The Mitogenic Activity of Human T-Cell Leukemia Virus Type I Is T-Cell Associated and Requires the CD2/LFA-3 Activation Pathway

JASON T. KIMATA,¹ THOMAS J. PALKER,² AND LEE RATNER^{1*}

Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110,¹ and Department of Medicine, Duke University School of Medicine, Durham, North Carolina 27710²

Received 20 November 1992/Accepted 23 February 1993

The presence of a high number of activated T cells in the bloodstream and spontaneous proliferation of peripheral blood mononuclear cells *in vitro* are striking characteristics of human T-cell leukemia virus type I (HTLV-I) infection. The HTLV-I regulatory protein Tax and the envelope protein gp46 have been implicated in mediating the activation process. In this study, HTLV-I-producing cell lines and purified virus from the cell lines were examined for the ability to activate peripheral blood lymphocytes (PBLs) and Jurkat cells. Antisera and monoclonal antibodies against several cellular adhesion proteins involved in T-cell activation and against viral proteins were used to identify which molecules may be participating in the activation process. First, neither virus from a T-cell line, MT2, nor virus produced from the human osteosarcoma cell line HOS/PL was able to induce PBLs to proliferate. In contrast, both fixed and irradiated HTLV-I-producing T-cell lines induced proliferation of PBLs; HOS/PL cells did not activate PBLs. Second, HTLV-I-positive T-cell lines were capable of activating interleukin-2 mRNA expression in Jurkat cells. Induction of interleukin-2 expression was inhibited by anti-CD2 and anti-lymphocyte function-associated antigen 3 (LFA-3) monoclonal antibodies but not anti-human leukocyte antigen-DR, anti-CD4, anti-LFA-1, or anti-intercellular adhesion molecule 1. Similar results were obtained with PBLs as the responder cells. Furthermore, monoclonal antibodies and antisera against various regions of the HTLV-I envelope proteins gp46 and gp21 as well as p40^{tax} did not block activation. These data indicate that HTLV-I viral particles are not intrinsically mitogenic and that infection of target T cells is not necessary for activation. Instead, the mitogenic activity is restricted to virus-producing T cells, requires cell-to-cell contact, and may be mediated through the LFA-3/CD2 activation pathway.

Spontaneous proliferation *in vitro* is a notable characteristic of freshly isolated peripheral blood mononuclear cells (PBMCs) from individuals with human T-cell leukemia virus type I (HTLV-I)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and asymptomatic HTLV-I carriers (19, 20, 39). The association of HTLV-I gene expression with this immune activation (16, 39) and detection of viral DNA in the blood T-cell population (32) suggest that infected T cells regulate this proliferation. The HTLV-I regulatory protein Tax and the extracellular domain of the envelope protein gp46 may have critical roles in the spontaneous proliferation of PBMCs. First, since the proliferation of PBMCs is interleukin-2 (IL-2) dependent and associated with HTLV-I gene expression, it has been suggested that Tax stimulates an autocrine growth loop by *trans* activating IL-2 and its receptor (39). Tax has been shown to *trans* activate these two genes and several others (e.g., *c-fos*, the granulocyte-macrophage colony-stimulating factor gene, *c-myc*, and the major histocompatibility complex protein class I and II genes) that are commonly associated with the early stage of T-cell activation (13, 14, 26, 34, 36, 41). Second, virus particles concentrated from conditioned supernatants of HTLV-I-producing T-cell lines have been shown to activate T cells by direct contact (15). HTLV-I patient antisera and a monoclonal antibody (MAb), 0.5

alpha, against gp46 neutralize the mitogenic activity of the virus, suggesting that gp46 is a T-cell mitogen.

We have demonstrated that an HTLV-I-producing T-cell line induces an IL-2 dependent proliferation of peripheral blood T cells during the early stage of *in vitro* immortalization and that infection of the responder cells and secretion of a soluble factor by the HTLV-I-producing cells are not prerequisites for activation (22). Cell-to-cell contact was found to be sufficient for T-cell activation. This observation led us to hypothesize that cell surface contact between HTLV-I-expressing T cells and resting T cells may be important for activation of proliferative responses not only in the early stages of T-lymphocyte infection and immortalization but also in the spontaneous proliferation observed in PBMCs from HTLV-I-infected asymptomatic carriers and individuals with HAM/TSP. Therefore, we evaluated the contribution of viral particles and cell contact to T-lymphocyte activation by examining the ability of purified HTLV-I particles and infected T-cell and non-T-cell lines to induce proliferation of peripheral blood lymphocytes (PBLs) and to induce IL-2 mRNA expression in the leukemic cell line Jurkat. Furthermore, we attempted to define the possible cellular and viral proteins that may be involved in this activation process by using MAbs and antisera against several viral proteins and cellular adhesion molecules known to modulate T-cell activation. The results suggest that the mitogenic activity is associated with virus-producing T cells and mediated in part via the CD2/lymphocyte function-associated antigen 3 (LFA-3) activation pathway.

* Corresponding author.

MATERIALS AND METHODS

Cell lines and culture. HTLV-I-positive T-cell lines MT2, HUT102, and MT4 as well as the uninfected T-cell lines H9, HUT78, and Jurkat 25 (kindly provided by S. Luria and P. Berg) were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml (RPMI complete). The HTLV-I-infected HOS/PL cell line and the parental HOS line (8) were passaged in Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter), 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, penicillin, and streptomycin.

Antisera and MAbs. Generation and analysis of reactivity of rabbit antisera against synthetic peptides of hydrophilic regions of the HTLV-I envelope glycoproteins gp46 and gp21 have been previously described (29, 30). MAbs against gp46 have been previously described: LAT-27 (1 mg/ml) and REY-30 (1 mg/ml) (generously provided by Y. Tanaka [38]) and 0.5 alpha (2 mg/ml) (provided by S. Matsushita [27]). The anti-Tax (1 mg/ml) antibody was a gift of R. Gartenhaus. Antiserum to IL-2 was purchased from Genzyme. Leu 4 was purchased from Becton Dickinson. Anti-CD4 and anti-human leukocyte antigen (HLA)-DR were obtained from Ortho Diagnostics. Anti-intercellular adhesion molecule 1 (ICAM-1) (RR1/1) was a gift from T. Springer. Hybridomas that produce MAbs LFA-1 (TS1/22), CD2 (TS2/18), and LFA-3 (TS2/9) were obtained from the American Type Culture Collection (33). Hybridomas were maintained in Dulbecco's Modified Eagle's medium with high glucose, 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, penicillin, and streptomycin. Conditioned supernatants were used as a source of the antibodies. Presence of the MAbs was tested by Western immunoblotting and flow cytometry (fluorescence-activated cell sorting).

Virus concentration and purification by sucrose density sedimentation. Virus particles were purified by a modification of the method of Ratner et al. (31). Conditioned supernatants from the HTLV-I-producing cell lines HOS/PL and MT2 were clarified by centrifugation at 2,000 rpm for 10 min at 4°C. The supernatants were then spun at 20,000 rpm in a Beckman SW28 rotor for 2.5 h at 4°C. Supernatants were removed, and the pellets were resuspended in STE (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA), loaded onto 20 to 60% continuous sucrose gradients in STE, and spun at 20,000 rpm in a Beckman SW28 rotor for 16 h at 4°C. Fractions were collected, tested, and quantitated for HTLV-I p19^{gag} activity with an antigen enzyme-linked immunosorbent assay (ELISA) according to the protocol of the manufacturer (Cellular Products, Inc.). The density of sucrose gradient fractions was determined from refractive indices obtained by using a Abbe refractometer.

Western blot for viral structural proteins. Ten-microliter samples from culture supernatants or sucrose gradient fractions were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and transferred to nitrocellulose. HTLV-I envelope gp46 and other structural proteins were detected by using the ProtoBlot Western blot alkaline phosphatase system (Promega) according to the manufacturer's specifications. 0.5 alpha (1:500 dilution) and TSP patient serum (1:200 dilution) were used as primary antibodies for the detection of gp46 and other structural proteins of HTLV-I. Goat anti-human alkaline phosphatase conjugate (1:5,000 dilution; Promega) served as the secondary antibody.

Activation assay. PBMCs were isolated from normal

healthy adult donors by Ficoll-Hypaque (Sigma Chemical Co.) centrifugation. The cells were washed twice with phosphate-buffered saline (PBS), resuspended in RPMI complete, then seeded into plastic culture flasks, and incubated at 37°C for 2 h. Nonadherent cells were used for cocultivation with HTLV-I particles or irradiated (6,000 R) or fixed HTLV-I-infected cells in the presence and absence of antibodies. Cocultivation with irradiated cells was performed as previously described (22). For fixation, cell lines were incubated in 1% paraformaldehyde in PBS for 1 h and washed twice with RPMI complete prior to cocultivation.

To monitor activation, 10⁵ PBLs were mixed with viral particles or 10⁵ fixed HTLV-I-positive or -negative cell lines in wells of a 96-well plate in triplicate and cultured for 5 days. During the last 12 h of cultivation, 1 µCi of [³H]thymidine (Amersham Corp.) was added to each well. Cells were harvested with a Skatron cell harvester, and [³H]thymidine incorporation was monitored by standard scintillation counting. In addition, proliferation was monitored by counting viable cells of duplicate cultures with trypan blue.

Induction and detection of IL-2 mRNA expression in Jurkat 25 cells. HTLV-I-producing and -nonproducing cell lines were fixed as described above. Cells (2.5 × 10⁵) were mixed with 2.5 × 10⁵ Jurkat 25 cells in wells of a 96-well plate in the presence and absence of antibodies. After 6 h, the cells were directly lysed in guanidine isothiocyanate, and total RNA was prepared according to the method of Chomczynski and Sacchi (6). IL-2 mRNA expression was examined by reverse transcriptase (RT)-polymerase chain reaction (PCR) followed by Southern blot analysis of the PCR products as previously described (22). RT-PCR amplification of the constitutively expressed aldolase A mRNA served as a control. A cDNA clone of IL-2 (a gift from S. Arya and R. C. Gallo) and an oligonucleotide to the C1 exon of aldolase A (5) served as probes for hybridization to the respective PCR products. One hundred picomoles of each primer was used for RT-PCR. The amplification was as follows: denaturation at 94°C for 2 min, annealing at 56°C for 2 min, and extension at 72°C for 3 min. The number of cycles ranged from 20 to 40, depending on the number of cycles found to generate products in the linear range of amplification with respect to the initial cDNA concentration. The level of IL-2 mRNA expression was quantitated by laser densitometry of autoradiographs; the ratio of the signals from IL-2 mRNA expression to aldolase A mRNA expression of cocultivated cells was compared with the ratio of those signals detected in phytohemagglutinin P (PHA) (1 µg/ml)-phorbol myristate acetate (PMA) (50 ng/ml)-stimulated Jurkat 25 cell total RNA samples diluted at 10-fold serial dilutions.

RESULTS

Activation of PBL by HTLV-I. Previously, concentrated HTLV-I particles had been demonstrated to be mitogenic for purified T cells, and the activity was mediated by the envelope protein gp46 (15). However, it was unclear whether the mitogenic activity was due to the virus particles or contaminating membranes from the virus-producing T cells, since the source of the virus particles was pelleted material from conditioned supernatants concentrated by ultracentrifugation. Cellular membrane-associated proteins could have mediated the activity, as we had found that an HTLV-I-infected T-cell line induced proliferation of PBLs (22). Thus, to determine whether HTLV-I particles were mitogenic for PBLs, we first purified virus particles from conditioned supernatants of MT2 cell cultures by sucrose density cen-

FIG. 1. Activation of PBLs by HTLV-I. (A) Sucrose density gradient purification of HTLV-I virus particles from MT2 cell conditioned supernatant. Gradient fractions were diluted $1:2 \times 10^4$ for the antigen ELISA for p19^{gag}. Fraction 1 is the bottom of the gradient, and fraction 30 the top. (B) Immunoblot of the HTLV-I envelope glycoprotein gp46 in fractions from the sucrose gradient shown in panel A. gp46 was detected with MAb 0.5 alpha. (C) Proliferation of PBLs induced by purified or concentrated HTLV-I particles. Equal amounts of p19^{gag} (5 μ g/ml) were added to unstimulated PBLs for 5 days. Proliferation was assessed by [³H]thymidine incorporation. The values shown are the mean counts per minute $\pm$ standard error of the mean of triplicate cultures. HUT78 conc., HOS/PL conc., and MT2 conc. represent resuspended material from supernatants of those respective cell lines that had been subjected to ultracentrifugation as described in Materials and Methods. MT2 particles were from fraction 17 of the gradient shown in panel A. PBL + PHA, PBLs stimulated with PHA; PBL alone, unstimulated PBLs. The inset shows an immunoblot of 3- and 4-day conditioned culture supernatants from MT2 cells and HOS/PL cells, respectively. The blot was screened with anti-HTLV-I antiserum from a TSP/HAM patient; sizes of markers (M) are indicated in kilodaltons. (D) Proliferation of PBLs as measured by viable cell number. Unstimulated PBLs were cultured alone or with PHA, concentrated material from HUT78 supernatant, concentrated HOS/PL virus particles, or sucrose gradient-purified virions from MT2 cells. Viable cell number was determined by trypan blue exclusion. The values shown are the averages of duplicate cultures. (E) Proliferation of PBLs induced by HTLV-I-infected cell lines. Unstimulated PBLs were cultured with the indicated paraformaldehyde-fixed cell line, with PHA, or without stimulation for 5 days. Proliferation was monitored as in panel C. (F) Cocultivation of PBLs with irradiated T-cell lines. The indicated cell lines were irradiated (irr) (6,000 R) and cultured with unstimulated PBLs as previously described (22). The viable cell number was determined by trypan blue exclusion.

trifugation (Fig. 1A). HTLV-I *gag* protein p19^{gag} was detected in fractions 15 to 17. These fractions had a density of 1.15 to 1.16 g/ml, a range similar to values previously reported for HTLV-I particles (35). This finding indicated that the majority of p19^{gag} found in culture supernatants was virion associated. The envelope protein gp46 was detected in fractions 15 to 17 as well as in fraction 30 (top of the gradient) by immunoblotting (Fig. 1B), indicating that gp46 was also virion associated but that some may have been stripped off during the purification process.

We compared the purified virus particles (MT2 particles) with concentrated virus particles from MT2 and HOS/PL cells for the ability to activate PBLs by [³H]thymidine incorporation and viable cell number (Fig. 1C and D). The inset to Fig. 1C shows that 4-day conditioned supernatant of HOS/PL cells contained amounts of the viral structural proteins p19^{gag}, p24^{gag}, and gp46 similar to those found in 3-day conditioned supernatants from the MT2 cell line. We confirmed the presence of virus particles in the HOS/PL supernatants by sucrose density centrifugation (data not shown). Purified virions (5 μ g of p19^{gag} per ml) did not induce either thymidine incorporation or an increase in cell number. In contrast, an equal amount of p19^{gag} concentrated from MT2 supernatant by ultracentrifugation increased thymidine incorporation 10-fold over unstimulated PBLs (2,417

$\pm$ 541 cpm versus 235 ± 30 cpm); this level was 10-fold less than the proliferation induced by PHA ($26,700 \pm 2,092$ cpm). Furthermore, neither pelleted material from an equal amount of conditioned supernatant of the HTLV-I-negative mature T-cell line (IL-2 receptor⁺, HLA-DR⁺) HUT78 (17) nor HTLV-I particles from the HTLV-I-producing human osteosarcoma cell line HOS/PL (5 μ g of p19^{gag} per ml) induced proliferation of PBLs. Virus from HOS/PL also did not induce an increase in viable cell number.

In contrast to the purified virions, HTLV-I-producing T-cell lines induced proliferation of PBLs (Fig. 1E). Paraformaldehyde-fixed HTLV-I-producing T-cell lines MT2, HUT102, and MT4 induced a level of [³H]thymidine incorporation in PBLs 20 to 35% of that induced by PHA. Similar to the uninfected T-cell line Jurkat and parental cell line HOS, fixed HOS/PL cells did not induce thymidine incorporation.

When proliferation of PBLs was monitored by determination of the number of viable cells over a 20-day period, only the irradiated HTLV-I-producing cell line MT2 induced PBLs to divide continuously (Fig. 1F), even though less than 5% of the PBLs were productively infected by HTLV-I during this stage of infection, as determined by immunofluorescence for p19^{gag}. PHA induced proliferation for 10 days. Unstimulated PBLs and cocultures of irradiated HTLV-I-

negative T-cell lines Jurkat (resting phenotype) and H9 (activated phenotype) with PBLs did not proliferate and continuously decreased in viable cell number. This result indicated that the proliferation induced by MT2 was not due to a mixed lymphocyte reaction. Finally, the irradiated cells did not divide and died within 2 weeks. Similar results were obtained with use of different PBL donors in separate experiments (data not shown). Taken together, the data from Fig. 1 suggest that HTLV-I viral particles are not mitogenic for PBLs. Instead, the mitogenic activity is associated with HTLV-I-producing T-cell lines.

Antibodies against the envelope glycoproteins and Tax do not inhibit proliferation. Envelope gp46 and extracellular Tax protein have been suggested as having roles in HTLV-I-induced proliferation (11, 15, 25). To determine whether either of these proteins was a mediator of activation of PBLs by HTLV-I-producing T-cell lines, we cocultivated fixed MT2 cells with unstimulated PBLs in the presence and absence of antibodies recognizing various regions of gp46, gp21, and p40^{tax} (Table 1). In the two experiments, MT2 cells induced a level of proliferation 25 to 50% of that of the positive control PHA-stimulated PBLs. The HTLV-I-negative cell lines Jurkat and HUT78 did not induce proliferation. Furthermore, none of the rabbit antisera (1:40 dilution) against the indicated regions of the envelope proteins or the anti-gp46 MAbs LAT-27 (50 µg/ml, final concentration) and 0.5 alpha (33.3 µg/ml, final concentration) inhibited proliferation. Of particular interest, regions of gp46 corresponding to amino acids 86 to 107 and 190 to 209 have been demonstrated to be neutralizing domains for syncytium formation (29), 0.5 alpha (33.3 µg/ml) has been shown to block the mitogenic activity of HTLV-I particles (15), and LAT-27 inhibits syncytium formation and infection of T lymphocytes by HTLV-I at concentrations of 2.5 and 10 µg/ml, respectively (38). Anti-synthetic peptide antiserum to amino acids 86 to 107 (anti-SP-2) at a dilution as low as 1:10 was not inhibitory (data not shown). Although the anti-envelope antibodies (anti-SP-2, anti-SP-4A, 0.5 alpha, and LAT-27) did not inhibit T-cell proliferation at neutralizing conditions, they were capable of binding envelope protein on immunoblot assays (Fig. 1B and data not shown). Lastly, the anti-Tax antibody (10 µg/ml, final concentration) also did not inhibit proliferation, though this concentration of antibody neutralizes the activity of a soluble form of Tax.

Anti-CD2 and anti-LFA-3 inhibit HTLV-I-induced proliferation of PBLs. Antigen-specific activation of T cells occurs through the T-cell receptor (TCR) (9, 42). The signals initiated through the TCR can be augmented by binding of accessory surface molecules to their ligands. Among these molecules are CD2 and LFA-1 and their ligands LFA-3 and ICAM-1, respectively (2, 10, 37, 40). In addition to its costimulatory effect with the TCR, CD2 is also capable of transducing an activation signal independent of engagement of the TCR by its ligand LFA-3 plus a MAb or a combination of MAbs against distinct epitopes (3, 12, 18, 28). Furthermore, it has been shown that T cells can be activated by CD2/LFA-3 and ICAM-1/LFA-1 interactions in autologous T-lymphocyte reactions for which TCR engagement is not believed to be required (4). To determine whether any of these molecules participate in activation of PBLs by HTLV-I infected T cells, fixed MT2 cells were cocultivated with PBLs in the presence of MAbs directed to CD2, LFA-3, ICAM-1, LFA-1, HLA-DR, and IL-2. Antibodies directed to LFA-3 (TS2/9), CD2 (TS2/18), and IL-2 blocked proliferation by 80, 93, and 63.7%, respectively (Table 2). Anti-

TABLE 1. Failure of MAbs and antisera against the HTLV-I envelope and Tax proteins to inhibit activation mediated by HTLV-I-producing cells

Culture ^a	Antibody reactivity ^b	[³ H]thymidine incorporation ^c
Expt 1		
PBL alone		261 ± 11
PBL + PHA		21,027 ± 152
J25 alone		101 ± 15
PBL + J25		207 ± 41
HUT78 alone		135 ± 15
PBL + HUT78		650 ± 166
MT2 alone		141 ± 7
PBL + MT2 +:		
No antibody		5,337 ± 532
LAT-27	191-196	6,971 ± 1,878
REY-30		6,221 ± 707
Anti-SP-2 Pre		7,000 ± 942
Anti-SP-2I	86-107	7,743 ± 933
Anti-SP-10 Pre		4,950 ± 540
Anti-SP-10 I	462-480	4,494 ± 536
Anti-SP-11 Pre		4,809 ± 291
Anti-SP-11 I	475-488	5,550 ± 772
Expt 2		
PBL alone		523 ± 154
PBL + PHA		13,539 ± 484
MT2 alone		70 ± 9
PBL + MT2 +:		
No antibody		6,022 ± 411
0.5 alpha	186-195	7,660 ± 345
Anti-SP-1	33-47	7,528 ± 569
Anti-SP-4	129-149	8,984 ± 285
Anti-SP-3 Pre		6,904 ± 415
Anti-SP-3 I	176-189	9,478 ± 384
Anti-SP-4A Pre		8,232 ± 620
Anti-SP-4A I	190-209	8,190 ± 325
Anti-SP-6	296-312	9,545 ± 503
Mouse IgG		5,459 ± 166
Anti-Tax		8,204 ± 1,423

^a Paraformaldehyde-fixed T-cell lines were cocultivated with unstimulated PBLs in the presence and absence of MAbs and rabbit antisera to gp46, gp21, and Tax. The concentrations of antibodies used were as follows: 1:40 dilution of rabbit preimmune (Pre) and immune (I) sera to synthetic peptides (SP) of the HTLV-I envelope glycoproteins gp46 and gp21; 50 µg each of LAT-27 and REY-30 per ml; 33.3 µg of 0.5 alpha per ml; 10 µg of anti-Tax per ml; and a 1:40 dilution of mouse immunoglobulin G (IgG).

^b Amino acids of envelope against which the antisera or MAbs react.

^c Mean counts per minute ± standard error of the mean of triplicate cultures.

LFA-1 (TS1/22), anti-ICAM-1 (RR1/1), and anti-HLA-DR did not inhibit proliferation significantly.

Although the response of the PBLs to PHA or HTLV-I-producing T-cell lines varied from donor to donor, we consistently reproduced the results shown Table 1 and 2 in three to four independent experiments using either paraformaldehyde-fixed or irradiated cell lines (data not shown). These data suggest that the CD2/LFA-3 activation pathway, and not the envelope glycoproteins or Tax, mediates the mitogenic activity of HTLV-I-producing T cells and that proliferation occurs in an autocrine fashion involving IL-2.

Activation of Jurkat cells by HTLV-I-producing T-cell lines. To circumvent donor variability in the activation response and eliminate the possibility of a mixed lymphocyte reaction due to contaminating monocytes/macrophages, we used Jurkat 25 cells as responder cells in activation assays. The response of Jurkat cells to mitogenic stimuli mimics that of

TABLE 2. Evidence that antibodies against LFA-3 and CD2 inhibit activation of PBLs by an HTLV-I-infected cell line

Culture	[³ H]thymidine incorporation ^a
PBL alone	1,024 ± 236
PBL + PHA	41,069 ± 4,603
Jurkat 25 alone	130 ± 42
PBL + Jurkat 25	1,404 ± 148
HUT78 alone	112 ± 37
PBL + HUT78	1,570 ± 525
MT2 alone	118 ± 24
PBL + MT2 + ^b :	
No antibody	13,092 ± 876
Mouse IgG	15,674 ± 3,316
TS2/9 (anti-LFA-3)	2,414 ± 623
TS2/18 (anti-CD2)	941 ± 290
TS1/22 (anti-LFA-1)	22,963 ± 904
RR1/1 (anti-ICAM-1)	14,294 ± 717
Anti-HLA-DR	10,867 ± 1,067
Anti-IL-2	4,746 ± 343
Normal rabbit serum	17,283 ± 673

^a Mean counts per minute ± standard error of the mean of triplicate cultures.

^b The amount of antibody added to each 200-μl culture was as follows: 50 μl of conditioned hybridoma supernatants; 1:100 dilution of RR1/1; 1:40 dilution of anti-IL-2 and normal rabbit serum; 1:40 dilution of anti-HLA-DR; and 1:40 dilution of mouse immunoglobulin G (IgG).

resting T cells (42, 43). For this assay, we used RT-PCR to monitor activation of Jurkat 25 cells by HTLV-I-producing cell lines and uninfected cell lines. Induction of IL-2 mRNA expression was assayed for 6 h after cocultivation of fixed cell lines with Jurkat 25 cells. Figure 2 shows that MT2 cells induced IL-2 mRNA expression that was at least 1,000-fold greater than that of unstimulated Jurkat cells. Cell lines HUT78, HOS, and HOS/PL did not induce IL-2 mRNA expression. Neither IL-2 mRNA nor aldolase A mRNA was detectable in the paraformaldehyde-fixed MT2 cell line (Fig. 3B, lane MT2 alone). Similar observations were made for paraformaldehyde-fixed HUT78 and HUT102 cells. Also, concentrated virus from MT2 supernatant was capable of stimulating IL-2 mRNA expression in Jurkat cells, whereas sucrose gradient-purified particles from MT2 (1 to 15 μg of p19^{ag} per ml) and concentrated virions from HOS/PL did not induce IL-2 mRNA expression, even at concentrations approximately 10- to 12-fold greater than the amount of concentrated virus from MT2 supernatants (data not shown). Thus, as with the studies using PBLs, this finding indicates that HTLV-I alone is not mitogenic.

FIG. 2. Induction of IL-2 mRNA expression in Jurkat 25 cells by HTLV-I-producing T cells. (A) IL-2 mRNA expression in Jurkat 25 cells (J25) cultured alone or with the indicated fixed cell line; (B) IL-2 mRNA expression of PHA (1 μg/ml)-PMA (50 ng/ml)-stimulated Jurkat 25 cells diluted at 10-fold serial dilutions with total RNA from unstimulated Jurkat cells. Ald A, aldolase A.

FIG. 3. Evidence that induction of IL-2 mRNA expression in Jurkat 25 cells by HTLV-I is mediated through the CD2/LFA-3 pathway. (A and B) Induction of IL-2 mRNA expression by the HTLV-I-producing T-cell lines HUT102 (A) and MT2 (B) is inhibited by anti-CD2 (TS2/18) and anti-LFA-3 (TS2/9). Fixed HUT78, HUT102, and MT2 cells were mixed with Jurkat cells in the presence and absence of the indicated antibodies; total RNA was harvested after 6 h for analysis of IL-2 expression. (C) Fixation of anti-LFA-3 to MT2 cells reduces its mitogenic potential. The indicated antibodies were preincubated with MT2 cells on ice for 0.5 h, washed twice with PBS to remove excess antibody, and fixed in 1.0% paraformaldehyde for 1 h on ice. Cells were then washed twice with RPMI complete and mixed in a 1:1 ratio with Jurkat 25 cells. After 6 h, the total RNA was prepared and analyzed as described in Materials and Methods. In panels A to C, the gel on the right shows IL-2 mRNA expression of PHA-PMA-stimulated Jurkat 25 cells diluted at 10-fold serial dilutions as done in Fig. 2B. (D) MAbs to LFA-3 (TS2/9) and CD2 (TS2/18) do not inhibit activation by PHA and PMA or Leu 4 and PMA. Jurkat cells were stimulated with PHA (0.01 μg/ml)-PMA (50 ng/ml) or Leu 4 (1:200 dilution)-PMA (50 ng/ml) for 6 h in the presence of the indicated antibodies. Analysis of IL-2 and aldolase A (Ald A) mRNA expression was carried out as described in Materials and Methods. IgG, immunoglobulin G.

Induction of IL-2 mRNA expression in Jurkat cells is blocked by anti-CD2 and anti-LFA-3. To determine whether cellular adhesion molecules were mediating the activation of IL-2 mRNA expression in Jurkat cells, we cocultivated paraformaldehyde-fixed MT2 and HUT102 cells in the presence and absence of MAbs to cellular adhesion molecules (Fig. 3A and B). MT2 and HUT102 cells induced IL-2 mRNA expression greater than 1,000-fold over unstimulated Jurkat 25 cells. In contrast, HUT78 cells did not induce IL-2 mRNA expression. Interestingly, anti-CD2 (TS2/18) and anti-LFA-3 (TS2/9) blocked expression of IL-2 mRNA. Anti-ICAM-1 (RR1/1), anti-LFA-1 (TS1/22), anti-HLA-DR, and anti-CD4 did not inhibit expression. The inhibition by anti-CD2 and anti-LFA-3 was not due to negative signalling, as these MAbs did not inhibit activation of Jurkat 25 cells by PHA and PMA or Leu 4 (anti-CD3) plus PMA (Fig. 3D).

To determine which of the adhesion molecules on the HTLV-I-producing cell lines were mediating the activation of Jurkat cells, we preincubated the MAbs with MT2 cells and removed the excess antibodies by washing the cells twice with PBS prior to fixation. Figure 3C shows that in comparison with the negative control mouse immunoglobulin G, anti-LFA-3 (TS2/9) inhibited IL-2 mRNA expression approximately 90-fold. None of the other antibodies had a negative effect. Hence, as with HTLV-I-induced proliferation of PBLs, activation of Jurkat 25 cells to express IL-2 mRNA by HTLV-I is T-cell associated and mediated by the CD2/LFA-3 pathway. Antibodies directed to gp46, gp21, p40^{ax}, p19^{gag}, and TSP patient antisera did not inhibit activation of Jurkat cells by HTLV-I-infected cells or concentrated virus particles from MT2 cells (data not shown).

DISCUSSION

In this study, we have examined the mitogenic activity of HTLV-I by using concentrated and sucrose gradient-purified viral particles as well as virus-producing cell lines. Gazzolo and Duc Dudon reported that concentrated viral particles can directly activate resting T lymphocytes and that the envelope protein gp46 mediates this effect (15). While we also find that concentrated virus produced from a T-cell line acts as a weak mitogen for PBLs, the same virus strain after sucrose gradient purification is incapable of stimulating proliferation. Furthermore, we demonstrate the presence of gp46 on purified viral particles; this finding indicates that the lack of stimulation cannot be accounted for by an absence of gp46 due to stripping during the purification process. In light of these data, we conclude that virion-associated gp46 is not a T-cell mitogen. Lastly, the inability of concentrated virus from HOS/PL cells to induce proliferation of PBLs confirms that HTLV-I virions are not intrinsically mitogenic.

A possible explanation for these results is that an inhibitor of the mitogenic activity is present in purified virus particles from MT2 cells and concentrated virions from HOS/PL cells but absent from concentrated virus particles from the MT2 cell line. Although we cannot completely exclude this as an explanation, we feel that it is a remote possibility.

As we and others have shown (22, 44) and we have demonstrated here, the HTLV-I-associated mitogenic activity is cell associated, requires direct cell contact, and is IL-2 dependent. In this study, we also show that the type of virus-producing cell is important: only the HTLV-I-infected T-cell lines were capable of activating PBLs or Jurkat cells; the HTLV-I-producing human osteosarcoma cell line HOS/PL did not activate PBLs or Jurkat cells. Importantly, we could reproduce the results by using different PBL

donors for cocultivation with virus-positive and -negative cell lines. It is therefore unlikely that the proliferation is simply due to a mixed lymphocyte reaction involving macrophages/monocytes. The fact that we faithfully reproduced this activation with use of Jurkat cells as responders provides additional evidence that T-cell^{HTLV-I+}-to-T-cell contact is sufficient for activation.

The virus-producing T-cell lines used in this study are positive for p40^{ax} expression and produce different amounts of virus (23). For example, 3-day conditioned culture supernatants from MT2 and HUT102 cells contain 1,400 and 70 ng of p19^{gag} per ml, respectively, yet they are equally able to activate PBLs and Jurkat cells. Hence, not only is the mitogenic potential dependent on cell type, it is also independent of the amount of virus released from the infected cells. Thus, it is likely that a nonviral protein(s) is critical for mediating T-cell activation and that the mitogenic activity originally attributed to HTLV-I is due to contaminating cellular membranes and not viral particles.

We have used MAbs and antisera against HTLV-I proteins and cellular adhesion molecules to inhibit activation of PBLs and Jurkat cells by HTLV-I-infected T-cell lines. Antibodies to the structural proteins gp46, gp21, and p19^{gag}, an antibody to the *trans* activator Tax, and anti-HTLV-I patient antisera did not neutralize activation. The data suggest that the viral proteins recognized by the antibodies do not function in this particular activation process.

Of particular interest were the anti-envelope antibodies anti-SP-2, LAT-27, and 0.5 alpha, which have been shown to inhibit infection by a vesicular stomatitis virus-HTLV-I pseudotype, HTLV-I-induced syncytium formation and infection, and T-cell activation, respectively (15, 29, 38). Their inability to block activation of PBLs or Jurkat cells by HTLV-I-infected T cells further suggests that the envelope glycoprotein gp46 is not a T-cell mitogen. These data corroborate and extend the findings of Wucherpfennig et al. (44), who also reported that the Mab 0.5 alpha did not inhibit T-cell activation by HTLV-I. It, however, remains possible that anti-SP-2 and LAT-27 block infection but are nonneutralizing for the mitogenic effect. Along these lines, it is of interest to note that while 0.5 alpha had previously been demonstrated to inhibit the mitogenic effect of concentrated virus particles (15), it does not neutralize syncytium formation (27, 30, 38).

While a soluble form of Tax has been demonstrated to induce proliferation of PHA-stimulated PBLs (25), we believe that the activation of PBLs and Jurkat cells observed in this study are not the result of *trans* cellular activation by Tax. First, we found that paraformaldehyde-fixed HTLV-I-producing T-cell lines are mitogenic for PBLs and Jurkat cells, whereas the virus-producing HOS/PL cell line does not activate either of these types of cells. Presumably, Tax is fixed and not released. If *trans* cellular activation occurs, it is unclear why HOS/PL does not exhibit the same effect as do the HTLV-I-producing T-cell lines. Second, the anti-Tax antibody did not neutralize the mitogenic activity. Third, soluble Tax has been demonstrated to induce proliferation of only PHA-stimulated PBLs; it does not activate unstimulated PBLs to proliferate (25). Fourth, Tax alone is ineffective at *trans* activating IL-2 expression; it acts synergistically with additional mitogenic stimuli to potently induce IL-2 expression (26, 36, 41). Counter to points three and four, we observe activation of unstimulated PBLs and induction of IL-2 expression in Jurkat cells without the addition of other mitogens.

In contrast to the anti-HTLV-I antibodies, MAbs to CD2

and LFA-3 block activation of PBLs and Jurkat cells by the HTLV-I-producing T-cell lines. This neutralizing effect implies that CD2 and its ligand LFA-3 are essential for mediating T-cell activation by HTLV-I-infected T cells. LFA-3 is not detectable on purified virus particles or HOS/PL cells by immunoblotting with TS2/9 (anti-LFA-3) (23); this might explain their lack of mitogenic activity. Duc Dudon et al. reported that the mitogenic activity of concentrated virus could be inhibited by MAbs directed to CD2 (11). However, they concluded that an envelope gp46/receptor complex may be interacting with CD2. Our data and conclusions contrast with their data but are in agreement with the finding of Wucherpfennig et al. (44) that LFA-3 and not an HTLV-I envelope glycoprotein gp46/receptor complex associates with CD2. On the other hand, unlike Wucherpfennig et al., we did not find that MAbs to either ICAM-1 or LFA-1 blocked HTLV-I-mediated T-cell activation. Therefore, we suggest that lymphocyte activation by HTLV-I-infected T-cells is not a result of cooperativity between CD2/LFA-3 and LFA-1/ICAM-1.

Adhesion molecules other than CD2/LFA-3 and LFA-1/ICAM-1 play a role in costimulating T-cell activation through the CD3/TCR complex, including V-CAM-1/VLA-4/ β_1 integrin and B7/CD28 (10). We have not tested antibodies against these adhesion proteins and therefore cannot rule out their potential participation in lymphocyte activation by HTLV-I. However, the fact that anti-LFA-3 and anti-CD2 strongly block proliferation of PBLs induced by HTLV-I-infected T cells (80 and 93%, respectively) and completely block activation of IL-2 mRNA expression in Jurkat cells demonstrates that the CD2 pathway plays a central role in this activation. Furthermore, T-cell activation via CD2 can occur without engagement of the CD3/TCR complex (3, 4, 12, 18, 28). We found that anti-HLA-DR and anti-CD4 did not inhibit IL-2 mRNA expression in Jurkat cells. However, we cannot completely rule out ligand engagement with the CD3/TCR complex. We have tested the ability of HTLV-I-infected T cells to activate IL-2 mRNA expression in a mutant CD3⁻ CD2⁺ Jurkat cell line. As others have shown with different stimuli (3), we have found that activation by HTLV-I infected T cells requires the expression of the CD3/TCR complex (23).

Although it does not appear that either the envelope glycoprotein gp46 or gp21 or extracellular Tax protein is mediating T-cell activation by HTLV-I-infected T cells, another viral protein or *trans* activation of cellular genes by Tax may be important for conferring this activity upon infected T cells. Brod et al. (4) have shown that early after activation, T cells could stimulate proliferation of resting T cells in an autologous reaction. Presumably, this occurred in an antigen-independent fashion. Interestingly, Kelly et al. (21) have demonstrated that HTLV-I infection of T cells stabilizes an early-activation phenotype which is normally transient. They suggest that Tax and perhaps other pX-encoded proteins modulate the cell state. In the context of these data, it may be that the mitogenic activity of HTLV-I-infected T cells results from maintaining an early-activation phenotype via expression of a viral regulatory gene product(s).

On the other hand, as LFA-3 alone does not activate T cells (12), a viral protein involved in virus-cell interactions might act as a costimulator of T-cell activation with LFA-3. Several open reading frames capable of encoding novel proteins have been reported for HTLV-I (1, 7, 24). Korallnik et al. (24) speculate that these potential proteins may have roles in regulating viral replication and in virus-cell interac-

tions. Determining the function of these potential proteins and construction of a replication-competent molecular clone of HTLV-I may provide insight into which viral genes are involved in conferring the mitogenic activity and the mechanism of T-cell activation via direct cell contact between infected and uninfected T cells. In any case, in addition to *trans* activation of cytokine genes by Tax, T-lymphocyte activation by cell-to-cell contact may be another mechanism by which HTLV-I infection contributes to the immune activation observed in HTLV-I-infected asymptomatic and HAM/TSP patients.

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