

## Spontaneous Establishment of an Epstein-Barr Virus-Infected Fibroblast Line from the Synovial Tissue of a Rheumatoid Arthritis Patient

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**An Epstein-Barr virus (EBV)-infected fibroblast line, designated DSEK, was spontaneously established from synovial tissue of a patient with rheumatoid arthritis (RA). DSEK cells expressed EBV nuclear antigens EBNA-1 and EBNA-2 and latent membrane protein LMP-1. Cell surface markers of DSEK cells were similar to those of EBV-negative fibroblast clones derived from synoviocytes and were negative for lymphocyte and macrophage markers. DSEK cells expressed CD44, CD58, and HLA-DR antigens and spontaneously produced interleukin-10 basic fibroblast growth factor and transforming growth factor  $\beta$ 1. These results indicate that rheumatoid synoviocytes can be a target for EBV infection and suggest that EBV may play a role in the pathogenesis of RA.**

The cause of rheumatoid arthritis (RA) remains unknown, and several factors may be causally associated with it. Bacteria and viruses remain suspect (15). Candidate viruses include rubella virus (4), the B19 strain of parvovirus (6), hepatitis B virus (11), and Epstein-Barr virus (EBV). EBV, a ubiquitous human herpesvirus, has been detected in several human malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, gastric carcinoma, and AIDS-associated lymphoma (16). RA patients have a higher frequency and higher level of antibodies against EBV nuclear antigens EBNA-2, -3a, -3b, and -3c in their sera than normal individuals (9, 19, 23). It has been reported that the number of circulating B lymphocytes infected with EBV is elevated in RA patients and that EBV-specific T-cell immunity is reduced (8, 13, 20, 21). These findings suggest an association between EBV infection and RA. However, no convincing evidence has yet been provided to support EBV infection in RA synovial tissue. Here, we report spontaneous establishment of an EBV-infected fibroblast line from the synovial tissue of an RA patient.

**Isolation of synovial cell clones.** Synovial tissue from 12 RA patients was investigated in this study. All RA patients met the revised criteria of the American College of Rheumatology (1). Three of these 12 RA patients, IA, IIA, and IIIA, are the main subjects of the present paper. Immediately after surgical removal, synovial tissue from the knee joints of the RA patients was plated in Iscove's modified Dulbecco modified Eagle medium (IMDM) supplemented with 10% fetal calf serum (FCS). After treatment with type I collagenase (5 mg/ml) and DNase (0.15 mg/ml), the synovial cells were resuspended at  $2 \times 10^6$  cells/well in six-well plates and cultured for 7 days at 37°C in a 7% humidified CO<sub>2</sub> atmosphere. The adherent synoviocytes were collected and cloned at 1, 5, 20, 50, and 100 cells/well in 96-well flat-bottomed plates. Mitomycin-treated autologous adherent synoviocytes (10,000 cells) were added to each well as

feeder cells. The cultures were maintained for 4 weeks and subjected to recloning at 0.7 cells/well. After 2 months, the growing clones were isolated and maintained in complete culture medium without adding feeder cells. As a result, synovial cell cultures were isolated from 11 RA patients. Morphologically the cell clones from eight patients were fibroblastoid, while those from three patients were lymphoblastoid.

**EBV detection in synovial cell clones.** The synovial cell clones from each patient were analyzed to ascertain whether the cells were infected with EBV. Three lymphoblastoid cell clones derived from three patients and one fibroblastoid cell clone were virtually 100% positive for EBNA by anticomplement immunofluorescence, but the other seven fibroblastoid cell clones were negative. The EBNA-positive fibroblastoid cell clone, referred to as DSEK, and three lymphoblastoid cell clones became capable of growing continuously in IMDM culture medium containing 10% FCS without adding feeder cells. In contrast, the other seven EBNA-negative fibroblastoid cell clones failed to grow within 4 months after isolation even when feeder cells were added. Ten DSEK subclones were successfully established from this parental cell clone by a recloning procedure, and all of them were EBNA positive (Fig. 1A and C).

Southern analysis indicated that all 10 subclones of DSEK cells contained EBV DNA. Three lymphoblastoid cell lines (LCLs) were positive for EBV DNA, and seven EBNA-negative fibroblastoid cell clones were negative. Figure 2 shows the results of the representative clones (KF6-6 isolated from patient IIIA for LCLs and OM-fb isolated from patient IIA for EBNA-negative fibroblastoid cell clones).

Further analysis of EBV-positive DSEK and KF6-6 cells by immunoblotting demonstrated that all cell clones were positive for EBNA-1, EBNA-2, and latent membrane protein LMP-1 (Fig. 3). In contrast to the KF6-6 cells, the DSEK cells expressed EBV early antigens (EA). The immunofluorescence assay indicated that these DSEK cells were 5 to 8% and 4 to 7% positive for EA (Fig. 1D) and EBV capsid antigen (VCA), respectively.

Southern blot analysis of the DSEK cells with a probe for the termini of EBV DNA showed a single band consistent with a

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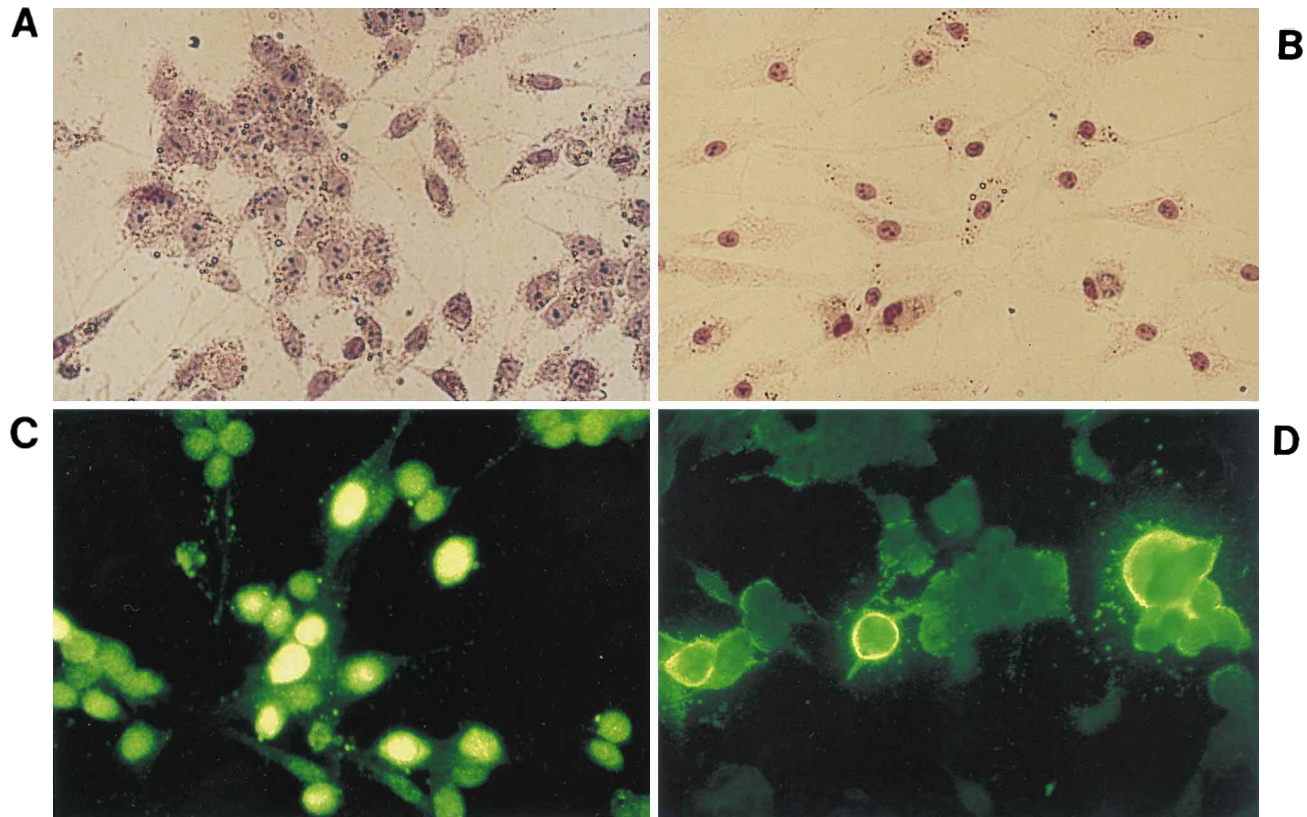


FIG. 1. Morphology of fibroblastoid synovial cell clones DSEK and OM-fb and EBV antigen expression in DSEK cells. DSEK cells (A) and OM-fb cells (B) show fibroblastoid morphology and are stained with Giemsa. (C) EBNA expression in DSEK cells stained by the anticomplement immunofluorescence method, using an EBNA-positive human serum. Cell smears were incubated with a polyvalent human antiserum containing complement at 37°C for 45 min, washed, and reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-human C3c at 37°C for 30 min. (D) EA expression in DSEK cells stained by the indirect immunofluorescence method, using an EA-positive human serum. The smears of cells were incubated with serum from a patient with nasopharyngeal carcinoma (EA titer, 1:1,280) at 37°C for 45 min, washed, and reacted with FITC-conjugated goat anti-human immunoglobulin. Magnification,  $\times 200$ .

monoclonal proliferation of EBV-positive cells and the ladder of lytic bands that are seen in productive EBV infection (data not shown).

**Morphology and growth characteristics.** The DSEK cells were less slender and less elongated than the OM-fb cells and they formed homotypic clusters (Fig. 1A and B). In addition, DSEK cells grew well in the medium with 0.1% FCS and

reached maximum cell density at around  $2 \times 10^6$  cells per ml, whereas OM-fb cells did not grow at all. When  $10^4$  cells of each clone were seeded in 3 ml of medium containing 0.33% agarose in a 60-mm-diameter Petri dish,  $78 \pm 18$  colonies of DSEK cells grew to be macroscopically visible, whereas none of the OM-fb cells grew to macroscopically visible colonies.

**Cell surface markers.** To characterize the clones we had established, we analyzed the expression of various cell surface antigens with a flow cytometer. The results defined the phenotype of representative subclones of DSEK cells (cl-2, cl-18, and cl-22) to be highly positive for CD44, CD58, and HLA-DR and negative for CD11a, CD19, CD20, CD21, CD22, CD23, CD45, CD54, CD62L, HLA-DQ, and immunoglobulin M (IgM) (Table 1). The OM-fb cells had a pattern similar to those of the DSEK cells, including CD11a, B-lymphocyte markers (CD19, -20, -21, -22, -23, and IgM), CD44, CD45, CD58, CD62L, and HLA-DQ expression. The difference was that the DSEK cells were strongly positive for HLA-DR antigen and virtually negative for CD54 antigen, whereas OM-fb cells expressed a moderate level of CD54 antigen and had very low amounts of the HLA-DR molecule. KF6-6 cells, on the other hand, possessed all the surface phenotypes of activated B lymphocytes. Additionally, these DSEK cells, OM-fb cells, and KF6-6 cells were not stained by anti-CD3, -CD10, -CD11b, -CD11c, -CD14, -CD16, or -CD106 (data not shown). These staining patterns were representative of 10 DSEK subclones, 7 EBV-negative fibroblast cell clones, and 3 LCLs, respectively.



FIG. 2. Detection of EBV DNA in synovial cell clones by Southern blotting. Ten micrograms of cellular DNAs was digested with *Bam*HI restriction enzyme, blotted, and hybridized with  $^{32}$ P-labeled *Bam*HI K fragment of EBV DNA.

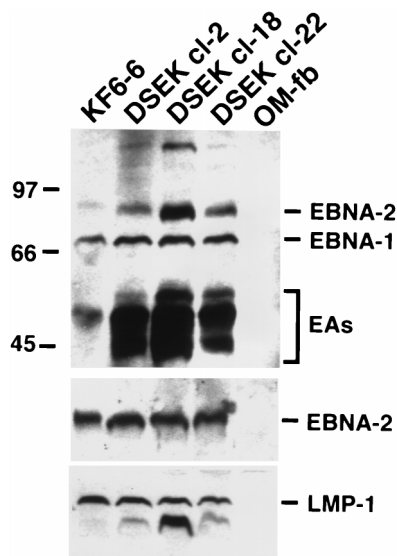


FIG. 3. Detection of EBV proteins in synovial cell clones by immunoblot analysis. For immunostaining, the top blot was treated with a standard EBV-positive human serum and peroxidase-labeled protein A. The middle blot was treated with an EBNA-2 monoclonal antibody (PE2) (24) and peroxidase-labeled anti-mouse immunoglobulin. The bottom blot was treated with an LMP-1 monoclonal antibody (CS1-4) (17) and peroxidase-labeled anti-mouse immunoglobulin. After immunostaining, the blots were developed by the enhanced chemiluminescence method (Amersham) according to the manufacturer's protocol. Size markers (in kilodaltons) are on the left. The smaller band seen in DSEK cell clones on the bottom blot is a deleted form of LMP-1 that is expressed in productive EBV infection.

**Measurement of cytokine levels.** The ability of DSEK cells, OM-fb cells, and KF6-6 cells to produce various cytokines was studied at the protein level. All three DSEK subclones (cl-2, cl-18, and cl-22) produced interleukin-10 (IL-10), basic fibroblast growth factor (bFGF), and transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) in the culture supernatants without any stimulation but did not produce detectable levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-4,

IL-6, tumor necrosis factor alpha (TNF- $\alpha$ ), or TNF- $\beta$  (Table 2). The cytokine production pattern of OM-fb cells was almost the same as those of DSEK subclones. The differences were that the DSEK cells produced IL-10, while the OM-fb cells did not, and that the DSEK cells secreted much higher levels of bFGF and TGF $\beta$ 1 than the OM-fb cells did. In contrast, KF6-6 cells produced higher amounts of IL-1 $\beta$ , IL-6, IL-10, TNF- $\alpha$ , and TNF- $\beta$  and had a significant level (1.1 ng/ml) of TGF $\beta$ 1 but did not produce IL-1 $\alpha$ , IL-4, or bFGF. These cytokine profiles were representative of 10 DSEK subclones, 7 EBV-negative fibroblast cell clones, and 3 LCLs, respectively.

In this study, we spontaneously established an EBV-infected synovial cell line, designated DSEK, from the synovial tissue of a patient with RA and first provided the evidence that rheumatoid synoviocytes can be a target for EBV infection. The EBV-positive synoviocyte cell clones were definitely fibroblasts, not B lymphocytes, with the morphology, surface phenotypes, cytokine profiles, and immunoglobulin rearrangement of a *JH* gene (data not shown).

EBV infection must be responsible for the continued proliferation of the DSEK cell clones, since the other EBV-negative fibroblast clones became extinct within 2 to 4 months after isolation. Rheumatoid synovial fibroblasts, including DSEK cells, are negative for the EBV receptor, CD21 antigen, and are not susceptible to experimental EBV infection in vitro. The possibility that the cell line was a fusion of two cells was excluded by chromosome analysis (data not shown). The mechanism of EBV entry into synovial fibroblasts remains to be clarified.

Both DSEK and OM-fb cells had similar surface marker characteristics. There were only three differences between DSEK and OM-fb cells: (i) DSEK, but not OM-fb, expressed HLA-DR, (ii) OM-fb, but not DSEK, expressed CD54 (ICAM-1), and (iii) DSEK expressed higher levels of CD58 (LFA-3) antigen than OM-fb. Since it has been reported that the LMP-1 gene induces up-regulation of surface activation markers and adhesion molecules, including HLA-DR (25), LFA-3, and ICAM-1 (22), we speculate that the LMP-1 expressed in DSEK clones is responsible for increased HLA-DR and LFA-3 expression. It is also well known that rheumatoid synovial fibroblasts have been observed to convert from negative to positive for HLA-DR expression within 48 h of induction with gamma interferon (5). However, it is not known why ICAM-1 expression is not induced in the DSEK cells. A previous report has shown that nasopharyngeal carcinoma cells exhibit increased expression of ICAM-1 but not LFA-3 molecules (3). Therefore, the induction of these adhesion molecules might be intriguing in view of the difference of the target cells for EBV infection.

The expression of LMP-1 and EBNA-2 genes in DSEK cells may even be a liability in vivo, because these EBV proteins are targets of EBV-specific cytotoxic T lymphocytes (16) and induce cell-to-cell interaction through up-regulated surface molecules, such as HLA-DR and LFA-3, on the cells, which may lead to an inflammatory process in the rheumatoid synovium.

DSEK cells produced IL-10, bFGF, and TGF $\beta$ 1. The quantitative difference in the amounts produced by DSEK and OM-fb cells suggested stimulation of cytokine synthesis by EBV infection. IL-10 production is strongly associated with EBV infection in a human B-cell line (2). IL-10 levels have been reported to be elevated in the synovial fluid of RA patients, which may contribute to dysregulation of cellular and humoral responses by both T and B lymphocytes infiltrating into the rheumatoid synovium (7). bFGF is a potent fibroblast and endothelial growth factor (12) that can induce the synovial hyperplasia and neovascularization characteristic of rheuma-

TABLE 1. Surface molecule expression on synovial cell clones<sup>a</sup>

Surface molecule	% Positive expression of indicated cells:				
	DSEK cell clone			OM-fb	KF6-6
	2	18	22		
CD11a	0.2	0.6	0.8	1.3	94.2
CD19	0.0	0.8	0.0	1.8	97.4
CD20	0.8	0.0	0.0	0.4	72.8
CD21	0.6	0.0	0.0	0.0	27.6
CD22	0.0	0.3	0.0	2.8	66.9
CD23	0.0	0.0	0.0	1.0	81.3
CD44	88.0	98.8	94.9	96.4	85.9
CD45	0.2	0.1	0.1	0.3	99.6
CD54	0.9	0.0	0.0	42.7	92.8
CD58	84.0	76.7	89.7	38.0	69.4
CD62L	0.0	0.5	0.0	2.3	94.0
HLA-DR	99.0	99.5	99.2	3.4	99.5
HLA-DQ	0.2	0.7	0.0	1.7	99.5
IgM	0.0	0.2	0.0	0.0	86.6

<sup>a</sup> The expression of surface molecules was analyzed on a log fluorescence scale by FACScan. Monoclonal antibodies for CD11a (LFA-1 $\alpha$ ), CD19 (Leu12), CD20 (Leu16), CD21 (B2), CD22 (Leu14), CD23 (Leu20), CD44 (CBL154), CD45 (HLA-1), CD54 (Leu54), CD58 (LFA-3), CD62L (Leu8), HLA-DR (L243), HLA-DQ (Leu10), and IgM were used as direct staining reagents or in combination with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for indirect immunofluorescence analysis as described previously (18).



TABLE 2. Spontaneous production of various cytokines by synovial cell clones

Cell	Cytokines in culture supernatant <sup>a</sup>								
	IL-1 $\alpha$ (pg/ml)	IL-1 $\beta$ (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	bFGF (pg/ml)	TNF- $\alpha$ (pg/ml)	TNF- $\beta$ (pg/ml)	TGF- $\beta$ 1 (ng/ml)
DSEK									
cl-2	<7.8	<0.125	<31.3	<0.156	21.7	190	<0.5	<15.6	1.7
cl-18	<7.8	<0.125	<31.3	<0.156	22.0	320	<0.5	<15.6	1.9
cl-22	<7.8	<0.125	<31.3	<0.156	18.8	240	<0.5	<15.6	1.2
OM-fb	<7.8	<0.125	<31.3	<0.156	<0.5	80	<0.5	<15.6	0.75
KF6-6	<7.8	0.34	<31.3	0.571	23.6	<50	47.7	1260.0	1.1

<sup>a</sup> The culture supernatant of each DSEK cl-2, cl-18, or cl-22, OM-fb, and KF6-6 clone was collected after 3 days of culture of 10<sup>6</sup> cells in 1.5 ml of IMDM with 10% FCS. The amount of each cytokine in the duplicate culture supernatants was measured with an enzyme-linked immunosorbent assay kit for each clone, according to the manufacturer's instructions. Results are expressed as mean values.

toid inflammation. TGF $\beta$ 1 promotes inflammation through its effects on mononuclear phagocytes and affects synovial fibroblasts by slowing colony growth, increasing collagen production, decreasing collagenase expression, and inducing hyaluronan synthesis (10, 14).

Expression of EA and VCA on the surfaces of the DSEK cells indicates an ability to infect other cells, such as B lymphocytes, T lymphocytes, and synoviocytes in the host. This suggests that immune responses are also directed against EBV-infected synovial cells. Moreover, the lytic infection induces a cytopathic effect in infected cells that eventually leads to cell death.

These findings suggest that EBV-infected synovial fibroblasts play a role in the migration, proliferation, and/or differentiation of synovial cells in the inflammation of RA. This evidence supports the hypothesis that EBV is a cofactor in the pathogenesis of RA.

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