Decreased T cell precursor frequencies to Epstein-Barr virus glycoprotein gp110 in peripheral blood correlate with disease activity and severity in patients with rheumatoid arthritis

Eric Toussirot, Daniel Wendling, Pierre Tiberghien, Janos Luka, Jean Roudier

Abstract

Objectives—Rheumatoid arthritis (RA) is a chronic joint disease associated with certain HLA-DR alleles expressing the QK/RRAA motif or shared epitope. The Epstein-Barr virus (EBV) has been suspected to be a causative factor for RA. The EBV gp110, a glycoprotein of the replicative cycle that contains a copy of the shared epitope, constitutes an important target in the immune control of EBV replication. This study evaluated the specific T cell response to EBV gp110 in patients with RA expressing or not the shared epitope and examined whether this immune cellular response might be related to disease activity and severity.

Methods—25 patients with RA were studied and compared with 25 healthy controls. Disease activity was assessed by biochemical markers of inflammation (erythrocyte sedimentation rate (ESR) and C reactive protein (CRP) levels). Disease severity was defined by extra-articular disease (vasculitis, subcutaneous nodules, or other organ disease). The frequencies of peripheral blood T cells specific for EBV gp110 and a control protein (total protein extract from Escherichia coli) were determined by direct limiting dilution analysis without preliminary bulk culture.

Results—The gp110 precursor frequencies ranged from 0 to 20 × 10^−6 in patients with RA and controls. The mean gp110 T cell precursor frequency was lower in patients with RA (SD 3.2 (4.4) × 10^-10^-6) than in healthy controls (4.1 (3.8) × 10^-10^-6) (p = 0.02). No difference was found for the control protein (p = 0.09). Both shared epitope positive and negative patients with RA responded to gp110, without significant difference. A negative correlation between both ESR and CRP levels and the gp110 T cell response was found (r = −0.71, p<0.0001 and r = −0.42, p = 0.038, respectively). Finally, patients with extra-articular disease displayed the lowest immune cellular response to EBV gp110.

Conclusion—These results suggest that patients with RA have a decreased T cell response to EBV gp110. Since gp110 is an important protein in the control of EBV replication, this might lead to a poor control of EBV infection, chronic exposure to other EBV antigens, and thus to a chronic inflammatory response in patients with RA.

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease of unknown cause. Both genetic and environmental factors are thought to have a role in its development. Indeed, RA is strongly associated with certain HLA-DR alleles containing in the third hypervariable region of their β chains the QK/RRAA sequence or shared epitope. Among environmental factors, an association between a virus and RA has long been suspected. For 15 years Epstein-Barr virus (EBV) has been suspected to be a causative factor for RA and to contribute to its pathogenesis. Indeed, RA sera contain increased titres of antibodies precipitating a nuclear antigen present in EBV infected cells and patients with RA have a higher percentage of EBV infected peripheral blood B cells than controls. In addition, it was shown that T cell control of the outgrowth of EBV infected cells was impaired in patients with RA. Furthermore, it was recently found that a large fraction of T cells infiltrating the synovium from patients with RA recognised EBV proteins of the replicative cycle and EBV DNA was detected in the synovial membrane of patients with RA. Moreover, the EBV genome encodes a protein in its BamHI C restriction fragment open-reading frame or viral interleukin 10 that shares protein homology and biological properties with human interleukin 10.

An explanation for the link between an infectious agent and autoimmune or inflammatory disease might be the molecular resemblance between a microbial antigen and a host antigen, a mechanism usually called molecular mimicry. EBV is a good candidate for RA pathogenesis as several EBV proteins share similar sequences with normal human proteins: the EBV nuclear antigen EBNA-1 shares sequence identity with human type II collagen, the EBNA-6 protein contains a sequence found in the HLA-DQB1*0302 molecule and the EBV glycoprotein 110 (gp110) carries the QK/RRAA sequence or shared epitope. GP110 is a glycoprotein of...
the replicative cycle and constitutes a B and T cell epitope in normal controls. GP110 is the EBV equivalent of gB proteins of other herpes viruses (herpes virus and cytomegalovirus) and these proteins have an important role in the control of the virus infection.

Rheumatoid arthritis is strongly associated with certain HLA-DR antigens expressing the shared epitope, and EBV gp110 contains a copy of the shared epitope. Additionally, there is a defect in the T cell control of EBV infection in RA. Thus it is relevant to assess the EBV gp110 T cell response in patients with RA. In this study we evaluated this specific T cell response in patients with RA expressing or not the shared epitope and we examined whether this cellular response might be related to disease activity or severity, or both. The results show that patients with RA, particularly those with active or severe disease, have a lower cellular response to EBV gp110 than healthy controls.

Patients and methods

Patients
Twenty five patients satisfying the 1987 American Rheumatism Association criteria for RA were studied. Analysis of patients' clinical records included disease duration, history of reconstructive joint surgery (hip or knee joint surgery), extra-articular organ disease (vascularitis, subcutaneous rheumatoid nodules, rheumatoid lung disease—pleuritis, bronchiectasis, fibrosing alveolitis—secondary Sjögren's syndrome and pericarditis). All the patients were taking non-steroidal anti-inflammatory drugs and most were using disease modifying drugs; the need for immunosuppressive drugs (cyclophosphamide, chlorambucil) or immunoregulatory agents (intravenous methotrexate or cyclosporin A) was also recorded. A functional disability index (such as the Health Assessment Questionnaire) was not systematically determined and articular indexes were calculated by different investigators and thus could not be used. Laboratory activity was assessed by the Westergren erythrocyte sedimentation rate (ESR) and the C reactive protein (CRP) level. The presence of rheumatoid factors was determined by nephelometry (positive titres >20 IU/l). Patients were considered seronegative if two determinations were negative.

The presence of erosions was evaluated in the phalangeal joints, and wrists according to the American Rheumatism Association criteria. Patients receiving corticosteroid treatment with a daily dose higher than 10 mg were excluded, as were those treated with immunosuppressive drugs at the time of examination. Patients with recent disease (disease duration <1 year) were also excluded.

Controls
Twenty five healthy controls with no previous history of RA were chosen from the staff at the Besançon blood transfusion centre. To compare the T cell reactivity between the two groups, patients and controls were HLA-DR matched as far as possible.

HLA-DR Typing
After extraction of genomic DNA from peripheral blood cells, HLA-DR genotyping was performed using previously described methods—namely, polymerase chain reaction and sequence-specific oligonucleotide probes (PCR-SSP, Biotest Elpha, Buc France). People who typed as DRB1*01 and DRB1*04 had a subtyping for HLA-DRB1*0101 to *0104 and DRB1*0401 to *0422, respectively (sequence specific primers PCR-SSP, Dynal SSP Products, Compiègne France).

Detection of Previous EBV Infection
Previous EBV infection was recorded in patient and control sera with EBV IgG viral capsid antigen serology.

Antigens Tested in the Proliferation Test
GP110 glycoprotein (858 aa), encoded by the BALF4 open reading frame of EBV, was purified by immunoaffinity as previously described. The control antigen was an endotoxin-free total protein extract from E.coli (OM PHARMA, Geneva, Switzerland).

T Cell Proliferation Assay and Limiting Dilution Analysis
We used direct limiting dilution analysis without previous bulk culture. Peripheral blood mononuclear cells were isolated by isopycnic centrifugation through Ficoll-Hypaque and washed three times in RPMI 1640 medium. Cells were cultured in 96-well plates in 200 microlitres of RPMI 1640 medium supplemented with 1% l-glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin, and 10% self serum. For each subject we measured the proliferative response for 10 wells containing 4 × 10⁵, 2 × 10⁵, 10⁵, or 5 × 10⁴ cells in the presence of gp110, control protein, or no antigen. GP110 concentration in the wells was 0.25 mg/ml, control protein concentration was 10 mg/ml. Cell proliferation was assessed by [³H]thymidine incorporation after five days of culture: one mCi of [³H]thymidine was added to each well 18 hours before cell harvesting on glass fibre filters. Positive wells were defined as having counts per minute higher than the mean plus two standard deviations of the cpm obtained for the 10 control wells. T cell precursor frequencies were calculated from the percentage of negative wells as previously described.

Statistical Analysis
The Mann-Whitney test was used to compare the T cell precursor frequencies in controls and patients. The Spearman rank order test assessed the correlation between ESR, CRP, and the antigen T cell precursor frequencies in patients with RA. A χ² test with Yates's correction, or Fisher's exact test, when appropriate,
was used for comparison of qualitative variables. A p value <0.05 was considered significant.

Results

Table 1 gives details of the clinical and biological characteristics of the 25 patients with RA (20 female, five male; mean age 61.9 (SD 10.2)). Six patients had had arthroplasty and eight had previously taken immunosuppressive/immunoregulatory agents. All patients presented radiological erosions (mean (SD) Larsen score 41.6 (31.8); range 9–112). Four-suggested radiological erosions (mean (SD) Larsen score 41.6 (31.8); range 9–112). Four-suggested radiological erosions (mean (SD) Larsen score 41.6 (31.8); range 9–112).

Table 2 shows the treatments given at the time of assessment. All the patients and the control subjects had positive EBV viral capsid antigen serology. Table 2 gives the demographic data and HLA-DRB1* alleles of the controls (19 female, six male; mean age 48.2 (7.5)). Twelve control subjects expressed the shared epitope (one copy (eight controls), two copies (four)).

Patients with RA had a poor T cell response to EBV gp110

The peripheral blood T cells from both patients with RA and from healthy controls proliferated in response to gp110 and the control protein. In patients with RA the mean gp110 T cell precursor frequency was 3.2 \( \times 10^{-6} \) (range 0–20 \( \times 10^{-6} \)) and in control subjects the mean frequency of T cells able to proliferate to gp110 was 4.1 \( \times 10^{-6} \) (range 0–20 \( \times 10^{-6} \)) (p = 0.02). No significant difference was observed for the control protein (mean T cell precursor frequencies (SD) in RA vs controls were 8.2 (11.2) \( \times 10^{-6} \) vs 6.5 \( \times 10^{-6} \) (p = 0.09). Patients with RA, with or without the shared epitope, responded to gp110 (mean gp110 T cell precursor frequencies (SD) in RA shared epitope positive vs RA shared epitope negative were 2.4 \( \times 10^{-6} \) vs 2.5 \( \times 10^{-6} \) (p = 0.47) (tables 1 and 2).

Frequency of T cells specific for EBV gp110

To determine the effect of specific immune cellular response to gp110 on disease activity, we then examined the relation between laboratory indexes of disease activity (ESR

\[ \text{Mean (SD) ESR: 3.2 (4.4) vs 8.2 (11.2)} \]

\[ \text{Median 63 CRP: 2 vs 2.5} \]
response specific for the control protein did not correlate with either ESR or CRP. Additionally, we found no correlation between the Larsen score and gp110 or control protein T cell response.

A DECREASED EBV GP110 T CELL RESPONSE DISTINGUISHED PATIENTS WITH SEVERE RA

We then examined whether a relation between disease severity and the immune cellular response to EBV gp110 could be found. For this analysis the patients were categorised as low or high responders to gp110 according to the mean T cell precursor frequencies to this antigen (low responders had a T cell response to gp110 <3.2 × 10⁻⁶ and high responders >3.2 × 10⁻⁶). It was therefore evident that all the patients with RA with extra-articular disease were in the low responder group (Fisher’s test, \( p = 0.02 \)) (fig 2). Similarly, a large proportion of patients who had a history of joint replacement or had been previously treated by immunosuppressive/immunoregulatory agents were low responders, but not significantly (\( \chi^2 \) test with Yate’s correction, \( p = 0.67 \) and \( p = 0.6 \), respectively). Finally, the T cell precursor frequencies to the control antigen did not differ between patients with and without extra-articular disease (Fisher’s test, \( p = 0.6 \)).

Discussion

In this study we examined the status of the immune cellular response to EBV gp110 in patients with RA, and its influence on disease activity and severity. Therefore, we quantified the T cells able to proliferate to gp110 using a direct limiting dilution analysis without preliminary bulk culture. We thus obtained an unamplified evaluation of the frequency of T cell precursors specific to this antigen in peripheral blood. Our results showed that patients with RA had a significantly poor immune cellular response to this antigen compared with healthy controls. No difference was seen in the level of cellular response to gp110 between patients with or without the shared epitope. However, only a few patients (25) were tested. In addition, a negative correlation was found between the gp110 T cell response and the laboratory indexes of disease activity (ESR and CRP levels), suggesting a relation between this specific EBV immune cellular response and the inflammatory process and, therefore, the disease activity. Consistent with these data was the observation of an impact of the defect in the gp110 cellular response on clinical features as patients who displayed the lowest T cell precursor frequency for gp110 had the most severe disease with systemic disease such as vasculitis or nodules.

However, a depressed immune cellular reactivity has been described in RA. Indeed, a reduction of delayed-type hypersensitivity and of proliferative responses in vitro to recall antigens and mitogens has been reported in patients with established disease.18 This phenomenon is still not well understood and has been associated with reduced CD4+ and
increased CD8+ T cells in peripheral blood, and with disease activity as determined by ESR.19 Accordingly, our results showed that reactivity to EBV gp110 was related to ESR or CRP levels, or both. The depressed immune cellular reactivity to EBV gp110 in the patient group may reflect the general decreased responsiveness of T cell reactivity in RA. However, this seems unlikely as we did not find a similar decreased reactivity to the control antigen in RA as compared with healthy subjects.

Additionally, this study determined the frequencies of T cells able to proliferate to EBV gp110 and to a control antigen. Only T helper responsiveness was evaluated and it would also be relevant to examine cytotoxic precursor frequencies to the same antigens.

Our results support the hypothesis that altered T cell response to EBV antigens may influence the course of the disease and the clinical features. Indeed, gp110 is a late replicative antigen and it has been shown that it is an important target of the immune response during primary EBV infection or infectious mononucleosis.18 A T lymphocyte defect in the control of EBV is well known in RA as it has been shown that RA T cells had a reduced ability to inhibit EBV-induced outgrowth.20 Thus, conceivably, a poor immune cellular response to gp110 may impair the immune control of EBV, favouring a reservoir of latently infected cells. A later reactivation of these infected cells might then lead to a T cell response to different EBV antigens. In this connection, a recent study found that RA synovium was enriched in CD8+ T cells capable of recognising proteins of the lytic cycle of EBV,15 and this antiviral response might then contribute to inducing and perpetuating chronic joint inflammation.

In our series most patients with a high Larsen score had a low gp110 T cell precursor frequency, but the results were not significant (data not shown; p = 0.5). In this study we evaluated the immune cellular response to the whole gp110 protein, and synthetic peptides of gp110 (containing or not the shared epitope) have not been tested. However, previous studies have shown that the gp110 fragment containing the shared epitope is the main target during immune cellular response to gp110.10 11 20 22 Another study evaluated the T cell response specific for EBV gp110 protein and peptides from this protein containing the shared epitope.22 This was evaluated by classical lymphocyte proliferation assays in patients with RA with early disease (disease duration <1 year) and thus without erosions or joint damage. This study found strong proliferative T cell responses to gp110 in patients expressing the shared epitope as compared with those without the shared epitope and healthy controls. The relation of disease activity with biochemical markers was not examined. This seems in contradiction with our results. However, our study included patients with definite RA who had longstanding (mean disease duration of 9.2 years) and erosive disease. It is thus likely that both immune cellular and humoral responses to EBV differ with the stage of the disease.

It is well known that EBV induces B cell proliferation and polyclonal production of antibodies.3 4 The association of immunoglobulin production by mononuclear cells from patients with RA with disease severity (assessed by joint damage) has been studied: high production of immunoglobulins by lymphocytes in response to EBV stimulation was associated with severe disease and development of joint erosions. These data are in accordance with our results and support the hypothesis of a relation between the status of the EBV immune response and the clinical course of the disease. Similar results have been found in patients with juvenile rheumatoid arthritis who displayed a strong cellular and humoral immune response to dnaJ, a heat shock protein from Escherichia coli that contains the shared epitope;23 the cellular immune response to this bacterial antigen was related to disease activity as defined by the presence of synovitis.

Our findings are consistent with an altered immune cellular response to EBV gp110, suggesting another defect in the T cell control of EBV in patients with RA. The hypothetical consequence may be a persistent exposure to EBV antigens in RA, a situation that might lead to chronic inflammation. This is supported by the negative correlation between the T cell response to gp110 and the laboratory indexes of disease activity. This is also consistent with a decreased T cell response to gp110 in patients with severe disease. However, it would also be relevant to examine the EBV gp110 T cell response in joints of patients with RA.
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