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J Clin Invest. 1993;92(4):1957-1966. <https://doi.org/10.1172/JCI116789>.

Research Article

The present study was performed to clarify the relationship between human T cell lymphotropic virus type I (HTLV-I) infection and chronic inflammatory arthropathy. To determine the ability of HTLV-I to infect synovial cells and the effect on synovial cell proliferation, synovial cells were cocultured with the HTLV-I-producing T cell lines (MT-2 or HCT-1). After coculture with HTLV-I-infected T cells, the synovial cells expressed HTLV-I-specific core antigens, and HTLV-I proviral DNA was detected from the synovial cells by polymerase chain reaction. These cocultured synovial cells with HTLV-I-infected T cells proliferated more actively than the synovial cells cocultured with uninfected T cells. This stimulatory effect of HTLV-I-infected T cells on synovial cell proliferation seems necessary to contact each other. After being cocultured with MT-2 cells, synovial cells proliferated more actively than control cells even after several passages. Furthermore, HTLV-I-infected synovial cells produced significant amounts of granulocyte/macrophage colony-stimulating factor. These results suggest that HTLV-I can infect synovial cells, resulting their active proliferation and may be involved in the pathogenesis of proliferative synovitis similar to that found in rheumatoid arthritis.

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Infection of Human Synovial Cells by Human T Cell Lymphotropic Virus Type I

Proliferation and Granulocyte/Macrophage Colony-stimulating Factor Production by Synovial Cells

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Abstract

The present study was performed to clarify the relationship between human T cell lymphotropic virus type I (HTLV-I) infection and chronic inflammatory arthropathy. To determine the ability of HTLV-I to infect synovial cells and the effect on synovial cell proliferation, synovial cells were cocultured with the HTLV-I-producing T cell lines (MT-2 or HCT-1). After coculture with HTLV-I-infected T cells, the synovial cells expressed HTLV-I-specific core antigens, and HTLV-I proviral DNA was detected from the synovial cells by polymerase chain reaction. These cocultured synovial cells with HTLV-I-infected T cells proliferated more actively than the synovial cells cocultured with uninfected T cells. This stimulatory effect of HTLV-I-infected T cells on synovial cell proliferation seems necessary to contact each other. After being cocultured with MT-2 cells, synovial cells proliferated more actively than control cells even after several passages. Furthermore, HTLV-I-infected synovial cells produced significant amounts of granulocyte/macrophage colony-stimulating factor. These results suggest that HTLV-I can infect synovial cells, resulting their active proliferation and may be involved in the pathogenesis of proliferative synovitis similar to that found in rheumatoid arthritis. (*J. Clin. Invest.* 1993;92:1957-1966.) Key words: human T cell lymphotropic virus type I (HTLV-I) • chronic inflammatory arthropathy • synovial cells • granulocyte/macrophage colony-stimulating factor

Introduction

Human T cell lymphotropic virus type I (HTLV-I)¹ is known to be the etiologic agent of adult T cell leukemia/lymphoma (ATLL) (1) and is also associated with a chronic neurodegenerative syndrome termed HTLV-I-associated myelopathy (HAM) (2) or tropical spastic paraparesis (TSP) (3). Re-

cently, we and other investigators have reported that proliferative synovitis is seen in patients with ATLL (4, 5) and HAM/TSP (6). On the other hand, patients with chronic inflammatory arthropathy are shown to be sero-positive for anti-HTLV-I antibodies with high frequency in the southwest of Japan, which is one of the endemic area of HTLV-I (7, 8). Kitajima et al. (9) detected HTLV-I proviral DNA and viral gene expression in synovial cells from polyarthritic patients with anti-HTLV-I antibodies. More recently, Iwakura et al. (10) demonstrated that transgenic mice that carry the HTLV-I genome developed proliferative synovitis, which resembles rheumatoid arthritis. These findings suggest that HTLV-I has tropism for synovial cells and is one of the etiologic agents of chronic arthropathy. But the details of mechanism of the pathological association between arthropathy and HTLV-I remain to be clarified. To resolve this problem, we sought to determine whether synovial cells can be infected with HTLV-I and whether such infection may affect the synovial cell proliferation and the production of cytokines. We demonstrate here that synovial cells cocultivated with an HTLV-I-producing T cell line expressed HTLV-I viral antigens and proviral DNA. Moreover, the HTLV-I-infected synovial cells produce GM-CSF and these cells might trigger active proliferation of synovial cells.

Methods

Preparation of human synovial cells. Synovial tissues were obtained from patients with rheumatoid arthritis (RA) who were underwent arthroscopic synovectomy or corrective surgery. Their seronegativity for anti-HTLV-I antibody was confirmed by Western blot analysis using HTLV-I antigens derived from the MT-2 cell line (Eitest ATL-WB, Eisai Inc., Tokyo, Japan) (11). Synovial cells were obtained by enzymatic digestion as reported elsewhere (12). In brief, after removing adipose tissue, synovial tissues were washed in HBSS. The synovial membranes were minced aseptically, then dissociated enzymatically with 100 µg/ml collagenase (Sigma Chemical Co., St Louis, MO) and 3.3 mg/ml dispase (Godo Shusei Co., Tokyo, Japan) in HBSS for 30 min at 37°C and stirred gently. After washing with HBSS, the cells were suspended in RPMI 1640 supplemented with 10% heat-inactivated FCS (Gibco Laboratories, Grand Island, NY) and were plated in culture dishes (Falcon 3003, Becton Dickinson & Co., Oxnard, CA). To eliminate nonadherent cells from the synovial cell preparations, the plated cells were cultured for 18 h at 37°C in humidified 5% CO₂ in air, and then washed intensively with HBSS. Adherent synovial cells were removed by adding trypsin-EDTA HBSS. Synovial cells from the second to sixth passage were used in the following experiments. Fewer than 1% of the synovial cells reacted with monoclonal antibodies to CD3 (present on all mature T cells) (Coulter Immunology, Hialeah, FL), Leu-M3 (an antigen on monocytes and macrophages) (Becton Dickinson & Co.), CD20 (a pan B cell antigen), (Coulter Immunology, Hialeah, FL), and anti-human Von Willebrand factor (present on vascular endothelial cells) (Immunotek, Marseille, France).

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Received for publication 12 June 1992 and in revised form 5 April 1993.

1. *Abbreviations used in this paper:* ATLL, adult T cell leukemia/lymphoma; HTLV-I, human T cell lymphotropic virus type I; HAM, HTLV-I-associated myelopathy; RA, rheumatoid arthritis; TSP, tropical spastic paraparesis.

J. Clin. Invest.

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0021-9738/93/10/1957/10 \$2.00

Volume 92, October 1993, 1957-1966

Cocultivation of human synovial cells and HTLV-I-infected T cells. MT-2 and HCT-1 cells were used as the HTLV-I-infected T cell lines. MT-2 was obtained by coculturing peripheral leukemic cells from ATLL patient with normal umbilical cord leukocytes (13), and HCT-1 is a T cell line established from cells in the cerebro-spinal fluid of a patient with HAM (14). HCT-1 cells proliferate dependently on interleukin 2 and are positive for CD4 antigens on the cell surface. Both cell lines contain proviral HTLV-I DNA and produce viral particle. These cells were treated with 100 $\mu\text{g}/\text{ml}$ of mitomycin C for 1 h at 37°C (15). After washing three times with HBSS, they were cultured with an equal number of human synovial cells in RPMI 1640 containing 5% FCS. A total of 5×10^5 of each of the cells were added to culture dishes (Falcon 3003; Becton Dickinson & Co.), or 1.5×10^4 of each of the cells were added to 24-well flat-bottomed culture plates (Costar, Cambridge, MA). This gave the same cell density under both sets of culture condition. The culture medium was changed every 3–4 d. MOLT4 and CEM cells were used as the uninfected T cell lines. In some experiments, synovial cells were cultured with mitomycin C-treated T cells in a Millicell (Millipore Products Division, Bedford, MA) equipped with the transparent, 0.4- μm pore membrane. In this system, synovial cells were cultured without contact of T cells, while having the same medium.

Immunostaining. Immunohistochemical staining was performed by the avidin-biotin-immunoperoxidase method as reported elsewhere (16), and by indirect immunofluorescence. First, the cells were cultured in eight-chamber glass slides (Nunc, Naperville, IL). 1.5×10^4 of cells were incubated in each well for 3 d, then the cells were fixed with cold acetone for 10 min. For immunohistochemical staining, endogenous peroxidase was inactivated by placing the slide into 0.3% H_2O_2 in HBSS for 10 min at room temperature. The cells were then incubated with a diluted solution of the primary anti-human monoclonal antibodies; anti-CD3, anti-Leu-M3, and anti-CD29 (reactive with the VLA- β chain) (Coulter Immunology), anti-human Von Willebrand factor, and Gin14 (monoclonal mouse antibody reactive with the HTLV-I core proteins p19 and p28) (Fujirebio, Inc., Tokyo, Japan) (17), and using the Histofine staining reagents kit (Nichirei Co., Tokyo, Japan). The sections were treated with biotinylated goat anti-mouse IgG for 12 min. After being washed, the treated sections were incubated with streptavidin-peroxidase conjugates, after which they were incubated in 0.5 mg/ml of diaminobenzidine (Sigma Chemical Co.) prepared in 5 mM Tris buffer solution, pH 7.6, containing 0.03% H_2O_2 , then stained with hematoxylin. The control sections were treated routinely with mouse IgG (Coulter Immunology) instead of a specific monoclonal antibody. Furthermore, the cells were analyzed by a double immunofluorescence as described in detail previously (18). Briefly, the cells were exposed to a fluorescein isothiocyanate-conjugated polyclonal rabbit anti-GM-CSF (anti-human GM-CSF; Polyvalent, Endogen, MA) and a rhodamine-conjugated GIN14 monoclonal antibody for 1 h at 4°C. Rabbit antibody against human alpha-1-fetoprotein (Dako Corp., Carpinteria, CA) and mouse IgG were used for control antibodies. After each section was washed, positive cells were determined by a fluorescence microscopy (Micro Systems, Zeiss LSM, Germany).

Isolation of DNA. High-molecular weight DNAs of synovial cells or T cells were isolated by the method described previously (19) with some modification. Briefly, cells were lysed and digested by 0.5% SDS and 0.5 mg/ml of proteinase K, followed by phenol and chloroform. The extracted DNA was precipitated with ethanol and finally dialyzed against TE buffer.

Detection of HTLV-I proviral DNA. To detect the pX region of HTLV-I proviral DNA in synovial cells, PCR was used as previously described (20) with some modifications. The oligo nucleotides used as primers were synthesized (380B; Applied Biosystems, Inc., Foster City, CA), and included pX1 (+) (base pairs 7324–7348), pX 2 (-) (7503–7526), pX8 (+) (7363–7382), and pX9 (-) (7463–7482). Sample DNA, 1 μg each, was added to a cocktail adjusted to final concentrations of 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, 0.2 mM of each deoxynucleotide triphosphate, 100 pmol of each primer, and 1.25 U of Taq DNA polymerase (Perkin Elmer Cetus,

Norwalk, CT) in a total volume of 50 μl . Sample DNA was amplified 30 cycles using the outer primers. Then, $1/10$ of these PCR products were subjected to 30 cycles of amplification using inner pX primers. In each cycle of PCR, the mixture was denatured at 94°C for 2 min (3 min for first cycle), annealed at 60°C for 2 min, and then extended at 72°C for 2 min on a DNA thermal cycler (PC-500; Astec, Fukuoka, Japan). 10 μl of each final PCR product was loaded on a composite gel containing 1% NuSieve/1% Seaken agaroses (FMC Co., Rockland, ME), electrophorated, and visualized with ethidium bromide fluorostaining. Finally, the DNA in the agarose gel was transferred to a Zeta-Probe nylon membrane (Bio-Rad Laboratories, Richmond, CA) and hybridized with a ^{32}P -labeled genomic HTLV-I pX probe (base pairs 7421–7459).

Synovial cell-proliferation assay. Synovial cells (1.5×10^4 /well) were cultured with or without mitomycin C-treated HTLV-I-infected or uninfected T cell lines in 24-well flat-bottomed culture plates (Costar) in RPMI 1640 containing 5% FCS for 7 d. The data were obtained by quadruplicate experiments. 24 h before terminating the culture, 0.4 μCi [^3H]thymidine (New England Nuclear, Boston, MA) was added to each well. At the end of incubation, unincorporated [^3H]thymidine was removed by washing the plates with HBSS. Then the cells were treated twice with a cold 10% solution of TCA for 5 min. TCA-insoluble material was harvested in 500 μl of 5% SDS. The radioactivity of each sample was determined with a liquid scintillation counter. The results were expressed as the mean counts per min (cpm) or a stimulation index (SI), where: $\text{SI} = \text{mean cpm of synovial cells cocultured with T cells} / \text{mean cpm of synovial cells uncultured}$. Furthermore, the number of viable synovial cells was also counted by trypan blue dye exclusion.

Cytokine measurement. The cells, 1.5×10^4 /well, were incubated in a 24-well plate with the medium supplemented with 5% FCS. After 5 d the supernatants were collected and used for assay. IL-1 α , IL-1 β , TNF- α , and GM-CSF were measured by an immunoenzymetric assay. Briefly, plates precoated with monoclonal antibody to each cytokine were incubated with samples, incubated further with polyclonal rabbit anticytokine antibody, and then reacted with goat anti-rabbit Ig conjugated to horseradish peroxidase. Between each step excess reactants were removed by washing three times with 0.01 M phosphate buffer (pH 7.4) containing 1% bovine serum albumin. Addition of enzyme substrate produced a chromogenic product whose absorbance was measured at 490 nm. The sensitivity of the cytokine determination was as follows: IL-1 α , 10 pg/ml; IL-1 β , 20 pg/ml; TNF- α , 20 pg/ml; and GM-CSF, 100 pg/ml.

Statistical analysis. The statistical significance of any difference was calculated using student's *t* test.

Results

Detection of HTLV-I antigens in synovial cells cocultured with HTLV-I-infected cells. Synovial cells were obtained from RA patients who were sero-negative for anti-HTLV-I antibodies confirmed by Western blot analysis. These synovial cells were cocultured with either an HTLV-I-infected T cell line (MT-2) or an uninfected T cell line (CEM) in culture dishes. After cocultivation for 7 d with exchanges of fresh medium every 3–4 d, the cells were washed and passaged. After several passages, the synovial cells were harvested to assess by immunohistochemical staining for expressing HTLV-I viral antigens. Although specimens after the second passage of synovial cells included $\sim 0.2\%$ CD3-positive MT-2 cells, the synovial cells of the third passage and beyond contained $\leq 0.01\%$ CD3-positive MT-2 cells. No anti-Leu M3, anti-CD20, or anti-Von Willebrand factor monoclonal antibody-reacted cells were observed by immunoenzymetric and indirect immunofluorescent methods. More than 99% of cells were fibroblastic in morphology, suggesting these were all synovial cells. As shown in Figs. 1 A

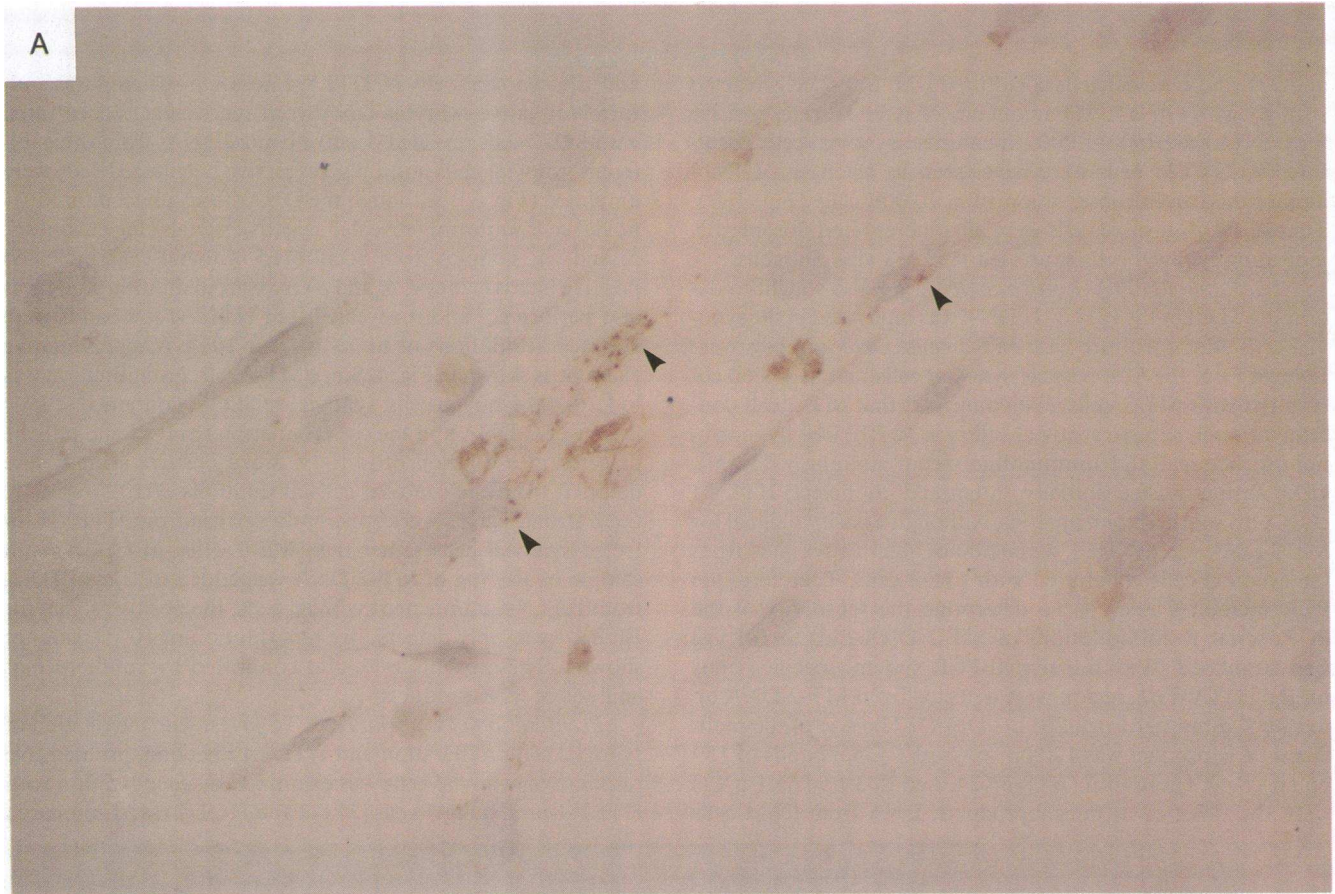


Figure 1. Detection of HTLV-I gag protein by immunohistochemical staining. (A) Synovial cells were cocultured with the HTLV-I-infected cell line (MT-2) for 7 d. After washing extensively, the synovial cells were passaged and then cultured in a chamber slide for 3 d. After incubation, the cells were treated with G1N14 monoclonal antibody and stained by avidin-biotin immunoperoxidase technique. Finally, they were counterstained with hematoxylin. Positive staining is evidenced by a brown deposit (*arrow*) ($\times 100$). (B) Synovial cells cocultured with CEM cells were not reacted with G1N14 ($\times 40$).

and 2 *A*, the synovial cells cocultured with MT-2 cells reacted strongly with GIN14. The frequency of synovial cells positive for GIN14 was 1:500–1,000. In contrast, synovial cells cocultured with CEM cells were never seen to react with GIN14 despite the careful observation of > 10,000 cells (Fig. 1 *B*). These GIN14-positive cells were all adherent and fibroblastic, indicating synovial cells expressed HTLV-I viral antigens.

Detection of HTLV-I proviral DNA. We also sought to determine whether the HTLV-I cDNA was integrated in the synovial cells after coculture with MT-2 cells. DNA samples were isolated from the fifth-passage synovial cells that had been cocultured with MT-2 cells. We confirmed that MT-2 cell contamination in sample synovial cells was < 1:10,000 by immunohistochemical and immunofluorescent staining with anti-CD3 antibody. We initially attempted to detect HTLV-I cDNA by Southern blot analysis. Although a positive signal for MT-2 DNA was detected, we could not obtain any signal from the synovial cells cocultured with MT-2 cells. Therefore, the PCR method was used. To determine the sensitivity of the PCR, serial 10-fold dilutions of MT-2 DNA (1.0 to 10^8 μg) were amplified. With our nested PCR system, specific bands for the HTLV-I pX region were detected from MT-2 DNA up to a 10^{-6} dilution (1 pg of MT-2 DNA). The size of the main target PCR product was 120 bp (pX8-pX9), but bands of 159 bp (pX1-pX9), 164 bp (pX8-pX2), and 203 bp (pX1-pX2) were also detected as the subproducts. DNA from CEM cells

and the synovial cells of HTLV-I-negative patients was uniformly negative, whereas DNA from synovial cells cocultured with MT-2 cells revealed positive bands. DNAs from three different synovial cells and their respective cocultured cells were amplified (Fig. 3). Although the DNAs from uncultured synovial cells uniformly revealed negative signals (Fig. 3, lanes 3, 5, and 7), samples from cocultured synovial cells indicated positive bands (lanes 4, 6, and 8). A 10-fold dilution study was also performed, and it revealed that HTLV-I pX bands were detected at dilutions of up to 10^{-3} or 10^{-4} . A representative example is shown in Fig. 4. Since MT-2 cell contamination was < 1:10,000 synovial cells, synovial cell DNA contained < 10^{-4} μg of MT-2 DNA and the contamination band should only be detected at a dilution of $\leq 10^{-2}$. Since positive signals were detected from the synovial cells at dilutions of 10^{-3} or 10^{-4} , these strong signals were apparently derived from HTLV-I-infected synovial cells rather than MT-2 cells. In fact, when a sample of 10^{-4} μg of MT-2 DNA was added to 1 μg of DNA from HTLV-I-uninfected synovial cells, the positive signal for HTLV-I was only detected up to a dilution of 10^{-2} (data not shown). These results were also confirmed by Southern blot analysis of PCR products.

Proliferation of synovial cells. To investigate the relation of synovial cell proliferation and HTLV-I infection, proliferative response of synovial cells was examined by cocultivation with HTLV-I-producing T cells (MT-2 and HCT-1), and compared

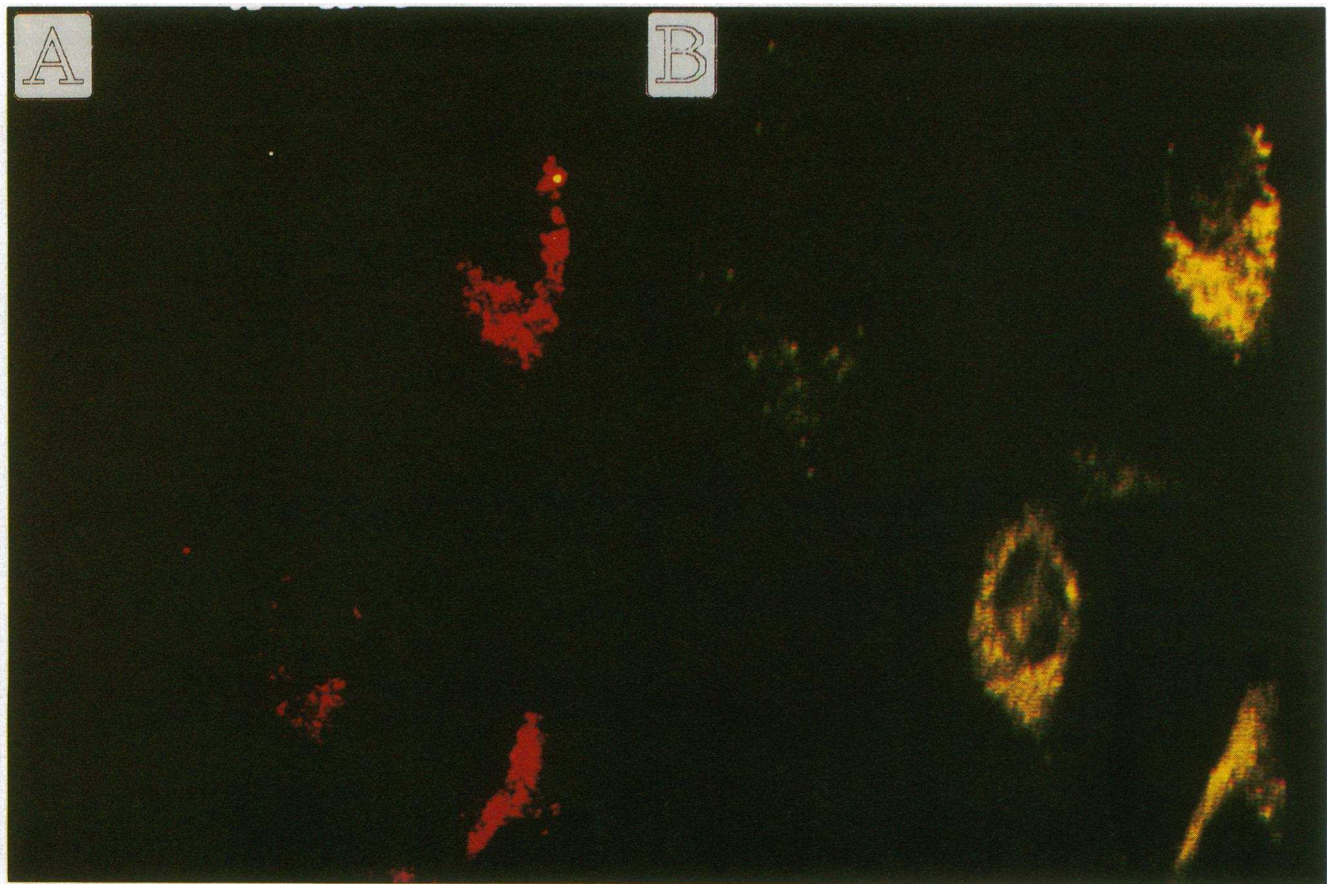


Figure 2. Detection of HTLV-I gag protein and GM-CSF by double-immunofluorescence staining. Synovial cells were cocultured with an HTLV-I-infected T cell line (MT-2) for 7 d. After incubation, the cells were passaged four times. The cells on the chamber slide were stained with rhodamine-conjugated GIN14 monoclonal antibody and fluorescein isothiocyanate-conjugated anti-GM-CSF polyclonal antibody. (*A*) Cells positive for GIN14 (reactive with HTLV-I gag proteins, p19 and p28) ($\times 400$). (*B*) Cells positive for anti-GM-CSF monoclonal antibody as in *A*.

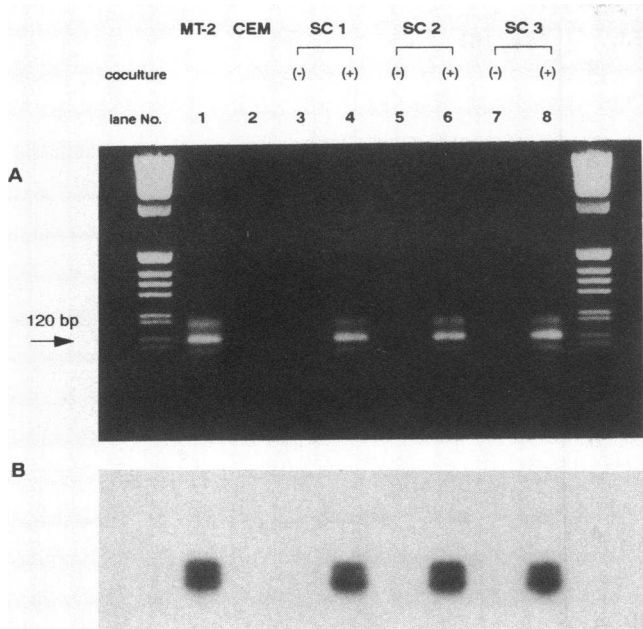


Figure 3. Detection of HTLV-I proviral DNA by PCR. (A) Synovial cells were obtained from three RA patients. Each sample was cocultured with mitomycin C-treated MT-2 cells. DNAs were extracted from the synovial cells in the fifth passage after coculture. They were amplified for HTLV-I pX region by nested PCR. 1 μ g of each PCR product was electrophorated on 1% NuSieve/1% Seaken agarose gel and visualized by ethidium bromide staining. DNA molecular markers are 1-kb ladder DNA (Bethesda Research Laboratories, Gaithersburg, MD). (B) Southern blot analysis. The amplified DNAs were hybridized with 32 P-labeled HTLV-I probe. Lane 1, MT-2 cell; lane 2, CEM cell; lanes 3, 5, and 7, uncultured synovial cell; lanes 4, 6, and 8, cocultured synovial cell with MT-2.

with that of synovial cells cocultured with uninfected T cells (CEM and MOLT4). As T cell lines were intensively pre-treated with mitomycin C, these mitomycin C-treated T cells could not proliferate, as determined by [3 H]thymidine incorporation and reduced cell number. [3 H]Thymidine incorporation during the first 24-h culture by mitomycin C-treated MT-2 cells were always < 150 cpm and decreased day by day. Alive MT-2 cells determined by trypan blue staining were counted, and they rapidly decreased from 1.5×10^4 /well to $0.67 \pm 0.18 \times 10^4$ /well after 4 d of culture, $0.18 \pm 0.18 \times 10^4$ /well after 7 d culture, to 0/well on day 14. The proliferative response of synovial cells was first examined by culture with varying numbers of MT-2 cells (1.5×10^4 synovial cells/well and 1.5×10^3 to 1.5×10^5 MT-2 cells/well). The peak proliferative response was observed on day 5 in cultures containing 1.5×10^4 or 3×10^4 MT-2 cells per well (data not shown), so subsequent coculture experiments were done with the same number of cells. Fig. 5 shows a representative kinetic study as indicated by stimulation index of [3 H]thymidine incorporation. The proliferative response of synovial cells reached a peak at culture day 5 (stimulation index, 5.5 ± 0.9). Because we did not supply fresh medium and did not passage the cells during culture, the diminished proliferation of synovial cells at the end of the experiments may be due to either lack of fresh medium or contact inhibition. Synovial cells were also stimulated to proliferate when cocultured with HCT-1 cells, whereas HTLV-I-uninfected T cells (CEM and MOLT4) did not stimulate the proliferation of synovial cells (Figs. 5 and 6).

Next, we investigated cytokine release by HTLV-I-infected T cell. As shown in Table I, these cells released various cytokines (IL- 1α , TNF- α , and GM-CSF) into the culture supernatant. Release was somewhat decreased when they were treated with mitomycin C during incubation for 5 d in 5% FCS-supple-

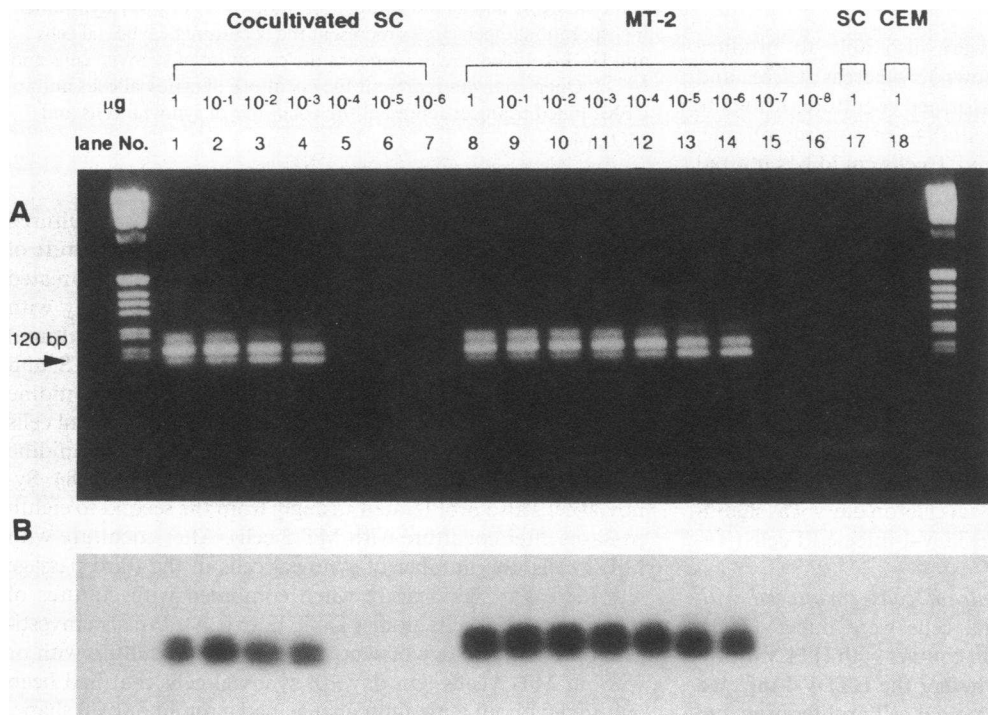


Figure 4. A 10-fold dilution study in PCR. (A) 1 μ g of each DNA sample from cocultured synovial cells was examined by a 10-fold dilution study. A representative example is shown. (B) Southern blot analysis. lanes 1-7, synovial cell cocultured with MT-2 ($1-10^{-6}$ μ g); lanes 8-16, MT-2 cell ($1-10^{-8}$ μ g); lane 17, HTLV-I-negative synovial cell; lane 18, CEM cell.

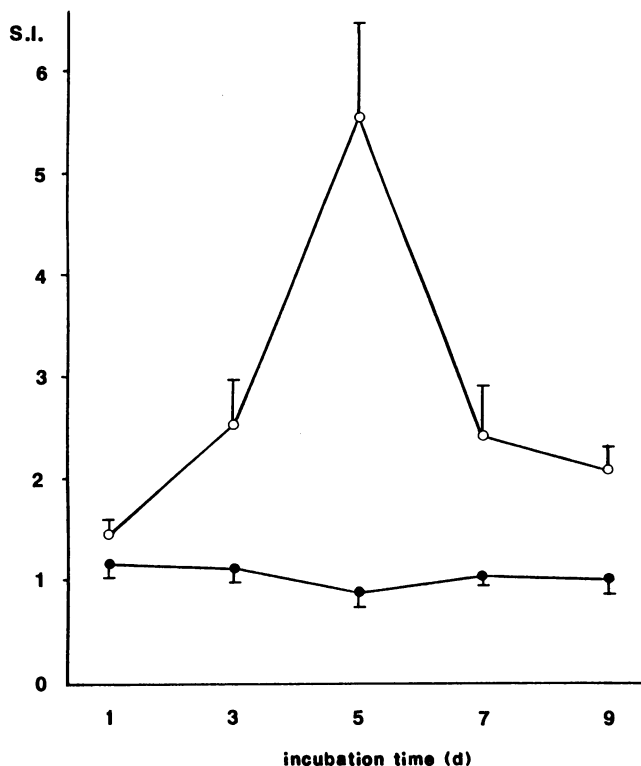


Figure 5. Proliferation of human synovial cells stimulated by HTLV-I-infected T cells (MT-2) or uninfected T cells (CEM). Synovial cells were cultured in the presence or absence of mitomycin C-treated T cells for various periods. 24 h before terminating the culture, [^3H]-thymidine was added to each well and the radioactivity in each sample was determined in a liquid scintillation counter. Data are expressed as the stimulation index (SI). Open circles showed the SI of synovial cells stimulated by MT-2 cells. Filled circles showed the SI of synovial cells stimulated by CEM cells. The results are expressed as the mean \pm SD of quadruplicate cultures.

mented medium. HCT-1 cells were also able to produce various cytokines (data not shown), whereas CEM and MOLT4 cells did not. Then we performed cocultures using the Millicell equipped with a transparent $0.4\text{-}\mu\text{m}$ pore membrane. Using this system, synovial cells and T cells could be cultured in the same medium without coming into contact with each other, so the effect of cytokines released from the T cells could be investigated. As shown in Fig. 7, synovial cells cocultured with HTLV-I-infected T cells in Millicell proliferated more actively than control synovial cells. However, their proliferative response was significantly less than that of synovial cells in standard coculture with HTLV-I-infected T cells. In contrast, when synovial cells were cultured with HTLV-I-uninfected T cells, there were no significant differences in proliferation between standard cocultures and control synovial cell cultures. These proliferative responses were confirmed by determining the cell numbers. The number of synovial cells on day 7 was significantly increased by coculture with HTLV-I-infected T cells (Table II, exps. 1 and 2).

Persistent proliferation of synovial cells cocultured with HTLV-I-infected T cells. Synovial cells were infected with HTLV-I and proliferated actively in contact with HTLV-I-producing T cells. We investigated whether the HTLV-I-infected synovial cells had a role in such synovial cell proliferation and

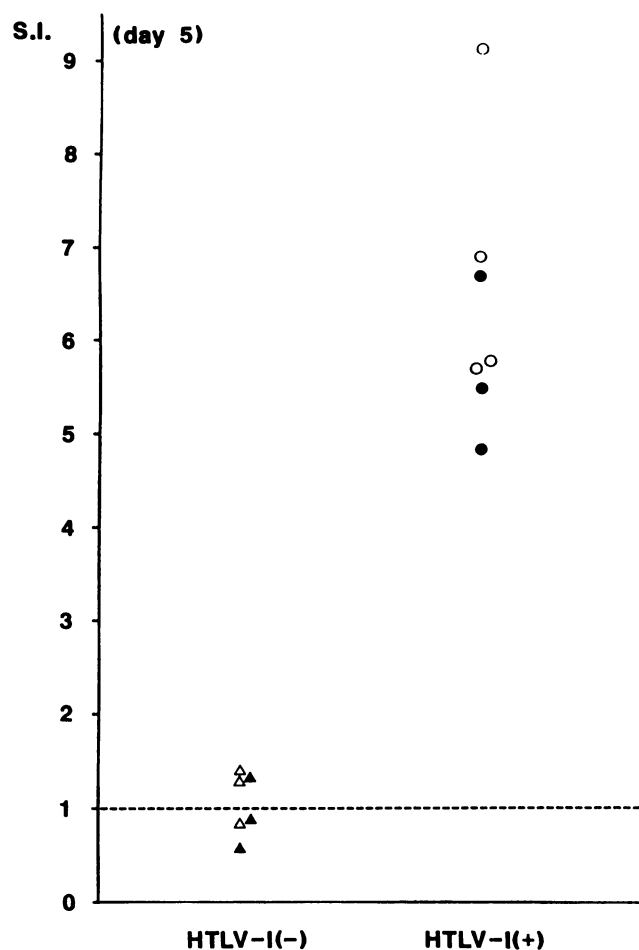


Figure 6. Proliferative responses of human synovial cells stimulated by the HTLV-I-infected T cell lines (MT-2 and HCT-1) and the uninfected T cell lines (CEM and MOLT4). Synovial cells were cultured with or without HTLV-I-infected or uninfected T cell lines for 5 d. Data are expressed as the stimulation index (SI) of [^3H]thymidine incorporation. Open circles represent the coculture of synovial cells and HCT-1. Filled circles represent the coculture of synovial cells and MT-2. Open triangles represent the coculture of synovial cells and CEM. Filled triangles represent the coculture of synovial cells and MOLT4.

whether this active proliferation of synovial cells cocultured with HTLV-I-infected T cells was persistent. After coculture of synovial cells for 7 d with or without mitomycin C-treated MT-2 cells, the synovial cells were washed intensively with HBSS and then passaged. The synovial cells were next cultured in 24-well flat plates containing RPMI 1640 with 5% FCS, and their proliferative activity was determined by [^3H]thymidine incorporation and cell counting. Second passage synovial cells cocultured with MT-2 cells incorporated more [^3H]thymidine than second passage synovial cells cultured alone (Fig. 8). Synovial cells also proliferated strongly from the second to eighth passages after coculture with MT-2 cells. After coculture with MT-2 cells, the number of synovial cells in the third passage was increased significantly when compared with cultures of control synovial cells alone (Table II, exp. 3). We also investigated the morphology of synovial cells after coculture with or without MT-2 cells. On day 10, synovial cells that had been cultured without a medium change had a round or oval shape,

Table I. Detection of Cytokines in the Supernatant of Synovial Cells Stimulated by HTLV-I-infected T Cell Line

Cells	IL-1 α	IL-1 β	TNF- α	GM-CSF
MT-2				
Mitomycin C treated	21	n.d.	185	388
Mitomycin C untreated	64	n.d.	1111	329
CEM				
Mitomycin C treated	n.d.	n.d.	n.d.	n.d.
Mitomycin C untreated	n.d.	n.d.	n.d.	n.d.
Synovial cells				
Unstimulated	n.d.	n.d.	n.d.	n.d.
Cocultured with CEM	n.d.	n.d.	n.d.	n.d.
Cocultured with MT-2	n.d.	n.d.	n.d.	322
First passage of synovial cells				
Unstimulated	11	n.d.	n.d.	n.d.
Stimulated by MT-2	17	n.d.	n.d.	496
Second passage of synovial cells				
Unstimulated	n.d.	n.d.	n.d.	n.d.
Stimulated by MT-2	21	n.d.	n.d.	408

Synovial cells (1.5×10^4 /well) were cultured with CEM and MT-2 for 5 d. MT-2 and CEM cells (1.5×10^4 /well) with or without mitomycin C treatment were cultured in the same way. The supernatants were collected and used for cytokine assay. After 7 d of coculture of synovial cells and mitomycin C-treated MT-2 cells, the cells were passaged serially. Synovial cells (1.5×10^4 /well) were incubated for 5 d and the supernatants were collected and used for assay. Cytokines, including IL-1 α , IL-1 β , TNF- α , and GM-CSF, were measured by an immunoenzymetric assay. n.d., not detectable.

and some of them were detached from the culture dish. In contrast, synovial cells cocultured with MT-2 cells grew to confluence and overgrew in some parts of the culture wells. These cocultured synovial cells were spindle-like and adherent to the culture dishes (Fig. 9). HTLV-I-infected synovial cells were detected by immunostaining with GIN14 after each passage. It was found that proliferative stimulation of synovial cells cocultured with MT-2 cells persisted through several passages, suggesting that not transient cell-to-cell contact but HTLV-I infection of synovial cells caused their proliferation.

GM-CSF production by HTLV-I-infected synovial cells. Some of the synovial cells became infected by HTLV-I when cocultured with HTLV-I-producing T cells. Therefore, we next investigated the functioning of such HTLV-I-infected synovial cells. Initially, we measured the cytokines in the culture supernatants of HTLV-I-infected synovial cells by immunoenzymetric assays. When synovial cells were cocultured with MT-2 cells, the medium contained a considerable amount of GM-CSF, although the medium of unstimulated synovial cell cultures did not. As for IL-1 α , IL-1 β , or TNF- α , none of these cytokines was detected in the medium of either cocultured or uncultured synovial cells. The same results were obtained after one or two passages (Table I).

Cultured synovial cells were stained by the double-immunofluorescence method. Fig. 2 shows that synovial cells reacting

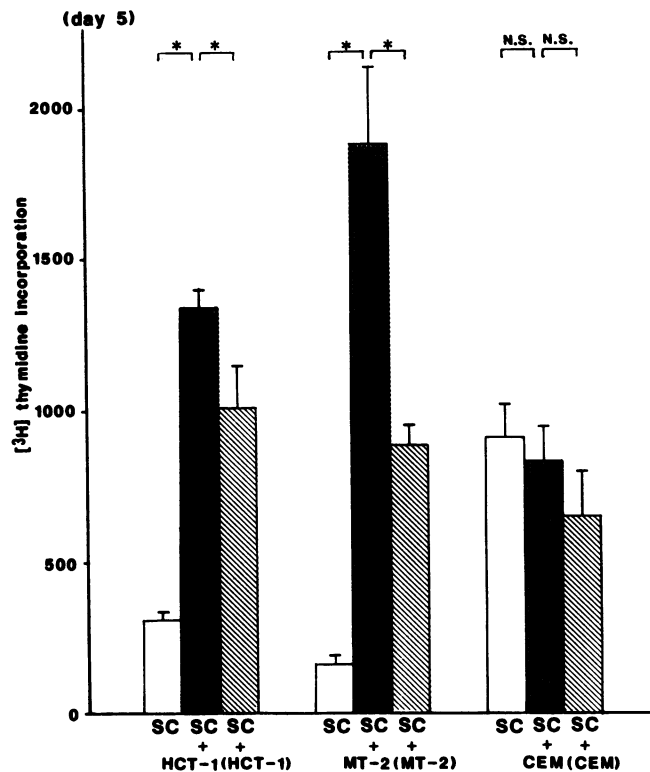


Figure 7. Proliferation of synovial cells cocultured with T cells in Millicells. Synovial cells were cultured with the mitomycin C-treated T cells in Millicells for 5 d. The membrane in the Millicell separated the synovial cells from the mitomycin C-treated T cells. 24 h before terminating the culture, [3 H]thymidine was added to each well and the radioactivity in each sample was determined in a liquid scintillation counter. Data are expressed as the mean \pm SD cpm. Open columns represent [3 H]thymidine incorporation by unstimulated synovial cells. Dotted columns represent [3 H]thymidine incorporation by synovial cells cocultured with mitomycin C-treated T cells. Hatched columns represent [3 H]thymidine incorporation by synovial cells cocultured with mitomycin C-treated T cells in Millicells. Statistical analysis was performed between [3 H]thymidine incorporation by synovial cells cocultured with T cells vs. that by unstimulated synovial cells and that by synovial cells cocultured with T cells in Millicells vs. that by synovial cells cocultured without Millicells. * $P < 0.01$.

with rhodamine-conjugated GIN14 were also stained by fluorescein isothiocyanate-conjugated anti-GM-CSF polyclonal antibodies. Only the synovial cells positive for GIN14 were stained by anti-GM-CSF, suggesting that GM-CSF was mainly produced by the HTLV-I-infected synovial cells. These findings were confirmed up to the fifth passage, and the cultures did not contain anti-CD3-positive MT-2 cells.

Discussion

The present study demonstrates that HTLV-I genome can be transmitted into human synovial cells from an HTLV-I-producing T cell line. Immunohistochemically, the monoclonal antibody GIN14 detected HTLV-I gag proteins within synovial cells cocultured with mitomycin C-treated MT-2 cells. In this method synovial cells could be easily distinguished by their morphology from MT-2 cells. HTLV-I has the specific gene complex, called pX, that promotes its own replication and

Table II. The Number of Synovial Cells Stimulated by HTLV-I-infected T Cell Line

Exp.	Synovial cells	Mitomycin C-treated cells	No. of synovial cells $\times 10^4$
1	1×10^4 /well	HCT-1 cells (1×10^4 /well)	$1.64 \pm 0.04^*$
	1×10^4 /well	CEM cells (1×10^4 /well)	1.26 ± 0.04
	1×10^4 /well	(—)	1.23 ± 0.05
2	2×10^4 /well	HCT-1 cells (2×10^4 /well)	$2.92 \pm 0.21^*$
	2×10^4 /well	CEM cells (2×10^4 /well)	2.31 ± 0.17
	2×10^4 /well	(—)	2.33 ± 0.15
3	2×10^4 /well	MT-2 cells	$2.57 \pm 0.21^*$
	2×10^4 /well	CEM cells	2.12 ± 0.20

Synovial cells were cultured with mitomycin C-treated HCT-1 or CEM cells for 7 d. After incubation, the number of synovial cells was counted by trypan blue dye exclusion (Exps. 1 and 2). After cell passages for twice, the number of synovial cells was counted in the same way (Exp. 3). * $P < 0.01$, number of synovial cells stimulated by HTLV-I-infected T cells vs. number of synovial cells stimulated by CEM (Exps. 1 and 2) or unstimulated synovial cells (Exp. 3).

transactivates various cellular genes (21, 22). By PCR, the HTLV-I pX genome was also detected in the sample DNA. The efficiency of infection by this method was relatively low, and $\sim 1:500$ – 1000 synovial cells were infected with HTLV-I in the immunohistochemical study. This level of infection corresponded to the results of the PCR, as indicated by the 10-fold dilution study, which showed a 10^2 - to 10^3 -fold difference from MT-2 DNA. Thus, it was reasonable that HTLV-I signal could not be detected by direct Southern blot analysis.

To the best of our knowledge, this is the first demonstration that HTLV-I can infect human synovial cells in vitro. HTLV-I has tropism to $CD4^+$ lymphocyte, but nonlymphoid cells such as vascular endothelial cells (23, 24), fibroblasts (25), human lung cell line, and human uterine cervical carcinoma cell line (26) were reported to be infected by HTLV-I when these cells are cocultured in vitro with irradiated HTLV-I-producing T cells. Recently HTLV-I viral antigens and tax_1 / rex_1 messenger RNA were detected in the fresh synovial tissues and cultured synovial cells from patients with HTLV-I-associated chronic inflammatory arthropathy (9). This finding supports strongly our results that HTLV-I can be transmitted to synovial cells from the infected T cells and the integrated HTLV-I genes can be transcribed and expressed.

HTLV-I-transformed T cells have been reported to produce a multitude of cytokines, including IL- 1α (27), IL-2 (28, 29), IL-3 (27), IL-6 (30), interferon- γ (8), and transforming growth factor β (31). These cytokines may affect the proliferation of synovial cells. In fact, synovial cells cocultured using Millicell proliferated more actively than the control synovial cells. This is probably due to the effect of strong growth factors such as IL- 1α and TNF- α (32, 33), which were detected in the supernatant from mitomycin C-treated HTLV-I-producing T cell line. However, the proliferative response of synovial cells cocultured in the Millicell was significantly lower than that seen in standard cocultures. On the other hand, synovial cells cocultured with HTLV-I-uninfected T cells showed no significant differences in proliferation between standard cocultures

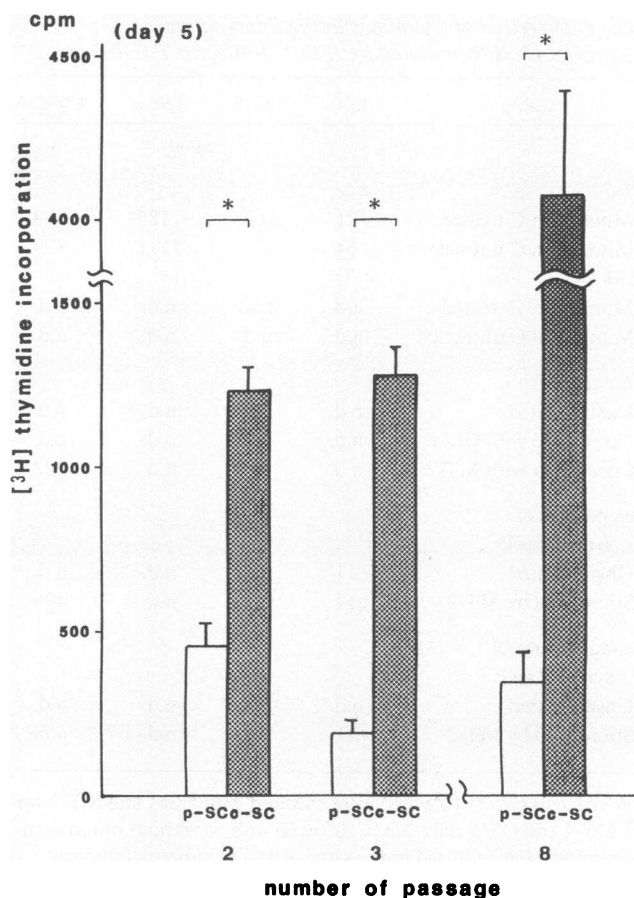


Figure 8. Proliferation of synovial cells after several passages. Synovial cells were cultured with or without mitomycin C-treated MT-2 cells. After incubation for 7 d, the cells were passaged serially. On the fifth day after the indicated passages, 24 h before terminating the culture, [3 H]thymidine was added to each well and radioactivity in each sample was determined in a liquid scintillation counter. Data are expressed as the mean \pm SD cpm. Open columns represent the unstimulated synovial cells. Dotted columns represent the synovial cells cocultured with mitomycin C-treated MT-2 cells. The left depicts the second passage. The middle depicts the third passage. The right depicts the eighth passage. * $P < 0.01$ for [3 H]thymidine incorporation by synovial cells cocultured with mitomycin C-treated MT-2 cells vs. that by unstimulated synovial cells.

and control cultures, suggesting that cell-to-cell attachment alone was not sufficient to enhance the proliferation of synovial cells and that contact with HTLV-I-infected T cells was much more important. It is known that HTLV-I genome transmission occurs only through cell-to-cell contact, and not via free materials (34). Therefore, the active proliferation of synovial cells may have been related to HTLV-I infection. To confirm our speculation that HTLV-I infection of synovial cells caused their active proliferation, the persistence of this proliferation was examined. Synovial cells showed active proliferation and morphological changes even after several passages. We excluded the effects of mitomycin C-treated HTLV-I-infected T cells and their products in this experiment, therefore, it appears that HTLV-I infection of synovial cells may have some roles in their enhanced proliferation.

The HTLV-I gene product, Tax *trans*-regulatory protein, is encoded in the pX region of the HTLV-I provirus and is trans-

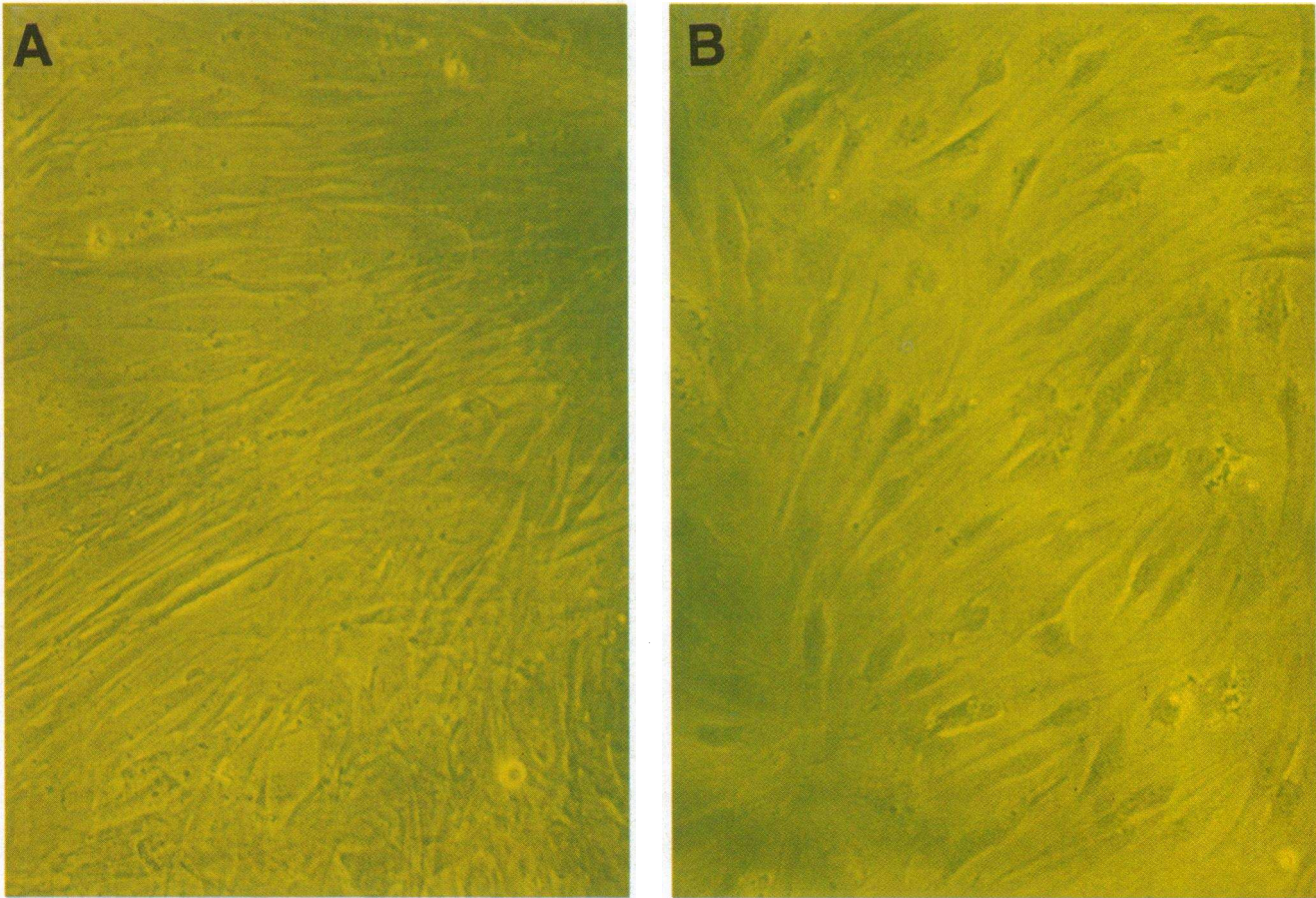


Figure 9. Morphology of synovial cells cocultured with (A) or without (B) MT-2 cells ($\times 100$).

lated from the doubly spliced tax/rex transcript (35). Tax protein increases the transcription of viral and cellular genes through at least two host transcription factor pathways. One is the cAMP-responsive element binding protein (CREB) and activating transcription factor (ATF) family of DNA-binding proteins, and the other is the pleiotropic transcription factor NF- κ B, which activates transcription of a variety of cellular and heterologous viral promoters that contain NF- κ B enhancer elements (36). These include the promoters of the IL-2, IL-2R α , long terminal repeat of HIV-I.

Transcription of GM-CSF is regulated by tax protein in HTLV-I-infected T cells (37). Expression of GM-CSF gene is induced through the action of specific cellular transcription factors that can interact with tax protein (38, 39). Synovial fibroblasts may potentially express GM-CSF when stimulated by IL-1 or TNF- α (40) as well as lung fibroblasts, monocytes, and endothelial cells (41, 42), but we only detected GM-CSF in the HTLV-I-infected synovial cells by double-immunofluorescence staining. This finding may suggest the possibility that tax protein might operate the transcription of GM-CSF in synovial fibroblasts the same way as in HTLV-I-infected T cells.

Rheumatoid synovitis is histologically characterized by mononuclear cell infiltration, neovascularization, and synovial cell proliferation. The present study demonstrated that HTLV-I infection of synovial cells caused their proliferation in vitro, however, the mechanism of this increased capacity of the proliferation is unknown. The role of GM-CSF is reported to in-

crease the expression of HLA class II molecules on monocytes (43). The expression of HTLV-I proteins in synovial cells, which is related to GM-CSF production, might have some effects on activation or proliferation of synovial cells. Therefore, HTLV-I infection of synovial cells could be involved in the pathogenesis of chronic inflammatory arthropathy in a subset of patients.

Acknowledgments

The primers for PCR and probe oligonucleotide were kindly provided by Dr. Katamine (Department of Bacteriology, Nagasaki University School of Medicine, Nagasaki, Japan). We also thank Dr. Yasuichi Omoto (Otsuka Pharmaceutical, Tokushima, Japan) for the detection of IL-1 α , IL-1 β , TNF- α , and GM-CSF.

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