

Defective Epstein-Barr virus specific suppressor T cell function in progressive systemic sclerosis

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SUMMARY Several immunoregulatory defects of Epstein-Barr virus (EBV) induced B cell activation have been described in patients with rheumatoid arthritis (RA), suggesting that EBV may have a role in the pathogenesis of RA. We assessed EBV specific T cell regulation in 20 patients with progressive systemic sclerosis (PSS) and immune to EBV and in 10 control subjects also immune to EBV by comparing the secretion of IgM into supernatants of 16 day cultures of B cells alone and cocultures of B and autologous T cells. In control subjects autologous T cells mediated a significant decrease in the secretion of IgM by B cells at 12 and 16 days of culture. Analysis of individual responses showed the existence of two subgroups of patients with PSS: group I (10 patients) had a suppressor T cell function similar to that of controls; group II (10 patients) had a defective T cell function. Differences in the duration or severity of the disease, the slow acting therapeutic agents, and anti-inflammatory drugs could not account for these subdivisions. These results suggest that several immunoregulatory defects of EBV induced B cell activation exist in different connective tissue diseases.

The aetiology and the pathogenesis of progressive systemic sclerosis (PSS) have not been well elucidated.^{1 2} PSS is characterised by vascular and microvascular abnormalities,¹⁻⁴ excessive fibroblastic activity,^{5 6} and collagen deposition in numerous organs.^{1 2 7} A wide range of immunological abnormalities have been described in patients with this disorder.^{8 9} These findings include decreased numbers of circulating T lymphocytes,¹⁰⁻¹⁵ decreased lymphocyte proliferation to mitogens,^{16 17} impaired antibody dependent, cell mediated cytotoxicity,^{18 19} increased cell mediated immunity to skin extracts and collagen,²⁰ and increased OKT4⁺/OKT8⁺ ratio with depressed numbers of OKT8⁺.^{15 21} The existence of hypergammaglobulinaemia,²² antinuclear antibodies,²³⁻²⁵ and immune complexes²³ suggests a persistent state of enhanced B cell activation in progressive systemic sclerosis.

The Epstein-Barr virus (EBV) is an ubiquitous virus of the herpes group, which is persistent and capable of altering immune responses. EBV is

considered to be a polyclonal B cell activator.²⁶ Thus EBV could be responsible for at least part of the sustained B cell activation in PSS.

Several reports have shown a defective EBV specific suppressor T cell function in rheumatoid arthritis (RA), suggesting that EBV may have a role in the pathogenesis of RA.²⁶⁻²⁹ We and others have recently shown that several immunoregulatory defects exist in subgroups of RA patients.^{30 31}

In this study we examined EBV specific T cell regulation in patients with PSS and in controls. Our results show that 50% of PSS patients have a strikingly defective suppressor T cell function of EBV induced B cell activation.

Patients and methods

PATIENTS

Twenty patients with progressive systemic sclerosis and diffuse scleroderma (mean age 49.8±9.2 (SD) years; 18 women, two men) and 10 control subjects (mean age 56.6±16.3 years; eight women, two men) were studied. All patients satisfied the American Rheumatism Association preliminary criteria for classification of definite systemic sclerosis.³² None of the patients had the CREST syndrome variant

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(calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia) of PSS.³³ Eight patients were taking non-steroidal anti-inflammatory agents. Four patients received 10 mg or less prednisone daily. All 20 patients had stopped slow acting therapeutic agents for at least two months.

Control subjects had sciatica (nuclear herniations) (seven patients) and osteoporosis (three patients); six of them were taking non-steroidal anti-inflammatory agents.

VIRUS

The B95-8 marmoset lymphoblastoid line served as the source of transforming EBV. B95-8 cells were seeded at a concentration of 0.5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum (Flow Laboratories). The cell free supernatants of these cultures were harvested after 14 days, filtered through a $0.22 \mu\text{m}$ Millipore filter, stored in small aliquots at -80°C , and thawed only once on the day of use.

CELL SEPARATION

The mononuclear cell population was obtained from 60 ml heparinised blood by a standard density gradient centrifugation with a mixture of Ficoll-Paque (density 1.076) (Pharmacia Laboratories, Uppsala). B and T cells were further separated by rosetting with aminoethylisothiuronium bromide treated sheep red blood cells (AET-SRBC)³⁴ at 4°C for two hours. The mixture was separated by the use of Ficoll-Paque into an interphase population of non-rosetted cells and a pellet of rosetted cells. The rosetted lymphocyte population was treated with ammonium chloride lysing buffer to remove SRBC. This cell population was tested with OKT monoclonal antibodies (Ortho-mune, Raritan, NJ) and was found to contain 95–98% OKT3⁺ cells; the percentages of OKT4⁺ and OKT8⁺ cells were variable. The non-rosetted population referred to as the 'B cell population' contained less than 2% OKT3⁺ cells, a variable number of monocytes (20–35%) identified with an anti-OKM1 monoclonal antibody (Ortho-mune), and 65–70% B cells (surface immunoglobulin positive cells, sIg⁺, identified with an antihuman immunoglobulin fluorescein conjugated serum (Meloy Laboratories, Springfield, VA).

LYMPHOCYTE CULTURES

For each patient and control two cell populations were infected with EBV: (a) B cells alone (5×10^6 cells) and (b) B cells (5×10^6) and autologous T cells (5×10^6). The cells were incubated for one hour at 37°C with 200 μl of B95-8 supernatant (EBV).

Thereafter, cells were washed twice with RPMI 1640 (Dutch modification, Flow Laboratories) medium and resuspended in 10 ml of RPMI 1640 medium supplemented with 15% fetal calf serum (Flow Laboratories), 2mM glutamine, 1mM pyruvate, non-essential amino acids (Flow Laboratories), 5×10^{-5} M 2-mercaptoethanol, and antibiotics (Gentamicin) to a final concentration of 0.5×10^6 B cells/ml.

Thus for each patient and control two parallel cultures were done: (a) cultures of B cells alone (0.5×10^6 cells/ml) and (b) cocultures of B cells (0.5×10^6 cells/ml) and autologous T cells (0.5×10^6 cells/ml). Lymphocytes were cultured at 37°C for 16 days in 25 ml Falcon flasks (Becton-Dickinson, Palo Alto, CA) in a humidified atmosphere containing 5% carbon dioxide. Half of the supernatant was removed from each culture on the eighth day and replaced with an equal volume of fresh complete medium. The same procedure was subsequently performed at 12 and 16 days of culture. The supernatants were filtered through a $0.45 \mu\text{m}$ Millipore filter and stored at -20°C .

DEPLETION OF ADHERENT CELLS

Ten million 'B' cells were incubated for 60 minutes at 37°C in plastic culture dishes in RPMI 1640 medium supplemented with 15% fetal calf serum. Non-adherent cells were recovered by washing the dishes with warm medium. The recovered cells were 95–98% sIg⁺ OKM1⁻.

IGM SECRETION

IgM secretion into the culture supernatants was assessed by a laser-nephelometry assay (laser-nephelometer PDQ instrument, coupled to a mini-computer, Hyland, Belgium),³⁵ as an index of EBV induced B cell activation. The sensitivity of this method allows the detection of IgM at a minimal concentration of 3 $\mu\text{g/ml}$. Goat antibodies (Hyland) were used to avoid interference with rheumatoid factor that could occur with rabbit antisera. The reference serum used in the nephelometry assay had been tested (Hyland) with a World Health Organisation reference serum. Testing of the same supernatant at one week intervals gave reproducible results. The nephelometry assay was compared with an enzyme linked immunosorbent assay in some patients and gave similar results. For some patients several identical cultures were done and gave reproducible results during the 16 day cultures. Since half of the culture supernatant was substituted with fresh complete medium every four days the actual secretion of IgM during these four day intervals was calculated taking into account this dilution.

For each patient and control subject the secretion of IgM in the two parallel cultures (a) and (b) described above was compared. Furthermore, a suppression ratio was calculated as follows: $B-(B+T)/B$, with B representing IgM secretion in cultures of B cells alone and (B+T) the secretion of IgM in cocultures of B and autologous T cells.

ANTIBODIES TO EBV SPECIFIC ANTIGENS
Antibody titres to the virocapsid antigen (VCA) were determined by an immunofluorescence technique as previously described.³⁶ Antibody titres to Epstein-Barr virus nuclear antigen were not analysed since 13 of the 20 PSS patients had anti-nuclear antibodies.

STATISTICS

The statistical significance of differences between IgM secretion in B cell cultures and in autologous B and T cell cocultures was determined with the two tailed Student's *t* test.

For IgM secretion in B cell cultures alone statistical analysis was performed with two way analysis of variance for repeated measures³⁷ in order to test the overall group and time effects, and one way analysis of variance to test the group effect at each time. Pairwise comparisons between group (and/or time) means were made by the Newman-Keuls method and with the subject within group variance as an error term for group effect. All tests were performed with the SAS statistical analysis package. For clinical characteristics one way analysis of variance and χ^2 analysis were performed. A value of $p=0.05$ was considered significant.

Results

AUTOLOGOUS T CELL MEDIATED REGULATION OF IgM SECRETION BY EBV ACTIVATED B CELLS IN VITRO

The results of IgM secretion (mean \pm SD) into the supernatants of cultures of B cells alone and cocultures of B cells and autologous T cells are shown in Table 1.

In control subjects autologous T cells induced a significant suppression of IgM secretion by EBV activated B cells at 12 and 16 days of culture; the mean and individual results are shown in Fig. 1a.

In PSS patients autologous T cells also induced a significant suppression at 12 days of culture. At 16 days of culture, however, the difference of IgM secretion between B cell cultures and autologous B and T cell cocultures was not statistically significant (Table 1).

The suppression ratio in PSS patients was lower

Table 1 IgM secretion in B cell cultures and in B and autologous T cell cocultures in control subjects and patients with progressive systemic sclerosis (PSS)[†]

Culture	Control subjects (n=10)	PSS		
		All patients (n=20)	Group I (n=10)	Group II (n=10)
12 days				
B cell	10.3 \pm 7.1	6.0 \pm 5.6	5.4 \pm 5.8	6.6 \pm 5.6
B+T cell	4.0 \pm 4.2*	3.4 \pm 4.7*	0.9 \pm 1.9*	5.9 \pm 5.4
16 days				
B cell	8.0 \pm 3.5	7.8 \pm 5.1	6.8 \pm 3.9	8.8 \pm 6.2
B+T cell	3.0 \pm 2.8**	5.9 \pm 6.7	2.7 \pm 3.2*	9.1 \pm 7.9

* $p<0.01$; ** $p<0.001$.

[†]Values given are mean IgM secretion (\pm SD, μ g/ml) in B cell cultures and in B and autologous T cell cocultures during four day intervals in control subjects and PSS patients. The differences in IgM secretion between B cell cultures and autologous B and T cell cocultures were analysed with the Student's two tailed *t* test.

than in control subjects (mean \pm SD 0.29 \pm 0.50 v 0.56 \pm 0.36 at 12 days, and 0.34 \pm 0.50 v 0.62 \pm 0.33 at 16 days, respectively), but the differences did not reach statistical significance.

Analysis of individual responses showed the existence of two subgroups of PSS patients: PSS patients with a suppression ratio higher than 0.50 were included in group I; PSS patients who had a suppression ratio lower than 0.50 were in group II.

In PSS group I (10 patients) autologous T cells induced a significant suppression of IgM secretion by EBV activated B cells at 12 and 16 days of culture (Table 1 and Fig. 1b). In PSS group II (10 patients) autologous T cells did not induce a significant suppression of IgM secretion by EBV activated B cells (Table 1 and Fig. 1c).

Thus a suppression ratio lower or higher than 0.50 at 12 and 16 days of culture allowed the classification of PSS patients into two groups with definitely different T cell regulations. Group II was characterised by a defective T cell regulation. In contrast, group I had a T cell regulation similar to that of control subjects.

This in vitro study was repeated at six month intervals in seven patients (three patients in group I, four patients in group II). None of these patients was taking prednisone or slow acting therapeutic agents. Four of the seven patients were taking non-steroidal anti-inflammatory drugs; the treatment had remained unchanged during this six month period. In each patient the results of these vitro assays were similar at six month intervals: all patients remained in the same subgroups (data not shown). Thus the classification of each individual

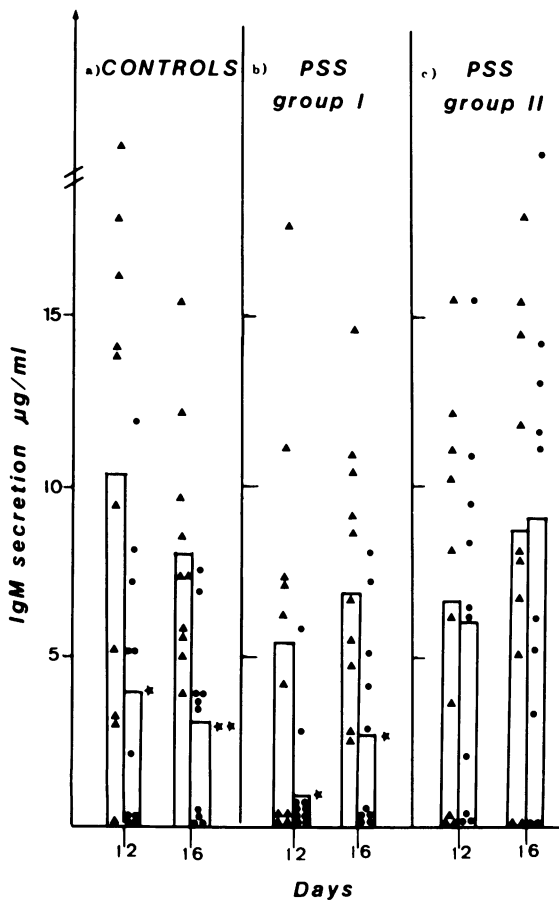


Fig. 1 IgM secretion over a 16 day period in Epstein-Barr virus (EBV) stimulated cultures from control subjects (a) and from patients in progressive systemic sclerosis (PSS) group I (b) and group II (c). ▲ = B cells alone ($0.5 \times 10^6/\text{ml}$); ● = autologous B cell ($0.5 \times 10^6/\text{ml}$) and T cell ($0.5 \times 10^6/\text{ml}$) mixtures. The individual data points represent IgM secretion during four day intervals in 10 controls, 10 PSS patients in group I, and 10 PSS patients in group II. The open bars represent the mean IgM secretion. * $p < 0.01$ and ** $p < 0.001$, with the Student's two tailed t test.

PSS patient into one particular subgroup according to the in vitro abnormalities of EBV induced B cell activation seems to be a constant finding.

ROLE OF THE T:B CELL RATIO

It may be questioned whether different T:B cell ratios could influence these results. Parallel experiments were performed in 10 PSS patients in group I and five PSS patients in group II with three different T:B cell ratios: (a) 1:1; (b) 2:1; (c) 4:1 (B cells were at concentration of $0.5 \times 10^6/\text{ml}$ and T cells were

$0.5 \times 10^6/\text{ml}$ (a), or $1.0 \times 10^6/\text{ml}$ (b), or $2.0 \times 10^6/\text{ml}$ (c)). In each patient the results of these in vitro assays were similar with the three T:B cell ratios used: all patients remained in the same subgroup (data not shown). Thus the T cell regulation defect of EBV induced B cell activation in PSS group II was not modified when different ratios of immunoregulatory cells were used.

IgM SECRETION BY EBV ACTIVATED B CELLS ALONE IN VITRO

In PSS patients the mean IgM secretion in cultures of B cells alone was lower than in control subjects at 12 days, but the difference did not reach statistical significance (Table 1). The mean number of B cells (sIg^+ , $OKM1^-$) in cultures from control subjects, all 20 PSS patients, PSS group I, and PSS group II did not differ significantly.

ROLE OF MACROPHAGES

Since B cell cultures contained 65–80% B lymphocytes (sIg^+) and 20–35% monocytes ($OKM1^+$) it

Table 2 Clinical characteristics of the two progressive systemic sclerosis (PSS) patient subgroups[†]

	PSS group I	PSS group II
Number of patients	10	10
Sex (female:male)	9:1	9:1
Age (years)	53.1 ± 6.9	46.6 ± 10.3
Duration of disease (years)	10.1 ± 7.5	12.2 ± 9.4
Raynaud's phenomenon [‡]	10	10
History of digital ulcers [‡]	5	4
Oesophageal dysmotility ^{‡*}	9	8
Pulmonary involvement ^{‡**}	5	3
Sjögren's syndrome [‡]	3	3
Prednisone [‡]	2	2
Non-steroidal anti-inflammatory drugs [‡]	5	3
ESR [§] (mm/1st h)	32.7 ± 13.8	$18.3 \pm 13.6^*$
Haemoglobin (g/dl) [¶]	12.2 ± 1.7	13.4 ± 1.3
WBC [§] $\times 10^{-3}$ (cells/mm ³) [¶]	8.3 ± 2.8	6.5 ± 1.9
Peripheral blood lymphocytes $\times 10^{-3}$ (cells/mm ³) [¶]	2.1 ± 1.2	1.9 ± 0.9
Serum creatinine ($\mu\text{mol/l}$)	73.3 ± 15.4	77.9 ± 16.3
Latex test [‡]	3	2
ANA ^{§§}	7	6

* $p < 0.05$.

[†]Values with \pm are mean \pm SD. The differences between the two PSS subgroups were not statistically significant, except for the ESR ($p < 0.05$) (one way analysis of variance and χ^2 analysis).

[‡]Figures give the number of patients.

[§]ESR = erythrocyte sedimentation rate; WBC = white blood cell count; ANA = antinuclear antibodies.

[¶]SI conversion: haemoglobin— $\text{g} \times \text{dl} \times 10 = \text{g/l}$; WBC, blood lymphocytes— $\text{cells}/\text{mm}^3 \times 10^6 = \text{cells/l}$.

^{¶¶}Oesophageal dysmotility assessed by manometric study.

**Pulmonary involvement: mild or moderate restrictive lung disease (forced vital capacity) and decrease in DLco (single breath diffusing capacity for carbon monoxide).

may be questioned whether macrophages could have a role in this *in vitro* system and whether the immunoregulatory defect in PSS group II could be explained by an abnormality in adherent cells.

To test these hypotheses we performed depletion experiments in four PSS patients in group I and five PSS patients in group II. In these patients four parallel cultures were done: (a) B cells; (b) B and T cell mixtures; (c) B cells with depletion of adherent cells; (d) B cells with depletion of adherent cells, and T cell mixtures.

Depletion of adherent cells in group I and in group II did not significantly modify the results (data not shown), suggesting that adherent cells do not have an essential role in this *in vitro* system and that the regulation defect in group II was not due to a macrophage abnormality.

EVIDENCE OF PRIOR INFECTION WITH EBV
All patients with PSS and all control subjects had detectable antibodies to VCA, indicating that all had previously been infected with EBV. The geometric mean antibody titre to the VCA did not differ significantly between control subjects (190×3.95), all PSS patients (94×3.39), PSS group I (80×3.32), and PSS group II (107×3.73).

CLINICAL CHARACTERISTICS OF THE PSS PATIENT SUBGROUPS

The clinical differences between the two PSS subgroups were not statistically significant, except for the erythrocyte sedimentation rate, which was lower in PSS group II (Table 2).

Discussion

The addition of EBV to lymphocytes *in vitro* induces the transformation of infected B lymphocytes; they become blasts, begin active DNA synthesis and rapid cell division, secrete immunoglobulin of all classes (but predominantly IgM), and finally, they produce lymphoblastoid cell lines. Thus EBV is considered to be a polyclonal B cell activator.²⁶⁻³⁸ The activation by EBV of B lymphocytes does not require the participation of helper T cells,³⁸⁻³⁹ though T cells can negatively regulate this process.²⁹ In normal subjects a T cell mediated suppression of immunoglobulin synthesis induced by EBV appears to be dependent on a cytotoxic or suppressor T cell population.⁴⁰

Several immunoregulatory abnormalities of EBV induced B cell activation *in vitro* have been shown in patients with rheumatoid arthritis.^{26-31 41-44} Tosato *et al* have shown by means of a reverse haemolytic plaque assay that RA patients immune to EBV had an impaired EBV specific suppressor T cell

function.²⁸ The existence of such a specific immune defect suggested that in the absence of generalised immunoregulatory abnormalities EBV may have a role in the pathogenesis of RA.

In a previous study we assessed IgM secretion by EBV activated B cells from RA patients and showed that the T cell regulation defect was not found in all RA patients.³⁰ analysis of individual results showed that using the value of the suppression ratio higher or lower than 0.50 RA patients could be divided into two groups having a normal or defective suppressor T cell function.

In the present study we used a similar method to assess a possible immunoregulatory abnormality of EBV induced B cell activation in patients with PSS and diffuse scleroderma.

Our data clearly show that 50% of PSS patients have a strikingly defective T cell suppression of EBV induced B cell activation. We assessed T cell regulation by comparing IgM secretion into the supernatant of 16 day cultures of B cells alone and cocultures of autologous T and B cells. In all PSS patients studied as a group the suppression ratio was lower than that in control subjects at 12 and 16 days of culture. We have previously observed similar results in RA patients.³⁰

Analysis of individual results showed that using the value of the suppression ratio at 12 and 16 days of culture PSS patients could be classified into two subgroups: PSS group I was defined by a suppression ratio higher than 0.50 and PSS group II by a suppression ratio lower than 0.50. In PSS group I (10 patients) a significant T cell suppression was obvious, similar to that of controls. In contrast, PSS group II (10 patients) was characterised by a strikingly defective T cell suppression of EBV induced B cell activation as compared with control subjects. These results are similar to those obtained in our previous study of RA patients.³⁰ Thus a suppression ratio higher or lower than 0.50 allows the classification of PSS patients into two groups characterised by a normal (group I) or a defective (group II) T cell suppression of EBV induced B cell activation.

This classification of PSS patients according to the *in vitro* abnormalities of EBV induced B cell activation seems to be a constant finding since similar results were obtained in PSS patients from both groups at six month intervals.

Furthermore, the T cell regulation defect could not be explained by a low T:B cell ratio since the use of higher (two- or fourfold) T:B cell ratios did not modify the results.

Other investigators have reported that some suppressor T cell functions in different *in vitro*

systems are normal in patients with PSS.⁹ Alarcon-Segovia *et al* examined T cell subpopulations and their immunoregulatory circuits in patients with PSS and failed to show abnormalities in the function of post-thymic T cell precursors or in the concanavalin A induced or spontaneously expanded suppressor cell function.⁴⁵ Keystone *et al* investigating antigen specific suppressor cell activity in patients with PSS failed to show any difference between the results for PSS patients and those for age matched controls.⁴⁶ These findings suggest that the striking deficiency in EBV specific suppressor T cell function does not reflect a broader immunoregulatory T cell defect in patients with PSS.

In contrast with RA patients,^{30 47 48} an intrinsic B cell defect could not be shown in PSS patients in this *in vitro* system.

In other *in vitro* systems an abnormal macrophage mediated activity was shown in PSS patients.^{49 50} In the present investigation macrophages did not seem to have an essential role and could not account for the defective suppressor activity in PSS group II since depletion of adherent cells did not significantly modify the results in patients in both PSS groups. These results accord with those of previous studies, which have shown that the elimination of adherent cells does not dramatically affect the polyclonal activation of B cells by EBV.^{30 51}

It may be questioned whether disease activity, anti-inflammatory drugs, and previous *in vivo* exposure to EBV affected the results or even accounted for subdivision.

The clinical differences between the two groups of PSS patients were not statistically significant (Table 2). The only significant difference was a decreased mean ESR value in PSS group II. Thus the defective T cell regulation in PSS group II could not be explained by differences in the duration or severity of the disease.

Hasler *et al* have shown that in rheumatoid arthritis patients the defective production of gamma interferon in the RA autologous mixed leucocyte reaction is, at least in part, the result of enhanced sensitivity of RA T lymphocytes to adherent cell produced prostaglandins, and that indomethacin corrects this *in vitro* defect.⁵² In our study the number of patients receiving anti-inflammatory drugs (and the doses used) were similar in the two PSS groups (Table 2) and in the control subjects. Thus the T cell regulation defect in PSS group II could not be explained by differences in therapy with anti-inflammatory agents. We³⁰ and other investigators^{29 42 43} have also reported that T cell regulation defects in patients with RA were not caused by therapy with anti-inflammatory drugs.

Furthermore, in the present study previous administration of slow acting therapeutic agents (D-penicillamine) did not differ significantly between the two PSS subgroups.

Finally, all patients and controls in our study were immune to EBV and the geometric antibody titres in the two PSS subgroups and the control group did not significantly differ; thus the immunoregulatory defect in PSS group II could not be explained by differences in prior *in vivo* exposure to EBV.

It is yet to be determined whether the immunoregulatory defect we found is a primary phenomenon that can be implicated in the initiation or perpetuation of PSS in some cases, or whether it develops secondarily as a consequence of chronic disease.

The mechanisms, the cell subpopulations, the lymphokines, and other soluble factors possibly involved in the immunoregulatory defect are currently under study.

Other investigators have reported similar immunoregulatory defects in systemic lupus erythematosus.⁵³ These findings suggest that EBV regulation defects are not unique to rheumatoid arthritis and can be found in subgroups of patients with different connective tissue diseases.

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