crassa. A single DNA fragment in the *A* mating-type strains of *N. discreta, N. intermedia, N. sitophila*, and *N. tetrasperma* hybridized to the *A* probe from *N. crassa* (Fig. 4). Hybridization of the *N. crassa a*-specific probe to *A* and *a* genomic fragments could be detected only in the *a* genomes of the related heterothallic and pseudohomothallic species. Our data show that the two mating-type sequences are conserved among *Neurospora* heterothallic and pseudohomothallic species, although there is some variability of restriction sites within and around the mating type–specific regions. As in *N. crassa*, there is only a single copy of a mating-type sequence in any haploid, homokaryotic strain of the related heterothallic and pseudohomothallic species, and no unexpressed copy of the opposite mating type.

Six homothallic strains, denominated as five species, have been placed in the genus *Neurospora* (*1, 12*). Haploid ascospore isolates from any of these species are capable of completing the sexual cycle. In the homothallic yeasts, mating occurs by the rapid switching of the **a** or α cassette to the active locus by a transposition event (*10*). To determine whether such a switching mechanism could also be responsible for homothallism in the genus *Neurospora*, we isolated DNA from the six homothallic strains and probed restriction digests at high and low stringency with the cloned nonhomologous segments of the *A* and *a* mating-type regions of *N. crassa*. Five homothallic strains contained fragments that hybridize only with the *A*-specific *N. crassa* mating-type probe (Fig. 5A); no hybridization to *a*-specific sequences was detected (Fig. 5B). In all of these homothallic species the size of the fragment that hybridizes to the *A*-specific probe of *N. crassa* is identical. Thus, in five of the six homothallic strains all the events involved in sexual development can occur with the genetic information associated with only the *A* mating type of heterothallic species; the products of the *a* and *A* genes are not both required for sexual development. The genome of the sixth homothallic strain, *N. terricola*, contains a segment that hybridizes to the *A*-specific probe and a second segment that hybridizes to the *a*-specific probe (Fig. 5, A and B). However, unlike the situation in yeast, there is only one copy of each mating type–specific sequence. This suggests that homothallism may function by two different mechanisms in the genus *Neurospora*, neither of which involves mating-type interconversion. In addition, our findings suggest that expression of *A* may be sufficient to activate the genes or gene products necessary for perithecium formation and meiosis in at least some homothallic strains.

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HIV-1–Infected T Cells Show a Selective Signaling Defect After Perturbation of CD3/Antigen Receptor

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The binding of antigen or monoclonal antibody to the T cell receptor for antigen or the closely associated CD3 complex causes increases in the concentration of intracellular ionized calcium and subsequent cell proliferation. By measuring second messenger production in primary cultures of human immunodeficiency virus (HIV-1)–infected T cells stimulated with monoclonal antibodies specific for either CD3 or CD2, a specific impairment of membrane signaling was revealed. The HIV-1–infected T cells were unable to mobilize Ca^{2+} after stimulation with anti-CD3, whereas CD2-induced calcium mobilization remained intact. Furthermore, the HIV-1–infected cells proliferated poorly after CD3 stimulation, although the cells retained normal DNA synthesis in response to interleukin-2 stimulation. These results show that the signals initiated by CD2 and CD3 can be regulated independently within the same T cell; uncoupling of signal transduction after antigen-specific stimulation provides a biochemical mechanism to explain, in part, the profound immunodeficiency of patients with HIV-1 infection.

THE MOST CONSPICUOUS ABNORMALITY associated with HIV-1 infection is a quantitative deficiency of the CD4 helper/inducer T cell (*1*). The CD4 cell is a major source of lymphokines and is important for most immunologic functions, including interactions with nonlymphoid cells. Among the immunologic defects associated with HIV-1 infections are cutaneous anergy and decreased T cell proliferative responses after stimulation by soluble antigens such as tetanus toxoid (*1*). This T cell defect appears to be qualitative in that immunologic unresponsiveness in HIV-1–seropositive patients can occur despite normal numbers of circulating CD4 T lymphocytes (*2*). We have investigated early transmembrane signaling events in an effort to further understand the basis for the impaired response of HIV-1–infected T cells to antigen.

In T lymphocytes, the binding of monoclonal antibody (mAb) to the CD3 complex mimics activation via the antigen receptor, resulting in the production of inositol 1,4,5-trisphosphate (IP_3), increased intracellular ionized Ca^{2+} concentration ($[Ca^{2+}]_i$), and

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subsequent proliferation (*3*). An antigen-independent pathway of T cell activation involving the CD2 (E rosette receptor) molecule has also been described (*3–5*). The binding of the ligand LFA-3, CD2 mAbs, or sheep erythrocytes to the CD2 molecule results in a cascade of biochemical events similar or identical to the events initiated through the CD3 pathway (*4, 5*).

Purified $CD4^+$ T cells from normal donors were infected with HIV-1 and maintained in interleukin-2 (IL-2)–supplemented medium for up to 60 days (*6*). Uninfected $CD4^+$ T cells were maintained under identical culture conditions. The time course of HIV-1 replication and the effects of HIV-1 on cell surface antigen expression were similar to previous reports (*7*). Cells from the HIV-1–infected and uninfected cultures were periodically analyzed for CD2- and CD3-induced calcium mobilization by using the fluorescence indicator indo-1 in conjunction with flow cytometry (*8*). This procedure, which allows the simultaneous measurement of $[Ca^{2+}]_i$ and membrane antigen fluoresence of single cells, is more sensitive than previous techniques that used quin2. Infection of primary cultures of CD4 cells results in a noncytopathic persistent infection (*7*); the infected cells show decreased surface expression of CD4, presumably because of the formation of cytoplasmic CD4-gp120 complexes, but they continue to produce mRNA for CD4 and retain normal expression of the CD2 and CD3 antigens (*7, 9*). In addition, a subpopulation of $CD8^+$ T cells emerges in primary cultures of highly enriched $CD4^+$ cells after infection with HIV-1 (*7*). Thus, we analyzed only the $CD4^+$ T cells or the $CD4^-$ $CD8^-$ T cells (Fig. 1).

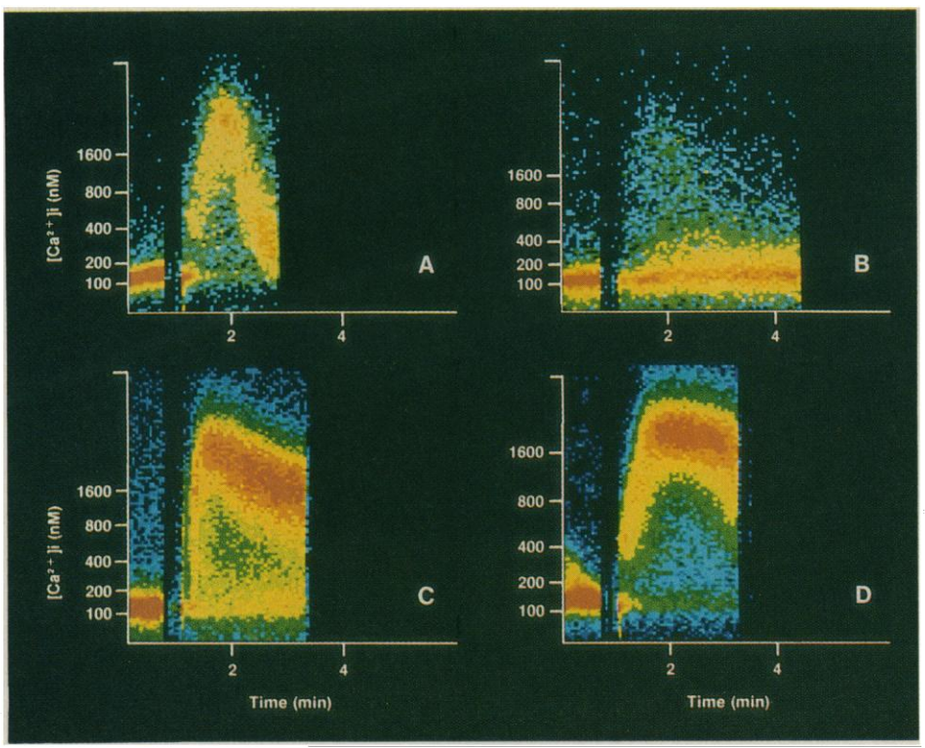

Fig. 1. Measurement of $[Ca^{2+}]_i$ in uninfected (**A** and **C**) and HIV-1–infected (**B** and **D**) T cells. $CD4^+$ T cells (98% pure) were isolated from peripheral blood lymphocytes obtained from normal donors by negative selection with magnetic particles coated with CD8-, CD11-, CD16-, and CD20-specific mAbs (*18*). On day 19 after infection, HIV-1–infected cells (B and D) or uninfected cells from companion cultures (A and C) were incubated with the acetoxymethyl ester of indo-1, stained with phycoerythrin (PE)-conjugated CD8 mAb, and stimulated with optimal amounts of CD3 mAb (A and B) or CD2 mAb (C and D) (*19*). The cells were analyzed at ~250 cells per second by means of a dual-laser fluorescence-activated cell sorter as previously described (*8*). The indo-1 ratio of violet and blue fluorescence (directly proportional to $[Ca^{2+}]_i$) was digitally calculated in real time for each individual cell and is displayed on the *y*-axis versus time on the *x*-axis. We excluded PE-fluorescent cells from analysis by electronic gating, taking advantage of the fact that in the peripheral blood, the $CD4^+$ and $CD8^+$ T cells represent reciprocal, nonoverlapping subsets of CD2-positive T cells (*20*). The results are displayed on a 100 × 100 pixel grid on which the number of cells per pixel is represented by a 16-color progression according to an exponential function where 0 to 1 cells are assigned a color of black, 2 to 10 cells shades of blue, 11 to 80 cells shades of green, and 81 to 600 cells shades of yellow to red. The gap in the analysis occurred when the CD3 or CD2 mAb was added to the cell suspension.

On day 19 of culture, the T cells from the HIV-1–infected culture (Fig. 1B) had a severely impaired response after CD3 stimulation compared to the control cells (Fig. 1A). More than 95% of the control cells responded to CD3 stimulation with the peak mean $[Ca^{2+}]_i$ reaching ~1.6 μM, whereas only 20% of the HIV-1–infected cells had a $[Ca^{2+}]_i$ that was >0.2 μM. The impaired response to CD3 stimulation appeared to be generalized in the HIV-1–infected CD4 cells, because the response of <5% of cells reached the mean response of the uninfected cells, suggesting that no subpopulation of $CD8^-$ T cells was resistant to HIV-1. In contrast, the response of HIV-1–infected cells was nearly normal after CD2 stimulation when compared to control cells (Fig. 1, C and D). The basal $[Ca^{2+}]_i$ was

Table 1. The effects of HIV-1 infection of $CD4^+$ T cells on cell surface antigen expression during a 58-day experiment. Purified CD4 T cells were activated with CD3 mAb (day −3), infected with HIV-1 (day 0), and propagated in IL-2 (*6*). On day 42 after infection, cells were restimulated with immobilized CD3 mAb for 2 days and harvested, and culture was continued in IL-2–supplemented medium. The mean fluorescence intensity of the cell surface antigen and the percentage of positive cells of 5000 cells analyzed were monitored with FITC-conjugated mAbs (Leu3a, Leu4, Leu5b, WT31, Becton Dickinson; T8, Coulter Immunology) and flow cytometry. Similar results were obtained in three independent experiments. UNF, uninfected; HIV-1, HIV-1–infected; ND, not done.

Cell surface antigen expression	Percent positive (mean fluorescence intensity)									
	4 days		11 days		21 days		25 days		58 days	
	UNF	HIV-1	UNF	HIV-1	UNF	HIV-1	UNF	HIV-1	UNF	HIV-1
CD2	96 (109)	95 (107)	98 (129)	95 (131)	99 (140)	99 (138)	98 (131)	98 (132)	97 (122)	97 (138)
CD3	95 (122)	98 (116)	99 (140)	99 (144)	99 (152)	99 (156)	99 (156)	99 (155)	99 (153)	99 (158)
CD4	96 (117)	95 (114)	75 (102)	28 (58)	90 (136)	7 (41)	92 (132)	4 (37)	78 (123)	2 (36)
CD8	4 (37)	5 (37)	8 (46)	13 (52)	10 (50)	24 (67)	12 (58)	16 (58)	22 (65)	36 (86)
TCR α/β	94 (106)	91 (105)	ND	ND	94 (140)	93 (120)	97 (131)	94 (116)	95 (130)	30 (75)

identical in control or HIV-1–infected cells, and T cells from HIV-1–infected and uninfected cultures responded equally well (>2000 n*M*) to the calcium ionophore ionomycin, further indicating that impaired viability could not explain the defective response of HIV-1–infected cells to CD3 stimulation.

We determined whether the impaired response after CD3 stimulation was qualitative or quantitative. Cells from the control culture responded to CD3 mAb in a concentration-dependent manner, with the mean maximum $[Ca^{2+}]_i$ reaching a plateau of 2000 n*M* after stimulation with more than 1 μg/ml of CD3 mAb G19-4 (Fig. 2). In contrast, 1 μg/ml of CD3 mAb stimulated only a 200-n*M* increase in $[Ca^{2+}]_i$ in the HIV-1–infected cells 26 days after infection. Stimulation of HIV-1–infected cells with an optimal concentration of CD2 mAb resulted in a Ca^{2+} response that was comparable to that seen in control cells. At suboptimal amounts of CD2 mAb, the response of infected cells was only slightly impaired. Thus, the impaired response of HIV-1–infected cells to stimulation by CD3 is not due to a shift in the concentration dependence, and both peak and net calcium mobilization are impaired after CD3 stimulation.

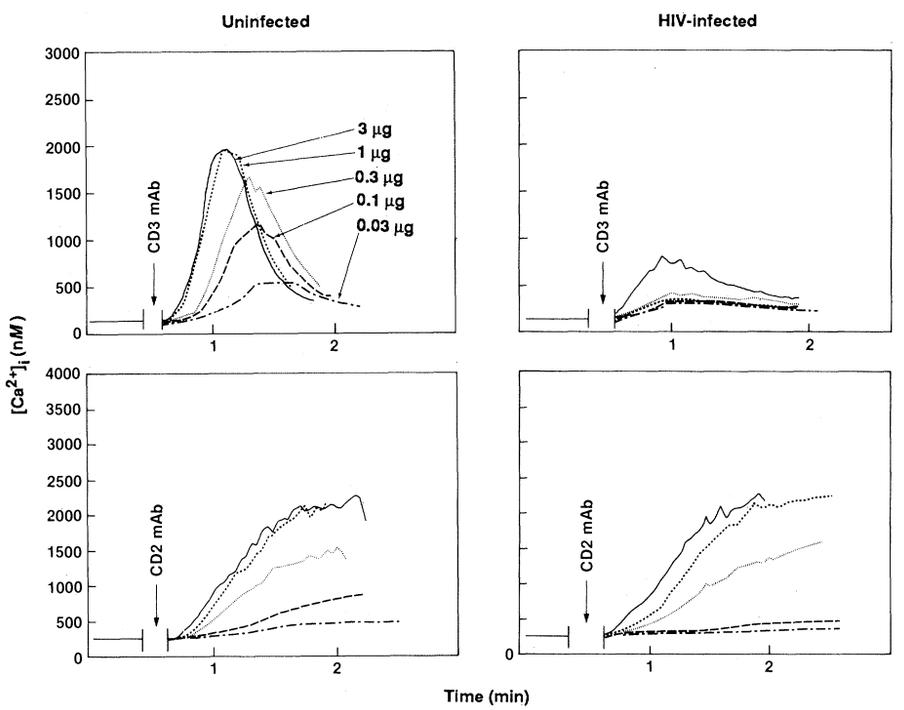

Fig. 2. Relation between calcium signaling and CD2 or CD3 mAb concentration in uninfected and HIV-1–infected CD4 cells (day 26 after infection). Cells were loaded with indo-1, stained with PE-conjugated CD8 mAb, and analyzed as described in Fig. 1. PE-fluorescent ($CD8^+$) cells were excluded from analysis by electronic gating. After measuring the basal $[Ca^{2+}]_i$, half-log dilutions of CD3 or CD2 mAbs were added at the indicated concentration. The indo-1 fluorescence ratio at 404 to 485 nm was individually stored for each cell, and subsequently, the mean $[Ca^{2+}]_i$ was plotted against time. Each of the data points represents the mean $[Ca^{2+}]_i$ value of ~1000 cells analyzed during the 4 seconds that constitute each data point and, therefore, intra-assay estimates of error about the mean are not displayed. Data are representative of more than 100 assays from four independent experiments.

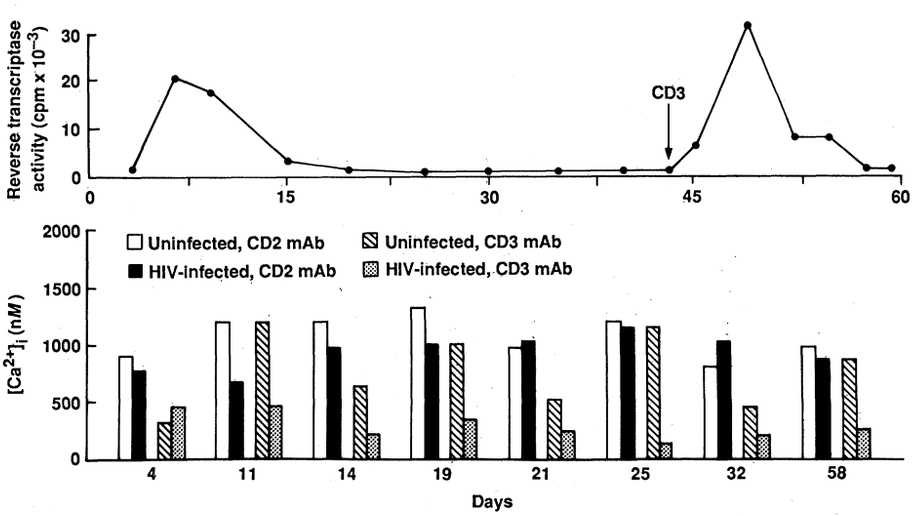

Fig. 3. The time course of RT activity and CD2- and CD3-induced $[Ca^{2+}]_i$ signaling after infection with HIV-1. The data are derived from the experiment described in the legend to Table 1. Reverse transcriptase assays of cell-free supernatants were performed in duplicate as described (7). The mean maximal $[Ca^{2+}]_i$ response of T cells that are not CD8-positive from HIV-1–infected or companion control culture was determined after optimal stimulation by CD2 or CD3 mAbs as described in the legends to Figs. 1 and 2. Similar results were obtained in three independent experiments.

Cultures were monitored for 58 days after HIV-1 infection for the following: (i) expression of lymphocyte-specific cell surface antigens by flow cytometry, (ii) HIV-1 expression by reverse transcriptase (RT) assays, and (iii) calcium signaling after stimulation by CD3 or CD2 mAbs. Peak expression of HIV-1 occurred 7 to 12 days after infection as monitored by RT assay (Fig. 3) and the presence of virally induced cytopathic effects. On day 8 after infection, approximately 40% of cells in the infected culture had detectable cytoplasmic HIV-1 antigen as shown by staining of cells fixed in methanol and acetone with a virus-specific p17 (*gag*) mAb and counting positive cells by fluorescence microscopy. By day 30, RT was no longer detectable in the HIV-1–infected cultures. On day 4 of culture, the $[Ca^{2+}]_i$ response of HIV-1–infected cells was normal (Fig. 3). Defective calcium signaling was first observed 8 to 12 days after infection with HIV-1, and the onset of this defect was strongly associated with modulation of the CD4 molecule (Table 1). During the peak of virus replication, the CD2 response of infected cells is also inhibited (day 11); however, as the RT activity declined the response to CD2 became similar to that of uninfected cells (*10*). In contrast, the $[Ca^{2+}]_i$ response of HIV-1–infected cells to CD3 mAb was impaired at day 11 and remained low throughout the 58-day culture period.

On day 42 of culture all cells from each group were restimulated with immobilized CD3 mAb so that we could continue propagation of the cultures in IL-2 and test for latent viral infection (7). That the cells harbored latent virus was shown by the second peak of RT activity at 6 days after restimulation (Fig. 3). The results shown in Figs. 1 to 3 indicate that the calcium transients of cells after stimulation by CD3 are severely im-

paired after HIV-1 infection, whereas the response to CD2 remains intact, and that this defect persists as long as the cells can be maintained in culture (*11*).

Analysis of surface antigens revealed that the HIV-1–infected cells had begun to modulate CD4 by day 11, and by day 25 of culture, only 4% of the infected cells remained CD4-positive (Table 1). Of interest, CD3 and CD2 expression were not affected in the HIV-1–infected cell population, indicating that the defective transmembrane signaling was probably not due to decreased amounts of the antigens on the plasma membrane. On day 58 only 30% of the HIV-1–infected cells were positive for α/β T cell receptor (TCR) while 66% of the cells from the HIV-1–infected culture expressed the δ chain of the TCR (*12, 13*).

At various times after HIV-1 infection, T cells from HIV-1–infected and uninfected cultures were assayed for their ability to initiate DNA synthesis in response to CD3 mAb or recombinant human IL-2 (Table 2). Cells from the HIV-1–infected culture proliferated poorly in response to CD3 stimulation compared to control T cells. It is remarkable that HIV-1–infected T cells responded normally to IL-2, indicating that the ability of HIV-1–infected cells to synthesize DNA is not impaired in response to a stimulus that acts late in the cell cycle (*14*), and therefore, certain activation pathways appear to remain intact.

The above results indicate that HIV-1 infection has a profound effect on calcium transients after CD3 stimulation of primary cultures of CD4 cells. These studies, although technically more demanding, were done on normal lymphocytes rather than on leukemic T cell lines that can differ in the requirements for cell activation and in the regulation of cell calcium levels. Therefore, it is likely that these results are more relevant to the pathogenesis of HIV-1–induced immune deficiency, although an inherent weakness of these studies is that we were unable to study synchronized infections in cloned cells. The effects of HIV-1 on normal lymphocytes provides the first indication that calcium transients after CD2 and CD3 stimulation might be regulated independently within a single cell. The contribution of the differences in the TCR expression to the CD3 signaling defect requires further study. Initial reports suggest that second-messenger generation in α/β T cells is indistinguishable from γ/δ T cells after CD3 stimulation (*15*).

Several recent reports suggest that the CD4 molecule may serve to enhance or inhibit CD3-induced signaling (*16*). CD4 appears to be the cell receptor for gp120, a product of the *env* gene, and the expression of CD4 antigen is characteristically down regulated in HIV-1–infected T cells (*7*), at least in part by the formation of HIV-1 envelope–CD4 cytoplasmic complexes (*9*). Thus, the present results suggest that the loss of CD4 may cause the uncoupling of CD3 from signal transduction and, furthermore, that CD4 is required for efficient signal transduction after CD3 stimulation and not after CD2 stimulation.

The contribution of the CD3-specific signaling defect to the overall state of immunosuppression in patients with acquired immunodeficiency syndrome (AIDS) remains to be clarified. In preliminary experiments with CD4 cells from patients with AIDS-related complex (ARC) or with AIDS, we have also observed impaired transmembrane signaling (*17*). The present demonstration of a qualitative defect in antigen-specific signaling in HIV-1–infected T cells raises the possibility that immune responses may be restored with effective antiviral therapy in patients infected with HIV-1.

Table 2. Effects of HIV-1 infection on cells stimulated with mAb to CD3 or exposed to IL-2. On day 14 after infection, $CD4^+$ HIV-1–infected or control cells were cultured in quadruplicate (5×10^4 viable cells per well) in flat-bottom wells in RPMI 1640 plus 10% fetal bovine serum. The cells were stimulated with either recombinant human IL-2 (Amgen) or CD3 mAb G19-4 that had been immobilized to the culture well. Thymidine incorporation (arithmetic mean ± 1 SD) was determined on day 3 of culture.

Stimulus	Thymidine incorporation (cpm × 10^{-3})	
	Uninfected	HIV-1–infected
CD3		
100 ng	44.0 (±3.8)	3.5 (±0.6)
50 ng	8.6 (±5.5)	1.0 (±0.2)
25 ng	3.4 (±1.6)	0.8 (±0.1)
12 ng	1.2 (±0.4)	0.9 (±0.2)
rIL-2		
2 U/ml	61.6 (±17.1)	101.0 (±7.2)
1 U/ml	50.2 (± 9.8)	95.5 (±1.6)
0.25 U/ml	41.3 (± 3.3)	54.6 (±7.1)

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6. Peripheral blood lymphocytes (PBL) obtained from healthy, HIV-1–seronegative donors were separated on Ficoll-Hypaque gradients, and purified CD4 T cells were obtained as described (*18*). After activation of the cells with immobilized CD3 mAb G19-4 for 3 days, the cells were collected, washed, infected with HIV-1 (LAV-1 isolate at a multiplicity of infection of ~0.001 for 2 hours at 37°C), and maintained in medium supplemented with IL-2 (Electronucleonics). LAV-1, originally characterized by F. Barré-Sinoussi *et al.* [*Science* **220**, 868 (1983)], was provided by T. M. Folks. Uninfected CD4 cells were maintained under identical culture conditions.
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10. It is likely that impaired cell viability explains the impaired response of HIV-1–infected cells to both CD2 and CD3 on day 11; viability of HIV-1–infected cells estimated by trypan blue exclusion was ~60% during the period of maximal RT release into the culture supernatant. Viability was >95% in both HIV-1–infected and control cultures after day 15 of infection. In addition, effects on cell viability are minimized in the calcium assay because indo-1 only loads into viable cells.
11. There was substantial unexplained day-to-day variation in the magnitude of the calcium transients after CD3 stimulation, probably because of the use of different preparations of CD3 mAb. However, the data in Table 1 represent the optimal calcium signal of uninfected and HIV-1–infected cells, and for each day depicted in Table 1, a dose-response evaluation was done as shown in Fig. 2. The impairment of CD3 response would thus appear more impressive if the results after stimulation by suboptimal amounts of anti-CD3 were shown.
12. Expression of the TCR α/β heterodimer was monitored with the WT31 mAb that binds to a nonpolymorphic determinant of the β chain and with δ-1 mAb that binds a nonpolymorphic determinant of the δ chain (*13*). Since expression of α/β TCR and γ/δ TCR are mutually exclusive (*13*), on day 58 in the HIV-1–infected culture, ~30% of the cells were $CD4^-$ $CD8^+$ α/β TCR^+, ~65% were $CD4^-$ $CD8^-$ γ/δ TCR^+, and ~5% of the cells were surface or cytoplasmic $CD4^+$ $CD8^-$ α/β TCR^+.
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19. The following azide-free murine mAbs were used for experiments to study $[Ca^{2+}]_i$ of cells. Anti-CD3 mAb (38.1, immunoglobulin M) was sterile-filtered before use. For CD2 stimulation, mAbs 9.6 and 9-1 were used as previously described by June *et al.* (*5*).
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