Brief Definitive Report

Formation of Simian Immunodeficiency Virus Long Terminal Repeat Circles in Resting T Cells Requires Both T Cell Receptor- and IL-2-dependent Activation

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Summary

Although immunodeficiency viruses can enter resting $CD4^+$ T lymphocytes, activation of T cells is required for complete viral cDNA synthesis and transport of double-stranded viral DNA to the nucleus. Cross-linking T cell receptors (TCRs) on resting $CD4^+$ T cells induces reverse transcription of full-length simian immunodeficiency virus (SIV) genomes, but TCR engagement alone is insufficient to stimulate SIV DNA to move to the nucleus and form long terminal repeat (LTR) circles. Neither ligation of TCR or CD28 receptors nor interleukin 2 (IL-2) alone induces formation of LTR circles; however, the combination of TCR ligation with either CD28 ligation or IL-2 does. Anti-IL-2 serum inhibits the formation of LTR circles induced by cross-linking CD3 and CD28, but has no effect on the induction of increased viral reverse transcription. Thus, two signals appear to be required for immunodeficiency viruses to move to the T cell nucleus, one from the TCR to promote reverse transcription of the viral genome, the other through an IL-2-dependent process leading to formation of LTR circles.

The molecular events required for human or simian immunodeficiency virus (HIV or SIV) RNA to replicate and be established as proviruses in $CD4^+$ T cells are not well defined (1). After entering T cells and uncoating, these viruses reverse transcribe virion RNA into a duplex linear DNA, which in turn is transported to the nucleus via an energy-dependent, cell division-independent process (2). The nucleus of infected cells contains both the linear viral DNA synthesized in the cytoplasm and two forms of circular DNA with either one or two copies of long terminal repeat (LTR) (3). Since these forms are produced only after synthesis and transport to the nucleus of linear viral DNA and are essential for completion of the immunodeficiency virus life cycle (4), they can serve as a convenient marker to monitor early events in the virus life cycle and the nuclear localization of a structurally distinct viral DNA (2, 4–6).

Although initiation of HIV or SIV reverse transcription can be detected in resting T cells, (*a*) completion of viral DNA synthesis in the cytoplasm, (*b*) transport of reverse-transcribed viral genomes (viral cDNA) to the nucleus, and (*c*) chromosomal integration of viral DNA do not occur (7–10) unless the T cells are stimulated with mitogens (5, 9). It is unclear which physiologic T cell activation signals are required for each of these steps in the viral infection. T cell activation is initiated by cross-linking of the CD3/TCR complex (11), but for cell division to be induced, T cells normally must receive additional "costimulatory" signals, e.g., through accessory receptors such as CD28 or from cytokines such as IL-2 (12, 13). Transcription of full-length viral DNA is induced in resting $CD4^+$ T cells activated with mitogens or CD3 mAb but not with CD28 mAb or IL-2 alone (7–10). Here, we report that while cross-linking of CD3/TCR receptors with soluble mAbs stimulate transport of SIV DNA to the nucleus to form LTR circles, circularization of nuclear SIV DNA is induced only in infected T cells stimulated via both CD3/TCR and CD28 receptors by an IL-2-dependent process.

Materials and Methods

Cells, Viral Preparations, and Infection. Dense resting blood T lymphocytes from normal macaques (*Macaca nemestrina*) were isolated and prepared as described (10). Virus used for infection was derived from the cell culture supernatant of a Hut-78 cell clone, clone E11s, of SIV/Mne (14, 15). Details of viral preparations have been described (10). SIV-infected T cells were incubated in media only (unstimulated) or stimulated with 5–20 μg/ml of mAb FN18 anti-macaque CD3 (16) and/or with 20 μg/ml of CD28-specific mAb 9.3 (17), which binds and stimulates macaque T cells (18). Recombinant IL-2 and rabbit IgG anti-human IL-2 were obtained from Genzyme (Cambridge, MA). Rabbit IgG, aphidicolin (stock 10 mg/ml in DMSO), and L-mimosine (10 mM stock in PBS were obtained from Sigma Immunochemicals (St. Louis, MO). Aphidicolin was diluted 1:50 in RPMI 1640 medium just before use (to

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200 μg/ml) and then added to microtiter well cultures to make a final concentration of 2.5–5 μg/ml, a dose in excess of that needed to arrest DNA synthesis (19). L-mimosine was used at 100–200 μM/liter. Cyclosporin A (CsA; Sandoz, Hanover, NH) stock was prepared at 10 mg/ml in ethanol and was stored at −20°C until use.

DNA Preparations and PCR. Total DNA for amplification was prepared as described (10). Integrated DNA and unintegrated DNA were prepared from cells extracted by the procedures of Hirt (20) and Chinsky (21). 1 μg of DNA from each sample was amplified using PCR conditions as published (10). The oligonucleotide primers used for SIV DNA detection were derived from the nucleotide sequence of SIV/Mac (15) and included (*a*) a primer pair specific for the LTR region RO1 position 61-84 (sense) and U51 228-251 (antisense), yielding an amplified 191-bp product; (*b*) a primer pair specific for the envelope region env10 7191-7211 (sense) and env12 7541-7561 (antisense), yielding an amplified 370-bp product; (*c*) a primer pair for the LTR-gag region S1 228-251 (sense) and S8 536-559 (antisense, yielding an amplified product of 330 bp; and (*d*) a pair of primers complementary to the human β-globin gene, PCO4 54-73, and GH20 195-176 (Perkin-Elmer Corp., Norwalk, CT), which amplifies a 268-bp product. Circular molecules of SIV DNA were detected using two rounds of PCR with nested primers. The first round was run with primers for the nef region (NEFP, 8841-8861, sense) and gag region (S8, 536-559, antisense). These primers amplify circles with one or two LTR. A sample dilution of the first round was used in the second round with primers inside the LTR (RO1/U51). One oligonucleotide of each complementary pair was 5′ end-labeled with [^{32}P]ATP, and the ^{32}P-labeled PCR products were analyzed by electrophoresis and visualized by autoradiography as described (10). The copy numbers of SIV included in the standard curve generally ranged from 30 to 3,000 copies in the reaction, and the individual standard curves used are indicated in the figures. In some experiments, the bands revealed by autoradiography were quantified using phosphorimager analysis; then the envelope signal (elongation)/LTR signal (initiation) ratio was determined as described (10).

Results and Discussion

Compared with unstimulated controls, resting macaque T cells stimulated with PMA, anti-CD3, or anti-CD3 plus anti-CD28 mAbs at the time of SIV infection had increased levels of SIV DNA initiation, as detected by R/U5 primers (10). However, the amount of R/U5 DNA detected after CD3 stimulation alone vs ligation of both CD3 and CD28 receptors was similar. Full-length viral DNA was detectable 6–9 h after infection in stimulated cells but not in unstimulated cells (10).

In a series of experiments, we monitored SIV DNA levels after T cell activation using primers for env and LTR-gag; as in our previous study (10), we detected little or no differences using primers to env vs LTR-gag. For this study, results using env are shown. To monitor the next step in virus replication, migration to the nucleus and integration, we used a PCR assay and primers that detect circular DNA because circularization of retroviral DNA only occurs in the nucleus (1, 3), and detection of circular DNA molecules is a con-

Table 1. *CD28 mAbs Overcome CsA-mediated Inhibition of SIV Elongation*

Stimulus*	CsA*	Ratio of env DNA/LTR DNA (percent of control) Experiment 1	Experiment 2
Anti-CD3	−	0.11	0.08
	+	0.04 (36)	0.03 (38)
Anti-CD3 + Anti-CD28	−	0.09	0.07
	+	0.07 (78)	0.05 (71)

* Dense macaque T cells were cultured for 24 h with FN18 anti-CD3 mAb alone or with 9.3 anti-CD28 mAb in the presence or absence of 1.0 μg/ml CsA. The ratio of env/LTR DNA was calculated as described (10).

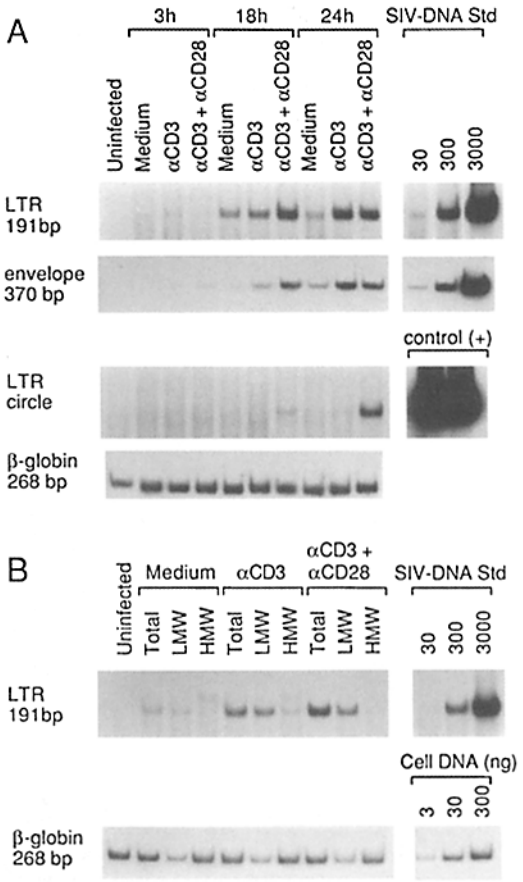

Figure 1. Resting T cells stimulated by ligation of CD3 and CD28 receptors at the time of SIV infection produce LTR circles. (*A*) Dense macaque T cells were infected at a multiplicity of infection of 0.01 tissue culture infectious dose of SIV/Mne and either were not stimulated (*Medium*) or were stimulated with 5 μg/ml FN18 anti-CD3 mAb alone (αCD3) or in combination with 20 μg/ml 9.3 anti-CD28 mAb (αCD3 + αCD28). After 3, 18, or 24 h, DNA was isolated from infected cells and levels of initiated SIV LTR, envelope or LTR circles were quantified by PCR as described in the Materials and Methods. β-globin and SIV DNA standards (*Std*) were run in parallel. Representative of four experiments. (*B*) In similar experiments at 24 h after infection, DNA was isolated from infected cell groups and separated by the Hirt method (19) into low molecular weight (*LMW*) and high molecular weight (*HMW*) fractions before PCR analysis of SIV LTR levels.

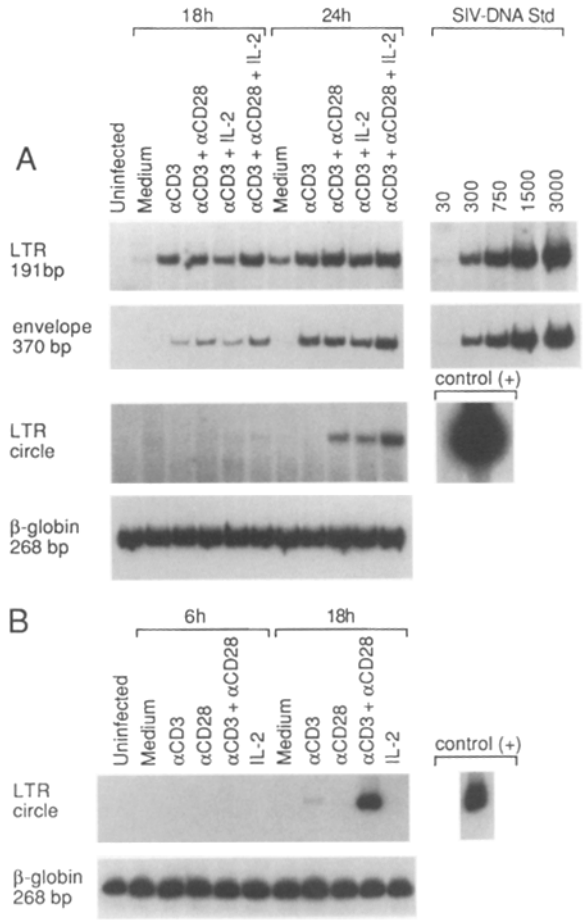

Figure 2. Either CD28 ligation or IL-2 induces the production of LTR circles in anti-CD3–activated T cells. (*A*) Dense macaque T cells were infected with SIV/Mne and either were not stimulated (*Medium*) or were stimulated with CD3 mAb alone as in Fig. 1, CD3 mAb + CD28 mAb as in Fig. 1, CD3 mAb plus 150 U/ml recombinant human IL-2 (αCD3 + IL-2), or CD3 mAb with both CD28 mAb and IL-2 (αCD3 + αCD28 + IL-2). After 18 or 24 h, initiated SIV, SIV envelope DNA, and SIV LTR circles were measured by PCR as described in Fig. 1. Representative of three experiments. (*B*) Similar experiments as in *A* illustrating that LTR circles are not induced in resting T cells 6 or 18 h after SIV infection by CD28 mAb only (*αCD28*) or with IL-2 only (*IL-2*).

venient method to monitor nuclear localization of viral DNA (2, 4–6).

As others have reported (8), we initially found that when we cultured T cells under conditions that drive them to proliferate rapidly, e.g., high doses of solid-phase bound CD3 mAb or combinations of solid-phase bound CD3 mAb plus either CD28 mAb or PMA, circular SIV DNA and integrated SIV DNA was detectable by 24 h after infection (data not shown). Low doses of CsA, a potent immunosuppressive agent, profoundly inhibit anti–CD3-dependent proliferation of resting macaque T cells and anti–CD3-induced elongation of viral DNA (10). Since CD28 induces a CsA-resistant signal in T cells (12), we tested whether CD28 ligation could overcome the inhibitory effect of CsA on CD3-induced SIV cDNA synthesis. Without CsA, CD3-stimulated T cells and T cells stimulated with CD3 plus CD28 mAb had similar levels of elongated SIV DNA (Table 1). However, CsA inhibited SIV elongation in CD3-stimulated T cells by an average 63%, but they only inhibited elongation by 25% in cells infected in the presence of both CD3 and CD28 mAb.

These initial experiments suggested that CD28 ligation may normally regulate immunodeficiency virus infection in activated T cells. To test this hypothesis, we infected resting T cells with SIV and cultured them with either medium only, soluble CD3 mAb only (which activates T cells but does not induce proliferation [11, 18]), or anti-CD3 plus anti-CD28 mAb for 3, 18, or 24 h before monitoring SIV DNA synthesis (Fig. 1 *A*). At 18 h after infection, cells stimulated with anti-CD3 plus anti-CD28 had more DNA initiation (LTR, 191 bp) and elongation into the envelope region than cells stimulated with CD3 mAb, but this difference was not evident by 24 h after infection. However, circular molecules of SIV DNA in the nucleus were detected only when T cells were co-stimulated with both CD3 and CD28 mAb (Fig. 1 *A*). Under these conditions of stimulation, 24 h after T cell activation, most or all the reversed transcribed viral genome was not integrated since, after separating genomic DNA into high molecular weight and low molecular weight fractions, we detected SIV DNA only in the low molecular weight fractions (Fig. 1 *B*). As noted previously (10), levels of initiated LTRs increased over time, even in resting T cells treated with medium, most likely because of low levels of spontaneous T cell activation over time. Reverse-transcribed viral genomes and LTR circles, however, were not detected in cells treated with medium only.

Ligation of CD28 on CD3-activated T cells increases IL-2 production and IL-2 mRNA levels (12). Therefore, we tested whether IL-2 could mimic the effect of CD28 ligation on circularization of SIV DNA; it could (Fig. 2 *A*). Again, at 24 h after infection, there were no significant differences in the elongation of SIV (envelope, 370 bp) in resting T cells stimulated with CD3 mAb with or without CD28 mAb or IL-2. Circular SIV molecules, however, were induced in anti-CD3–activated cells when either anti-CD28 or IL-2 was present (Fig. 2 *A*). Neither CD28 mAb nor IL-2 alone stimulates significant initiation or elongation of SIV (10) and, as expected, neither alone stimulated formation of LTR circles (Fig. 2 *B*).

To ascertain whether the CD28-induced formation of LTR circles was dependent on IL-2, we compared levels of envelope DNA sequences or LTR circles in resting T cells stimulated with CD3 and/or CD28 mAb in the presence of rabbit IgG anti–IL-2 vs IgG only (Fig. 3). Under conditions that had little or no effect on induction of genome reverse transcription detected with primers to the env region, anti–IL-2 dramatically reduced the amount of LTR circles synthesized in T cells that had been stimulated with both CD3 and CD28 ligation.

CD3 mAb with either CD28 mAb or IL-2 over time can induced macaque (18) or human (12, 13) T cells to divide. Therefore, it was possible that T cell proliferation might be required for SIV LTR circle formation. On the other hand, unlike with oncoretroviruses, passage through mitosis is not

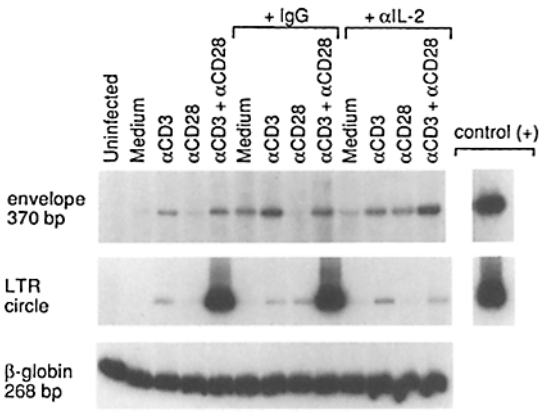

Figure 3. Anti-IL-2 antisera block the synthesis of SIV circular DNA induced by either CD28 costimulation of anti-CD3-activated T cells. Dense macaque T cells were infected with SIV/Mne and either were not stimulated or were stimulated as in Fig. 1 for 24 h, and then SIV envelope DNA and LTR circles were measured. Treatment of T cells with 100 μg/ml rabbit IgG (+ *IgG*) had no effect on envelope or LTR circles induced; anti-IL-2 (100 μg/ml) also had no effect on envelope DNA levels but significantly reduced LTR circles in T cells stimulated with both CD3 and CD28 mAb. One of three representative experiments.

required for HIV integration (22), and Bukrinsky et al. (2) found that an HIV-infected T cell line arrested in the G1/S stage of the cell cycle with aphidicolin still formed HIV LTR circles, implying that entry into the cell cycle is not necessary for LTR circle formation. To test if this was also the case for SIV, we stimulated resting macaque T cells in the presence of medium only, the G1 inhibitor mimosine, or the G1/S inhibitor Aphidicolin (23), and we measured levels of env or LTR circle DNA 18–24 h later. While mimosine blocked induction of new env or LTR circle DNA, aphidicolin, as previously shown for HIV-1 (2, 19), had no effect (data not shown). Therefore, IL-2–induced SIV LTR circle production is likely to be mediated by activation of processes preceding DNA synthesis. IL-2 binding to its high affinity receptors on T cells rapidly activates protein tyrosine kinases, the phosphatidylinositol 3-kinase, and the cyclin-dependent kinase (Cdk) 2–cyclin E complexes (13, 24, 25). IL-2 also induces the elimination of the Cdk inhibitor $p27^{Kip1}$ via a rapamycin-sensitive process (26). One or more of these or other IL-2–induced signals may be required for the establishment of a "reservoir" (5) of SIV or HIV in T lymphocytes. High levels of unintegrated retroviral DNA have been associated with the presence of HIV encephalitis (27). Our study focused on monitoring the early nuclear import of virus before detectable integration of proviruses (Fig. 1). Further studies will be necessary to clarify which IL-2–dependent signals or other lymphocyte signals regulate levels of unintegrated vs integrated viral DNAs.

Activation of T cells through CD3/TCR, CD28, or IL-2 receptors not only affects SIV or HIV genome reverse transcription and the transport of LTR circles to the nucleus, but it also can activate viral RNA transcription and production of mature virions (28–30). Thus, each phase of the viral life cycle in T cells is affected by lymphocyte activation signals. Agents such as CsA, rapamycin, or CTLA4.Ig fusion proteins that inhibit distinct steps in T cell activation may be worth considering in combination to block establishment of immunodeficiency virus infection and viral spread (10, 29).

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