Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11094–11098, December 1993
Immunology

Costimulation of T-cell activation and virus production by B7 antigen on activated CD4+ T cells from human immunodeficiency virus type 1-infected donors

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Communicated by Leon E. Rosenberg, August 3, 1993 (received for review April 29, 1993)

ABSTRACT **Infection with the human immunodeficiency virus type 1 (HIV-1) requires T-cell activation. Recent studies have shown that interactions of the T-lymphocyte receptors CD28 and CTLA-4 with their counter receptor, B7, on antigen-presenting cells are required for optimal T-cell activation. Here we show that HIV-1 infection is associated with decreased expression of CD28 and increased expression of B7 on CD4+ T-cell lines generated from seropositive donors by alloantigen stimulation. Loss of CD28 expression was not seen on CD4+ T-cell lines from seronegative donors, but up-regulation of B7 expression was observed upon more prolonged culture. Both T-cell proliferation and interleukin 2 mRNA accumulation in HIV-1-infected cultures required costimulation with exogenous B7 because these events were blocked by CTLA4Ig, a soluble form of CTLA-4 that binds B7 with high avidity. In contrast, levels of HIV-1 RNA were not affected by CTLA4Ig, indicating that regulation of virus transcription in these cultures did not depend upon CD28–B7 engagement. Infected T cells could present alloantigen to fresh, uninfected CD4+ T cells, leading to increased proliferation and virus spread to the activated cells. Both of these events were blocked by CTLA4Ig. Thus, chronic activation of HIV-1-infected CD4+ T cells reduces expression of CD28 and increases expression of B7, thereby enabling these T cells to become antigen-presenting cells for uninfected CD4+ T cells; this might be another mechanism for HIV-1 transmission via T-cell–T-cell contact.**

Infection with the human immunodeficiency virus type 1 (HIV-1) leads to a reduction of CD4+ T cells and a loss of immune competency (1). HIV-1-infected individuals exhibit a marked deficiency in their ability to respond to recall antigens (e.g., tetanus toxoid) (2). Analysis of HIV-1-infected cells *in vitro* has shown defects in T-cell activation and signal transduction (3). Despite the immune defects caused by HIV-1 infection, induction and spread of the virus requires T-cell activation (4–6). Thus, HIV-1 infection is intimately involved with function of the immune system.

Optimal antigenic stimulation of CD4+ T cells requires (*i*) engagement of the T-cell receptor–CD3 complex by antigenic peptides presented in the context of major histocompatibility complex (MHC) molecules, and (*ii*) additional costimulatory signals provided by engagement of accessory receptors on the T-cell surface with counter receptors on antigen-presenting cells (for review, see ref. 7). In addition to lymphocyte function-associated antigen 1 (LFA-1) and CD2 (for review, see ref. 7), recent work has elucidated the importance of the CD28 receptor in T-cell-dependent immune responses (8, 9). A counter receptor for CD28 is the B7 molecule expressed on activated antigen-presenting cells (10, 11). B7 also binds to cytolytic T-lymphocyte-associated sequence (CTLA-4) (34), a protein structurally related to CD28 but only expressed on T cells after activation (12). CTLA-4 acts cooperatively with CD28 to bind B7 and deliver T-cell costimulatory signals (13).

Because of the importance of the CD28/CTLA-4 and B7 interactions in immune responses, it is likely that these interactions are also important during HIV-1 infection. Studies with anti-CD28 monoclonal antibodies (mAbs) suggested a role for CD28 in up-regulating HIV-1 long terminal repeat-driven transcription of a reporter gene in leukemic cell lines (14). Groux *et al.* (15) showed that CD28 triggering with specific mAbs prevented activation-induced cell death in T-cell cultures from HIV-1-infected donors. The role of B7 in HIV-1 infection has not been studied, but cultured cell lines infected with another human lymphocyte-specific retrovirus, human T-cell lymphotropic virus type 1 (HTLV-1), expressed the B7 protein on their surface (16). More recently, Freeman *et al.* (17) showed that several HTLV-1-transformed T-cell lines expressed mRNAs for B7 and CTLA-4 but not for CD28.

We studied the expression and function of CD28/CTLA-4 and B7 during HIV-1 infection. Specifically, we investigated whether infection with HIV-1, like infection with HTLV-1, was associated with increased expression of B7 on T cells, and if so, whether this might have functional consequences. We generated primary cultures of CD4+ T cells from HIV-1-infected individuals by alloantigen stimulation, which provided a homogeneous population of primary T cells with a high frequency of HIV-1 infection.

MATERIALS AND METHODS

mAbs and Soluble Receptors. Origins of the mAbs used, G17-2 (anti-CD4), G10-1 (anti-CD8), 60.1 (anti-CD11b), 63D3 (anti-CD14), FC2 (anti-CD16), 1F5 (anti-CD20), HB10a (anti-MHCII), and 9.3 (anti-CD28) have been presented (11, 18). mAb 11D4 (anti-CTLA-4) has been described (13). Anti-CD3 mAb Leu-4 conjugated to peridinin chlorophyll protein (PerCP) and anti-B7 mAb BB1 were purchased from Becton Dickinson. Anti-HIV-1 mAb 25-3 was reactive with the HIV-1 core protein p24 (Genetic Systems, Seattle). Human CTLA4Ig (the soluble form of CTLA-4 that binds B7) has been described (12).

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Abbreviations: HIV-1, human immunodeficiency virus type 1; HTLV-1, human T-cell lymphotropic virus type 1; mAb, monoclonal antibody; IL-2, interleukin 2; CTLA-4, cytolytic T-lymphocyte-associated sequence; FITC, fluorescein isothiocyanate; CTLA4Ig, soluble form of CTLA-4 that binds B7; LFA-1, lymphocyte function-associated antigen 1; MLC, mixed lymphocyte cultures; MHC, major histocompatibility complex; EBV-LCL, Epstein–Barr virus-transformed B cell(s).

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Generation of Alloantigen-Primed T-Cell Lines. $CD4^+$ T lymphocytes were isolated from peripheral blood mononuclear cells from HIV-1-seronegative and -seropositive donors, as described (18). Purified $CD4^+$ T cells (>90% pure) were cocultured with irradiated (10,000 rads; 1 rad = 0.01 Gy) Epstein–Barr virus-transformed B cells (EBV-LCL) from a seronegative donor at weekly intervals. After the initial stimulation, the ratio of stimulators to responders was altered from 1:2 to 1:5 for all subsequent stimulations, and culture medium (RPMI 1640 medium/10% human serum) was supplemented with human interleukin 2 (IL-2) at 10 units/ml. Expanded T cells were collected and depleted of $CD8^+$ T cells by complement lysis after mAb G10-1 binding.

Cocultivation and Mixed Lymphocyte Response Assays. Irradiated (10,000 rads) infected alloreactive T cells were used as stimulator cells in mixed lymphocyte cultures (MLC) with freshly isolated uninfected $CD4^+$ T cells. The responder cells were derived from the same seronegative donor as the B cells used in the alloactivation of the HIV-1-infected cells. The MLC were maintained in culture medium with or without CTLA4Ig (10 μg/ml). Cell proliferation was measured by [^{3}H]thymidine incorporation, and HIV-1 production was measured by evaluating the p24 concentrations in the culture medium using specific enzyme immunoassay (5).

Immunostaining of Cell Surface-Associated Proteins. Cell-surface expression of proteins was evaluated by multicolor immunofluorescent staining. After staining, cells were washed with phosphate-buffered saline and fixed overnight with 2% paraformaldehyde solution, pH 7.2, at 4°C. Intact cells were analyzed by using either an EPICS V (Coulter) or FACScan (Becton Dickinson) instrument.

RNA Blot Analysis. RNA was prepared from the alloreactive cell lines at various times during the fourth round of stimulation, fractionated by electrophoresis, and transferred to nitrocellulose filters as described (9). The filters were probed for various RNAs as described (9, 13). The HIV probe was a 3.1-kb DNA fragment excised from the gag open reading frame by digestion of the plasmid pMgtre (provided by Alan Senear, Bristol–Myers Squibb) with *Bss*HII and *Xba* I.

PCR Analysis. Presence of B7-specific message was evaluated by PCR of cDNA generated from reverse transcription of cellular RNA fractions (19). The primers used were as follows: for B7, 5′-CTGAGCTCTATGCTGTTAGC-3′ (sense) and 5′-ACAGAATTCTGCGGACACTGTTATACAGG-3′ (antisense); for CD19, 5′-CTGAACCAGAGCCTCAGCCAG-3′ (sense) and 5′-TGCTCGGGTTTCCATAAGACG-3′ (antisense); for hypoxanthine phosphoribosyltransferase 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ (sense), and 5′-GATTCAACTTGCGCTCATCTTAGGC-3′ (antisense).

RESULTS

HIV Infection Specifically Alters the Expression of the T-Cell Costimulatory Receptor CD28. Primary T-cell lines were generated by alloantigen stimulation of $CD4^+$ T lymphocytes purified from HIV-1-seropositive (Z29, Z36, Z39, Z44, and Z64) and seronegative (400, 742, 806, 851, and 948) donors. Proliferation of the lines required the addition of antigen-presenting cells. Cell-surface expression of various T-cell activation-associated receptors was evaluated using specific mAbs. Fig. 1*A* shows that surface reactivity of cells from the seropositive donors Z29, Z39, and Z44 with anti-CD28 mAb was significantly reduced compared with the surface reactivity of cells from the seronegative donor 806 when assayed after 4 weeks of allostimulation. This decrease in CD28 reactivity was evident in T-cell lines from the five seropositive donors (Fig. 1 *A* and *B* and data not shown). In contrast, cell-surface reactivity with mAb to CD2 and LFA-1 were not

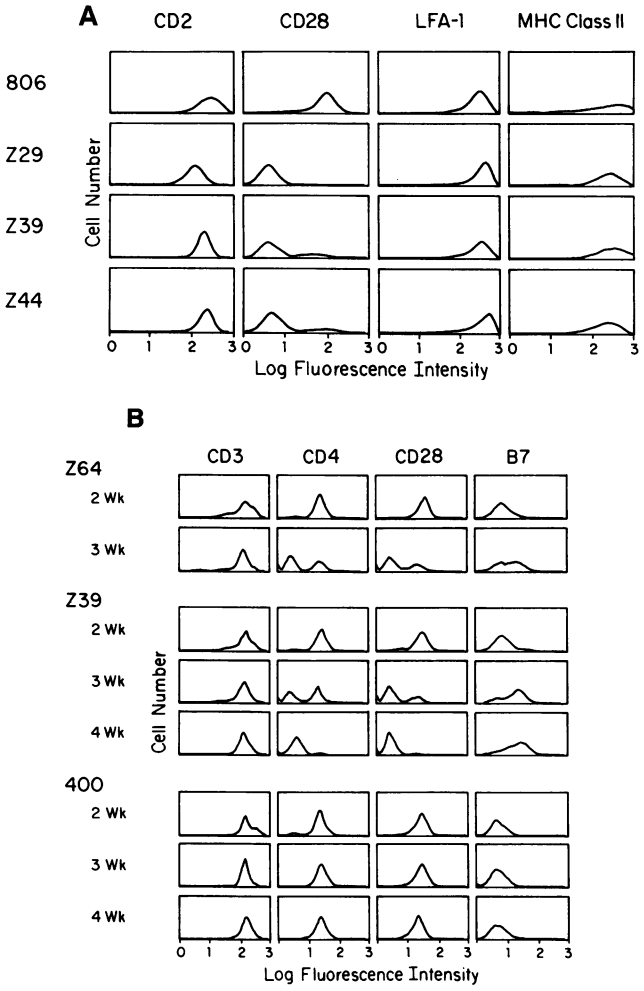

FIG. 1. (*A*) Cell-surface expression of specific receptors in HIV-1-infected and -uninfected alloantigen-primed $CD4^+$ T-cell lines. Cell-surface expression of accessory molecules CD2, CD28, and LFA-1, as well as MHC class II protein, was evaluated in HIV-1-infected (Z29, Z39, and Z44) and uninfected (806) lines at day 7 of the fourth round of stimulation using fluorescein isothiocyanate (FITC)-conjugated mAbs. (*B*) Kinetic analysis of down-regulation of CD28 and up-regulation of the counter receptor B7. Cells from infected (Z29 and Z64) and uninfected (400) lines were assayed for expression of CD28 and B7 in parallel with CD3 and CD4 at day 7 of various rounds of stimulation, as indicated using FITC-conjugated mAbs. CTLA4Ig conjugated to FITC was used for B7-specific staining.

altered in either the seropositive lines Z29, Z39, and Z44 or the seronegative line 806 (Fig. 1*A*). Also presented in Fig. 1*A* is the relative expression of MHC class II molecules on the activated T cells from the various donors.

Fig. 1*B* shows that reduction in cell-surface expression of CD28 in HIV-1-infected cultures was gradual and paralleled the down-modulation of CD4 cell-surface reactivity (compare lines Z64 and Z39 with line 400). In contrast, cell-surface expression of CD3 was not affected. Loss of CD4 expression in HIV-1-infected cultures was shown to correlate with expression of viral proteins (20–23). Analysis of the alloactivated cultures showed increased virus production, and virus spread with increased periods of stimulation (data not shown). Levels of infection at round four of stimulation varied between lines, but HIV-specific immunofluorescent assays revealed that levels of infection as high as 50–90% could be achieved for some cell lines.

Expression of CD28 and its homologue CTLA-4 were evaluated in cells from donors Z29 and 948 during the fifth round of stimulation. Fig. 2 shows that the $CD4^+$ CTLA-4^+

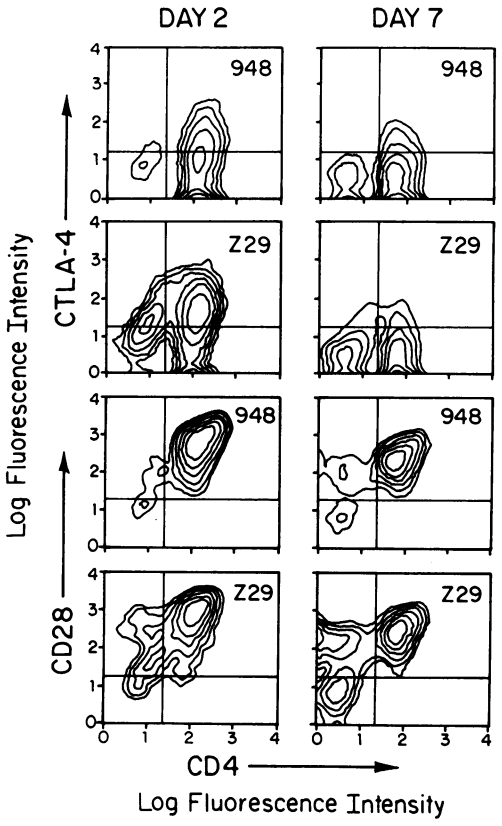

FIG. 2. Kinetic analysis of CD28 and CTLA-4 expression in primed T-cell lines. Three-color fluorescence was used to evaluate expression of CD28 and CTLA-4 at days 2 and 7 during the fifth round of stimulation of infected (Z29) and uninfected (806) lines. The anti-CD3 mAb Leu-4 was conjugated to peridinin chlorophyll protein (PerCP), the anti-CD4 mAb G17-2 was conjugated to FITC, the anti-CD28 mAb 9.3 and the anti-CTLA-4 mAb 11D4 were conjugated to biotin. After binding of mAbs, cells were incubated with streptavidin–phycoerythrin. Costaining of $CD3^+$ cells for CD4 and CD28 or CTLA-4 was evaluated.

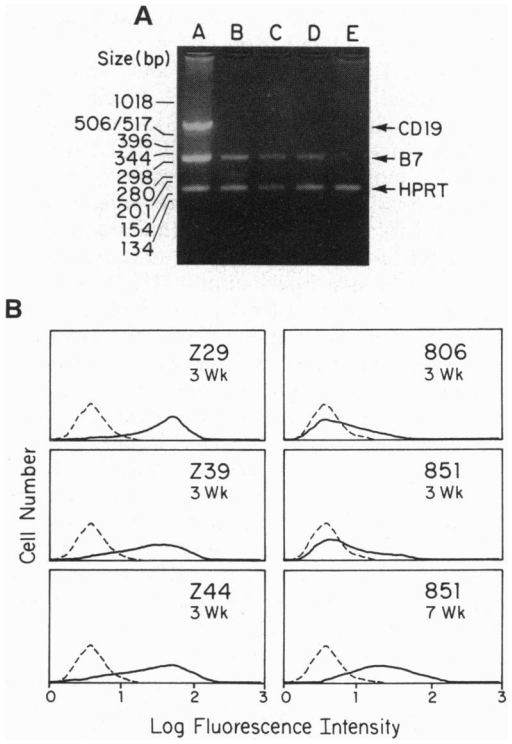

FIG. 3. (*A*) B7 mRNA is present in infected and uninfected lines. cDNA was generated by reverse transcription of RNA derived from infected (Z36, lane C, and Z44, lane D) and uninfected (400, lane B) cell lines after the fourth round of stimulation. B7 sequences were amplified by PCR, fractionated in agarose gels and visualized with ethidium bromide staining. RNA fractions from the EBV-LCL (lane A) and Jurkat cells (lane E) were used as positive and negative controls, respectively, for B7 mRNA expression. PCR analysis for CD19 mRNA (lane A) served as an internal control to confirm the absence of RNA from residual B cells in our T-cell fractions (lanes B–D). Evaluation of hypoxanthine phosphoribosyltransferase (HPRT) RNA confirmed equal loading of samples. (*B*) Relative expression of B7 on infected (Z29, Z39, and Z44) and uninfected (806 and 851) lines was evaluated after three rounds (3 Wk) and seven rounds (7 Wk) of stimulation using CTLA4Ig conjugated to FITC.

population represented 60% of the Z29 culture but only 28% of the 948 culture when evaluated 2 days after activation. At day 7 after activation, cell-surface reactivity with anti-CTLA-4 mAb returned to basal levels in both cultures. Two additional HIV-infected lines also showed enhanced expression of CTLA-4 (data not shown). In contrast, at day 2 postactivation the $CD4^+$ $CD28^+$ populations were comparable in both the Z29 and 948 cultures (81% and 95%, respectively). However, at day 7 after activation CD28 expression was reduced in the Z29 culture ($CD4^+$ $CD28^+$ = 58%) and correlated with loss of CD4 expression because $CD4^+$ $CD28^-$ cells were not detected (see lower right quadrant). Expression of CD28 in the 948 culture remained relatively stable.

Expression of CD28 Counter Receptor B7 on Activated T Cells. Infection of human T cells with HTLV-1 up-regulated B7 expression (16). Because HIV-1 and HTLV-1 are both retroviruses, we evaluated whether infection of T cells with HIV-1 also induces B7 expression. Fig. 1*B* shows that cell-surface reactivity with mAb to B7 was higher in the HIV-infected lines Z64 and Z39 compared with uninfected line 400. Moreover, the increase in B7 expression evident in the HIV-infected lines was gradual over several weeks of allostimulation (see donors Z64 and Z39). PCR analysis showed the presence of a B7-specific transcript in RNA fractions from infected and uninfected lines (Fig. 3*A*). However, as shown in Fig. 3*B*, several additional weeks of allostimulation were required to achieve levels of B7 expression on the seronegative cells comparable to those on seropositive cells (806 and 851 vs. Z29, Z39, and Z44, respectively). The B7 transcript was not derived from residual B cells because CD19 mRNA was not detected (Fig. 3*A*, lane A vs. lanes B, C, and D).

Function of CD28/CTLA-4 Costimulatory Molecules on HIV-1-Infected Lines. Costimulation of T-cell activation via the CD28 receptor results in accumulation of IL-2 mRNA and proliferation (9). Therefore, the effect of HIV-1 infection on activation of the alloantigen-primed T-cell lines was evaluated by assaying for IL-2 transcripts. Fig. 4 shows that the HIV-1-infected and -uninfected lines expressed equivalent levels of IL-2 mRNA. This response was inhibited in all cell lines equally by addition of CTLA4Ig to the cultures. Thus, regulation of IL-2 production by the CD28/CTLA-4 receptors was not greatly affected by HIV-1 infection in these primary T-cell cultures. The transient reduction in CD28 mRNA levels early after activation (see 6-hr time point) was recently reported (24) and was not observed for CTLA-4 mRNA (Fig. 4). However, the expression of CTLA-4 mRNA but not CD28 mRNA was blocked by CTLA4Ig when assayed at both 6-hr and 24-hr postactivation.

Normal induction and regulation of IL-2 mRNA in infected and uninfected cultures was consistent with the presence of relatively equivalent levels of CD28 and CD28 mRNA early in the stimulation cycle (donor Z29; Fig. 2, day 2, and Fig. 4, respectively, as compared with seronegative lines). In addi-

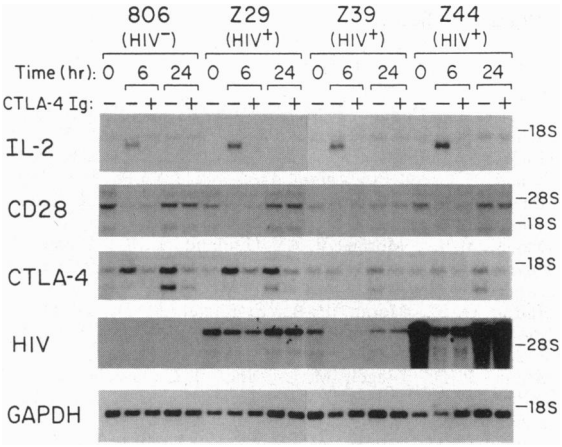

FIG. 4. Northern blot analysis of mRNA expression after activation of the primed T-cell lines. Cells from infected and uninfected cell lines (as indicated) were activated by addition of EBV-LCL in the presence or absence of CTLA4Ig at 10 μg/ml (as indicated). At 6 and 24 hr after activation RNA was extracted from the cells and analyzed by Northern blot for expression of specific mRNA using ^{32}P-radiolabeled probes. Equal loading of material was ensured by evaluating GAPDH mRNA levels.

tion, the enhanced expression of CTLA-4 in HIV-infected lines (Fig. 2 and data not shown) may contribute to normal activation of the T cells.

Although HIV-1 induction depends on T-cell activation (4–6), Fig. 4 shows that after a transient reduction at 6-hr postactivation, the expression and/or accumulation of HIV-1 genomic RNA (Z29, Z39, and Z44) were not altered in the presence of CTLA4Ig by 24-hr postactivation. The viral transcripts detected in these experiments represent cell-associated RNA that could encompass RNA derived from cell-adsorbed virions. Therefore, the levels of HIV-1 RNA in the alloantigen-primed T-cell lines were not regulated by CD28–B7 association.

Role of B7 Protein in T-Cell Activation and Virus Spread. The observation that HIV-1-infected cultures expressed both MHC class II (see Fig. 1*B*) and B7 suggested that these T-cell lines may be capable of presenting alloantigen to fresh CD4$^+$ T cells. This question was addressed by testing the ability of the HIV-1-infected lines to activate CD4$^+$ T cells in MLC. CD4$^+$ T cells were prepared from a seronegative donor (see *Materials and Methods*) and cocultured with irradiated seropositive stimulator cells for 7 days. Fig. 5 *Upper* shows that the responder cells proliferated as determined by incorporation of [^{3}H]thymidine. Magnitude of the response depended on the stimulator/responder cell ratios (see abscissa). In addition, virus production was amplified in these cocultures, indicating spread to the responder cells (*Lower*). Increased virus production required the addition of fresh cells because cultures containing only irradiated stimulator cells, carried in parallel, yielded basal levels of virus (≈1–2 ng of p24 per ml at the highest input of cells; data not shown). Virus spread further confirms the activated state of the responder cells, because HIV-1 cannot productively infect resting cells (25, 26). Fig. 5 also shows that both the proliferative response and virus production (*Upper* and *Lower*, respectively) were inhibited by the presence of CTLA4Ig, suggesting that both processes were costimulated by the B7 molecule.

The concentrations of CTLA4Ig resulting in 50% inhibition (IC_{50}) of proliferation and virus production were 0.31 μg/ml and 0.08 μg/ml, respectively, as determined in cultures using EBV-LCL stimulation of HIV-1-infected cells (Z64; data not shown).

DISCUSSION

We used allostimulated primary CD4$^+$ T-cell lines to investigate the effect of HIV-1 infection on the expression of

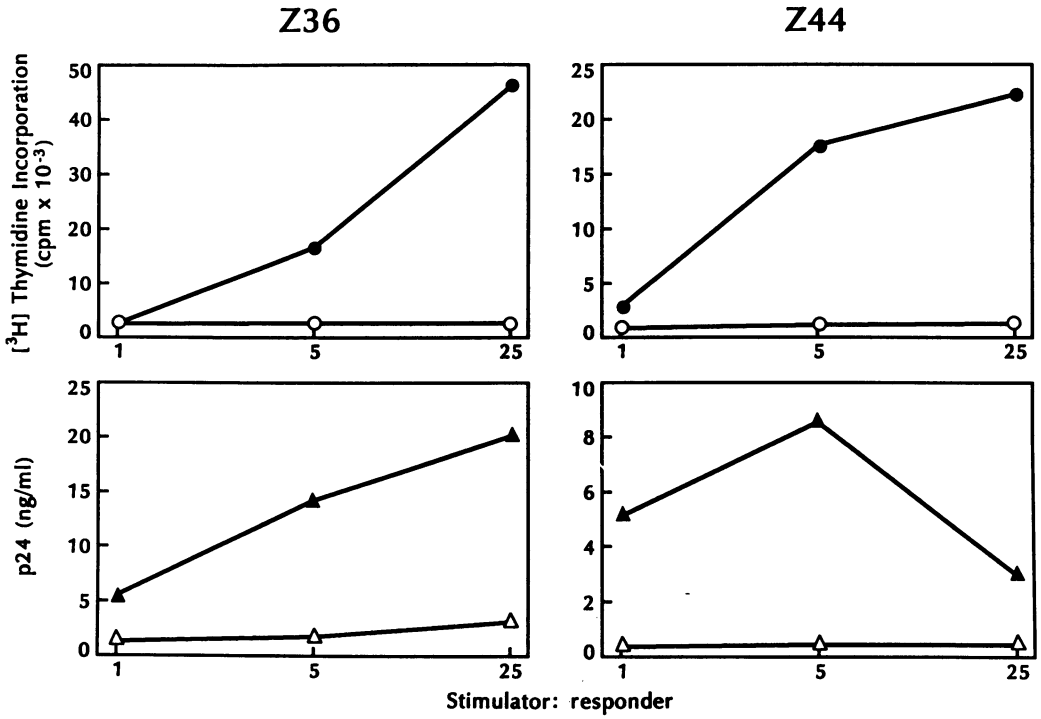

FIG. 5. B7 on HIV-infected T cells costimulates proliferation and virus production in MLC. Cells from Z36 and Z44 HIV-infected cultures were irradiated and used as stimulator cells in MLC with freshly isolated uninfected CD4$^+$ T cells. The cultures contained various stimulator/responder cell ratios, as indicated. Proliferation of the responder cells was evaluated by measuring [^{3}H]thymidine incorporation by cells (cpm × 10^{-3}, *Upper*) on day 3 of coculture, and virus production was evaluated by measuring HIV-1 p24 levels (ng/ml, *Lower*) in the culture supernatants on day 7 of coculture. The role of CD28–B7 in mediating these events was evaluated by assaying the effect of CTLA4Ig (10 μg/ml) (open symbols) in the culture.

specific T-cell costimulatory receptors and on T-cell function. Analysis of virus production by p24-specific enzyme immunoassay and antigen-specific immunofluorescence assay showed that virus spreads in the cultures with repeated stimulation (data not shown). By week 4 of stimulation high infection levels (50–90%) can be achieved with this system. In addition, cells in the alloreactive lines described here can be uniformly activated to produce HIV-1 by addition of the EBV-LCL. These properties rendered the system very well suited to addressing specific questions regarding HIV-1–$CD4^+$ T-cell interactions.

Our results indicate that HIV-1 modulates the expression of specific cell-surface proteins. Several reports have addressed the reduced expression of CD4 in the presence of HIV-1 (20–23). We showed that infection with HIV-1 resulted in the down-modulation of CD28 but not other accessory molecules, such as CD2 or LFA-1. Kinetic analysis during one cycle of stimulation suggested that down-regulation of CD28 expression occurred late in the cycle and most likely reflected expression or accumulation of viral proteins. The gradual reduction in CD28 expression in the cell cultures over several rounds of stimulation correlated with virus spread and increased number of infected cells. Expression of CTLA-4 was enhanced in several HIV-1-infected lines compared with the seronegative line 948 (Fig. 2 and data not shown). Because CTLA-4 is the high-affinity receptor for B7 (12), this enhanced expression may have compensated functionally for the down-regulation of CD28. Activation of the T-cell lines, as measured by induction of IL-2 mRNA and proliferation (data not shown), was not affected by the presence of HIV-1. Although stimulation of CD28 was shown to activate the HIV long terminal repeat-driven transcription of a reporter gene (14), our data show that early accumulation of HIV-1 genomic RNA in the primed T-cell lines was not dependent on CD28–B7 association because CTLA4Ig could not block the RNA responses to alloantigen.

We also showed that HIV-1 accelerates B7 expression in alloactivated T-cell lines. While this work was being prepared for publication, two reports presented the expression of B7 on $CD4^+$ T cells after prolonged activation *in vitro* (27, 28). The mechanism for induction of B7 in T cells is not known; however, expression of B7 in B cells is highly regulated and can be induced by cytokines (29), signaling through the MHC class II or the CD40 molecules (refs. 30 and 31, respectively) and by treatment with dibutyryl cAMP (32).

The presence of the B7 protein on the HIV-1 infected T cells *in vitro* together with the MHC class II molecule allowed the T cells to present alloantigen and activate fresh $CD4^+$ T cells. Treatment of $B7^+$ T cells with CTLA4Ig inhibited activation of the responder cells and virus spread in the culture, suggesting that the B7–CD28 interaction could be important for virus transmission from stimulator to responder T cells. Thus, the B7–CD28 adhesion system may facilitate virus spread by juxtapositioning of the T cells, as was shown for the LFA-1–intercellular adhesion molecule 1 system (33). Because HIV-1 does not productively infect resting T cells (25, 26), previous analysis of virus transmission in T-cell cultures relied on the use of T-cell lines or mitogen-activated $CD4^+$ T cells as targets in cocultivation assays. The ability of the $B7^+$ T cells described here to provide an activation signal in conjunction with virus ensures the productive infection of the target cells. This result defines an efficient mechanism for HIV-1 transmission between T cells that could contribute to increased pathogenesis of the virus.

We thank Drs. Steve Kanner and Jeff Ledbetter for their critical review of the manuscript. We also thank Joanne Greene and Kerry Klussman for technical assistance.

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