

# Possible involvement of IL-12 expression by Epstein-Barr virus in Sjögren syndrome

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## Abstract

**Aim**—To determine the correlation between interleukin 12 (IL-12) expression and Epstein-Barr virus (EBV) in Sjögren syndrome.

**Methods**—Indirect immunohistochemical technique, enzyme linked immunosorbent assay (ELISA), and immunoblot analysis were used to investigate IL-12 expression by EBV activation, using 13 surgical specimens and four B cell lines.

**Results**—Marked expression of IL-12 was found in the epithelial cells and the infiltrating B cells of salivary gland tissues from patients with Sjögren syndrome (six of 10 cases), but not in those from normal individuals (none of three cases). A striking topographic correlation between IL-12 and EBV was found. In addition, levels of IL-12 production by B cell lines were clearly enhanced by EBV activation in vitro.

**Conclusions**—IL-12 expression closely reflects the intracellular event of EBV activation in Sjögren syndrome, and may contribute to the T helper cell type 1 (Th1) cytokine overexpression seen in this disease.

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Keywords: IL-12; Epstein-Barr virus; Sjögren syndrome

Sjögren syndrome is an autoimmune disease characterised by lymphocytic infiltration into lacrimal and salivary glands leading to symptomatic dry eyes and mouth.<sup>1</sup> Immunohistochemical studies have shown that the majority of infiltrating lymphocytes around the labial salivary and lacrimal glands are CD4+ T cells.<sup>2</sup> It has been reported that salivary gland CD4+ T cells produced more interleukin (IL)-2,  $\gamma$  interferon (IFN- $\gamma$ ), and IL-10 mRNAs than peripheral blood CD4+ T cells obtained from the same patients, whereas they produced little IL-4 and IL-5 mRNA.<sup>3</sup> Similarly, Ohyama *et al* reported that raised levels of cytokine mRNA including IL-2, IFN- $\gamma$ , and IL-10 in the labial salivary glands of patients with Sjögren syndrome.<sup>4</sup> These results indicate that T helper cell type 1 (Th1) cytokines are essential in the induction or maintenance of Sjögren syndrome.

IL-12 is a heterodimeric cytokine originally identified and purified as a product of Epstein-Barr virus (EBV) transformed B cell lines.<sup>5</sup> In human peripheral blood, monocytes/macrophages are the major producers of IL-12, together with B cells and other minor populations of HLA-DR+ accessory cells.<sup>6</sup> It has been suggested that IL-12 has a role in the induction of IFN- $\gamma$  production, in the development of Th1,<sup>7,8</sup> and in the generation of cytotoxic T

lymphocytes.<sup>9</sup> EBV is a widely occurring virus of the herpes family that infects the epithelial cells of the salivary glands and oropharyngeal tissue and the B cells. After the primary infection, the virus remains latent in the host, and occasionally becomes reactivated.<sup>10-16</sup> EBV antigen and its DNA have been found in salivary gland tissues of patients with Sjögren syndrome at levels well above the background levels for latent infection.<sup>17-19</sup> Infectious EBV is present in both the saliva of Sjögren patients<sup>20</sup> and in culture supernatants of B cell lines established from such patients.<sup>21</sup> Patients with Sjögren syndrome have raised levels of antibodies against EBV RNA protein complexes and against EBV antigens.<sup>22,23</sup> Taken together, these observations suggest that a reactivated EBV infection may play a role in Sjögren syndrome, contributing to the initiation or perpetuation of an autoimmune response in the target organs. However, the pathological role of the virus remains obscure. Previous experiments identified an EBV gene BZLF1, the product of which—ZEBRA—switches the virus from a latent to a replicative state.<sup>24-26</sup> ZEBRA is itself sufficient to disrupt latency.<sup>27</sup> Thus evidence for an association between EBV activation and Sjögren syndrome has been accumulating, but our present study is the first to report ZEBRA expression in salivary glands from patients with Sjögren syndrome.

As IL-12 may induce environmentally stimulated inflammatory responses, we have investigated IL-12 expression by EBV in Sjögren salivary glands and whether or not EBV activation is able to induce IL-12 expression in vitro.

## Methods

### PATIENTS

All patients were seen at the Tokyo Medical University Hospital. Ten patients with primary Sjögren syndrome underwent minor salivary gland biopsies to confirm the diagnosis.<sup>28</sup> The biopsies revealed a focus score of > 2, but did not show evidence of lymphoma in routine histological analysis. Each of these patients had keratoconjunctivitis sicca with decreased tear production (< 5 mm on Schirmer test with anaesthesia). Corneal and conjunctival epithelia showed positive rose bengal and fluorescein staining. Decreased secretion of saliva was confirmed by gum test results of < 5 ml.<sup>29</sup> These patients had not received glucocorticoids or immunosuppressive agents for at least six months before biopsy. All were women, and their mean age was 47 years (range 23 to 66 years).

As controls, labial salivary gland biopsy specimens were also obtained from three patients with mucoceles who had no clinical or

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laboratory evidence of systemic autoimmune disease. These labial salivary glands were histologically normal.

#### IMMUNOHISTOCHEMICAL STAINING

The labial salivary glands were immediately placed in OCT compound (Miles), snap frozen in liquid nitrogen, and stored  $-80^{\circ}\text{C}$ . Cryostat sections ( $4\ \mu\text{m}$ ) from each block were transferred onto glass slides for immunohistochemical analysis. Briefly, frozen sections were air dried for 10 minutes, fixed in acetone for 10 minutes, rinsed in phosphate buffered saline (PBS), and incubated with an appropriate blocking agent (Vector) for 20 minutes. They were then incubated for 60 minutes with the following antibodies: purified mouse monoclonal antibodies to human IL-12 p35 (PharMingen), EBV encoded ZEBRA (Dako), CD3, CD4, CD8, CD14, CD20, CD21 (Coulter), or cytokeratin (Becton Dickinson). Antibodies of the same isotype with irrelevant antibody activity were used as negative controls. After rinsing, the sections were reacted with biotinylated goat antimouse (IgG + IgM) antibody (Tago), followed by peroxidase conjugated avidin-biotin complex (Vector) and then the substrate 3,3'-diaminobenzidine (Sigma). All antibodies were diluted to give optimal staining results. Sections were routinely counterstained with haematoxylin.

#### CELL LINES AND CULTURE CONDITIONS

Four B cell lines (Akata, B95-8, BJAB, and Ramos) were obtained from the American Type Culture Collection. All of these cell lines are of human origin except for B95-8, which is of marmoset monkey origin. All cell lines were cultured in RPMI 1640 containing penicillin (100 units/ml), streptomycin (100  $\mu\text{g/ml}$ ), glutamine (10 mg/ml), and 10% fetal calf serum (FCS; Gibco).

#### ELISA FOR IL-12 p40

Four B cell lines were stimulated ( $10^6$  cells/ml,  $37^{\circ}\text{C}$ ) in a 24 well plate with 12-O-tetradecanoylphorbol 13-acetate (TPA, 50 ng/ml) or antihuman IgG (100  $\mu\text{g/ml}$ ) at 24 and 48 hours. Then, for the detection of immunoreactive IL-12 p40 secreted by four B cell line cultures, a commercially available ELISA kit (R&D Systems) was used which enabled the specific detection of IL-12 p40 proteins.

#### IMMUNOBLOT ANALYSIS FOR ZEBRA

Two B cell lines, TPA treated B95-8 and anti-IgG treated Akata, were cultured ( $10^6$  cells/ml,  $37^{\circ}\text{C}$ ) at the indicated time points. They were then solubilised in lysis buffer (300  $\mu\text{l}$  PBS containing 1% Triton X-100 (Sigma), EDTA (1 mM), leupeptin (1 mg/ml; Sigma), aprotinin (5 mg/ml; Sigma), and PMSF (50 mg/ml; Sigma), pH 7.5. Following solubilisation, the samples were clarified by centrifugation at 10 000  $g$  for 10 minutes. An aliquot was then removed for protein assay using a commercially available kit (Bio-Rad), and the remainder of the lysate was frozen at  $-20^{\circ}\text{C}$  pending analysis. For electrophoretic fractionation, an equal volume of lysate

(containing 30  $\mu\text{g}$  of protein) or protein standards (Bio-Rad) was mixed with  $2 \times$  electrophoresis buffer containing sodium dodecyl sulphate (SDS; 4%), Tris-HCl (125 mM, pH 6.8), glycerol (20% vol/vol), and bromophenol blue (0.005%), and fractionated on a 10% Tris/glycine gel at 150 V. Following electrophoresis, the samples were transferred to PVDF membrane, blocked, and then incubated with mouse monoclonal antibody against human anti-IL-12 p40 (R&D Systems).

## Results

#### IMMUNOHISTOLOGICAL ANALYSIS OF IL-12

##### EXPRESSING CELLS IN SJÖGREN SALIVARY GLANDS

To analyse the expression of IL-12 in the salivary glands of patients with Sjögren syndrome, we stained frozen sections of salivary gland tissues with mouse anti-IL-12 antibody using an immunohistological technique. In six of 10 cases, Sjögren salivary gland tissues showed strong reactivity with anti-IL-12 antibody. Positive staining was present in the cytoplasm of most epithelial cells (acini or ductal structures) and many scattered lymphocytes (fig 1A, B, and C). Based on this reactivity in serial sections of tissue stained with anti-CD20 and anti-CD21 antibodies (B cell markers) and anticytokeratin antibody (epithelial cell marker), respectively, and their lack of reactivity with anti-CD3 antibody (T cell marker) and anti-CD14 (macrophage marker), the stained cells appeared to be epithelial cells and B cells. No staining with anti-IL-12 antibody was detected in the normal salivary glands ( $n = 3$ ), and no cells were stained an isotype matched rabbit IgG (fig 1D).

As the detection of EBV encoded EBV-ZEBRA was carried out on one of the serial sections, assessment of the relation between IL-12 expression and EBV-ZEBRA localisation was possible. A striking correlation between EBV-ZEBRA positive cells and IL-12 positive cells was observed in the Sjögren salivary glands. EBV-ZEBRA was detected in five of 10 Sjögren salivary gland biopsy specimens. In normal salivary glands, no EBV-ZEBRA expression was detected ( $n = 3$ ). EBV-ZEBRA positive cells were found among the many infiltrating lymphocytes and in most epithelial cells of the Sjögren salivary glands (fig 2A).

Interestingly, the localisation of EBV-ZEBRA positive cells (fig 2B) was almost the same as that of IL-12 positive cells (fig 2C). All the EBV-ZEBRA positive areas were also positive for IL-12.

#### INDUCTION OF IL-12 SECRETION IN B CELL LINES

The stimulatory effect of EBV activation on IL-12 secretion was investigated by ELISA. Four B cell lines were cultured ( $10^6$  cells/ml,  $37^{\circ}\text{C}$ ) at 24 and 48 hours with TPA or anti-IgG, and IL-12 levels in culture supernatants were determined (table 1). The results showed that an incremental change in IL-12 secretion occurred in both TPA treated B95-8 and anti-IgG treated Akata 24 hours after stimulation (mean SEM IL-12 secretion without TPA  $< 31.2$  pg/ml, with TPA 128 pg/ml ( $n = 5$ ),  $p < 0.005$ ; without anti-IgG  $< 31.2$

pg/ml, with anti-IgG 135 pg/ml (n = 5), p < 0.005). In contrast, no stimulatory effect was found in EBV negative Burkitt lymphoma BJAB and Ramos.

INVESTIGATION OF THE RELATION BETWEEN IL-12 AND EBV-ZEBRA BY IMMUNOBLOT ANALYSIS  
EBV activation was confirmed in TPA treated B95-8 and anti-IgG treated Akata, using the

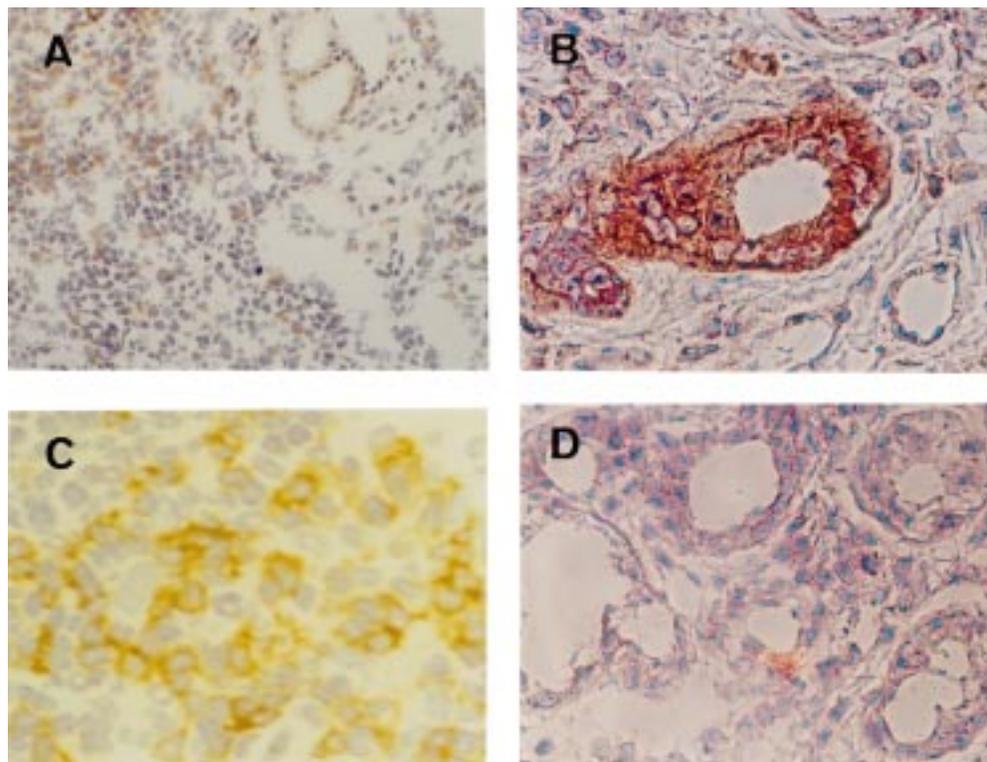


Figure 1 Immunostaining of salivary gland specimens from patients with Sjögren syndrome. Cryostat-frozen serial sections were fixed in cold acetone, stained with mouse anti-IL-12 antibody and mouse anti-ZEBRA antibody, and examined by immunohistological techniques. Numerous anti-IL-12 positive epithelial cells (acini and ductal structures) and lymphoid cells are seen in salivary gland biopsy specimens from patients with Sjögren syndrome (A-C). No cells stained with isotype matched mouse IgG (D). (Magnification  $\times 89$  in A,  $\times 359$  in B-D.)

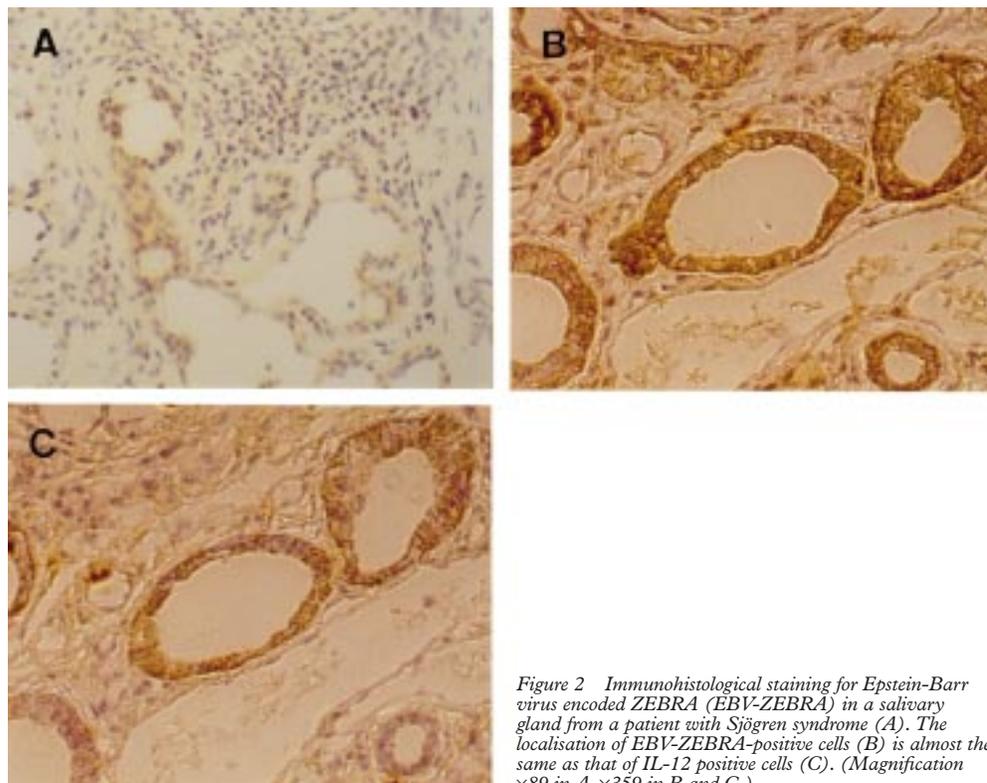


Figure 2 Immunohistological staining for Epstein-Barr virus encoded ZEBRA (EBV-ZEBRA) in a salivary gland from a patient with Sjögren syndrome (A). The localisation of EBV-ZEBRA-positive cells (B) is almost the same as that of IL-12 positive cells (C). (Magnification  $\times 89$  in A,  $\times 359$  in B and C.)

Table 1 Analysis of interleukin 12 (IL-12) secretion in B cell lines by enzyme linked immunosorbent assay (ELISA)

Responder cells	Unstimulated		Stimulated	
	24 h	48 h	24 h	48 h
Akata	<31.2	<31.2	135 (31)	141 (40)
B95-8	<31.2	<31.2	128 (24)	135 (32)
BJAB	<31.2	<31.2	<31.2	<31.2
Ramos	<31.2	<31.2	<31.2	<31.2

Values are pg/ml (mean (SEM)).

The expression of IL-12 p40 in four B cell lines is summarised. Unstimulated or 12-O-tetradecanoylphorbol 13-acetate (TPA, 50 ng/ml) stimulated B95-8 and BJAB, Ramos, and antihuman-IgG (100 µg/ml) stimulated Akata were cultured for 24 and 48 hours. The supernatants were collected at the indicated time and IL-12 p40 was measured by ELISA.

immunoblot assay for detection of ZEBRA. A substantial expression of ZEBRA was detected in both cell lines 10 hours after stimulation, and increased steadily thereafter (fig 3). In the absence of stimulation, no ZEBRA expression was observed in either cell line (data not shown).

### Discussion

Previous evidence for EBV reactivation in patients with Sjögren syndrome includes the presence of EBV antigens<sup>17</sup> and raised levels of EBV DNA<sup>17-19</sup> in salivary gland tissues and active production of virus in the saliva<sup>20</sup> or from B cell lines established from peripheral blood mononuclear cells.<sup>21</sup> Antibodies against EBV antigens are also raised in Sjögren sera.<sup>21-23</sup> Another defined manifestation of active EBV infection is the presence of circulating infected B cells that can transform into B cell lymphomas.<sup>30-32</sup> Thus Sjögren patients show many signs of active, uncontrolled EBV reactivation. In this study, we have shown that most epithelial cells and infiltrated B cells in Sjögren salivary glands had the capacity to express ZEBRA.

Investigation of the relation between IL-12 and EBV in closely parallel sections showed that EBV-ZEBRA positive samples were also positive for IL-12. A close topographic association of EBV-ZEBRA and IL-12 expression in Sjögren salivary glands was indicated. In relation to the production of IL-12 by non-haemopoietic cells, Aragane *et al* showed that stimulated human keratinocytes and keratinocyte cell lines have the capacity to express IL-12.<sup>33</sup> Moreover, Kang *et al* reported that epidermal Langerhans cells, in particular cultured Langerhans cells maturing into dendritic cells, expressed IL-12 mRNA.<sup>34</sup> They suggested that this was one mechanism behind the high potency of the dendritic antigen presenting cell for initiating Th1 type immune

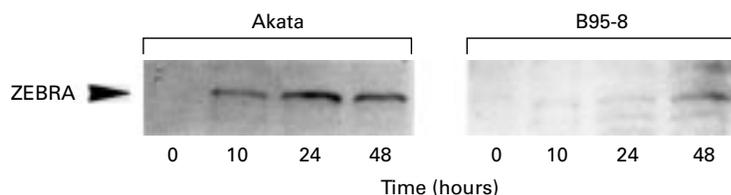


Figure 3 Immunoblot analysis of ZEBRA expression in two B cell lines. Immunoblotting was performed from the cell lysate of Akata, stimulated with anti-IgG, or of B95-8, stimulated with 12-O-tetradecanoylphorbol 13-acetate (TPA) at the indicated time.

responses under appropriate conditions. Interestingly, Fox *et al* reported the presence of HLA-DR antigens on salivary gland epithelial cells and showed that this induction might be caused by IFN- $\gamma$ .<sup>35</sup> Thus epithelial cells and infiltrated B cells in Sjögren salivary glands may be important mediators of IL-12 induced Th1 development.

Given that immunohistological data cannot be generated *in vivo*, we investigated the induction of IL-12 in Sjögren salivary glands using B cell lines. Our results showed IL-12 secretion from stimulated EBV infected lymphoma cell lines, Akata and B95-8, was associated with EBV-ZEBRA expression on western blot analysis. These findings suggest that there is a close relation between IL-12 production and EBV activation. Hsu *et al* reported that the virally encoded genes can modulate lymphokine expression,<sup>36</sup> and there is further evidence for an association between EBV infection and cytokine production.<sup>37-39</sup>

As previously reported, interaction between two inflammatory cytokines, such as IFN- $\gamma$  and tumour necrosis factor  $\alpha$ , induces IL-6 production from human salivary gland cell line.<sup>40</sup> In addition, Yoshida *et al* reported that IFN- $\gamma$  was capable of inducing IL-12 mRNA.<sup>41</sup> If EBV alone is not sufficient to lead to IL-12 expression, it may be one of many cofactors. It is known that Sjögren salivary glands express increased levels of cytokines compared with normal salivary glands.<sup>3</sup> Therefore IL-12 expression in Sjögren syndrome may result from EBV activation and cytokine network.

Several properties that may be relevant to the initiation and perpetuation of the pathogenesis of Sjögren syndrome are now known to be present in the salivary glands of affected patients,<sup>42-43</sup> but not in other salivary gland conditions or in normal salivary glands. These include intense lymphocytic infiltration, destruction of epithelial cells, abnormally high levels of EBV and EBV antigen, production of infectious EBV in saliva, *de novo* expression of HLA-DR on cell surfaces, and ectopic expression of IL-12. These observations are consistent with several scenarios in which EBV plays an important role in the initiation or continuation of immune system damage in salivary and lacrimal glands. We thus propose that IL-12 is a new member of the group of known factors that are overexpressed in Sjögren syndrome, and possibly induced by EBV activation.

### CONCLUSIONS

In summary, our study shows that epithelial cells and infiltrated B cells in salivary glands from patients with Sjögren syndrome have the capacity to express IL-12 associated with EBV activation. These findings suggest that the mutual stimulation of infiltrated lymphocytes and salivary gland epithelial cells through the production of cytokine maintains dominant expression of the Th1 type cytokine in Sjögren salivary glands, giving rise to the eventual destruction of the target organ. A more thorough understanding of the complex consequences of Sjögren syndrome might lead to a possible therapeutic application for cytokine

agonists and antagonists to disrupt the cytokine network and inhibit the initiation and progression of the disease.

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