Supplementary on-line material

HTLV-I spreads between lymphocytes by virus-induced polarization of the cytoskeleton

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Materials and methods

Patients and cells

Peripheral venous blood samples were donated by ten HTLV-I-infected subjects with a high proviral load of HTLV-I: nine with a clinical diagnosis of HAM/TSP, and one asymptomatic HTLV-I carrier. As controls, we used peripheral venous blood samples from healthy HTLV-I-seronegative subjects. All subjects were HIV-1 negative, and were free of other clinically evident infection at the time of sampling. All subjects gave informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated on a Histopaque-1077 (Sigma) density gradient and washed three times with phosphate-buffered saline (PBS). $CD8^+$ and $CD4^+$ T cell, B cell and NK cell microbead isolation kits (Miltenyi Biotec Ltd, Surrey, UK) were used according to the manufacturer's instructions to separate the respective PBMC subpopulations. Before conjugate formation, cells were cultured overnight at $1x10^6$ cells.mL^{-1} in R/10 medium: RPMI 1640 (GIBCO, Paisley, UK) supplemented with 10% fetal calf serum

(FCS) (Sigma), 2 mM glutamine (GIBCO), 100 IU/ml penicillin (GIBCO), and 100 mg/ml streptomycin (GIBCO). In experiments to examine transfer of HTLV-I proteins to uninfected cells, $CD4^+$ and $CD8^+$ T cells from the HTLV-I-infected individual were labelled with 0.5 microM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes).

Antibodies

The following HTLV-I-specific monoclonal antibodies (mAbs) were used (*1, 2*): mouse IgG2b anti-Gag p19 (clone GIN7) and rat IgG2a anti-Gag p19 (clone WAG19); rat IgG2a anti-Gag p15 (clone WAG15); rat IgG2a anti-Env gp46 (clone LAT27). The mouse anti-talin mAb was obtained from Sigma (Dorset, UK). The rat anti-tubulin alpha mAb was from Chemicon International (Temecula, California, USA), and the mouse anti-tubulin alpha mAb was from Santa Cruz Biotechnology (Delaware Santa Cruz, California, USA). The Cy5-conjugated rat anti-human CD8 mAb was obtained from Molecular Probes (Eugene, Oregon, USA). The FITC-conjugated mouse anti-human CD3 mAb was obtained from Santa Cruz (California, USA). Secondary antibodies conjugated to Texas Red and FITC were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA), and Alexa Fluoro 488 and 568 were obtained from Molecular Probes.

Conjugate formation, immunofluorescence and confocal microscopy

Positively selected $CD4^+$ and $CD8^+$ T cells, B cells and NK cells were incubated overnight at 37^{o}C, widely dispersed in 10 cm diameter tissue culture dishes (at 0.5 x.10^6 cells.mL^{-1}), to allow spontaneous expression of HTLV-I proteins (*29*). The cells were then washed in RPMI, and resuspended to a final concentration of ~5x10^6 cells.mL^{-1} in RPMI. To form cell-cell conjugates, the respective cells were mixed 1:1, then plated on to glass multiwell slides (HENDLEY-ESSEX, London, UK) and incubated at 37^{o}C for 30 to 120 min. Samples were fixed with 100% methanol (precooled to –20 ^{o}C) for 5 min, or 2% paraformaldehyde for 17 min, washed extensively in PBS, blocked in 1% BSA-PBS, and processed for immunofluorescence. Primary antibodies were added in the presence of 1% BSA-PBS for 40 min and washed extensively in PBS, 1% BSA-PBS (*3*). Secondary antibodies were added in the presence of 1% BSA-PBS for 40 min, washed extensively in PBS and mounted in PBS containing 90% glycerol and 2.5% DABCO. Samples were

examined using a Bio-Rad Radiance 2000 MP laser scanning microscope, with lasers exciting at 488, 543, and 637 nm. For 3D and z-axis image reconstruction, 30-40 confocal sections, 0.3 - 0.4 micrometer apart, were taken and assembled using Confocal Assistant version 4.02 software or Adobe Photo Shop LE version 5. Some samples were examined using an Optronics Magnafire cooled CCD camera (Optronics, Goleta, CA, USA) on a Leitz fluorescence microscope; these images were displayed using Magnafire software (Optronics).

Inhibitor test for microtubule assembly

$CD4^+$ T-cells were treated with 33nM nocodazole (Sigma) in RPMI 1640 medium supplemented with 10% fetal calf serum for 90 min at 37^{o}C (a treatment that depolymerizes the cytoplasmic microtubules). The cells were then washed and resuspended in medium containing 33 nM nocodazole. The $CD4^+$ T-cells were then used for immunofluorescence studies of conjugates.

Peptide nucleic acid – fluorescence in-situ hybridization (PNA-FISH)

Cells were adhered to silanized glass slides (DAKO USA), then fixed in PBS containing 4% paraformaldehyde (Sigma) for 10 min, and endogenous biotin reactivity was blocked by Endogenous Biotin-Blocking Kit (Molecular Probes). PNA probes were synthesized by Boston Probes (USA). The structure of the antisense probe was as follows: 5'-Bio-OO-CGTAGGCTCAACATA-Lys(Bio)-3' and the structure of the sense probe was as follows: 5'-Bio-OO-TATGTTGAGCCTACG-Lys(Bio)-3'. The base sequence corresponds to nt 1184 to 1199 of the Gag region of the HTLV-I genome (GenBank accession # J02029). Hybridization solution (DAKO) containing biotin-conjugated PNA probe (1ng/microliter) was mounted on the glass slide and then incubated for 90 min at 46^{o}C. The slide was washed twice with Stringent Wash Solution (DAKO) at 55^{o}C for 20 min. Alexa Fluor 568 conjugated Streptavidin (Molecular Probes) was used to detect hybridized probes.

Figure S1

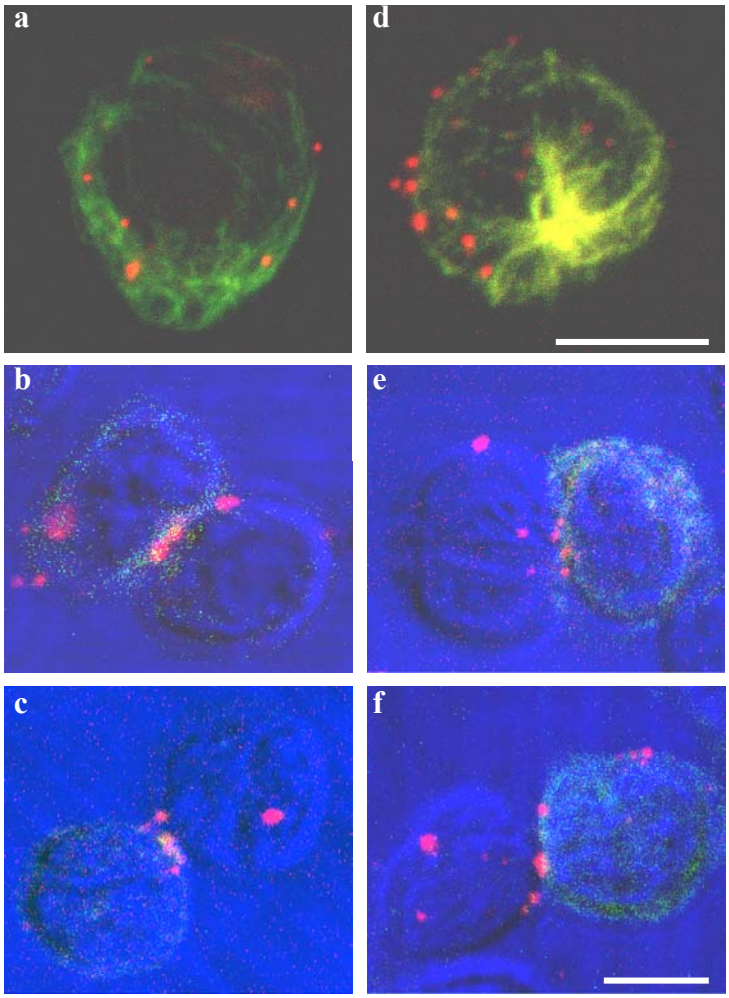


Figure S2

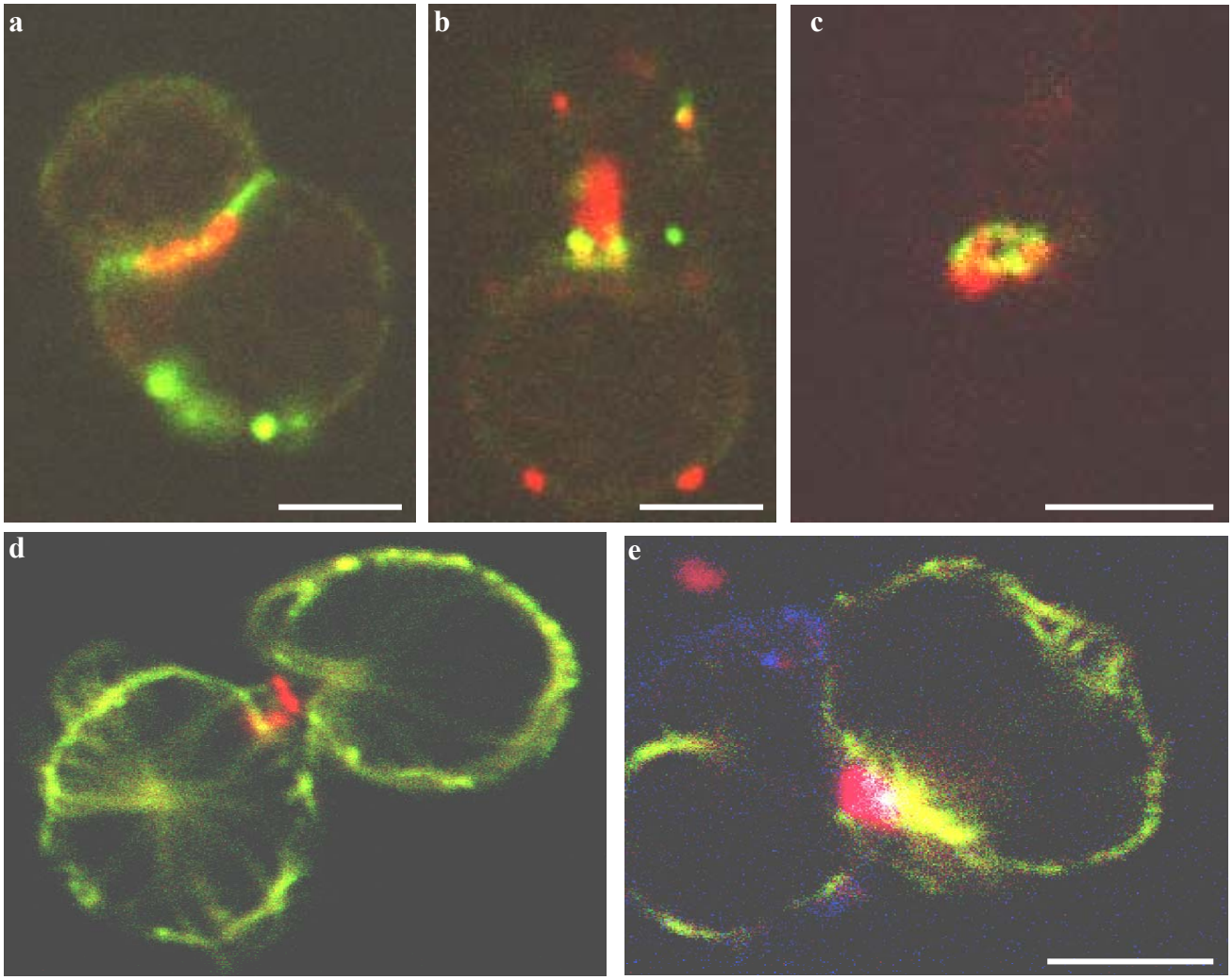
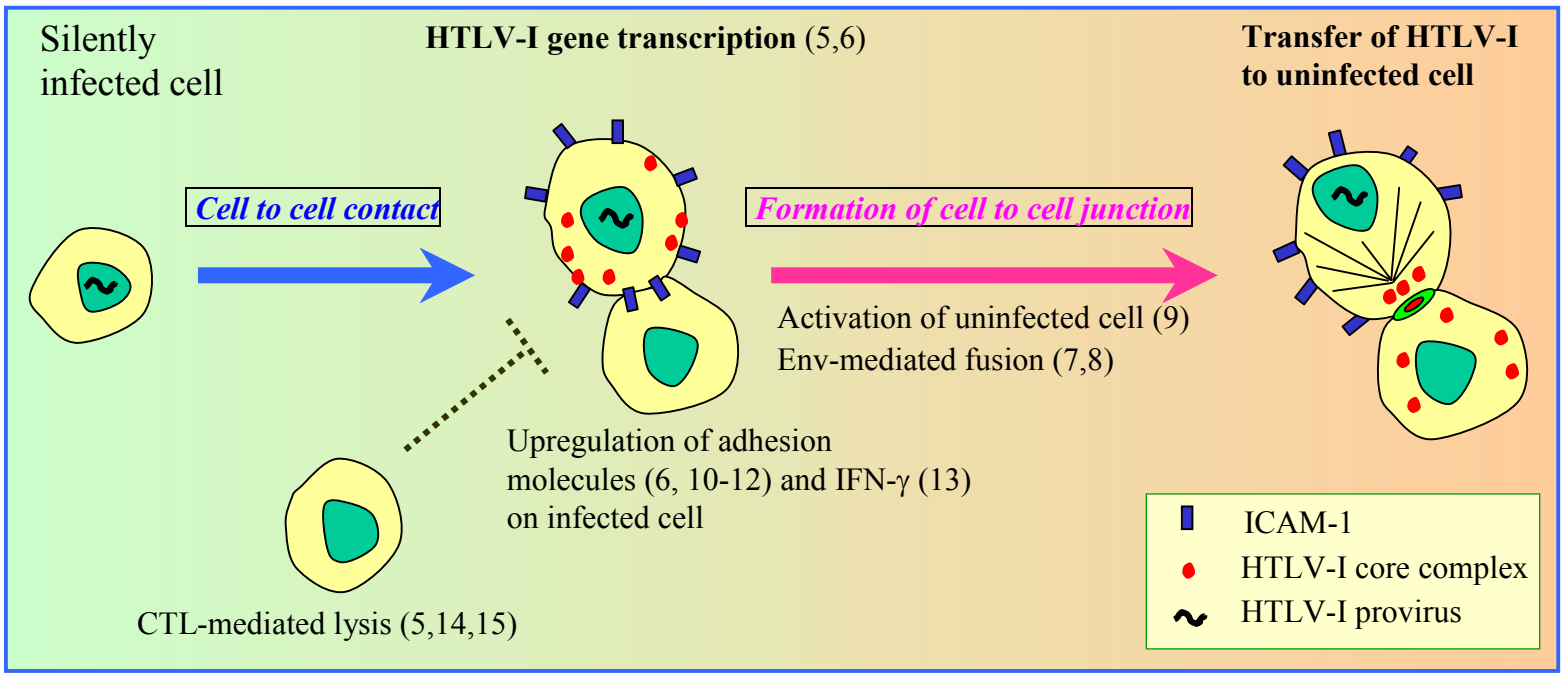


Figure S3


Silently infected cell
HTLV-I gene transcription (5,6)
Transfer of HTLV-I to uninfected cell
Cell to cell contact
Formation of cell to cell junction
Activation of uninfected cell (9)
Env-mediated fusion (7,8)
Upregulation of adhesion molecules (6, 10-12) and IFN-γ (13) on infected cell
CTL-mediated lysis (5,14,15)
ICAM-1
HTLV-I core complex
HTLV-I provirus

Legends to Figures

Figure S1. $CD19^+$ B cells and $CD56^+$ NK cells from an uninfected donor were allowed to form conjugates with CD4+ T cells from an HTLV-I-infected subject. When conjugated with autologous cells, both the B cells and the NK cells showed polarization of the Gag-staining material to the area of cell-cell contact (within 40 min) and subsequent transfer to the uninfected cell in the conjugate (within 120 min). However, the polarization was typically less complete in B-cell-T-cell and NK-cell-T-cell conjugates than in T-cell-T-cell conjugates (Fig. 1d-f, printed paper). As observed in $CD4^+$ T cell and non-T-cell conjugates, the polarized Gag protein was found adjacent to the $CD4^+$ T cell's microtubule organizing center (MTOC).
a, **d:** Confocal images showing isolated (non-conjugated) B cell and NK cell from a patient with HAM/TSP, tubulin-alpha (green) and Gag p19 (red). **a**; B cell, **d**; NK cell.
b, **c**, **e**, and **f** ; Confocal images showing transfer of Gag p19 protein from HTLV-I-infected T-cells to uninfected T-cells. Conjugation time 120 min; Gag p19 (red). HTLV-I-infected $CD4^+$ T-cells were counterstained with FITC-conjugated anti-CD3 antibody (green). The transmission picture (blue) is superimposed on a 0.3 micrometer confocal fluorescence single-section (red and green). **b, c**; Conjugate between HTLV-I-infected $CD4^+$ T cell and normal B cell. **e, f**; Conjugate between HTLV-I-infected $CD4^+$ T cell and normal NK cell. Scale bars represent 5 micrometers.

Figure S2. HTLV-I Gag protein and talin accumulate in distinct domains at the cell-cell junction (**a, b, c**); the microtubule organizing centre lies adjacent to the polarized HTLV-I Gag protein at the cell-cell junction (**d, e**). Conjugates were allowed to form for 40 min. **a, b, c**: Confocal images showing HAM autologous T cell conjugates stained with mAbs against talin (green) and HTLV-I Gag p19 (red). **a**; $CD4^+$-$CD8^+$ T cell conjugate. **b, c**; $CD4^+$-$CD4^+$T cell conjugate. **d**, **e**: Confocal images showing HAM autologous T cell conjugates stained with mAbs against tubulin-alpha (green) and HTLV-I Gag p19 (red). **d**; $CD4^+$ T cell conjugate. **e**; $CD4^+$-$CD8^+$ T cell

conjugate. $CD8^+$ T-cells were counterstained with Cy5-conjugated anti-CD8 antibody (blue). Scale bars represent 5 micrometers.

Figure S3. Schematic diagram of transfer of HTLV-I core complex at the cell-cell junction. The onset of HTLV-I provirus transcription may be spontaneous or stimulated by ligation of ICAM-1 or other adhesion molecules on the cell surface. The chief factor that limits the propagation of HTLV-I in vivo is the strong HTLV-I-specific cytotoxic T-lymphocyte response; antibody may also contribute some protection (*4*). Other references (*5-15*) are indicated on the Figure.

Table 1. HTLV-I-infected cells polarize their MTOCs to the cell-cell junction in $CD4^+$ T-cell conjugates.

MTOC orientation	uninfected control subject	HTLV-I-infected subjects			
		subject 1		subject 2	
		Gag $p19^-$	Gag $p19^+$	Gag $p19^-$	Gag $p19^+$
polarized (%)	79 (18.7)	85 (25.9)	**163 (58.2)**	45 (22.0)	**59 (53.2)**
not polarized (%)	322 (76.3)	**217 (66.2)**	111 (39.6)	**160 (78.0)**	52 (46.8)
not seen (%)	21 (5.0)	26 (7.9)	6 (2.1)	0 (0.0)	0 (0.0)
Total (%)	422 (100.0)	328 (100.0)	280 (99.9)	205 (100.0)	111 (100.0)
Odds ratio[a] (95% confidence interval) χ^2 (P << 0.001)	–	3.98 (2.83 – 5.61) $\chi^2 = 63.9$ (P << 0.001)		4.02 (2.45 – 6.64) $\chi^2 = 30.4$ (P << 0.001)	
		subject 1 and subject 2 combined : **4.07** (3.07 – 5.39) $\chi^2 = 99.0$ (P << 0.001)			

Two experiments were performed, each with fresh ex vivo $CD4^+$ T cells from an unrelated HTLV-I-infected subject. Conjugates were allowed to form for 30min (subject 1) or 60min (subject 2), then fixed and stained for HTLV-I Gag p19 (red

color) and tubulin alpha (green color). Only conjugates containing two cells were counted. The figures denote the number (percentage) of cells whose MTOC was polarized to the cell-cell junction. Odds ratio[a] of MTOC polarization in Gag $p19^+$ cells, comparing the numbers of polarized MTOCs with (polarized + not seen) MTOCs.

References

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