibody measurement system in kit format, optimized for sensitivity,
Epitopes in EBV ZEBRA protein

Fig. 3. Evaluation of disease specificity of optimized peptide analogues of peptides 1, 19 and 22. The peptides were tested in IgG ELISAs with 51 NPC sera, 31 EBV VCA-negative (EBV-) sera, 192 healthy donor (HD) sera, 65 sera from chronic fatigue syndrome (CFS) patients, 44 sera from infectious mononucleosis (IM) patients and 96 sera from lymphoma patients (LYMP).

Table 2. Proportion of positive sera for the three major ZEBRA epitopes ZEBRA-1 (in optimized version), ZEBRA-19 (in optimized version) and ZEBRA-22 (optimized version in kit format) in various patient groups*

<table>
<thead>
<tr>
<th>Patient group</th>
<th>ZEBRA-1</th>
<th>ZEBRA-19</th>
<th>ZEBRA-22</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive sera (%)</td>
<td>Positive sera (%)</td>
<td>Positive sera (%)</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>29</td>
<td>5</td>
<td>1, 6</td>
</tr>
<tr>
<td>NPC</td>
<td>47</td>
<td>0.01</td>
<td>29</td>
</tr>
<tr>
<td>IM</td>
<td>52</td>
<td>0.003</td>
<td>57</td>
</tr>
<tr>
<td>CFS</td>
<td>32</td>
<td>ns</td>
<td>0</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>44</td>
<td>0.01</td>
<td>5</td>
</tr>
<tr>
<td>Healthy donors (strongly positive)</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoma (strongly positive)</td>
<td>29</td>
<td>0.00002</td>
<td></td>
</tr>
</tbody>
</table>

* These results are also presented graphically in Fig. 3. The P values for significant differences compared to healthy normals, calculated using dichotomous analysis, are shown (ns, not significant). Since continuous analysis indicated that a suboptimal cut-off point was used for determination of ZEBRA-1 positivity among lymphoma patients, positivity for ZEBRA-1 was also determined using an arbitrary cut-off point for strong positivity.

stability of reagents and short assay time. The ZEBRA-22 ELISA kit had a clearly increased reactivity, in particular among the NPC patients (Table 1).

The best versions of peptides 1, 19 and 22 were evaluated using a panel of 51 NPC sera, 31 EBV VCA-negative sera, 192 healthy donor sera, 65 sera from
chronic fatigue syndrome patients, 44 sera from IM patients and 96 sera from lymphoma patients.

Peptide 19 showed very strong immunoreactivity with NPC and, above all, with IM patients' sera (Fig. 3). The non-specific reactivity with a few EBV-negative sera that had already been detected in the original peptide 19 (cf. Fig. 2) was still present, which resulted in very high cut-off values for determining positivity (0.895). ZEBRA-19 positivity was characteristic for IM and to a somewhat lesser extent for NPC (Fig. 3; Table 2), whereas very few EBV-positive normals or lymphoma patients were strongly positive.

ZEBRA-1 showed a clear EBV-specificity (Fig. 3), but the specificity for NPC compared to healthy EBV carriers was limited. Patients with IM, lymphomas or NPC all showed a somewhat elevated reactivity (Table 2).

ZEBRA-22 was outstanding both in having very little reactivity with EBV-negative sera and in showing a high degree of specificity for NPC as compared to healthy EBV-positive donors. The antibodies against ZEBRA-22 were also more frequent among patients with IM or lymphomas than among healthy donors (Table 2), albeit only NPC patients were regularly positive.

Analysis of the disease associations of all three antigens using the Mann–Whitney ranking test, which is independent of the cut-off level used to determine positivity, resulted in $P$ values for disease association that were similar to the $P$ values obtained in the dichotomous analysis (Table 2), except for lymphoma association of the anti-ZEBRA-1-response which was marginally elevated in the dichotomous analysis (Table 2) but highly significantly elevated in the Mann–Whitney test (not shown). We therefore analysed the lymphoma association of this antibody response also using an arbitrary cut-off for strong positivity of 1.0 (Table 2). With this higher cut-off, the results of the dichotomous and continuous analyses were similar.

**Discussion**

The characteristics of the antibody response to ZEBRA are unique among the EBV proteins. No other EBV protein has such striking disease association of its IgG response (Dillner, 1991). In contrast, an abundant disease-associated IgA response in NPC patients is a characteristic feature of many EBV proteins, such as the viral nuclease, ribonucleotide reductase, EBNA-1, VCA, EA, thymidine kinase, DNase and DNA polymerase (Chen et al., 1987; Foong et al., 1990; Ginsburg, 1990; Littler et al., 1990; Liu et al., 1989), whereas the IgA response against ZEBRA, as detected by immuno-fluorescence, is weak and seen in only 13% of NPC patients (Joab et al., 1991a). Also for the ZEBRA peptides, the IgA reactivity was very weak in comparison with the strong IgG responses seen against the same peptides and also in comparison with the IgA responses against EBNA-1-derived peptides (Cheng et al., 1991; Foong et al., 1990) that were analysed in parallel.

It is noteworthy that the poor IgA response was a feature of all the ZEBRA peptides, implying that the cause of the poor IgA response is a feature of the protein, e.g. absence of epitopes that are necessary for adequate T cell help in the induction of the IgA response, rather than a feature of the antibody-reactive epitopes. In contrast, the disease associations were frequently different for the different epitopes, implying that the disease associations are dependent on the intrinsic immunogenic properties of the B cell epitope itself.

The search for continuously improved EBV serological markers is of interest for several reasons. In the case of NPC, early detection is critical for the outcome and screening for NPC using EBV serology (IgA/VCA) has been thoroughly evaluated (Zeng et al., 1983, 1985). Both IgG anti-ZEBRA (Mathew et al., 1994) and IgA anti-EBNA-1 (Cheng et al., 1993) can be used to complement the IgA/VCA test, since they both have a high predictive value for NPC and since a substantial proportion of IgA/VCA-negative NPC cases are positive for IgG/ZEBRA or IgA/EBNA-1. However, both IgA/VCA (Marklund et al., 1986), IgG/ZEBRA (Joab et al., 1991a) and IgA/EBNA-1 (unpublished observation) are regularly positive in IM patients. It is therefore of particular interest that the ZEBRA-22 response described here was associated with NPC, but that the regular positivity in IM that is seen for the ZEBRA protein had been eliminated. The epitope that in its reactivity most resembles the characteristics of the IgG/ZEBRA protein is the ZEBRA-19 response, since it was regularly positive both in IM and in NPC. Epitope ZEBRA-1 was EBV-specific, but had limited disease specificity; this epitope is therefore probably not well-exposed in the reported assays based on ZEBRA protein. The fact that the delineation of the epitopes in the ZEBRA protein enabled the separation of the NPC-associated response in epitope 22 from the responses to other epitopes, such as epitope 1 and 19 that either have limited disease specificity or are also seen in primary EBV infection, further supports the concept that definition of individual epitopes can be used to optimize disease specificity of serological assays.

Serological markers of EBV reactivation are of interest in the study of several diseases. In BL, elevated IgG/VCA antibodies are present prior to disease development (de Thé et al., 1978). In Hodgkin's lymphoma, elevated IgG/VCA and EA antibodies are present even decades before disease develops (Lehtinen et al., 1993; Mueller et al., 1989). Also, in non-Hodgkin's lymphoma a fraction of patients have very high IgG/VCA titres (Masucci et
an aberrant EBV antibody profile is detectable also prior to disease development (Mueller et al., 1984) and an aberrant EBV antibody profile is detectable also prior to disease development (Mueller et al., 1991). In chronic mononucleosis, grossly aberrant EBV titres are present (Dillner, 1991; Schooley et al., 1986), whereas chronic fatigue syndrome is a less well-defined disease entity that may or may not have serological signs of EBV reactivation (Dillner, 1991). That chronic fatigue syndrome patients did not differ from normals in terms of ZEBRA responses was thus not entirely unexpected, whereas the association of ZEBRA antibodies with a fraction of lymphoma patients is in accordance with previous literature on serological markers of EBV reactivation. The ZEBRA antibodies are not only present in NPC, IM and BL, but are also found in asymptomatic HIV carriers (Joab et al., 1991b), a patient group that unquestionably has EBV reactivation (Birx et al., 1986) but where many serological markers of reactivation are negative (Joab et al., 1991b). Since the ZEBRA antibodies are rare among normals, they bear promise as a more sensitive and specific marker of EBV reactivation than traditional EBV serological assays. The defined epitopes identified in this study might therefore be useful in serological studies on the role of EBV reactivation in several human diseases.

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References


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