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A New Tactile Skin Sensor for Measuring Skin Hardness in Patients with Systemic Sclerosis and Autoimmune Raynaud's Phenomenon

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We used a new tactile sensor to measure the elastic properties of skin in patients with systemic sclerosis or Raynaud's phenomenon. The sensor consists of a piezoelectric vibrator with vibration pickup to measure frequency changes when the sensor is placed on the skin. The mean frequency change at the skin surface of the proximal third phalanx in patients with systemic sclerosis was significantly lower than in age- and sex-matched controls. The results in systemic sclerosis patients were statistically correlated to the Modified Rodnan Skin Thickness Score. This technique was also used to measure the therapeutic efficacy of salpogrelate, a new specific serotonin receptor antagonist. A greater mean frequency change was seen after treatment. We conclude that this new tactile sensor is useful for quantitatively measuring skin sclerosis and may help determine the efficacy of therapeutic treatments.

KEY WORDS: SKIN SENSOR, SKIN HARDNESS, SYSTEMIC SCLEROSIS, RAYNAUD'S PHENOMENON

Introduction

A number of studies have been published on the use of instruments for investigating the elastic properties of human skin,[1 - 6] and such instruments have facilitated many advances in the fields of dermatology and cosmetic science. In general, the ground substance in skin[7] shows thixotropic behaviour, that is it shows a reduction in viscosity as shear stress increases, and the decreased viscosity is recovered slowly on standing. For example, Finlay,[8] using a rotary displacement servo system, showed that human skin contains a thixotropic ground substance that enables gliding of the outer surface in a complex pattern. The viscoelastic and complex biomechanical structure of skin necessitates the use of simplified mechanical models to measure and assess its biomechanical properties.[9] Developing a tactile sensor and measuring system allowed detection of the stiffness- and elasticity-related properties of human skin.[10]

The sensor is based on a piezoelectric element and can measure displacement. In addition, it is hand-held and designed for easy and convenient use.

In patients with systemic sclerosis (SSc) the biomechanical properties of skin, such as elasticity and extensibility, are altered by fibrosis. We investigated whether this new tactile sensor is a useful tool for measuring skin hardness in patients with SSc, Raynaud's phenomenon or mixed connective tissue disease (MCTD; patients with MCTD usually have scleroderma and Raynaud's disease). The results were compared with those of the gold standard Modified Rodnan Skin Thickness Score (MRSTS). In addition, we investigated the use of the tactile sensor to measure the efficacy of salpogrelate treatment.

Patients and methods

PATIENTS

Systemic sclerosis patients who fulfilled the American College of Rheumatology (formerly the American Rheumatism Association) classification criteria[11] and patients with Raynaud's phenomenon diagnosed on the basis of characteristic triphasic digital colour changes[12] were included in the study. The SSc patients had suffered from diffuse atrophic or sclerotic skin changes for at least 5 years. Confirmation of the diagnosis of SSc was obtained from a forearm skin biopsy. Patients with MCTD were also investigated and the diagnosis was based on a high serum titre of anti-U1 ribonuclease-sensitive extractable nuclear antigen antibodies. The clinical features of MCTD were an overlap of diffuse connective tissue diseases (systemic lupus erythematosus, SSc and polymyositis).

Age- and sex-matched controls were also recruited; these included patients with other autoimmune diseases (except for MCTD) and healthy individuals without Raynaud's phenomenon.

Those enrolled in the study had no other skin disorders, did not use vibrating tools, and did not participate in any sport that affected the skin on their fingers. Informed consent was obtained from all the participants, but ethical approval was not sought.

THE TACTILE SENSOR

The tactile sensor consists of a piezoelectric vibrator (61 kHz) with vibration pickup to measure the change in frequency when the sensor is placed on the skin; it is connected to a computer equipped with the appropriate software. Integrated with the tactile sensor is a displacement sensor that shows the spring compression loading when the sensor element is placed against the skin during measurement. Under certain conditions (e.g. fixed contact pressure), the change in frequency reflects the acoustic impedance of the object and is related to the stiffness of the soft tissue.

MEASUREMENT OF SKIN HARDNESS

Briefly, the measurements were made with the tactile sensor oscillating at a resonance frequency of about 61 kHz. It was set to measure the change in resonance frequency (ΔF). $\Delta F = f_x - f_0$, where f_x is the resonance frequency of the sensor placed on the skin and f_0 is the non-contact frequency (61 kHz). A total of 150 measurements were made in sequence. To compare the degree of skin sclerosis quantitatively, the mean ΔF value between the 100th and 110th detection points, when the measurement became stable, was used.

Since the contact area of the sensor tip depends on the contact pressure, the resonance frequency of the piezoelectric element also changes according to how hard

the sensor tip is pressed against the surface of the skin. The contact pressure of the sensor element was held constant by the spring. The compression of the spring when the sensor probe touched the skin (and thus the movement of the sensor element) was continually measured by the displacement transducer.

The ΔF was measured at the skin surface of the proximal third phalanx. The mean ΔF was also measured at the locations assessed when calculating the MRSTS.[13-15]

MODIFIED RODNAN SKIN THICKNESS SCORE

The MRSTS was assessed by clinical palpation at multiple locations, assigning a score of 0 – 3 to each site (0, normal; 1, thickened; 2, thickened, unable to pinch; 3, thickened, unable to move).[13-15]

MEASUREMENT OF TREATMENT EFFICACY

The change in ΔF was measured in patients with autoimmune Raynaud's phenomenon, SSc or MCTD after administering the serotonin (5-hydroxytryptamine) receptor antagonist salpogrelate[16] at a dose of 300 mg/day for 4 weeks. Measurements were taken in the same way as described in the section on measuring skin hardness before and after salpogrelate therapy.

Salpogrelate therapy is used routinely in the clinic as therapy for these conditions.

STATISTICAL ANALYSIS

Results are given as the mean ± SD. Student's *t*- and *F*-tests were used for statistical comparison. A *P*-value < 0.05 was considered statistically significant. Statistical calculations were performed using the statistical software package STATVIEW II (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Five patients with SSc, 13 with Raynaud's phenomenon without SSc and MCTD, and five patients with MCTD were recruited to the study. There were a total of 23 age- and sex-matched controls, all of whom were female and ranged in age from 44 years to 66 years (mean ± SD 47.1 ± 15.8 years). The control women comprising six patients with systemic lupus erythematosus, three with Sjögren's syndrome, three with anti-phospholipid antibody syndrome, four with rheumatoid arthritis, two with Behçet's disease and five healthy individuals without Raynaud's phenomenon.

SKIN HARDNESS

The mean ΔF at the skin surface of the proximal third phalanx in patients with either atrophic or sclerotic SSc was significantly lower than in the age- and sex-matched controls (Table 1). The mean ΔF values at the locations used to calculate the MRSTS are shown in Table 2. MRSTS values were calculated for four patients, three with and one without SSc.

CORRELATION BETWEEN TACTILE SENSOR MEASUREMENTS AND MRSTS

To investigate the relationship between our measurements of skin hardness (using the tactile sensor) and the MRSTS, comparisons were made between MRSTS and mean ΔF values. Initial experiments demonstrated that the mean ΔF values associated with an MRSTS of 2 or 3 were statistically different from those associated with an MRSTS of 0 ($P < 0.001$). The mean ΔF value associated with an MRSTS of 1, however, was not statistically different from that associated with an MRSTS of 0 (Fig. 1).

MEASURING TREATMENT EFFICACY

We tested whether or not the sensor is useful for measuring treatment efficacy. The changes in ΔF measured after treating nine

TABLE 1
Demographic characteristics and change in frequency (ΔF) measurements obtained using the tactile sensor in patients with systemic sclerosis (SSc; $n = 5$), Raynaud's phenomenon ($n = 13$) or mixed connective tissue disease (MCTD; $n = 5$), and in age- and sex-matched controls (with other autoimmune disease [except MCTD] or healthy individuals without Raynaud's phenomenon)

Skin condition	Age (years)	Mean age (years)	Anti-Scl-70 antibody	Intraphalangeal ΔF (Hz)	Mean ± SD intraphalangeal ΔF (Hz)	Finger tip ΔF (Hz)	Mean ± SD Finger tip ΔF (Hz)
SSc	49			1167		1331	
SSc	62			1039		1016	
SSc	62		Positive	2148		779	
SSc	41		Positive	1585		1945	
SSc	45		Positive	1302		79	
		51.8			1448 ± 441[a]		1030 ± 771[a]
MCTD	30			1882		2853	
MCTD	66			2103		1507	
MCTD	67			2162		1502	
MCTD	43			2122		2415	
MCTD	28			2456		3055	
		46.8			2145 ± 205[a]		2266 ± 733
Raynaud's phenomenon		49			2070 ± 229[a] ($n = 13$)		2847 ± 315 ($n = 7$)*
Controls		55			2575 ± 199 ($n = 23$)		2712 ± 437 ($n = 8$)*

[a] $P < 0.005$ compared with the mean control value (Student's *t*-test).
*Finger tip ΔF was only measured in some patients with Raynaud's phenomenon and some controls.

Location	SSc1 (diffuse type)		SSc2 (diffuse type)		SSc3 (limited type)		Non-SSc*	
	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS
Finger								
Right	1479 ± 14	3	1475 ± 7.5	3	1754 ± 20	3	2282 ± 5	0
Left	1431 ± 11	3	1613 ±10	3	1618 ± 25	3	2344 ± 4	0
Hand								
Right	2089 ± 12	3	1582 ± 5.3	3	1582 ± 5	3	2217 ± 6	0
Left	1965 ± 15	3	1911 ± 3.7	3	1613 ± 10	3	2469 ± 5	0
Forearm								
Right	2270 ± 15	1	2084 ± 5.2	2	ND	2	2464 ± 50	0
Left	2329 ± 6	1	1691 ± 9	3	ND	2	2170 ± 11	0
Upper arm								
Right	2033 ± 3	2	2027 ± 1.6	3	2170 ± 11	2	2354 ± 6	0
Left	1944 ± 4	2	1885 ± 4.6	3	2186 ± 6.8	2	2234 ± 5	0
Anterior chest	1584 ± 33	3	2196 ± 5.1	1	2186 ± 6.8	0	2186 ± 7	0
Face (cheek)								
Right	1711 ± 32	3	1262 ± 9.1	3	2360 ± 7.9	3	2359 ± 8	0
Left	1893 ± 5	3	ND	ND	ND	3	2422 ± 4	0
Abdomen	1845 ± 14	3	1990 ± 14	3	2110 ± 1.6	2	2110 ± 2	0

TABLE 2 (continued)
Mean (± SD) change in frequency (ΔF) as measured by the tactile sensor and Modified Rodnan Skin Tightness Scores (MRSTS) at multiple locations in three patients with systemic sclerosis (SSc) and one control person

Location	SSc1 (diffuse type)		SSc2 (diffuse type)		SSc3 (limited type)		Non-SSc*	
	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS	Mean ± SD ΔF (Hz)	MRSTS
Thigh								
Right	1786 ± 18	3	1792 ± 1.3	3	2218 ± 6.5	0	ND	ND
Left	1683 ± 43	3	2021 ± 2	3	2469 ± 5.3	0	ND	ND
Leg								
Right	1976 ± 7	3	2083 ± 1.7	2	ND	0	ND	ND
Left	1822 ± 6	3	1709 ± 4.3	3	1789 ± 4.4	0	ND	ND
Foot								
Right	1842 ± 5	3	1742 ± 1.7	3	2021 ± 6.5	1	2193 ± 5	0
Left	1922 ± 8	3	1911 ± 3.7	3	2193 ± 5.1	1	2248 ± 20	0

ND, not done.
*The non-SSc patient (control) had interstitial pneumonia and a low Scl-70 serum titre.

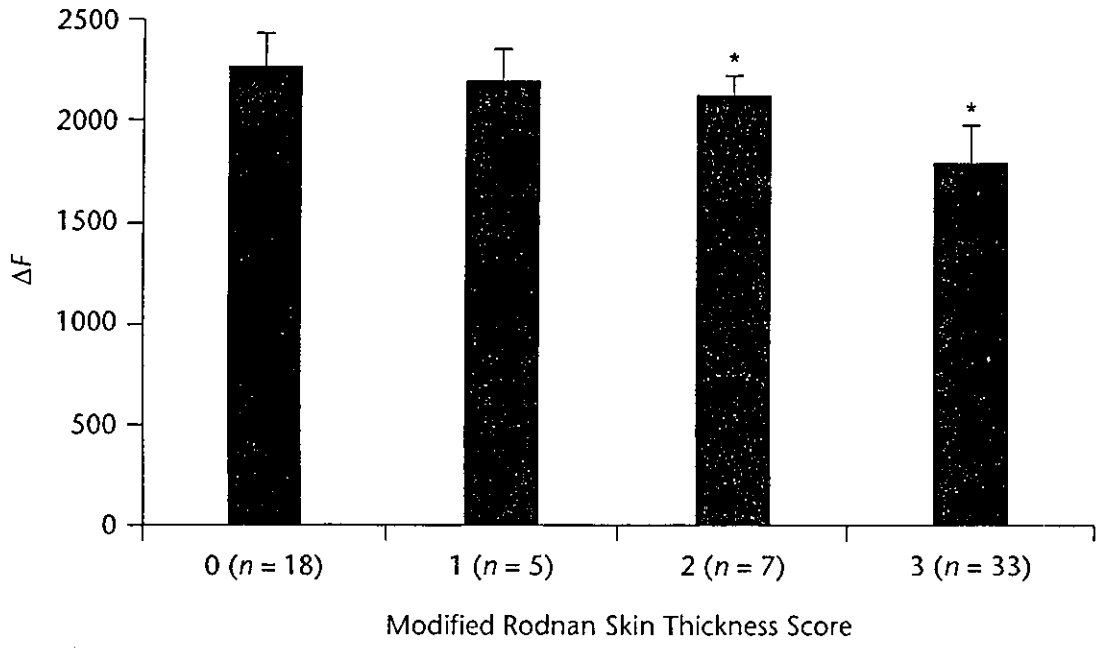

FIGURE 1: Correlation between the Modified Rodnan Skin Thickness Score and the tactile sensor change in frequency (ΔF) measurement. Values shown are the mean + SD. *$P < 0.001$ compared with an MRSTS of 0

patients (six with Raynaud's phenomenon, one with SSc and two with MCTD) with salpogrelate for 4 weeks are shown in Table 3.

In patient R2K, who suffered from unclassified connective tissue disease, ΔF was improved more (from 2040 to 2418) when treated with prostaglandin ointment and salpogrelate than with salpogrelate alone. In patient R6N who suffered from SLE, ΔF was not changed after taking low dose prednisone (from 1875 to 1822), but improved after taking salpogrelate and prednisolone (from 1822 to 2286). In this pilot study, salpogrelate improved the ΔF in the patients tested.

Discussion

There are several scores that use pinching to measure skin thickness, including the MRSTS, which is currently the gold standard. This test has been proven to be reasonably reliable,[14,17,18] but inexperienced general physicians may find it difficult to use. We report here a novel, non-invasive tactile sensor[19] that assesses physical properties such as stiffness and inverse compliance by measuring the change in resonance frequency when a vibrating ceramic rod touches the surface of an object. Preliminary results on measuring the physical properties of living tissue such as skin have given promising results.[20,21] It has been evaluated both in a standardized silicone gum model and in a rat testis model, and was compared with an impression method that measures interstitial pressure and water displacement in skin.[22] The two methods correlated well, and the tactile sensor was able to detect differences that correlated with silicone stiffness as measured by an international standard (DIN ISO 2137, 150 g hollow cone). We have further developed this tactile sensor and measuring system to detect the stiffness- and elasticity-related properties of human skin. The sensor is hand-held and designed for easy and convenient use. Experimental

TABLE 3
Change in frequency (ΔF) as measured by the tactile sensor in patients with autoimmune Raynaud's phenomenon, systemic sclerosis (SSc) or mixed connective tissue disease (MCTD) before and after treatment with salpogrelate (300 mg/day for 4 weeks)

Patient	Age (y)	Diagnosis	ΔF (Hz) Before salpogrelate	ΔF (Hz) After salpogrelate	Other therapy
R1K	42	UCTD, AP	2176	2447	Aspirin
R2N	65	UCTD, AP	1620	2418	Prostaglandin ointment (ΔF = 2040 with salpogrelate alone)
R3A	58	IP, Scl-70	2367	2976	Prednisone
R4T	23	SLE	1727	2263	Aspirin
R5C	45	RA	2133	2647	Prednisone
R6N	34	SLE, AIHA	1822	2286	Prednisone (ΔF = 1875 with prednisolone alone)
P1H	41	SSc, Scl-70	1585	1960	None
M1H	43	MCTD	2122	3029	None
M2M	28	MCTD	2456	2768	Prednisone

UCTD, unclassified connective tissue disease; AP, anti-phospholipid antibody; IP, interstitial pneumonia; Scl-70, anti-Scl-70 antibody; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; AIHA, autoimmune haemolytic anaemia.

results on silicone gum and in healthy Japanese and Swedish women indicated that the instrument was able to detect changes in the stiffness- and elasticity-related properties of human skin related to age, day-to-day variations and the application of cosmetics.[10]

In the atrophic phase an SSc patient's skin becomes sclerotic and, as a result, its elasticity and viscosity decrease. When we used this new tactile sensor to quantitatively measure sclerosis of the skin, it was shown to be very sensitive in detecting changes in skin properties in SSc patients. In addition, the results using this new method were statistically correlated to the gold standard MRSTS. We also found some decrease in ΔF in patients with autoimmune Raynaud's phenomenon. The reason for this was unclear, since histopathological data on the skin in these patients were not available. It has been suggested that autoimmune Raynaud's phenomenon may represent a pre-SSc state,[23] and that recurrent Raynaud's phenomenon results in fibrotic change in the small vessels. It is also possible that the tactile sensor might be influenced by the skin blood flow.

To investigate whether this new tactile sensor might be helpful in determining the efficacy of treatment, we used it to measure changes in the ΔF after treatment with

salpogrelate. The efficacy of this new specific serotonin receptor antagonist has been reported in Japanese patients with Raynaud's phenomenon.[24] In contrast, another serotonin antagonist, ketanserin, failed to show efficacy compared with placebo in a North American trial.[25] Salpogrelate and ketanserin have been shown to produce similar effects on serotonin-induced platelet aggregation and DNA synthesis of aortic smooth muscle cells due to their antagonism of 5-hydroxy-tryptamine-2 receptors.[26,27] Salpogrelate, however, does not exhibit an α_1-adrenergic receptor blocking action, unlike ketanserin,[28] and it is possible that the biological responses and side-effects of salpogrelate and ketanserin are not the same.

Our study shows that this new tactile sensor might be useful for quantitatively measuring sclerosis of the skin and assessing therapy efficacy. Further experiments are necessary to determine the efficacy of the sensor in relation to various other pathological states.

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Early Bone Marrow Hematopoietic Defect in Simian/Human Immunodeficiency Virus C2/1-Infected Macaques and Relevance to Advance of Disease

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To clarify hematological abnormalities following infection with human immunodeficiency virus (HIV), we examined the hematopoietic capability of bone marrow by using cynomolgus monkeys infected with pathogenic simian/human immunodeficiency virus (SHIV) strain C2/1, an animal model of HIV infection. The relationship between the progress of the infection and the CD4/CD8 ratio of T lymphocytes or the amount of SHIV C2/1 viral load in the peripheral blood was also investigated. A colony assay was performed to assess the hematopoietic capability of bone marrow stem cells during the early and advanced phases of the infection. Colonies of granulocytes-macrophages (GM) were examined by PCR for the presence of the SIVmac239 *gag* region to reveal direct viral infection. There was a remarkable decrease in the CFU-GM growth on days 1 and 3 postinoculation, followed by recovery on day 56. During the more advanced stage, the CFU-GM growth decreased again. There was minimal evidence of direct viral infection of pooled cultured CFU-GM despite the continuously low CD4/CD8 ratios. These results indicate that the decrease in colony formation by bone marrow stem cells is reversible and fluctuates with the advance of the disease. This decrease was not due to direct viral infection of CFU-GM. Our data may support the concept that, in the early phase, production of inhibitory factors or deficiency of a stimulatory cytokine is responsible for some of the bone marrow defects described in the SHIV C2/1 model.

It is generally known as a feature of human immunodeficiency virus (HIV) infection that CD4-positive T lymphocytes and monocytes infected with pathogenic HIV or simian/human immunodeficiency virus (SHIV) decrease in number and disappear. Infected hosts will thus become immunodeficient. Moreover, it has been reported that, after HIV infection is contracted, hematological abnormalities in the bone marrow and the peripheral blood such as anemia, lymphopenia, and thrombocytopenia ensue and correlate with the advance of the illness (36). Several possibilities have been noted as the cause of such hematological abnormalities: the apoptosis of virus-infected cells, changes in the hematological environment, dysfunction of the thymus or the lymphoid system, change of cell division, or dysfunction of hematopoietic progenitor cells (1). Furthermore, a few reports have shown that the bone marrow of patients with AIDS displays morphological alterations similar to those of patients with myelodysplastic syndrome (2, 31). The term "HIV myelopathy" has been used for this bone marrow pathology by some investigators (10, 22).

Reduced numbers of CFU (burst-forming units–erythrocytes [BFU-E] or CFU–granulocytes-macrophages [CFU-GM]) have been reported in bone marrow samples from patients infected with HIV (9, 16, 27). Moreover, the reduction in CFU-GM resembles that of an animal model of AIDS experimentally induced by simian immunodeficiency virus (SIV) (13, 30, 32). While the precise mechanisms of such hematopoietic abnormalities remain unclear, several hypotheses have been proposed: (i) decreased levels of appropriate cytokines secondary to altered numbers of T-cell subsets or macrophages, which are commonly seen in HIV type 1 (HIV-1) infection (28); (ii) production of inhibitory factors (14, 29); (iii) cytotoxic elimination of the precursor cells by the antibody-dependent cell-mediated cytolytic mechanism (7); and (iv) infection of hematopoietic precursor cells with viruses, which leads to death of these cells or their metabolic alteration (7). On the other hand, it has been suggested that primitive bone marrow progenitor cells are most likely not a major reservoir for HIVs (6, 13, 28).

Despite mounting data supporting the above-mentioned hypotheses, a unifying explanation remains elusive. We studied bone marrow samples from cynomolgus monkeys (*Macaca fascicularis*) experimentally infected with an SHIV strain in order to evaluate possible cellular and molecular events that affect hematopoiesis in SHIV infection.

MATERIALS AND METHODS

Animals. Twenty cynomolgus monkeys (nine males and 11 females) used in this study were maintained in our facility according to the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. All treatments were performed according to the standard operating procedures for monkeys for evaluation of human vaccines in the Tsukuba Primate Center, NIID, Tsukuba, Ibaraki, Japan. Their ages were 3 to 6 years, and their weights were approximately 3 to 5 kg (Table 1). Four sham-inoculated monkeys were included as a control. They were inoculated with saline alone instead of virus-containing saline solution. Two additional monkeys without sham treatment also served as a negative control. Low-dose ketamine (intramuscular dose of 10 mg/kg of body weight) was used as an anesthetic for blood and bone marrow sampling.

Viruses. A highly pathogenic SHIV strain, designated C2/1, was obtained by serum passages in cynomolgus monkeys. The SHIV C2/1 strain contains the *env*

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TABLE 1. Protocol for control and infection of cynomolgus monkeys with SHIV C2/1 and subsequent bone marrow harvesting[a]

Monkey no.	Age (yr)	Sex	Day of bone marrow harvesting	Virus administration	Dose of inoculated virus ($TCID_{50}$)
13	5	Male			
44	4	Male			
181	6	Male			
1037	5	Male			
1091	5	Male			
759	4	Male			
4345	4	Male	1	Intravenous	20
1	5	Female	3	Intravenous	20
2	5	Female	3	Intravenous	20
90c	5	Female	56	Intravenous	20
560	4	Female	56	Intrarectal	2,000
430	4	Female	56	Intrarectal	2,000
442	3	Female	56	Intrarectal	2,000
200	5	Female	56	Intravenous	20
944	5	Male	56	Intravenous	20
520	5	Female	56	Intrarectal	20
844	4	Female	56	Intrarectal	20
0634	4	Female	56	Intravenous	10
054	4	Female	113	Intravenous	2,000
039	4	Male	380	Intravenous	20

[a] Monkeys 13 to 759 were controls that were not infected with SHIV. Monkeys 4345 to 039 were inoculated intravenously or intrarectally with the doses of SHIV C2/1 shown in the table. Bone marrow harvesting was performed on the indicated day after inoculation. $TCID_{50}$, 50% tissue culture infective dose.

gene of pathogenic HIV-1 strain 89.6. This chimeric virus was propagated in concanavalin A-activated peripheral blood mononuclear cells (PBMC) from healthy monkeys or in a cell line, M8166. Cell-free virus stocks were stored at −120°C (25).

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated MAbs to monkey CD3 (NF-18; BioSource) and to human CD4 (Nu-T H/I; Nichirei), CD8 (Nu-T S/C; Nichirei), CD16 (3G8; Pharmingen), and CD20 (Leu-16; Becton Dickinson).

Preparation of bone marrow cells. Fourteen monkeys were infected with SHIV C2/1 at three 50% tissue culture infective doses by intravenous or intrarectal inoculation (Table 1). Bone marrow samples were aspirated from their femoral bone during autopsy. For a sham-inoculated control, monkeys received 0.5 ml of saline alone. One day or 3 days later, bone marrow samples were aspirated from their pelvic bones. Non-sham-control monkeys received only ketamine anesthesia for bone marrow aspiration.

Preparation of blood samples for cell surface antigen analysis by flow cytometry. Peripheral blood was mixed with lysis buffer (Becton Dickinson) and centrifuged at 300 × *g* for 5 min. Viable cells were counted by the trypan blue dye-exclusion method. The cell surface antigens CD3, CD4, CD8, CD16, and CD20 were stained with their respective MAbs. After being washed with staining buffer, 5×10^4 cells in each labeled sample tube were analyzed by a FACSCalibur flow cytometer (Becton Dickinson) with use of Cell Quest software (Becton Dickinson). Absolute PBMC count was determined as follows. Fifty milliliters of each whole-blood sample, containing FITC-conjugated anti-CD3 MAb (BioSource), PE-conjugated anti-CD4 MAb (Becton Dickinson), and peridinin-chlorophyll protein-conjugated anti-CD8 MAb (Becton Dickinson), was added to a TRUCOUNT tube and incubated at room temperature. Contaminating red blood cells were lysed, and each sample was analyzed by flow cytometry as described above. All measurements were made under the same instrumental setting.

Quantification of cell-associated and plasma viral load. Plasma viral RNA was extracted and purified using a QIAamp viral RNA minikit (Qiagen, Valencia, Calif.). For quantitative analysis of the RNA, reverse transcriptase-PCR (RT-PCR) was performed with primers and probes targeting the SIVmac239 *gag* region, designed by computer with the Primer Express software (PE Biosystems). The viral RNA was reverse transcribed and amplified using a Taqman EZ RT-PCR kit (PE Biosystems) with the designed primers (forward primer, 5′-ATGCAGAGCCCCAAGAAGAC-3′, and reverse primer, 5′-GGACCAAGGCCTAAAAAACCC-3′) and detected with a probe, FAM-5′-ACCATGTTATGGCCAAATGCCCAGAC-3′-TAMRA. Probed products were quantitatively monitored by their fluorescence intensity with ABI 7700 (PE Biosystems). For a positive-control RNA, SIVmac239 *gag* RNA was synthesized and purified using a MEGAscript kit (Ambion, Austin, Tex.) with template plasmid pKS460. This template contained the SIVmac239 *gag* sequence within the T7 promoter region. Plasma viral load, measured in duplicate, was estimated based on a standard curve of the control RNA and the RNA recovery rate (19).

Performance of colony assays on bone marrow specimens and detection of the SIVmac *gag* sequence by PCR in pooled cultured CFU-GM. Bone marrow samples (n = 20) were obtained by aspiration from the femoral or pelvic bones of monkeys. An approximately 10-ml bone marrow sample diluted with phosphate-buffered saline was slowly layered on top of 10 ml of sterile Ficoll-Hypaque in a 15-ml conical tube. The tubes were then centrifuged at 400 × *g* for 30 min at room temperature. With use of a pipette, a top plasma layer was removed, and a mononuclear cell layer was transferred in a small volume to a tube. After two washes with 2% fetal calf serum–Iscove's medium (code no. HBM-3160; Stem Cell Technologies Inc.), cell density was adjusted to 10^6 mononuclear cells/ml. The cell suspensions were then mixed with methylcellulose medium (Methocult HF4434; Stem Cell Technologies) so that it gave a final concentration of 10^5 cells per 1.1 ml for final plating. The cell culture was performed in duplicate in 35-mm-diameter plastic dishes at 37°C, 5% CO_2, and 100% humidity for 10 days, and colonies (BFU-E, CFU-GM, and CFU–granulocytes-erythroids-macrophages-megakaryocytes) were counted by inverted microscopy. CFU-GM were plucked from the methylcellulose culture and collected in pools and then subjected to PCR analysis by the method described above.

RESULTS

Figure 1 shows the relationship between the CD4/CD8 ratios of the peripheral blood T cells of infected monkeys and the postinoculation time. In general, the CD4/CD8 ratio decreased in 14 to 21 days after inoculation. It has been reported that monkeys inoculated with SHIV C2/1 had transient decreases of $CD4^+$ T lymphocytes within several days after infection (25). In this study, one monkey showed a decrease in CD4/CD8 ratio even within several hours: namely, the CD4/CD8 ratio of monkey 4345 decreased to 1.28 in 6 h after inoculation and went up to 1.80 in 24 h (Fig. 1). Control monkeys showed only negligible declines (Fig. 1).

The number of viral copies was estimated for four animals (200, 944, 520, and 844) by real-time PCR (Fig. 2). It has been

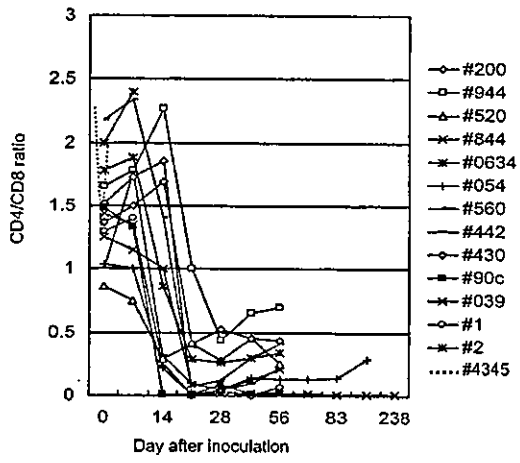

FIG. 1. Changes of CD4/CD8 ratio in monkeys inoculated with SHIV C2/1. All monkeys showed decreased CD4/CD8 ratios between day 14 and day 21 after inoculation. Monkey 4345 had a decrease in CD4/CD8 ratio in the first 24 h. Control monkeys showed only negligible declines in the first 24 h (preinoculation, 1.26 ± 0.400 [mean ± standard deviation]; 6 h, 1.24 ± 0.259; 24 h, 1.11 ± 0.323; n = 4).

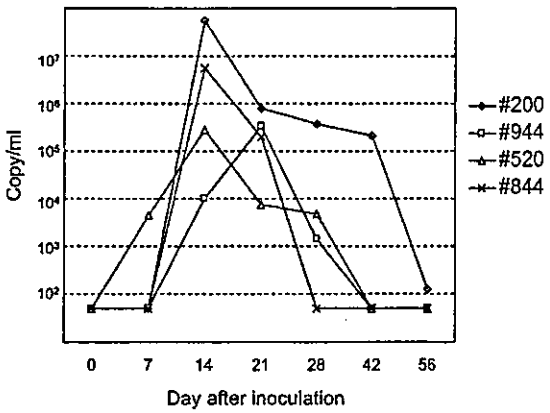

FIG. 2. Plasma viral load in the infected monkeys. Plasma viral RNA of four monkeys (200, 944, 520, and 844) was analyzed by PCR for the presence of the SIVmac239 *gag* region.

shown that the reduction of the CD4/CD8 ratio correlates with the increase of SHIV viral copies (15, 25). These four monkeys showed that the peak of viral copies occurred on the 14th day after inoculation and declined to 500 copies or less on the 56th day (Fig. 2).

We arbitrarily defined a postinoculation period of day 1 through 3 as "early stage" (covering monkeys 4345, 1, and 2 in Fig. 3) and that of day 56 or later as "advanced stage" (covering monkeys from 90c to 039 in Fig. 3). Monkey 4345 showed a remarkable reduction in the number of colonies in 24 h (Fig. 3). Monkeys 1 and 2 also had such a dramatic decline on day 3 (Fig. 3). However, nine monkeys (90c through 0634 in Table 1 and Fig. 3) maintained colony formation during the advanced stage at a level comparable to that of the control monkeys (Fig. 3). Compared with sham-inoculated controls, monkey 054 had a somewhat lower number of colonies on the 113th day. Monkey 039, which died of AIDS on day 238, showed more reduced colony formation, especially CFU-GM formation, than did monkey 054 or the sham-inoculated control monkeys (Fig. 3). At the advanced stage, no difference in the morphology or the number of colonies was noted between the noninfected and the infected monkeys (Fig. 4).

Taken together, a reduction of CD4/CD8 ratio and CFU-GM growth occurred in the early phase of the postinoculation period. However, the CFU-GM growth tended to increase following viremia while $CD4^+$ T lymphocytes continuously declined. The colony growth of the infected monkeys during the advanced stage recovered up to a level comparable to that of the control monkeys.

Infection of CFU-GM with SHIV C2/1 virus was tested by a PCR technique as described in Materials and Methods. Of the 14 cynomolgus monkeys infected with SHIV C2/1 virus, only three were positive, suggesting that the direct infection of bone marrow progenitor cells was minimal (Fig. 5). There was no positive case in the control monkey group.

DISCUSSION

Hematological abnormalities such as anemia, lymphopenia, and thrombocytopenia have been documented in a variety of retrovirus infections in both humans and experimental animals. While the precise mechanisms for such hematological abnormalities remain to be elucidated, several hypotheses have been postulated: (i) destruction of infected cells by a virus itself

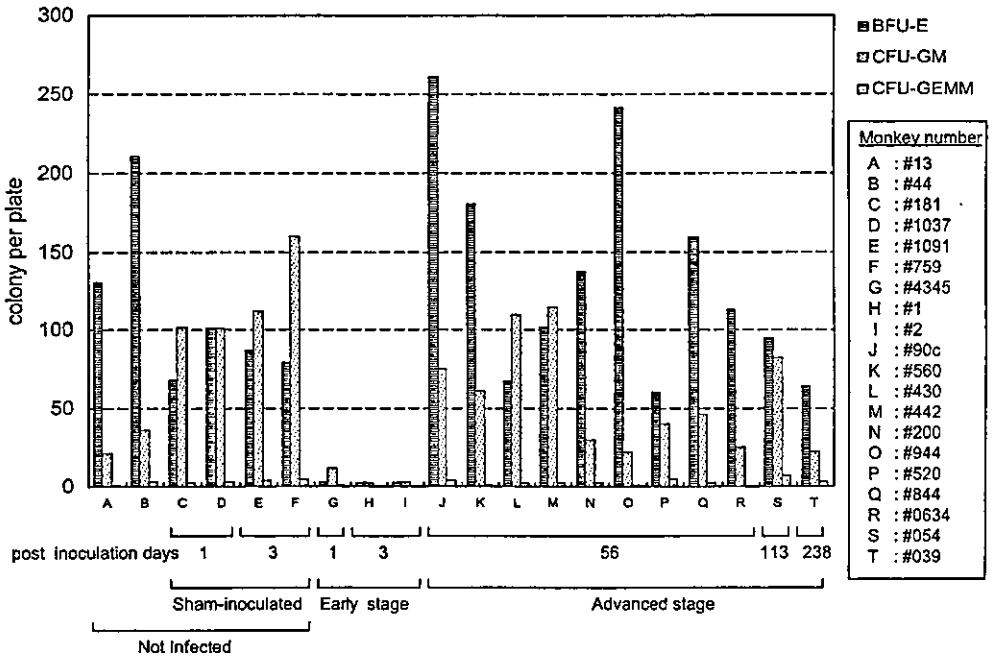

FIG. 3. Colony assay on monkeys inoculated with SHIV C2/1. A period of days 1 through 3 after inoculation was defined as early stage, whereas days 56 through 238 were defined as advanced stage. *P* was <0.005 for CFU-GM, and *P* was <0.02 for CFU-E in comparison of early stage and advanced stage and of virus-inoculated monkeys and sham-inoculated controls at days 1 and 3. *P* values were calculated according to Kruskal-Wallis analysis. There was no statistically significant difference between sham-inoculated controls and non-sham-treated controls by Mann-Whitney analysis.

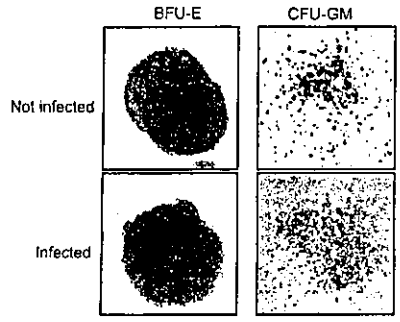

FIG. 4. Morphology of colonies produced by BFU-E and CFU-GM. Photographs of colonies cultured in nitrocellulose medium are shown at an ×75 magnification by a microscope. The left column shows BFU-E, and the right column shows CFU-GM. The upper section shows colonies from uninfected monkeys, while the lower shows colonies from monkeys infected with SHIV C2/1.

or by the antibody-dependent cell-mediated cytolytic mechanism, (ii) damage of the thymus or the lymphoid tissue, (iii) abnormal turnover of infected cells in the peripheral blood (i.e., apoptosis), and (iv) suppression of hematopoietic progenitor cells (23).

In this report, we showed that the remarkable decrease in the colony formation occurred during the early stage of infection with SHIV C2/1 (days 1 through 3 postinoculation). These results suggest that the hematopoietic progenitor cells are damaged or defective during such an early phase of infection. Furthermore, the CD4/CD8 ratio in monkey 4345 decreased within several hours, compared with controls (Fig. 1). We used ketamine for viral or saline inoculation, blood sampling, and autopsy. Ketamine has safely been used for bone marrow aspiration in humans and monkeys (21, 22, 26, 34, 35). It would be unlikely, therefore, that such bone marrow suppression occurred as a result of the anesthetic agent. However, our anesthetic procedure probably induces the release of corticosteroids in animals by the stress of capture and injection, which may have a negative impact on the colony formation. Our observation of the ability of bone marrow cultures from sham-inoculated controls to produce BFU-E and CFU-GM at days 1 and 3 proved otherwise.

After day 56, the ability of the bone marrow to form colonies recovered despite the preceding viremia and the continuing reduction of CD4/CD8 ratio. Furthermore, the colony formation was maintained at a level comparable to that of the control monkeys toward the terminal stage. Many reports have noted that CFU-GM growth continuously declines in SHIV infection, and such a decline appears to correlate with disease activity (20). However, CD4/CD8 ratio may not reflect the ongoing status of the bone marrow. A reason for the continuous reduction of CD4/CD8 ratio could be that infected T lymphocytes were destroyed in the peripheral blood more than they were produced in the bone marrow. This could be due to enhanced apoptosis or ongoing destruction of T cells by the antibody-dependent cell-mediated cytolytic mechanism in SHIV infection (24).

In contrast to previous reports, our results clearly showed that the decreasing CFU-GM growth recovered in the advanced stage, suggesting that the damage to colony formation during the early stage is reversible. We showed by PCR in this report that the direct infection of bone marrow progenitor cells with SHIV C2/1 was minimal (3, 5, 13, 18). It is possible, however, that the number of colonies was too low for detection of SHIV C2/1 virus or that SHIV C2/1 virus-infected cells were already removed by the host immune system before the assays (6, 8, 13).

It has been reported that the cellularity of the bone marrow from patients with HIV does not always correlate with the peripheral blood abnormalities (4). The commonly seen pancytopenia is often associated with hypercellular bone marrow where the increased number of lymphocytes, plasma cells, or histiocytes is seen. The latter finding suggests either dysmyelopoiesis or increased peripheral destruction of blood cells. Yoshino et al. have recently reported that atypical lymphocytes and monocytes were observed in the peripheral blood following intense viremia on the 10th to 14th days of SHIV infection (33). They further found erythroid multinucleation and atypical mononuclear cells in the hypercellular bone marrow, suggesting direct viral infection of hematopoietic progenitor cells (33).

As mentioned above, the colony formation in the bone marrow of the infected monkeys recovered spontaneously following viremia, suggesting that the reduced colony formation capability was reversible. It has been reported that inhibition of SIV replication in bone marrow macrophages resulted in increased colony growth of progenitor cells (32), and administration of recombinant human GM colony-stimulating factor could reverse leukocytopenia (11). Our data thus support the concept that, in the early phase, production of inhibitory factors or a lack or an inhibition of stimulatory cytokine production from lymphoid cells may be responsible for some of the bone marrow kinetic defects previously described in HIV (14, 28, 29). It is necessary to determine and verify what factor is participating in the regulation and recovery of the bone marrow CFU-GM growth.

The highly pathogenic SHIV C2/1 virus is an interesting tool to study the effect of HIV and SHIV infection on hematopoietic progenitor cells (15, 17, 25). Such studies will help us understand the pathophysiology of AIDS and contribute to the development of vaccines in humans (12).

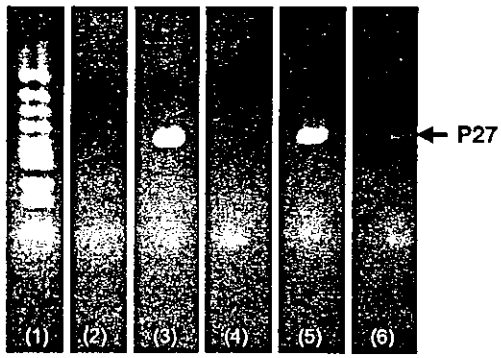

FIG. 5. Detection of SIVmac239 *gag* sequence in bone marrow colonies by PCR. Lanes: 1, a DNA ladder marker, HincII; 2, a negative control; 3, a positive control, a DNA sample from cell line M8166; 4, CFU-GM from monkey 4345 at the early stage; 5, CFU-GM from monkey 430 at the advanced stage; 6, CFU-GM from monkey 039 at the advanced stage (this monkey died of AIDS on day 238).

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Epstein-Barr virus etiology in rheumatoid synovitis

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Abstract

The etiology of rheumatoid arthritis (RA) has remained unknown, although it has been investigated and speculated that both genetic and environmental components contribute to the cause of this disease.

Epstein-Barr virus (EBV) has been a strong candidate about for over 25 years as environmental infectious agent(s). There are many circumstantial evidence for association between EBV and RA, but definite evidence is wanting.

In present article, we review an increase circumstantial proof which has been investigated so far and demonstrate direct evidence for the presence of EBV in inflamed synovial cells in patients with RA and discuss on the recent finding of signaling lymphocytic-activation molecule (SLAM)-associated protein (SAP), which opened a new approach to understand on impaired function of cytotoxic T cell for EBV in patients with RA.

Keywords: Epstein-Barr virus; Rheumatoid synovitis; EBV-encoded small RNA (EBER); Signaling lymphocytic activation molecule (SLAM); SLAM-associated protein (SAP)

Contents

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1. Why, Epstein-Barr virus and rheumatoid synovitis

The etiology of RA remains unclear, although the cause of RA has been attributed to several factors, such as genetic or environmental/infectious agents (bacteria, viruses) for over 25 years.

In twin studies, Silman et al. reported that the concordance rate for RA in a nationwide study of 91 monozygotic pairs was 15%, which is lower than the 30% figure usually quoted from a study 30 years ago and sets a limit on the potential genetic role in RA etiology [1]. Furthermore, they investigated the genetic contribution to the clinical features of RA by comparing RA-concordant monozygotic twin pairs, which highlighted the importance of non-genetic factors in RA susceptibility [2]. These data indicate that genetics is not the sole component and that both genetic and environmental/infectious factor(s) play important roles in the etiopathogenesis of RA. The current theory for the cause of RA is the RA shared epitope hypothesis, which is based on the fact that RA is associated with HLA-DRβ alleles containing the QKRAA amino acid sequence in their third hypervariable regions [3].

It was reported that RA patients have enhanced humoral and cellular immune responses to the EBV and to microorganisms that share the QKRAA sequence [4]. Immune reactivity to recombinant proteins encompassing the shared epitope was also seen in 22 monozygotic twin pairs discordant for RA, thus suggesting that either genetic or shared environmental/infectious agents were associated with the etiology of this disease.

Antibodies against EBV increased in patients with RA [5] and antibodies against an antigen in the nucleus of EBV-transformed B cells, designated RA-associated nuclear antigen (RANA), are also present in patients [6]. RANA is identical to EBNA-1 [7]. The antibody against EBNA-1 reacts with a 62-kDa protein in the synovium of patients with RA [8], and there is substantial homology in the amino acid sequences of gp110, which is a component of the EBV capsid protein, and HLA-DR4 [9]. These results suggest that EBV has a strong etiologic role in RA. Furthermore, the number of infected peripheral B lymphocytes in RA patients tends to be higher than in normal individuals [10,11] and RA patients show an impaired ability to generate EBV-specific cytotoxic T lymphocytes [11,12].

Taken together, the above results suggest that aberrant gene function and EBV may be present in patients with RA.

2. Evidence of Epstein-Barr virus in rheumatoid synovium

Fox et al. demonstrated that a monoclonal antibody, selected for reactivity with the EBV-encoded antigen EBNA-1, exhibited strong reactivity with synovial lining cells in joint biopsies from 10 of 12 patients with RA as well as with the adherent cells eluted from these tissues. No staining of RA synovial membrane or eluted synovial-lining cells was observed with monoclonal antibodies directed against antigens encoded by cytomegalovirus, herpes simplex viruses or human T cell leukemia virus type I. Among 12 osteoarthritis and normal synovial biopsies, few reactive cells were noted. These results suggest that the inflamed synovial cells in patients with RA contain EBV [8]. However, evidence of EBV DNA in rheumatoid synovial cells could not be demonstrated by Southern blotting [13]. The failure to detect EBV DNA may be due to insufficient sensitivity. Using polymerase chain reaction (PCR), EBV DNA was detected and confirmed in the synovial tissue of RA, including the RNA of several latent and lytic EBV genes [14–16] with also us, indicating that EBV is present in the RA synovial tissue. There were no differences in EBV gene expression between synovial tissues and peripheral blood when comparing RA with osteoarthritis and other disease controls because PCR is not able to identify the source of amplified signals and is typically a qualitative, rather than quantitative, form of analysis.

In order to identify of the cells in synovial tissue that EBV DNA was amplified from, we undertook the detection of EBV using in situ hybridization for the presence of EBV-encoded small RNA (EBER) in synovial cells and immunohistochemical staining for expression of CD21 molecules or latent membrane protein (LMP-1) and EBNA-2. EBER was identified in synovial cells and lymphocytes from RA patients (23.5%) but was not seen in any of the control synovial tissues (osteoarthritis and psoriasis). Interestingly, in some cases, we found that EBER was localized in the

synovial lining cells that were located at the apex of the villus proliferating lesions. Furthermore, LMP-1 molecules were also detected in synovial cells. However, CD19, CD21 and EBNA-2 were not observed. The incidence of EBV-positive cells in synovial cells with severe lymphocyte infiltration tended to be higher than in cells with moderate infiltration [17].

This study was very carefully performed in order to confirm EBV infection in patients with RA. Considerable evidence has been accumulated regarding the presence of EBV in synovial cells and lymphocytes in patients with RA [17–19], with the exception of a study by Niedobitek et al. [20].

EBV infection of synovial cells still eludes us, because CD21, a receptor for EBV, could not be detected on the synovial membrane. Cell fusion with EBV-infected lymphocytes has been suggested to play a role in viral infection of non-lymphoid cells [21]. It has recently been proposed that pre-synovial stem cells are recruited into the joints from bone marrow. Patients with rheumatoid arthritis may therefore have EBV-infected pre-synovial stem cells in their marrow and these cells move to the synovial membranes in the joints (Fig. 1).

3. Signaling lymphocytic activating molecule-associated protein (SAP) plays a crucial cytotoxic role in Epstein-Barr virus infection

In 1996, we cloned several cDNA clones from patients with IgA nephritis. The function of those

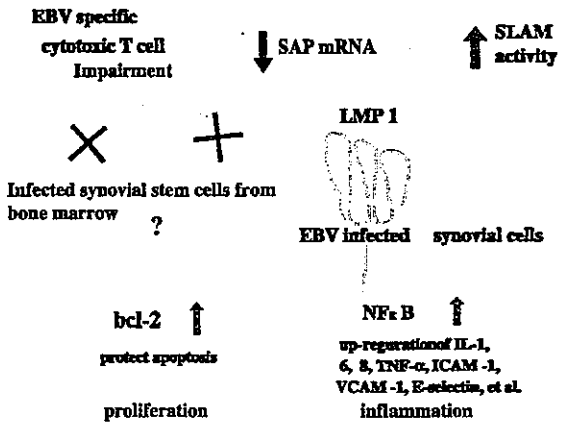

Fig. 1. Impaired SAP function may involve EBV infection to synovial cells and synovitis in patients with RA.

cloned gene has since been unclear. It was recently reported that Signaling lymphocytic activating molecule (SLAM) is a cell surface molecule (CD150) to which SAP binds, thus initiating a new T-cell signal-transduction pathway via the co-receptor molecule SLAM. One of these cDNA clones from patients with IgA was identified as SAP.

In T cells expressing SAP, SLAM–SLAM interactions trigger selective up-regulation of IL-4 secretion in response to antigen receptor stimulation [22]. Because defective SAP protein produced by a mutated SAP gene plays a crucial role in the pathogenesis of the inherited immunodeficiency X-linked lymphoproliferative syndrome (XLP) [23,24], we attempted to identify genes causing disturbances in the function of EBV cytotoxic T cells in RA patients, focusing particularly on the SAP encoded by the XLP gene.

Using quantitative real-time PCR, the expression levels of SAP transcripts in peripheral T lymphocytes or leukocytes were examined. SAP transcript levels in peripheral leukocytes of RA patients were significantly lower than those of normal individuals, those of inactive systemic lupus erythematosus patients, and those of chronic renal disease patients [25]. Decreased SAP transcript levels in patients with RA were also observed in peripheral CD2 T lymphocytes when compared with normal individuals. Furthermore, we found that the nucleotide sequence of SAP cDNA did not possess any mutations or deletions in the coding region when compared with wild-type SAP cDNA [25].

The role of this decrease in SAP transcripts is uncertain. Genomic polymorphism in the promoter or enhancer region might be present in RA patients or certain cytokines may inhibit SAP mRNA expression. Because SAP primarily exists in T cells, disturbances in SAP mRNA expression would affect SLAM-induced signal-transduction events in T cells. RA patients have impaired IFN-γ production by T cells, which indicative of Th2 type responses [26]. SLAM production during T cell activation induces IFN-γ production and redirects Th2 type cells to Th1 type cells, and an inadequate response in RA patients would result from impaired function of the SLAM/SAP pathway. An impaired SAP pathway may contribute to the failure to eliminate EBV-infected

cells by cytotoxic T lymphocytes or NK cells in patients with RA (Fig. 1).

This hypothesis is supported by the results of abnormally elevated EBV-infected B cells [10,11] and defective EBV-suppressive T cells in patients with RA [11,12]. The role of decreased SAP transcripts in patients with RA is unclear. Abnormal promoter or enhancer genes may contribute to this event in patients with RA. We investigated nucleotide mutations in the SAP promoter region in patients with RA, and identified a single nucleotide mutation (manuscript in preparation). This single nucleotide mutation lead to the failure of the immune system to eliminate Epstein-Barr virus infected synovial cells.

4. Summary

We are encouraged by Ollier, who stated that a causal link between EBV and RA cannot yet be supported, but it does seem increasingly likely that viruses, such as EBV, have a role in the progression or exacerbation of inflammatory responses within the RA joint, and if treatments can be developed that can limit or prevent reactivation of EBV, these may be beneficial for RA [27]. We believe that we are very close to identifying a link between EBV and RA.

Take-home messages

- Antibodies against Epstein-Barr virus (EBV) increased in patients with rheumatoid arthritis (RA).
- Number of infected peripheral B lymphocytes in RA patients tends to be higher than in normal individuals.
- Specific cytotoxic T lymphocytes against EBV show an impairment in patients with RA.
- EBV DNA was detected and confirmed in the synovial tissue in patients with RA by polymerase chain reaction.
- EBV gene and latent membrane protein were demonstrated and confirmed in synovial cells and lymphocytes in patients with RA by the methods of in situ hybridization and immunohistochemical staining.
- Decreased SAP transcript levels in patients with RA were observed in peripheral T lymphocytes when compared with normal individuals. An impaired SAP pathway may contribute to the failure to eliminate EBV-infected cells by cytotoxic T lymphocytes or NK cells in patients with RA.

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