RHEUMATIC DISEASE PATIENTS, PRONE TO SJÖGREN'S SYNDROME AND/OR LYMPHOMA, MOUNT AN ANTIBODY RESPONSE TO BHRF1, THE EPSTEIN–BARR VIRAL HOMOLOGUE OF BCL-2

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SUMMARY

The IgG response to Epstein–Barr virus (EBV) early antigens [BHRF1 (p17.1), the viral homologue of bcl-2, and BMRFL (p50.10), a DNA binding protein] was measured in patients with rheumatic disease to see whether there was any association with lymphoma. Patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), rheumatic disease patients with lymphoma, patients with lymphoma who did not have a rheumatic disease and normal individuals were tested for the presence of anti-EA peptide antibodies by ELISA. Whereas antibodies to early EBV peptides were detected only in one normal individual, patients with rheumatic diseases, especially those with either SS and/or lymphoma, had a much higher frequency of antibody detection. Antibodies to BMRFL p50.10 were found in 7–50% of patients, and to BHRF1 p17.1 in 4–27%, depending on the group studied. Patients with lymphoma lacking a rheumatic disease had a 2-fold lower frequency of anti-BHRF1 antibodies, compared to the lymphoma plus rheumatic disease group. The increased immune response to the EBV EA proteins in the rheumatic diseases probably reflects the presence of reactivated virus, and the BHRF1 protein (the viral homologue to bcl-2) could, via inhibiting apoptosis, contribute to the lymphoproliferative nature of these diseases.

KEY WORDS: BMRFL, Rheumatoid arthritis, Systemic lupus erythematosus.

The role of virus infection in the pathogenesis of rheumatic diseases has long been debated. Viruses have been associated with disease onset, exacerbation and other disease manifestations secondary to the primary rheumatic disease [1–14]. Epstein–Barr virus (EBV), a DNA virus of the herpes family, has been the subject of much research. Since the frequency of latent EBV infection in the general population is generally around 95% in those over 20 yr of age, antibodies to the proteins associated with latent infection have not been found to differ between groups of patients with rheumatic diseases and the unaffected population.

EBV has a complex relationship with man that is only now being discerned. When the virus infects B cells or epithelial cells, it initially undergoes a lytic cycle of replication. Early in this cycle, two major proteins can be detected: BHRF1 7 kDa protein (p17) (formerly EA-R), which is structurally and functionally similar to the proto-oncogene bcl-2 [15–17] (refer to Fig. 1) in that, like bcl-2, p17 has been shown to inhibit apoptosis [16]; and BMRFL, a 50 kDa protein (p50) (formerly EA-D), which has been found to bind to DNA and act as a cofactor of the viral DNA polymerase [18, 19]. The virus, after this initial burst of replication, enters a dormant phase or latency. During latency, a number of proteins including the nuclear antigens (EBNA) and latent membrane proteins (LMP) are expressed with, in general, a cessation of the production of the BMRFL/p50 and BHRF1/p17 proteins. Periodically, the virus is reactivated and one of the first proteins to be induced is p50 [20].

The salivary glands are a reservoir for EBV, where it can induce a lymphoproliferative disease, which is usually transient. Because a major site of pathology in Sjögren's syndrome (SS) of dry eyes and dry mouth coincides with this site of EBV infectivity, it has long been speculated that EBV may contribute to the pathogenesis of the disease [1, 7, 14]. Other viruses, such as cytomegalovirus (CMV), human herpes virus 6 (HHV-6), HTLV and HIV, have also been detected in the salivary glands and/or saliva [4, 10, 13, 14], and it is likely that such viruses may influence the inflammatory process at this site. SS can be the only rheumatic disease present, in which case it is referred to as primary SS, or it may be secondary to another disease such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), when it is referred to as secondary SS. It is, thus, not surprising that the EBV genome can be detected in the salivary tissue or whole saliva of patients with SS and, in addition, it can also be detected in patients with other rheumatic diseases without symptoms of SS, as well as in normal controls [13, 14, 21]. In a small subpopulation of patients, SS appears to develop in the absence of known viruses [14].

One of the consequences of SS (both primary and secondary) is the 40-fold increased risk of lymphoma
development [22-26]. With the recent demonstration that bcl-2 is associated with lymphoma [27] and the development of SLE-like disease in bcl-2 transgenic mice [28], it became important to see whether the EBV homologue to bcl-2 played a similar role in rheumatic diseases of man. Since it had previously been shown that an immune response (B cell and T cell) to BHRF1/p17 was detected in patients with non-Hodgkin’s lymphoma and Burkitt’s lymphoma [29], we have determined whether a similar immune response could be detected in patients with rheumatic diseases, focusing on patients with SS and lymphoma.

MATERIALS AND METHODS

Subjects

Sera were collected from 120 patients with RA, 70 patients with SLE, 27 with primary SS, each category fulfilling well-established criteria [30-32], and 31 normal individuals. The SLE patients studied included individuals with the full range of disease activity, and organ involvement. Cases of early rheumatic disease and disease of long duration of all patient categories are included in the study.

Thirteen rheumatic disease patients from practices in Montreal, Quebec, and London, UK, were identified who had B- or T-cell lymphoma, or another neoplasia. Of this group, seven had primary SS, three had secondary SS associated with RA, and there was one case each of RA, scleroderma and dermatomyositis. Nine of these patients had B-cell lymphomas (four of which were non-Hodgkin’s), two had T-cell lymphomas, one had nasopharyngeal carcinoma and one adenocarcinoma. A control group of 52 patients with lymphoma and/or neoplasia, not associated with a rheumatic disease, was also studied. Of these 52, 39 had B-cell lymphomas, six had T-cell lymphomas and the remaining seven had other neoplasias.

At the time of study, the sera from the SLE patients were tested for the presence of antinuclear antibodies (ANA), antibodies directed against Ro/SS-A, La/SS-B by ELISA (Therestest, Chicago, IL, USA), and DNA. Total serum IgG values were available for the RA and SLE patients. IgM rheumatoid factor (RF) was assayed by nephelometry for the RA group and by ELISA for the SS group. Disease activity (for RA, joint activity scores and extra-articular manifestations; for SLE, the SLEDAI), duration and therapy were determined.

Serological studies for viruses

Antibodies (IgG) to synthetic peptides encompassing antigenic epitopes of the EBV early antigens BHRF1 (peptide designated p17.1) and BMRF1 (peptide designated p50.10) associated with the lytic cycle were determined by ELISA in the above patients, as previously described [29, 33]. Sera were tested at a 1:10 dilution with a cut-off OD of >0.3 defined as positive. This is approximately twice the background of the negative sera for these epitopes. A greater number of sera in all groups were tested for the anti-p17.1 response than for anti-p50.10, due to its homology with bcl-2. In a small group of patients with rheumatic disease and normal controls, an ELISA with slight modifications from the previously described method was used. In brief, the synthetic peptide (5 μg/ml) of BHRF1 encompassing amino acids 63-77 (Fig. 1) (Chiron Mimotopes, NC, USA) was used to coat EIA II plus ELISA plates (ICN) overnight at 4°C in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After washing the plates three times with phosphate-buffered saline (PBS)/0.05% Tween 20, non-specific binding sites were blocked with 2% bovine serum albumin (BSA) (Gibco, BHRF1 2 AYSTREILLAL......................... CIROSRVKGNSTLMHPV 28

bcl-2 8 GYDNEIYMKYHXXLSQGYESWAGDVAGAPPAAAPAGFFSQQGHTP 57

BHRF1 29 LELEATPPLLPLSPEDT.................. VYLRHVLLEEIEIR 60

bcl-2 58 HFAASRDTPVARTESPQTAPAAGPAAAPSVPPPPVNLALRQAGDDFSRR 107

p17.1

BHRF1 61 NSETTFTETWRFITETRBDLDFNS [VLEHRGDPDDLQHAMA] 110

bcl-2 108 YRGDFAEEMSSQHLTLPFTARGAFAT [VEWE] 156

region required for apoptosis inhibition

BHRF1 111 ACRTLCNGQSTPPYVVDSVRGHELASEGDLGWQGWWSTLIDNIPG 160

bcl-2 157 MCTVESNRENSPVLONIALMTNEYRHLHHTQIDNGWDFAVLYGPS 205

BHRF1 161 SSRFSLTSFLFAGTLDLSLLVSCYL...ISR 188

bcl-2 206 MRPLFDSWLSSLKTLLSIALVGACTITLGAYLSD 238

Fig. 1.—Primary structure of BHRF1 [15] with the alignment of bcl-2 [17]. Peptide p17.1 is identified as the stippled area. The region that confers inhibition of apoptosis is boxed.
In the primary SS group, 10 of 22 patients had antibodies to Ro/SS-A and/or La/SS-B; however, there was no association between autoantibody status (including RF) and the presence of the anti-EBV EA antibodies. There was also no association between disease duration and the presence of anti-p17.1 or anti-p50.10 antibodies (data not shown). Incomplete information was available on the serum IgG levels in the SS patients, but where available there was no apparent association with total serum IgG and either antiviral antibody population (data not shown).

In the RA population, the group with compromised tear production, an indicator of SS, had a 3-fold increased frequency of anti-p17.1 antibodies compared to those patients with normal tear production. Of the anti-EA antibody-positive RA patients with compromised tear flow, 50% had definite secondary SS. There was no significant difference in disease duration between the anti-EA antibody-positive RA patients when compared to all of the RA patients in the study (data not shown). Although disease activity scores were slightly higher for the RA subgroup with anti-p50.10 and/or p17.1 antibodies (tenderness index 11.5 ± 4.5; swelling index 15.58 ± 4.22, vs 8.8 ± 2.4 and 8.9 ± 1.4, respectively, for the RA population), only the swelling index approached significance (P = 0.062). Of the RA patients who had antibodies to p17 and/or p50, one was receiving no therapy, three were receiving non-steroidal anti-inflammatory medications only, while the remainder were receiving disease-modifying drugs (Mtx, gold and prednisone with a maximum dose of 12.5 mg).

There was no significant difference in the serum IgG concentration between anti-p50.10 and/or anti-p17.1 antibody-positive individuals and those without anti-p50 or anti-p17 antibodies (RA patients, anti-p17+ vs negative, 12.4 ± 2.5 and 14.0 ± 2.3 mg/dl, respectively; RA patients anti-p50+ vs negative, 13.4 ± 1.9 and 14.0 ± 2.3 mg/ml, respectively). There was no correlation between the presence of RFs and anti-p17.1 or

### TABLE I

<table>
<thead>
<tr>
<th>Patient/group</th>
<th>Anti-BHRF1 (p17.1)</th>
<th>Anti-BMRF1 (p50.10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary SS</td>
<td>7/27†</td>
<td>26</td>
</tr>
<tr>
<td>RA Shirmer's  &gt; 10 mm</td>
<td>3/71</td>
<td>4/71</td>
</tr>
<tr>
<td>RA Shirmer's &lt; 10 mm</td>
<td>4/28</td>
<td>14/3/28</td>
</tr>
<tr>
<td>RA</td>
<td>8/120</td>
<td>7/899‡</td>
</tr>
<tr>
<td>SLE</td>
<td>12/70</td>
<td>19/43§</td>
</tr>
<tr>
<td>Rheumatic/lymphoma</td>
<td>3/11‡</td>
<td>27/2/5</td>
</tr>
<tr>
<td>Lymphoma/neoplasia</td>
<td>6/52+</td>
<td>12/10/52⁺</td>
</tr>
<tr>
<td>Normal</td>
<td>1/31†⁺</td>
<td>0/20/0§</td>
</tr>
</tbody>
</table>

(a) Includes those who also had lymphoma.
(b) Mean (both eyes) tear flow (mm/5 min).
(c) Patients with lymphoma or other neoplasia and rheumatic disease.
(d) No rheumatic disease.

* Not significant, \( \chi^2 \) analyses.
†P = 0.05.
‡P = 0.001.
§P = 0.004.
¶P = 0.08 (not significant).
TABLE III

Correlation of clinical features of patients with SLE and the detection of antibodies to epitopes on EBV IE proteins, BHRF1 (p17.1) and BMRF1 (p50.10)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Anti-BHRF1 (p17.1)</th>
<th>Anti-BMRF1 (p50.10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>³ 30 yr</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>&lt; 30 yr</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Disease duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 10 yr</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>5-10 yr</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>&lt; 5 yr</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Anti-Ro/SS-A ( + )</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>Anti-Ro/SS-A ( - )</td>
<td>23</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No.</th>
<th>(%)</th>
<th>No.</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>14(3)</td>
<td>17</td>
<td>15(3)</td>
</tr>
<tr>
<td>11</td>
<td>9(1)</td>
<td>3</td>
<td>2(7)</td>
</tr>
<tr>
<td>8</td>
<td>12(5)</td>
<td>4</td>
<td>50(0)</td>
</tr>
<tr>
<td>1</td>
<td>11(1)</td>
<td>4</td>
<td>44(4)</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>10</td>
<td>52(6)</td>
</tr>
<tr>
<td>2</td>
<td>13(0)</td>
<td>8</td>
<td>34(7)</td>
</tr>
</tbody>
</table>

anti-p50.10 antibodies, with six of the 13 positive for RF.

The SLE patients were found to have a significantly increased incidence of anti-EA antibodies, compared to the RA patients, especially for anti-p50.1 (Table II). There was, however, a significant association between the anti-p50.10 antibodies with an elevated serum IgG (P = 0.0045; the 18 SLE patients without anti-p50.10 had a mean IgG of 13.66 ± 0.94; 18 SLE patients with anti-p50 antibodies had a mean IgG of 18.13 ± 1.13). Since p50 is a viral DNA-binding protein, we were interested to determine whether anti-p50.10 antibodies correlated with anti-DNA or anti-Ro/SS-A antibodies. There was no significant correlation, although there was a significantly higher frequency of anti-p50.10 antibodies in those who were anti-Ro/SS-A positive (Table III).

From the information that was available on the secondary SS status of 20 of the SLE patients, 50% met the criteria for SS [32]. Of these 20 patients, two had anti-p17.1 antibodies and both had SS. Eleven of the 20 had antibodies to p50.10; however, there was no correlation with SS status (data not shown). Since the SLE patient population studied included individuals < 30 yr of age, there can be a lower incidence of EBV infection, we examined the frequency of antibodies to p17.1 and p50.10 according to age. As can be seen in Table III, there was a higher frequency of antibody positivity in the patients who were older. There was, however, no correlation between the frequency of anti-EA antibodies either to p17 or p50 and SLE disease duration. There was no correlation between the presence of anti-p17.1 antibodies and any autoantibody (anti-Ro/SS-A, anti-La/SS-B or anti-DNA) measured (Table III) in the SLE patients. There was no significant difference between the serum IgG in the patients with and without anti-p17.1 antibodies.

There was an elevated percentage of the rheumatic patients with lymphoma who mounted an immune response to BHRF1, and it was of interest to see whether the antibody presence correlated with disease features. Of the rheumatic disease patients with lymphoma/neoplasia investigated, the anti-BHRF1 antibody response was detected in only 2/9 (22%) of the B-cell lymphoma group. One patient with nasopharyngeal carcinoma (known to be associated with EBV) had antibodies to BMRF1/p50.1, but not to BHRF1/p17.1. In contrast, the two patients with T-cell lymphoma and the SS patient with metastatic adenocarcinoma were anti-EA antibody negative. Previous studies had determined that one of the T-cell lymphoma patients did not have detectable EBV genome in the lymphoma, but rather had CMV [34]. As a control, a group of non-rheumatic lymphoma patients was examined and, although the majority of the anti-EA-positive individuals fell into the B-cell lymphoma group (10 and 23% positive for anti-BHRF1 and anti-BMRF1, respectively; n = 39), 1/7 patients with T-cell lymphoma was positive. An acute EBV infection did precede the development of lymphoma in this patient. The anti-BHRF1 response was at a higher frequency in those who had active lymphoma (5/25) compared to those in remission (1/19), but this difference was not significant.

DISCUSSION

The aetiology and progression of autoimmune rheumatic diseases are multifactorial, with genetics and environmental factors (infectious agents, therapy) playing pivotal roles. In the present study, we demonstrate that a subset of patients with primary SS, those with lymphoma, patients with RA with sicca symptoms or SLE, mount an immune response to the EBV EAs, in particular to BHRF1/p17 and BMRF1/p50 which are associated with the lytic cycle of viral replication. Since the p17.1 peptide used to monitor the antibody response to the BHRF1 protein is not in the region that has been shown to be important for inhibition of apoptosis (Fig. 1) and has a very low level of amino acid identity to bcl-2 (only two of the 15 amino acids in identity, with two additional conservative substitutions), it is highly unlikely that antibodies directed to this region would bind to bcl-2.

It is interesting that patients with SS mount an immune response to the EA peptides to an extent not seen in the normal or non-rheumatic lymphoma controls. It is possible that immune response genes are important in this specific recognition. In previous studies, our laboratory and others have shown that there is no difference in the frequency of the immune response to EBV proteins, such as EBNA, that are associated with latent infection in patients with rheumatic disease and normal individuals [2, 6, 9, 11].

One previous study investigated the immune response to the EBV-EA(D) (BMRF1/p50) in SLE and found that 61% of the patients had antibodies to the polypeptides of the 52-54 complex (p50) by immunoblotting, compared to 5% of the patients with mixed connective tissue disease [5]. Normal controls were not included in that study. This agrees well with our findings; however, the influence of the elevated serum IgG in the patients with SLE in the study by Ngou et al. [5] was not investigated.

In other studies, we have found that the EBV EAs are expressed in mononuclear and epithelial cells from saliva of patients with rheumatic disease [35], and in
patients, primarily those who have SS with and without lymphoma, who mount an immune response to discontinuation of the therapy [46—48].

The majority of these 'induced' lymphomas reverse on and/or cyclosporin therapy, have been reported. The patients with RA or dermatomyositis, receiving Mtx screening of cells for BHRF1 expression and/or cell death induced by DNA-damaging agents [45], a particular Burkitt's lymphoma and nasopharyngeal carcinoma. EBV has also served as a useful tool to aid in the immortalization of human B cells. Characterization of the molecular state of the virus in the neoplasia has revealed differential expression of some viral proteins. It has been demonstrated that the EBV EAs can be detected in nasopharyngeal carcinoma [36], as well as in lymphoma and lymphoproliferative tissue associated with immunosuppression [37], and in the lymphomas associated with ataxia-telangiectasia [38]. BHRF1/p17, but not BMRF1/p50, was found, however, to be expressed in most cell lines established from lymphoma biopsies [39].

In the current study, we studied rheumatic disease patients with lymphoma, and found that those with disease that appeared to be associated with EBV mounted an immune response to BHRF1 and BMRF1. In a control group of lymphoma patients without rheumatic disease, there was a 2-fold lower incidence of antibodies to BHRF1 and to BMRF1, although the difference was not significant. This may reflect the presence of either a more fertile environment for EBV within the rheumatic diseases, possibly due to gene(s) as yet unidentified [40], and/or a more stimulated immune response.

It is possible that BHRF1/p17, by preventing apoptosis [16], can contribute to the accumulation of lymphocytes and/or epithelial cells [41] that are expressing this homologue of bcl-2. This accumulation of long-living cells may increase the chance of a neoplastic event. The expression of BHRF1 itself, however, has not been found to be essential for EBV-induced B-cell transformation [42, 43] and it is unlikely that p17 directly causes the neoplasia. It has been reported that there is defective repair of O\(^{\prime}\)-methylguanine DNA in SS patients predisposed to lymphoma [44]. Since BHRF1/p17 can protect against cell death induced by DNA-damaging agents [45], a scenario favouring neoplasm may be favoured.

It is important to identify patients who have an increased load of BHRF1/p17, either through direct screening of cells for BHRF1 expression and/or through anti-p17.1 monitoring, as it may be possible to reverse the accumulation of cells in the pathological tissue by antiviral therapy. A number of cases of EBV-associated lymphomas which developed in patients with RA or dermatomyositis, receiving Mtx and/or cyclosporin therapy, have been reported. The majority of these 'induced' lymphomas reverse on discontinuation of the therapy [46—48].

This study identifies a subgroup of rheumatic disease patients, primarily those who have SS with and without lymphoma, who mount an immune response to BHRF1, a protein that inhibits apoptosis. It is likely that the expression of BHRF1 in this setting may provide a pathological link to the lymphoproliferation or accumulation of lymphocytes unable to undergo normal apoptosis, that is commonly associated with SS, and could ultimately contribute indirectly to the development of lymphoma.

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