Distribution of epitopes within the amino acid sequence of the Epstein–Barr virus major envelope glycoprotein, gp340, recognized by hyperimmune rabbit sera

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Epstein–Barr virus (EBV) is a major human pathogen for which the development of an effective vaccine remains an important goal. Rabbits were immunized with one of a set of 10 fusion proteins representing protein fragments from the EBV receptor-binding ligand and candidate subunit vaccine gp340. Sera from recipients of fragments from the amino-terminal half of the polypeptide chain bound gp340 in Western blot assays and ELISA but were not virus-neutralizing. The fine epitope specificity of these sera, and of EBV-neutralizing rabbit sera raised against whole EBV and gp340-containing immune-stimulating complexes, were assessed in a peptide ELISA. All but two of these sera bound peptides located between positions 236 and 327 in the 907 amino acids of the gp340 polypeptide chain. Among these it was possible to identify regions containing candidate virus-neutralizing B cell epitopes. The use of a gp340 fusion protein affinity column to isolate antibodies from EBV-neutralizing rabbit sera specific for this region suggests the presence of both continuous and discontinuous B cell epitopes with potential roles in EBV neutralization.

Introduction

Epstein–Barr virus (EBV) is a human herpesvirus with which most individuals are infected by the time they reach adulthood. EBV infection is most commonly associated with asymptomatic seroconversion but is implicated in the aetiology of a number of human malignancies including endemic Burkitt’s lymphoma, lymphoma in immunodepressed individuals and undifferentiated nasopharyngeal carcinoma (reviewed in Miller, 1990). Prevention of primary infection through vaccination could result in a reduction in the incidence of such diseases (Morgan et al., 1989). Infection of the cottontop tamarin with a high dose of EBV invariably results in the appearance of EBV-induced lymphoma in the animal (Cleary et al., 1985). Vaccine preparations of the EBV major envelope glycoprotein and receptor ligand, gp340 (Tanner et al., 1988; Nemerow et al., 1989), provide protection from EBV-induced lymphoma in this model system (Epstein et al., 1985; Morgan et al., 1988a, 1989).

The immune basis for gp340-specific protection is unknown. In the animal model both cellular and humoral mechanisms, particularly virus-neutralizing antibody production, have been implicated (Epstein et al., 1986; Morgan et al., 1988b). The presence of gp340-specific EBV-neutralizing antibodies and gp340-specific cellular immunity has also been reported in healthy EBV seropositives (Pearson et al., 1978; North et al., 1982; Ulaeto et al., 1988; Wallace et al., 1991). Immunization with a gp340 subunit vaccine may yield a set of gp340-specific antibodies different to those seen to arise during the course of EBV infection. This has two important consequences for the development of subunit vaccines and for the search for neutralizing epitopes. The first is that the gp340-specific, virus-neutralizing antibodies associated with natural EBV infection may not be elicited by a subunit vaccine and so identification of such epitopes will probably be insufficient alone to allow such a response to be mimicked by a subunit vaccine. Second, and conversely, there may be regions of the gp340 molecule to which antibodies are not induced during infection, which are nevertheless capable of binding gp340, either on the virus envelope or the surface of infected cells, and influencing the course of infection through virus neutralization, complement fixation or promotion of antibody-dependent cell-mediated cytoxicity (ADCC) (Jondal, 1976; North et al., 1982; Beisel et al., 1985; Qualtière et al., 1982; Khyatti et al., 1991). Antibodies that fall into this category are perhaps more
likely to be elicited by the vaccination of people with subunit vaccines. Furthermore, we have shown that individuals with EBV-associated disease do have gp340-specific antibodies which exhibit a pattern of binding to gp340 fusion proteins indistinguishable from that of sera from healthy EBV-seropositives (Pither et al., 1992). Indeed, the anti-gp340 antibody titre is often higher in such people than in their healthy counterparts (Rocchi et al., 1973; Pearson et al., 1979). It may therefore be beneficial to induce an antibody response to epitopes against which antibodies are not normally produced in the course of natural infection.

The aim of this study was therefore to map the location of B cell epitopes within the polypeptide chain of gp340 to which antibodies were produced upon the immunization of rabbits with various preparations of gp340. In addition, progress was made in characterizing the EBV-neutralizing activity of serum IgG which bound continuous epitopes within the amino-terminal third of the gp340 polypeptide chain.

**Methods**

**Immunization of rabbits with gp340 fusion protein fragments.** Gp340 fusion proteins (Fig. 1) were prepared as described (Pither et al., 1992) and 150 µg in complete Freund's adjuvant, was administered subcutaneously to a single 6-month-old female New Zealand white rabbit at four sites close to the lymph nodes in the region of the neck. Subsequent injections of 150 µg were given at approximately 2 to 4 week intervals in incomplete Freund's adjuvant, and a serum sample (approximately 10 ml) was taken just prior to each injection. The serum was examined for fusion protein-specific antibody (see below) and, when such a response was optimal, usually after seven or eight injections, the rabbit was sacrificed and the serum collected and stored at −20 °C.

**Preparation and running of an FP2 gp340 fusion protein affinity column.** An FP2 affinity column was prepared by coupling the FP2 fusion protein to cyanogen bromide-activated Sepharose 4B and this was run as described previously (Pither et al., 1992). The test serum was diluted 1:1 with PBS and added to the column. The column was washed with 3 volumes of 50 mM-Tris-HCl pH 8.0 containing 0.5 M-NaCl and 3 volumes of 50 mM-Tris-HCl pH 9.0 containing 0.1 M-NaCl, then eluted with 5 volumes of 50 mM-triethanolamine pH 11.5 containing 0.1 M-NaCl. Fractions (2 ml) were collected and, along with wash eluates, tested for gp340-specific antibodies in Western blot assays. Positive fractions were pooled, concentrated using a Centricon 10 protein concentrator (Amicon) and stored at −20 °C.

**In vitro EBV neutralization assay.** This assay measures the ability of a serum to prevent EBV transformation of cord blood lymphocytes in vitro. The test serum was added to a range of EBV dilutions (10⁻² to 10⁻⁶) in a microtest plate, at a final concentration of 1/10, and the plate was incubated at 37 °C for 1 h. Cord blood lymphocytes (2 × 10⁶) were dispensed into glass test tubes and, at the end of the incubation period, 25 µl of the virus-serum mixture was added. The cells were mixed and incubated for a further 1 h at 37 °C prior to dilution to 2 ml with complete medium and plating out (5 × 0.2 ml). These plates were maintained at 37 °C for 4 to 5 weeks, with weekly feeding, until an endpoint could be satisfactorily read. This was taken as the virus dilution at which 50% of the wells showed outgrowth of EBV-transformed cells. A serum causing reduction in the virus transformation titre over the non-neutralizing control serum titre by 10-fold or more was considered to be EBV-neutralizing. Positive and negative control sera were included in each assay. The endpoint of this assay was determined using the Reed–Muench calculation (de Schryver, 1974).

**Mapping of B cell epitopes using overlapping peptides.** A set of 192 synthetic peptides was obtained. These were approximately 14 to 16 amino acids in length and overlapped by 12 amino acids, covering the amino acid sequence of gp340 (Wallace et al., 1991). Peptides were dissolved in a minimal volume of DMSO and diluted in carbonate ELISA coupling buffer at 10 or 50 µg/ml. Plates were coated with 50 µl per well and a standard ELISA was performed, using PBS/5% normal goat serum containing 0.5% BSA for blocking. A goat anti-rabbit IgG–horseradish peroxidase conjugate was used in conjunction with orthophenylenediamine. Plates were read at 490 nm. ELISAs were also performed using FPLC-purified gp340 diluted to 5 µg/ml in carbonate buffer.

**Results**

**Analysis of sera raised in rabbits against the gp340 fusion proteins**

Antisera were raised in rabbits to each of 10 cro-β-galactosidase–gp340 fusion proteins (Fig. 1) and the specificity and reactivity of the resulting sera were tested in a range of assays. Serum was taken prior to the immunization procedure (pre-bleed serum) and approximately 2 weeks after each administration of gp340 fusion protein. Each serum was tested by Western blotting and ELISA for reactivity to gp340. The results from these assays indicate that the sera raised against FP2, FP3 and FP4 were all capable of binding to gp340 on Western
blots (Fig. 2) and in ELISA, but those raised against FP1, FP5, FP6, FP7, FP8, FP9, FP10 and FP11 (a negative control serum raised against cro-β-galactosidase) were not. The binding of sera raised against FP2, FP3 and FP4 was comparable to that of a serum raised against FPLC-purified gp340 in an ELISA of a strongly EBV-neutralizing rabbit serum raised against a gp340–immune-stimulating complex (iscom). A positive control serum from a rabbit immunized with gp340–iscoms was included in each assay. All of the rabbits produced antibodies that bound cro-β-galactosidase in Western blots (data not shown).

The rabbit anti-gp340 fusion protein sera were screened for EBV-neutralizing activity in *in vitro* virus neutralization assays. Assays were performed on serum samples taken from the rabbits 2 weeks post-injection so that the appearance of neutralizing antibodies in their sera could be monitored. Sera from the test animals were examined in each assay at a final dilution of 1/10. Also included in the assay was a strongly neutralizing positive control serum from a rabbit immunized with gp340–iscoms and a negative control serum from an FP11-immunized rabbit. None of the anti-gp340 fusion protein sera was EBV-neutralizing in this assay, which is in contrast to the strong neutralizing activity shown by rabbit sera raised against gp340–iscoms or whole EBV.

Mapping of B cell epitopes on the gp340 polypeptide chain recognized by sera from rabbits immunized with gp340-containing immunogens, using a peptide ELISA

To define continuous epitopes recognized by rabbit sera raised against either the gp340 fusion proteins or other gp340-containing immunogens, the technique of peptide ELISA was employed. The sera chosen for examination in this way were those raised against FP2, FP3 and FP4, and EBV-neutralizing rabbit sera against gp340–iscoms and whole EBV. These gp340 fusion protein sera were selected because they exhibited gp340 specificity: sera FP2, FP3 and FP4 recognize gp340 on Western blots and in ELISA. This therefore implies that the epitopes recognized by these sera, present within the gp340 polypeptide chain, are exposed on the surface of the glycosylated gp340 molecule and thus may represent potential targets for a peptide-induced response. The other sera, which included gp340–iscoms and whole EBV, are both strongly EBV-neutralizing *in vitro* and display binding to specific gp340 fusion proteins in Western blots. The gp340–iscoms serum binds to FP2, FP3 and FP8; the whole EBV serum binds to FP2, FP4, FP5, FP8 and FP10 (data not shown). It was conceivable therefore that some of the epitopes recognized by these sera were linear in nature and that some may be important in virus neutralization.

A set of 192 overlapping 14 to 16 residue peptides covering the gp340 polypeptide chain was used in these assays. The test sera were diluted 1/1000 except for the serum raised against whole EBV which was diluted 1/200. Antisera raised against FP2, FP3 and FP4 were each tested against the peptides represented in their respective fusion proteins. Peptides spanning the FP2, FP3 and FP8 regions of the gp340 polypeptide chain were screened with the gp340–iscoms serum, and peptides spanning the entire gp340 polypeptide chain were screened with the serum raised against whole EBV. In each case it has been possible to map at least one binding site and the results are shown in Fig. 3 with the epitope-containing regions summarized in Table 1.
Table 1. Summary of the peptide-defined linear B cell epitopes in the gp340 polypeptide chain, detected in a peptide ELISA, recognized by rabbit gp340 immune sera

<table>
<thead>
<tr>
<th>Serum (immunogen)</th>
<th>Peptide bound</th>
<th>Peptide sequence</th>
<th>Gp340 fusion proteins carrying the epitope-containing region</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP2</td>
<td>199–213</td>
<td>199–213</td>
<td>FP2, FP3</td>
</tr>
<tr>
<td></td>
<td>297–311</td>
<td>KTEMLGNEIDIECM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>301–315</td>
<td>301–311</td>
<td>FP2, FP8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNIVFSDEIPA</td>
<td></td>
</tr>
<tr>
<td>FP3</td>
<td>142–156</td>
<td>148–156</td>
<td>FP2, FP3</td>
</tr>
<tr>
<td></td>
<td>145–159</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>148–162</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FP4</td>
<td>433–447</td>
<td>437–447</td>
<td>FP4, FP9</td>
</tr>
<tr>
<td></td>
<td>437–451</td>
<td>TGFAADPNTTGT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>236–249</td>
<td>ESHVPSGGLT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>301–315</td>
<td>301–315</td>
<td>FP2, FP8</td>
</tr>
<tr>
<td></td>
<td>313–327</td>
<td>SNIVFSDEIPASQDM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>317–331</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole EBV</td>
<td>248–262</td>
<td>251–262</td>
<td>FP2, FP8</td>
</tr>
<tr>
<td></td>
<td>251–265</td>
<td>VATPIGTGYAY</td>
<td></td>
</tr>
<tr>
<td></td>
<td>297–311</td>
<td>YCIQSNIVFSDEIPA</td>
<td></td>
</tr>
</tbody>
</table>

Owing to the overlapping nature of the peptides, the positions of the linear B cell epitopes identified in the above results can be more precisely defined. For example, the FP3 serum reacts strongly to the three peptides 142–156, 145–159 and 148–162. The epitope, or possibly multiple overlapping epitopes, therefore occurs between amino acids 148 and 156. It will be noted that some peptides are recognized by more than one serum, for example 301–315 is recognized by both the gp340–iscoms and FP2 sera. In these cases it appears that the epitopes recognized overlap but are not identical since the pattern of recognition of adjacent peptides is not always consistent. The implications of these observations are discussed below.

Affinity isolation and characterization of FP2-specific and peptide-specific antibodies from the sera of rabbits immunized with either gp340–iscoms or whole EBV

During the course of the epitope-mapping experiments described above, it became clear that only a very limited number of peptides were recognized by the two EBV-neutralizing rabbit sera that were studied, and in addition these peptides were all located within the FP2 region of the gp340 polypeptide chain. Therefore, if these sera contained neutralizing antibodies directed at continuous epitopes then such epitopes must be among those listed above. Furthermore, the fact that some of the linear epitopes identified overlap with those recognized by the FP2 serum, which has been shown not to mediate virus neutralization, allowed the potential virus-neutralizing linear B cell epitopes to be narrowed down to three regions: residues 236 to 247, 251 to 262 and 317 to 327. It was therefore decided to construct an affinity column using the FP2 gp340 fusion protein to isolate antibodies specific to this region.

Aliquots of each serum (2 ml), diluted to 4 ml with PBS, were applied to the FP2 column and 10 fractions collected for each serum. Each of these fractions was tested for FP2 and gp340 recognition in a Western blot.
were tested in a peptide ELISA for reactivity toward fractions 3 to 6 (data not shown) and so these were bound to gp340 in Western blots. The pooled fractions were tested in a peptide ELISA for reactivity toward peptides from the FP2 region against which each serum had previously been demonstrated to possess reactivity. This assay was performed using peptides diluted to 10 µg/ml and at a serum dilution (fractions 3 to 6) of 1/50. Fractions collected from the whole EBV serum bound to both the appropriate peptides and to gp340 in the ELISA. However, the gp340–iscoms serum showed no binding to appropriate peptides even though some binding to gp340 was apparent.

A neutralization assay was carried out on pooled fractions 3 to 6 from each serum, along with material that did not bind to the column and with unfractionated serum. FP2-specific antibodies from both sera were able to reduce the transforming titre of EBV in the single assay that was carried out.

Discussion

The results presented above clearly demonstrate that immunization of rabbits with gp340 leads to the generation of antibodies which are directed at continuous determinants on the gp340 polypeptide chain. Antibodies were produced to such epitopes regardless of whether gp340 was administered as part of an intact virus particle, FPLC-purified and incorporated into iscoms or as a non-glycosylated fragment of the gp340 polypeptide chain expressed as a cro–β-galactosidase fusion protein. The repertoire of antibody specificities is likely to be determined by both the nature of the immunogen and the genetic background of the animal (Milich, 1988; Celada & Sercarz, 1988; Francis et al., 1987).

Gp340 fusion proteins FP2, FP3 and FP4 elicited antibodies which bound FPLC-purified gp340 in ELISA or Western blots. Continuous B cell epitopes, mapped using the peptide ELISA protocol, were recognized by each of these sera (Table 1). Interestingly, all but two of these epitopes (amino acids 437 to 447 and 148 to 156) were located toward the amino terminus of gp340 between amino acids 236 and 327. Likewise, continuous B cell epitopes recognized by sera from rabbits immunized with either gp340–iscoms or whole EBV were located within the same region. Analysis of affinity-isolated antibodies, specific for the FP2 region of gp340, from the two EBV-neutralizing sera indicated that this region contains both continuous and discontinuous epitopes. This is demonstrated by the observation that the FP2-specific fraction from the gp340–iscoms serum did not bind the appropriate peptides in an ELISA assay whereas the whole EBV serum did. Nevertheless, the FP2-specific fractions from both sera bound gp340 in ELISA and exhibited some EBV-neutralizing activity. Data from the neutralization assays performed on sera raised against the gp340 fusion proteins allow, by a process of elimination, the exclusion of the majority of these continuous epitopes as targets for EBV-neutralizing antibodies. Indeed, the only possible candidates for continuous, EBV-neutralizing epitopes among those listed are between amino acids 235 to 248, 250 to 263 and 316 to 328. It should be noted that the use of single immunized animals may, due to non-responsiveness, lead to an apparent reduction in the true number of epitopes.

The concentration of continuous B cell epitopes in this amino-terminal domain may imply that this region is not concealed in the native gp340 molecule either as a result of protein folding or glycosylation (Morgan et al., 1984). The majority of epitopes defined above do not appear to be important in virus neutralization but could have some role in controlling the outcome of EBV infection by acting as targets in the membrane of lytically infected cells for ADCC or complement fixation (Beisel et al., 1985; Jondal, 1976; Qualtière et al., 1982; Khyatti et al., 1991; Sairenji et al., 1991). In a previous study we have shown that the serum from all healthy EBV-seropositive individuals contains IgG antibody directed at epitopes towards the carboxy terminus of the polypeptide chain of gp340 and, as these epitopes could not be mapped in a peptide ELISA, were likely to be discontinuous in nature (Pither et al., 1992). The prevalence of epitopes in the amino-terminal third of the gp340 polypeptide chain recognized by rabbit sera may reflect a difference in the gp340 immune response between immunized and naturally infected individuals and may thus be of importance in determining the repertoire of antibody specificities seen following vaccination. Alternatively, species variation in the immune response may account for the observed differences. Previously identified continuous epitopes within the gp340 polypeptide chain have not been shown to bind EBV-neutralizing antibodies (Modrow et al., 1988; Bertoni et al., 1990). It therefore appears that the virus-neutralizing epitopes of gp340 are, whether continuous or discontinuous, conformation-dependent such that a degree of native structure is required to elicit a neutralizing serum response (Hoffman et al., 1980; North et al., 1982; Qualtière et al., 1987).

It has been predicted that the vast majority of B cell epitopes are discontinuous in nature, composed of amino acid residues brought into juxtaposition by the tertiary folding of the protein but distal in the linear sequence.
(Barlow et al., 1986; Blundell et al., 1987). Additionally, carbohydrate can dramatically influence the reactivity of antisera to some glycoproteins (Alexander & Elder, 1984; Jemmersson, 1987). Nevertheless, examples exist of highly glycosylated viral proteins in which important functional epitopes have been defined within the polypeptide chain (Utz et al., 1989; Correa et al., 1990). Most notable is the gp120 molecule of human immunodeficiency virus which, like gp340, is approximately 50% carbohydrate by weight, and possesses a linear neutralizing epitope within its primary amino acid sequence, in addition to virus-neutralizing conformational and carbohydrate-dependent epitopes (Javaherian et al., 1990; Hansen et al., 1990; Ho et al., 1991). It is therefore evident that characterization of gp340 with respect to epitopes bound by both serum and salivary antibodies (Yao et al., 1991) and T cell epitopes (Ulaeto et al., 1988; Wallace et al., 1991) will provide an insight into the mechanism of gp340-specific EBV immunity and aid the rational design of future EBV vaccines.

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References


Continuous B cell epitopes of gp340


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