

# Identification of human T cell leukemia virus in a Japanese patient with adult T cell leukemia and cutaneous lymphomatous vasculitis

(thymus/lymphoma/human retrovirus)

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**ABSTRACT** We have identified a Japanese patient with adult T-cell leukemia (ATL) whose T cells *in vitro* produced the human T-cell leukemia virus (HTLV). This patient presented with lymphomatous arthritis and leukemia and subsequently developed skin lesions. Skin invasion by malignant T-cells was angiocentric and produced vessel wall destruction, resulting in necrotic cutaneous tumor nodules. Malignant T cells in peripheral blood, skin, and joint prior to culture *in vitro* did not express p19 HTLV-associated antigen. However, by electron microscopy, intracellular type C viral particles were seen in skin-infiltrating T cells. Peripheral blood malignant cells after 7 days in culture with T-cell growth factor-supplemented media expressed p19 antigen, and type C virus particles were seen by electron microscopy to be budding from malignant T lymphocytes. Mitomycin-C-treated peripheral-blood T cells induced the transformation of cord blood T cells into HTLV-infected p19<sup>+</sup> T cells. The demonstration of HTLV in malignant T cells from our patient confirms the association of HTLV with Japanese adult T-cell leukemia. Moreover, HTLV may be associated with a vasculitis-arthritis syndrome.

A syndrome of adult T-cell leukemia (ATL) has been recognized to be endemic in restricted areas of Japan (1, 2). A virus, ATL virus, has been detected in ATL-derived T-cell lines, and a close relationship between ATL and ATL virus has been suggested by serologic and biochemical analyses (3-5). In 1980, Poiesz and coworkers described human T-cell leukemia virus (HTLV) and have shown its association with a variety of clinical mature-T-cell lymphoproliferative syndromes in various areas of the world (6, 7). Recently, Gallo and colleagues have shown HTLV to be similar to ATL virus (8-10). In this report, we describe a Japanese patient with ATL with unusual clinical manifestations of the disease, namely arthritis and cutaneous lymphomatous vasculitis. Moreover, we have isolated a strain of HTLV from cultured peripheral blood (PB) T cells, thus confirming the association of HTLV with Japanese ATL.

## METHODS

**Patient Characteristics.** A 49-year-old Japanese woman (SD) was well until April 1982 when she developed swelling of her left index finger, right fourth proximal interphalangeal joint, and right ankle. One week later she developed a nodular rash on the skin of her neck, chest, back and right ear lobe. Whereas previous blood counts had been normal, on April 20, 1982, her white blood count was 20,000 with 54% lymphocytes. The only other abnormal laboratory test result at that time was the alkaline phosphatase (331 units; normal levels, <240 units).

By May 18, 1982, her skin lesions had begun to ulcerate (usually 5 to 7 days after initial appearance of individual lesions), and her PB white cell count was 24,000 cells per mm<sup>3</sup> with 90% abnormal-appearing lymphocytes, 91% of which rosetted with sheep erythrocytes. Because the patient was born in Sa-sebo, Kyushu, Japan, and lived there until age 23 (when she emigrated to the United States), the diagnosis of Japanese ATL was made. After admission to Duke University Hospital (June 8, 1982), physical examination demonstrated multiple 0.5- to 3-cm cutaneous nodular skin lesions, many of which had ulcerated centrally. The patient's left index and right fourth fingers were swollen at the proximal interphalangeal joints, and right tibiotalar ankle tenderness was present. Laboratory abnormalities included a PB white cell count of 27,000 cells per mm<sup>3</sup> with 85% lymphocytes and lactate dehydrogenase of 426 units (normal, 100-330 units/dl). Serum calcium level was 9.7 mg/dl (normal, 8.7-10.2 mg/dl).

**Preparation and Processing of Patient Cells or Tissue.** PB mononuclear cells were obtained from SD, purified on Hypaque/Ficoll gradients, stained with indirect immunofluorescence techniques, and assayed by visual fluorescence or cytofluorography as described (11). Four-micrometer frozen sections of skin were cut, fixed, and stained as described (12, 13). Joint fluid was processed for immunofluorescence as for PB cells. The panel of monoclonal antibodies used for phenotyping malignant T cells in skin, joint, and PB have been characterized and reviewed in detail (11, 14). Samples of cells in suspension or skin tissues were fixed in 2% glutaraldehyde and processed for electron microscopy by standard techniques. PB cells were cultured for 5-7 days in RPMI 1640 medium either supplemented with 20% fetal calf serum alone or supplemented with 20% fetal calf serum/50% crude T-cell growth factor (TCGF) (New England Enzyme Center, Boston, MA). SD PB cells cultured in TCGF were treated with mitomycin C (40 µg/ml) for 1 hr at 37°C and cocultured with cord blood lymphocytes in the presence of phytohemagglutinin (1 µg/ml) supplemented with TCGF-containing media. After 3 passages in TCGF-supplemented media, the transformed cord cells were assayed for intracellular HTLV-associated p19 antigen as described (15).

**Assays for HTLV-Associated Antigens.** Assay for intracellular p19 antigen in tissue and various cell suspensions was through indirect immunofluorescence with anti-HTLV p19 (anti-p19) monoclonal antibody 12/1-2 (the gift of M. Robert-Gur-off) and P3 × 63/Ag8 ascitic fluid as a control (15). Assay for HTLV p24 core antigen in tissue and various cell suspensions

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Abbreviations: ATL, adult T-cell leukemia; HTLV, human T-cell leukemia virus; TCGF, T-cell growth factor; PB, peripheral blood; anti-p19 and -p24 antibodies, antibodies against HTLV-associated p19 and p24 antigens.

was through indirect immunofluorescence with goat anti-HTLV p24 (anti-p24) antiserum (the gift of V. Kalyanaraman) and normal goat serum as a control (16). Surface or intracytoplasmic reactivity of SD's PB fresh or cultured cells with known HTLV-patient serum (T-1124, the gift of R. Gallo) or with autologous serum was determined by using serum diluted 1:10 in indirect immunofluorescence assays and developed with goat anti-human IgG (TAGO, Burlingame, CA). In each experiment, normal human serum (nonimmune to HTLV) was used as a control.

**Assay for Serum Anti-HTLV Antibodies.** Assay for serum anti-p19 and anti-p24 antibodies was performed by using SD serum, T-1124 serum (known HTLV antibody positive) or normal human serum to immunoprecipitate <sup>125</sup>I-labeled disrupted HTLV extract as described (16-18).

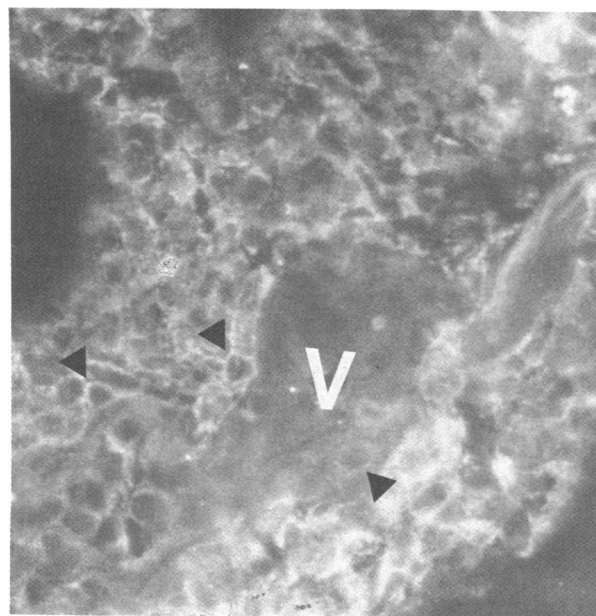
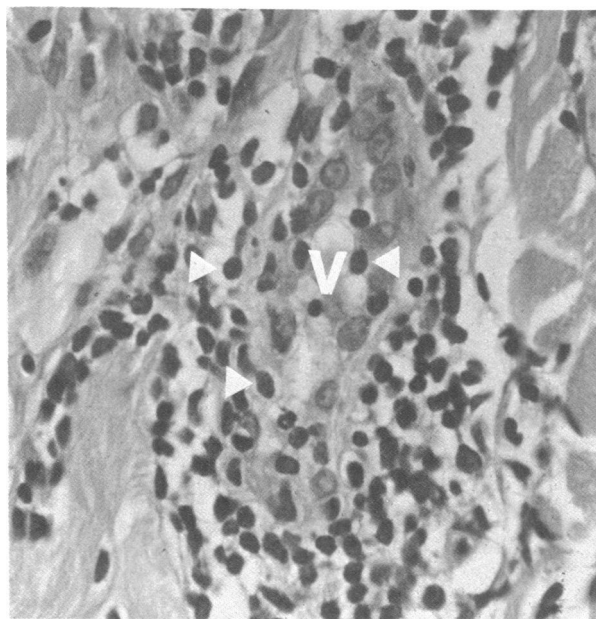


FIG. 1. Angiocentric and angioinvasive cutaneous lymphomatous vasculitis. (A) Dermal vessel (V) wall surrounded and invaded by lymphocytes (arrowheads). (hematoxylin and eosin stain; ×400.) (B) Indirect immunofluorescent stain of SD skin biopsy showing erythrocyte rosette\* T cells (arrowheads) (reactive with monoclonal antibody 35.1) around and in the wall of dermal vessel (V). (×400.)

Table 1. Phenotype of patient malignant T cells from PB and in skin

Monoclonal antibody (marker type)	PB leukemic cells*	Skin-infiltrating cells†
35.1 (E rosette, pan T)	+	+
Leu 4 (pan T, T3)	+	+
Leu 3a (inducer, T4)	+	+
Leu 2a (cytotoxic/suppressor, T8)	-	-
NA1/34 (cortical thymocyte)	-	-
3A1 (T-cell subset)	-	-
BA-1 (B cell)	-	-
3F10 (monomorphic HLA)	+	+
L-243 (monomorphic Ia)	-	+
5E9 (anti-transferrin receptor)	-	+

E, erythrocyte.

\* Analysis performed by using indirect immunofluorescence and cytofluorographic analysis.

† Analysis performed by using indirect immunofluorescence on frozen tissue sections.

## RESULTS

**Histology of Skin Lesions.** Histologic examination of SD skin lesions showed marked infiltration of the dermis and subcutaneous tissue with lymphocytes. In addition, numerous foci of lymphocytes were nested in the epidermis. Of particular interest was the angiocentric nature of the lymphocytic infiltrate with foci of malignant-appearing cells around virtually every vessel (Fig. 1A). Most vessels were in various stages of destruction with marked perivascular hemorrhage or intravascular thrombosis present. In areas close to the central ulceration of lesions, the lymphocyte infiltrate was confluent throughout the dermis.

**Phenotypic Characterization of PB and Skin Malignant T Cells.** By using a large panel of monoclonal antibodies to lymphocyte cells and to surface antigens Ia and HLA (13), the

Table 2. Expression of HTLV-associated antigens by patient malignant T cells

Source of cells (culture conditions)	% cells intracytoplasmic positive		
	Anti-p19*	Anti-HTLV <sub>T-1124</sub> †	Anti-p24‡
PB (uncultured)	0	ND	5
PB (cultured 7 days in medium alone)	90	ND	3
PB (cultured 7 days in medium/TCGF)	80	90§	6
Skin (uncultured)¶	0	ND	0
Joint fluid (uncultured)¶	0	ND	1

ND, not detected.

\* Determined by using mouse monoclonal 12/1-2 anti-p19 and indirect immunofluorescence on acetone fixed cytopreparations or tissue sections.

† Determined by using T-1124 serum and indirect immunofluorescence.

‡ Determined by using goat anti-p24 antiserum and indirect immunofluorescence.

§ Surface staining and cytofluorographic analysis of patient 7-day TCGF-cultured cells revealed 16% of the cells were positive for cell surface reactivity with T-1124 HTLV serum and 12% positive with surface staining with autologous (SD) serum. As controls, normal PB T cells did not have surface reactivity with T-1124 HTLV serum or SD HTLV serum. Normal PB T cells did not have any intracytoplasmic reactivity with T-1124 or SD HTLV serum or with anti-p19 or anti-p24 reagents.

¶ All cells present were T cells by reactivity with anti-erythrocyte-rosette monoclonal antibody 9.6 or 35.1.

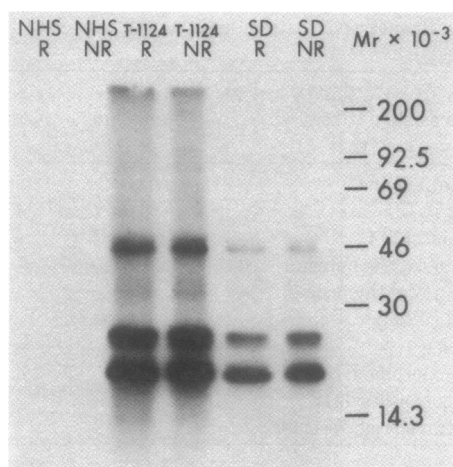


FIG. 2. Radioimmunoprecipitation of  $^{125}\text{I}$ -labeled HTLV proteins with patient sera. Three microliters ( $10\ \mu\text{g}$ ) of 0.5% Triton X-100 HTLV extract (16) was iodinated by using tubes coated with IODO-GEN (Pierce) (17). Patients' sera SD and T-1124 or normal human serum (NHS) were used to immunoprecipitate  $5 \times 10^5$  cpm of  $^{125}\text{I}$ -labeled HTLV proteins as described (16) with minor modifications. Briefly, iodinated HTLV suspended in  $50\ \mu\text{l}$  of radioimmunoprecipitation buffer (0.1% gelatin/300 mM NaCl/0.2% Triton X-100/0.01% Tween 80/10 mM sodium phosphate, pH 7.5/0.02%  $\text{NaN}_3$ ) was incubated for 1 hr at  $25^\circ\text{C}$  with  $3\ \mu\text{l}$  of serum samples in  $50\ \mu\text{l}$  of the same buffer. Antigen-antibody complexes were immunoprecipitated for 1 hr at  $4^\circ\text{C}$  with  $10\ \mu\text{l}$  of packed *Staphylococcus aureus* Cowan I strain (SACI) suspended in  $250\ \mu\text{l}$  of radioimmunoprecipitation buffer. The resulting pellets were washed three times with 1 ml of the same buffer, and the immunoprecipitates were analyzed by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis (18) on a 7–15% acrylamide gradient gel under both reducing (R) and non-reducing (NR) conditions. Molecular weights of protein standards are shown. Both T-1124 and SD sera contained antibodies reacting with HTLV-associated p19, p24, and p46 proteins.

phenotypes of both PB leukemic and skin malignant T cells were determined (Table 1). The phenotypes of the PB and skin-infiltrating T cells were near uniform, with greater than 90% of cells in both populations positive or negative for the markers listed in Table 1. Of particular interest was the observation that both PB and skin-infiltrating T cells were erythrocyte-rosette<sup>+</sup>, 3A1<sup>-</sup>, Leu 3a (T4)<sup>+</sup>, and Leu 2a (T8)<sup>-</sup> (Fig. 1B). This T-cell phenotype is consistent with that previously reported for patients with American cutaneous T-cell lymphoma (CTCL) leukemias (11), one of whom has subsequently been shown to be infected with HTLV (CR) (6). Regarding antigens T3, T4, and T8, the T3<sup>+</sup>, T4<sup>+</sup>, T8<sup>-</sup> PB phenotype is consistent with that reported for other Japanese ATL patients (19).

**Expression of HTLV-Associated Antigens by Patient Malignant T Cells.** PB SD cells from May 18, 1982, and leukapheresis cells from June 9, 1982, in culture *in vitro* for 7 days with TCGF expressed the HTLV-associated p19 antigen (i.e., p19<sup>+</sup>) (Table 2). Fresh PB or leukapheresis cells were p19<sup>-</sup>. Five percent of fresh PB cells and 6% of TCGF-cultured PB cells were HTLV p24<sup>+</sup>. Interestingly, PB cells in culture 6 days with media without TCGF also became p19<sup>+</sup>. Skin T cells, even though Ia<sup>+</sup> and transferrin receptor<sup>+</sup> [as are all transformed HTLV<sup>+</sup> T-cell lines *in vitro* (11)], were p19<sup>-</sup> and p24<sup>-</sup>. Fresh joint fluid T cells were also p19<sup>-</sup>, although 1% of the cells were p24<sup>+</sup>.

**Serum Anti-p19 and Anti-p24 Antibody Titers and Transmission of the HTLV Virus to Cord T Cells.** Serum HTLV anti-p19, anti-p24, and anti-p46 antibodies were demonstrated by radioimmunoprecipitation of  $^{125}\text{I}$ -labeled HTLV proteins followed by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis (20)

(Fig. 2). Assay for infectivity for HTLV for human cord blood T cells was performed by M. Popovic (National Institutes of Health); 40% of cord T cells were p19<sup>+</sup> after three passages in TCGF-supplemented media when cocultured with mitomycin C-treated SD PB malignant T cells.

**Electron Microscopy Analysis of SD Skin and Cultured T Cells.** Examination by electron microscopy of 7-day cultured T cells in either medium alone or medium supplemented with TCGF revealed budding type C HTLV particles (Fig. 3A–C). In addition, it was observed that the virus particles invariably were located in a polar distribution on the surface of malignant T cells (Fig. 3D). Even though skin T cells were p19<sup>-</sup> and p24<sup>-</sup>, intracellular type C particles were seen in skin-infiltrating T cells (Fig. 3E).

## DISCUSSION

We report a patient with Japanese ATL whose T cells harbor HTLV. PB malignant T cells in culture with and without TCGF expressed the HTLV-associated p19 antigen, and budding virus was seen by electron microscopy in cells from both types of cultures. Serum antibodies were present to p19, p24, and p46 HTLV antigens, and mitomycin C-treated SD malignant T cells were able to transform cord T lymphocytes.

In view of the similarity of Japanese ATL to HTLV-positive cases from the USA, sera from Japanese patients with ATL have been examined and found to contain high-titer antibodies to HTLV structural proteins (9). Further, nucleic acid homology analysis with HTLV-specific cDNA probes has shown that ATL virus is either identical to or a closely related strain of HTLV (10). Thus, the demonstration of anti-HTLV antibodies in SD serum, the expression of p19 in cultured SD PB cells, the expression of p24 antigen in cultured and fresh PB cells, the demonstration of the virus by electron microscopy, and the transmission of the virus to cord T cells confirms the association of HTLV with this case of Japanese ATL. Moreover, by using HTLV-specific cDNA probes, it was shown that SD PB malignant T cell DNA contains 1 copy per cell of HTLV proviral DNA (unpublished data).

The phenotype of SD skin and PB malignant T cells was usual for HTLV-associated T-cell leukemia in that they were 3A1<sup>-</sup>, T4<sup>+</sup>, T8<sup>-</sup> (11, 21). However, this patient is the first patient with a 3A1<sup>-</sup> leukemia in whom we have been able to assay simultaneously for 3A1 skin-infiltrating T cells. Because SD skin T cells were also 3A1<sup>-</sup>, and most if not all benign skin infiltrations are 3A1<sup>+</sup> (13), these data confirm that the 3A1<sup>-</sup>, T4<sup>+</sup> phenotype of skin-infiltrating T cells, when present, may be of use diagnostically for malignancy. It is important to note that the patient's clinical course was somewhat unusual in that she had cutaneous lymphomatous vasculitis and presented with arthritis (1, 2).

Of particular interest was the observation that SD malignant PB T cells cultured in media alone expressed HTLV p19 antigen and produced type C viral particles, suggesting that *in vivo* inhibitory factors rather than the *in vitro* requirement for TCGF might be relevant to the lack of tissue and PB HTLV antigen expression. In preliminary experiments, we showed that plasma but not serum from SD and other HTLV-positive cases inhibited the *in vitro* expression of HTLV-associated p19 antigen (unpublished data). The significance of intracellular but not budding type C retrovirus particles in SD skin T cells is not known because there were no detectable p19<sup>+</sup> or p24<sup>+</sup> T cells in skin. However, SD serum [and serum of most other HTLV-associated cases of T-cell malignancy (8, 9)] contain high titers of anti-p24 and anti-p19 antibodies, implying expression of these antigens at some sites in the body. Although skin-in-

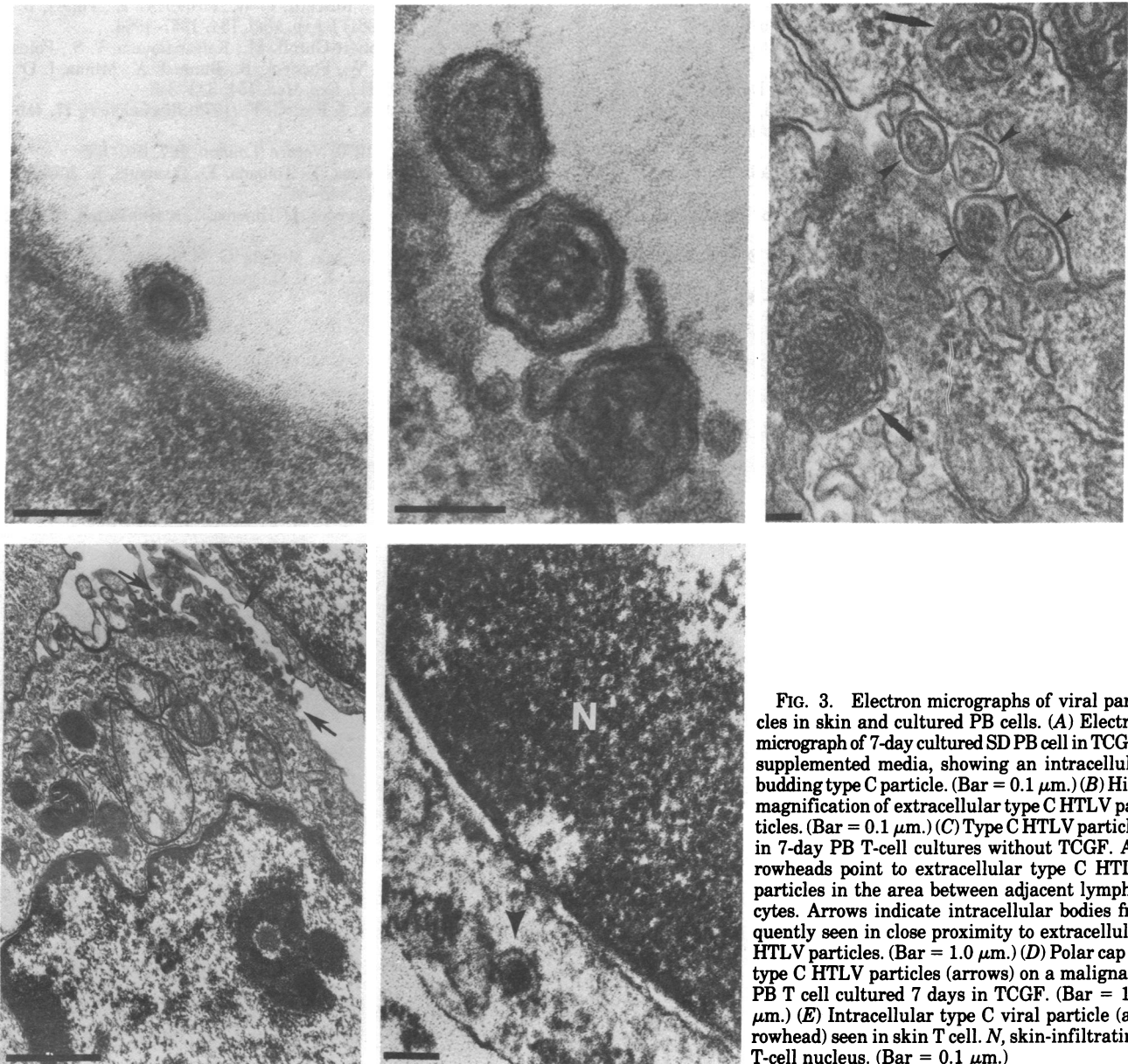


FIG. 3. Electron micrographs of viral particles in skin and cultured PB cells. (A) Electron micrograph of 7-day cultured SD PB cell in TCGF-supplemented media, showing an intracellular budding type C particle. (Bar = 0.1  $\mu\text{m}$ .) (B) High magnification of extracellular type C HTLV particles. (Bar = 0.1  $\mu\text{m}$ .) (C) Type C HTLV particles in 7-day PB T-cell cultures without TCGF. Arrowheads point to extracellular type C HTLV particles in the area between adjacent lymphocytes. Arrows indicate intracellular bodies frequently seen in close proximity to extracellular HTLV particles. (Bar = 1.0  $\mu\text{m}$ .) (D) Polar cap of type C HTLV particles (arrows) on a malignant PB T cell cultured 7 days in TCGF. (Bar = 1.0  $\mu\text{m}$ .) (E) Intracellular type C viral particle (arrowhead) seen in skin T cell. N, skin-infiltrating T-cell nucleus. (Bar = 0.1  $\mu\text{m}$ .)

filtrating malignant T cells were negative for HTLV p19 and p24, this negativity may reflect our inability to detect by fluorescence small but immunogenic amounts of these HTLV-associated antigens. Type C viral particles have been observed in Langerhans cells in the skin of mycosis fungoides patients but not in skin malignant T lymphocytes (21). It is important to note that small numbers of PB and joint-infiltrating T cells were p24<sup>+</sup> (Table 2) and may constitute sites of *in vivo* HTLV expression. However, a critical question remains as to where the primary sites of *in vivo* HTLV expression are located.

Because cDNA hybridization studies have shown that HTLV from SD is highly related and probably identical to HTLV originally isolated from patient CR (designated HTLV-1<sub>CR</sub>) (M. Reitz and R. C. Gallo, personal communication), we have named the isolate from our patient HTLV-1<sub>SD</sub>.

Thus, the demonstration of HTLV (HTLV-1<sub>SD</sub>) in malignant T cells from our patient confirms the association of HTLV with Japanese ATL.

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