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PENETRATION OF CD4 T CELLS BY HIV-1

The CD4 Receptor Does not Internalize with HIV, and CD4-Related Signal Transduction Events Are not Required for Entry

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Receptor binding of HIV to the CD4 molecule is required for efficient infection of T cells, but the post-binding steps that result in penetration of HIV are not well understood. CD4 is induced to internalize upon T cell activation, and mAb to CD4 modify signal transduction and T cell activation as does HIV in some systems. It is not known whether HIV binding triggers CD4 endocytosis or whether signal transduction events are required for penetration. Selected inhibitors of signal transduction were evaluated for their effects on penetration using two assays that are dependent on penetration. After short term exposure to inhibitor and HIV, cells were analyzed for reverse-transcribed HIV DNA (DNA amplification assay), or productive infection is monitored (infectivity assay). Viral penetration was tested in the presence of H7 (protein kinase C inhibition), EGTA (extracellular Ca^{2+} chelation), cyclosporine A (inhibition of Ca^{2+}/calmodulin-dependent activation), or pertussis toxin (inhibition of G protein function). All agents were used at concentrations that were inhibitory for their respective signal transduction pathways. None of the inhibitors affected viral penetration. We tracked the CD4 molecule with fluorescent probes that do not interfere with HIV binding in a system where CD4 T cells were saturated with HIV and the penetration event was relatively synchronized. Under conditions where detection of CD4 was more sensitive than the detection of HIV, HIV internalization was readily detected but CD4 internalization was not.

The HIV infects CD4 T cells, but details of the events that occur after attachment and before reverse transcription of viral RNA into DNA remain largely unknown. Enveloped viruses enter cells either by direct fusion of viral and cell membranes or indirectly by endocytosis. In the endosome, the low pH environment favors fusion and penetration into the cytoplasm. It is likely that a given virus uses one or the other mechanism or that both occur, but only one results in successful viral replication. In the case of HIV, electron micrographic studies have not been particularly helpful, and one can find evidence supporting either mode of entry (1, 2). In thorough studies, Stein et al. (2) and McClure et al. (3) could not rule out the occurrence of endocytosis but did show that the low pH of the endosome is not required for entry. The observation that opposing membranes of HIV-infected cells and uninfected CD4 cells bind and fuse with each other at neutral pH supports the direct fusion model, providing the mechanism of cell-cell fusion is analogous to that of penetration by cell-free HIV. Nevertheless, the endocytosis mechanism has a certain appeal in that the receptor for HIV, CD4, has been shown to undergo endocytosis subsequent to T cell activation (4, 5), a phenomenon dependent on PKC[2] activation and phosphorylation of the cytoplasmic segment of CD4 (6, 7). HIV has been reported to induce phosphorylation of CD4 via a PKC-dependent pathway, and inhibitors of PKC markedly diminish HIV replication in vitro (8, 9). The former finding is controversial (10), and the latter does not distinguish a requirement for PKC activation during penetration vs other steps in viral replication. Moreover, experiments with cells that express mutated CD4 molecules indicate that the capacity for PKC-induced phosphorylation and internalization of CD4 is not necessary for infection (6, 11). However, it remains possible that HIV induces internalization of CD4 independent of any requirement for PKC-dependent phosphorylation. Independent ligand-induced and PKC-dependent pathways of internalization have been described for the receptor of epidermal growth factor (12).

We tracked the CD4 molecule in a system where CD4 T cells were saturated with HIV and the penetration event was relatively synchronized. In addition, we explored the possibility that some CD4-associated or secondary signaling event is required for entry. Selected inhibitors of signal transduction were tested for their effects on penetration using two assays. Appropriate controls for effects on other steps in viral infection were performed. We find no evidence that the CD4 molecule is internalized with HIV nor does penetration require or involve PKC activation, extracellular Ca^{2+} influx, CsA-sensitive, and Ca^{2+}-dependent activation events, or pertussis toxin-sen-

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[2] Abbreviations used in this paper: PKC, protein kinase C; CsA, cyclosporine A; PCR, polymerase chain reaction; TPA, 12-O-tetradecanoylphorbol 13-acetate.

sitive G protein activation, although some of the inhibitors affected later stages of viral replication.

MATERIALS AND METHODS

Monoclonal antibodies and soluble CD4. The mAb OKT3, OKT4, OKT4a, OKT8, and their FITC conjugates were obtained from Ortho Diagnostics (Raritan, NJ). Leu 3a and its FITC and phycoerythrin conjugates were obtained from Becton Dickinson (Sunnyvale, CA). Polyclonal FITC-conjugated anti-mouse Ig was from Caltag (South San Francisco, CA) and monoclonal FITC-conjugated anti-mouse Ig was from Pandex (Mundelein, IL). For all experiments where cells were cultured after reaction with mAb, the mAb were dialyzed against three changes of PBS before use. Soluble CD4 was obtained from Progenics Therapeutics (Tarrytown, NY).

Cells and cell lines. PBL from healthy HIV-seronegative donors were obtained from the mononuclear cell fraction of whole blood by Ficoll-Hypaque sedimentation. PHA-stimulated lymphoblasts were obtained by culturing PBL (2×10^6/ml) in RPMI medium (GIBCO, Grand Island, NY) containing 10% v/v FCS (GIBCO), 100 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine with 10 μg/ml PHA (Difco, Detroit, MI) for 3 days. The E5 subline of CEM (13) was maintained in RPMI, 10% FCS, with penicillin, streptomycin, and L-glutamine.

Infectivity assays and HIV preparations. The microculture system for the titration of infectious HIV has been described in detail (14). Briefly, 1×10^6 PBL, 1×10^6 PHA-stimulated PBL, or 5×10^5 CEM cells are incubated with serial 10-fold dilutions of an HIV-containing culture supernate (LAV prototype) for periods of time as indicated in text, washed by centrifugation, trypsinized (as indicated), and dispensed into 10 μl containing 0.25 ml culture medium. For CEM cells, culture medium is the RPMI/10% FCS/penicillin/streptomycin/L-glutamine medium described above. For PHA-stimulated PBL, the same medium was supplemented with 10% v/v IL-2 (Cellular Products, Buffalo, NY). For unstimulated PBL, three media were used. All were antibiotic/L-glutamine-supplemented RPMI. The inoculation medium contained 10% autologous serum. The initial plating medium contained 10% FCS, 10% IL-2, and 10 μg/ml PHA, and the same medium, minus PHA, was used for feeding. Every 4 days, 100 μl of supernate were removed and replaced with fresh medium. Culture supernates from day 8 and 12 of culture were tested for HIV Ag by an ELISA Ag capture assay (14). A culture supernate is considered positive for HIV if the OD reading is more than the mean + 3 SD of 10 control cultures (cultures that are not inoculated with HIV and are included on every microculture plate). The ID-50 titer is the reciprocal of the HIV dilution at which 50% of the cultures are positive (14).

Culture supernate from bulk culture of PHA-stimulated PBL was the source of HIV for infectivity titrations. Culture supernate was harvested at day 6 to 7 of culture, centrifuged at $300 \times g$ to remove cells, centrifuged at $1500 \times g$ for 20 min, and stored in 0.5 ml volumes at −70°C. For some experiments, the virus-containing supernate was dialyzed against three changes of PBS at 4°C overnight.

A concentrated preparation of HIV (HB2 strain) was obtained from Hillcrest Biologicals (Cypress, CA). Its protein content was determined by the Bradford method (15) using bovine albumin as standard (Quantatest, Quantimetrics, Hawthorne, CA).

Trypsin treatment. Cells to be treated with trypsin were pelleted by centrifugation (1×10^6 cells/tube), and resuspended in 0.5 ml PBS containing 0.5 mM EDTA in an ice bath. An equal volume of freshly diluted trypsin stock (ICN, Cleveland, OH) in PBS/EDTA was added, and the tubes were moved to a 37°C water bath for exactly 15 min. The tubes were returned to the ice bath, and 2.0 ml of ice-cold medium containing 7.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) was added. The cells were pelleted by centrifugation and resuspended in medium. In all experiments, the result of trypsin treatment was assessed by cytofluorography with OKT4a mAb (trypsin-sensitive epitope of CD4) and OKT4 mAb (trypsin-resistant epitope of CD4) (16). Cell loss from the procedure was 10 to 30%. Because of variation in the effectiveness of and sensitivity to trypsin treatment (particularly for PHA-stimulated PBL), we predetermined the optimal dose for each batch of cells on the day they were used. Portions of the cells were treated with 0.2, 0.1, 0.05, and 0.025% final concentrations of trypsin, and cell recovery and CD4 epitope density was determined. For the ID-50 experiments, this was done while the rest of the cells were incubating with HIV (the first 4-h incubation).

Predetermination of inhibitor dose. Ten-fold dilutions of the phorbol ester TPA (Sigma) beginning at 1 μg/ml were incubated with PBL, PHA-stimulated PBL, or CEM cells (1×10^6/ml). After 1, 2, 4, 8, and 18 h of incubation at 37°C, cell surface CD4 density was determined by cytofluorography. A 100 ng/ml dose was selected, and this dose resulted in maximal down modulation of CD4 on PBL at 4 h and on CEM cells at 18 h.

The PKC inhibitor H7 and related isoquinoline derivatives, HA1004, H8, and H9 (Seikagaku America, St. Petersburg, FL), were tested at 1, 20, and 50 μM by adding them to cells 15 min before addition of TPA and determining CD4 density after 4 h (PBL and PHA-stimulated PBL) or 18 h (CEM). The 50-μM dose of H7 prevented TPA-induced CD4 down modulation whereas this dose of HA1004, H8, or H9 had no effect.

Medium containing 8 mM EGTA had no detectable free Ca^{2+} (<1 nM) before or after incubation with cells as judged by its failure to enhance the fluorescence emission spectrum of fluo-3, a Ca^{2+}-dependent fluor.

CsA (Sandoz, Hanover, NH) was tested for inhibition of proliferation by PBL in response to PHA (5 and 10 μg/ml), Con A (12.5, 25, and 50 μg/ml), and OKT3 mAb (50 ng/ml). Triplicate microcultures (1×10^5 cells in 0.2 ml) were incubated with 0.75, 1.5, 3, and 6 μg/ml CsA for 30 min before addition of mitogen or OKT3. After 4 h, one set of triplicate cultures was washed and resuspended in fresh medium. The other set was not washed. Cultures were incubated for 3 days at 37°C in a 5% CO_2 humidified incubator. Proliferative responses were measured after a 4-h pulse with 1 μCi ^{3}H-thymidine (New England Nuclear, Boston, MA). A dose of 3 μg CsA per ml was selected as it reduced the proliferative responses by greater than 95% for all culture conditions. A stock 1000-fold concentration was prepared in ethanol. Control cultures received the same amount of ethanol diluent.

Pertussis toxin (Calbiochem, LaJolla, CA) was incubated with cells in medium at 25, 50, 100, 400, and 800 ng/ml for 24 h. To test for completeness of ADP ribosylation, an ADP ribosylation assay was performed on membrane fractions in the presence of ^{32}P NAD and additional toxin. ^{32}P ADP-ribosylated products were analyzed by PAGE as described (17, 18). Further ADP-ribosylation was undetectable in cells preincubated with any of the doses tested. For infectivity experiments, the toxin was used at 100 ng/ml.

Cytochalasin E (Sigma) was used at 10 μg/ml. This concentration effectively prevented mAb-induced shedding of CD4 (assay described in *Results*). Colcemid (GIBCO) was used at 1 μg/ml, the concentration routinely used for metaphase arrest by the CDC genetics lab. NH_4Cl concentration (10 mM) was the same as that used by Stein et al. (2). Preincubations with cells were for 30 min.

Infectivity assay using PCR amplification of HIV DNA. PHA-stimulated PBL (3×10^6) were preincubated with inhibitors (concentrations and time indicated above) and incubated with 10 ml of an HIV inoculum at 37°C for 4 h. The multiplicity of infection, defined as ID_{50} U/cell, was 1 to 10. The HIV inoculum was preincubated at 37°C for 1 h with 23 U/ml DNAse I (Boehringer Mannheim, Indianapolis, IN) and the same concentrations of inhibitors were added to the inoculum before addition to cells. The cells were incubated 4 h at 37°C, washed twice, lysed in 0.5 ml buffer containing 50 mM KCl, 10 mM Tris, pH 8.3, 6 mM $MgCl_2$, 1 mg/ml gelatin, 0.45% Tween-20, 0.45% Nonidet P-40, and 6 μg/ml proteinase K (Boehringer Mannheim), and incubated at 56°C for 1 h, followed by a 10-min incubation at 95°C. A 30-μl sample (1.1 μg DNA) was amplified in 35 cycles by PCR (Perkin-Elmer-Cetus, Norwalk, CT) as described (19, 20). Primers were from the LTR sequence of the LAV isolate and correspond to base numbers 120 to 149 (+ strand) and 306 to 335 (− strand) (HIV-1-BRU sequence, Los Alamos data base) (21). Amplified DNA was electrophoresed in 1.2% agarose gels, transblotted to a filter (Genescreen, Du Pont, Wilmington, DE), and the amplified 216-base DNA fragment was detected using previously described methods (19, 20). The oligonucleotide detection probe was 5′-end labeled with ^{32}P using a commercial kit (Boehringer Mannheim) and corresponds to sequence numbers 177 to 196 (21).

Measurement of CD4 and HIV density after exposure to HIV or TPA. PBL, PHA-stimulated PBL, or CEM cells were preincubated with a concentrated preparation of HIV (20 μl containing 5 μg HIV/5 × 10^5 cells), with a high titer HIV containing supernate (2 ml/5 × 10^5 cells), with the phorbol ester TPA (Sigma) (100 ng/ml; 1×10^6 cells/ml), or with culture medium (volume and cell density the same as for the particular HIV preparation) for 1 h at 0°C. A portion of the HIV-preincubated preparation was washed once in ice-cold medium, and resuspended in medium to its original volume.

At 0 time, the cell preparations were moved to a 37°C water bath in a 37°C incubator. At various time intervals, a volume of cell suspension containing 5×10^5 cells was removed and added to a tube containing ice-cold diluent and wash buffer. Diluent and wash buffer is PBS, pH 7.2 to 7.4, containing 0.1% BSA, 2% v/v AB+ human serum, and 0.1% NaN_3. The cells were pelleted, and cell-surface CD4 or bound HIV were detected by immunofluorescence and cytofluorometry as previously described (13).

In some experiments, cells were prestained with saturating amounts of OKT4 mAb followed by FITC-conjugated antimouse Ig

mAb at 0°C. The cells were then processed for incubation with HIV, TPA, or medium as above. At various time intervals, two volumes containing 5×10^5 cells were removed and added to tubes containing ice-cold PBS, pH 7.2, or a low pH buffer (0.01 M citrate, 0.15 M NaCl, pH 3.0). The cells were washed once in PBS, fixed, and analyzed by cytofluorometry. Parallel measurements of fluorescence retention were obtained by fluorometry of cell lysates. Cells were lysed in PBS, pH 8.5, containing 1% Triton X-100. Fluorescence emission at 520 nm (excitation, 490 nm) was measured with a Perkin-Elmer LS-5 fluorometer.

Arbitrary units of CD4 density or HIV density were obtained by integration of the fluorescence histogram (10,000 cells) of cells stained with OKT4 mAb followed by FITC-conjugated anti-mouse Ig or of HIV-incubated cells stained with FITC-conjugated anti-HIV (24) and subtraction of the integrated background (buffer followed by FITC anti-mouse Ig or control cells stained with FITC anti-HIV). The FITC-conjugated human IgG anti-HIV reagent reacts with all major HIV proteins (14). It does not react with uninfected cells, either in suspension or after fixation for cytoplasmic staining (14); nor does it react with infected cells after absorbtion with cell-free HIV (13). Relative CD4 density or HIV density was calculated by dividing the density value at different time points by the respective 0 time density value. Linear-linear plots of cell number vs fluorescence intensity were used for these calculations; linear-log plots are shown in text. Parallel results were obtained when mean fluorescence intensity was used for calculation rather than integrated histograms.

RESULTS

Assay for penetration. A quantitative infectivity assay was developed whose readout is dependent on binding and penetration over a short culture period. During this time, potential inhibitors of penetration are tested. After the exposure, inhibitor and free virus are removed by washing the cells and virus that has bound (but not penetrated) is removed by trypsin treatment of the cells. Thus, subsequent replication of HIV is dependent on virus that penetrated during exposure to the potential inhibitor. The assay is performed as follows. Cells are preincubated with test agent, serial dilutions of HIV are added, and incubation is continued for 4 h. The cells are then washed and trypsinized to remove free and bound virus so that subsequent replication is dependent on HIV that has already penetrated. The cells are then incubated another 4 h in media during which a second set of cultures (previously incubated with HIV but not with agent) are incubated with agent. This "delayed addition control" differs from test cultures only in the sequence of exposure to HIV and inhibitor and serves as a control for residual effects of inhibitor on later steps in viral replication. The cells are then washed, plated in microculture, and HIV replication is monitored by Ag capture assay 8 and 12 days later. The following experiments are presented to support the basis for this assay. We show that trypsin treatment strips cell surface-bound HIV and that HIV binds but does not penetrate at 0°C. Using the 0°C incubation with HIV as a model for blocked penetration, we demonstrate the necessity of trypsin treatment for detecting blocked penetration and assess the sensitivity of the assay.

PBL were incubated with HIV, trypsin treated, and cell surface HIV and CD4 were detected by cytofluorometry. Under the conditions used, trypsin removes cell-bound HIV and the OKT4a epitope of CD4 but leaves the OKT4 epitope intact (Fig. 1). Trypsin also renders cell-free HIV noninfectious (22).

We determined that HIV binds but does not penetrate at 0°C. If cells are incubated with HIV at 0°C and trypsinized before plating in microculture, infectivity is orders of magnitude lower than that of cells incubated at 0°C but not trypsinized (Fig. 2*a*). Thus, the 0°C incubation allows HIV to bind, but it remains trypsin accessible. Note the necessity for trypsin treatment. Unless removed from the cell surface, virus that accumulates on the cells at 0°C may penetrate when placed in microculture at 37°C (resulting in ID_{50} titers only 1 log lower than cells incubated with HIV at 37°C).

There is little difference in infectivity for cells incubated with virus at 37°C whether they are trypsinized or not. Penetration must occur rapidly after binding at 37°C because, if there were a significant period of time between virus binding and penetration, we should have detected a difference in infectivity between trypsin-treated and untreated cells incubated at 37°C (Fig. 2*a*). To estimate the rate of penetration, we preincubated cells with HIV for 4 h at 0°C, and the temperature was raised to 37°C. Penetration was allowed to proceed until stopped at intervals by removal of cells to ice-cold buffer and trypsin treatment (Fig. 2*b*). In this protocol, penetration should limit the kinetics (because most binding has already occurred). Compared to the experiment in Figure 2*a* where kinetics are likely limited by binding, penetration kinetics appear to be more rapid (Fig. 2*b*). Although the kinetics of the infectivity assay appear to be limited by binding rather than penetration, the assay readout is dependent on the sequential occurrence of both. Considering the conditions of exposure to virus in this assay, it should not be surprising that binding kinetics rather than penetration kinetics appear to limit the assay readout. The forward rate of most ligand-receptor interactions is diffusion limited and therefore concentration dependent, and the endpoint of the ID_{50} assay measures virus replication after exposure to limiting concentrations of virus. To achieve a maximal ID_{50} determination with a given HIV inoculum, an 18- to 24-hour incubation is required. We selected a suboptimal incubation time (4 h) so that the assay would be sensitive to agents that perturb or alter the kinetics of initial exposure to virus.

To estimate the reduction in binding/penetration required to perturb the assay, we incubated sets of cells with HIV dilutions for a total of 4 h before trypsinizing and plating in microculture. Initial incubation was at 37°C, and at various times the cells were moved to 0°C to stop further penetration. Thus, total incubation time was the same but the opportunity for penetration was varied. A two-log reduction in ID_{50} titer resulted when penetration time (time at 37°C) was one quarter that of control (Fig. 2*c*).

Effect of inhibitors on HIV infectivity. We selected inhibitors based on their effects on pathways that may be involved in CD4 mobility or CD4-related signal transduction. To screen for effects on penetration, the infectivity assay described above was used. All compounds were tested at least twice using PHA-stimulated PBL. All compounds were also tested on the CD4 T cell line CEM and unstimulated PBL (usually twice). The latter were freshly prepared Ficoll-Hypaque separated PBL cultured in autologous serum. They were stimulated (with PHA and IL-2) in microculture after exposure to HIV and inhibitor. Results of repeated experiments with each compound were similar for all cell types, and statistical analysis was performed on the combined data (Wilcoxin rank sum test for paired data). Representative experiments are presented in Table I. A 50-μM concentration of the PKC

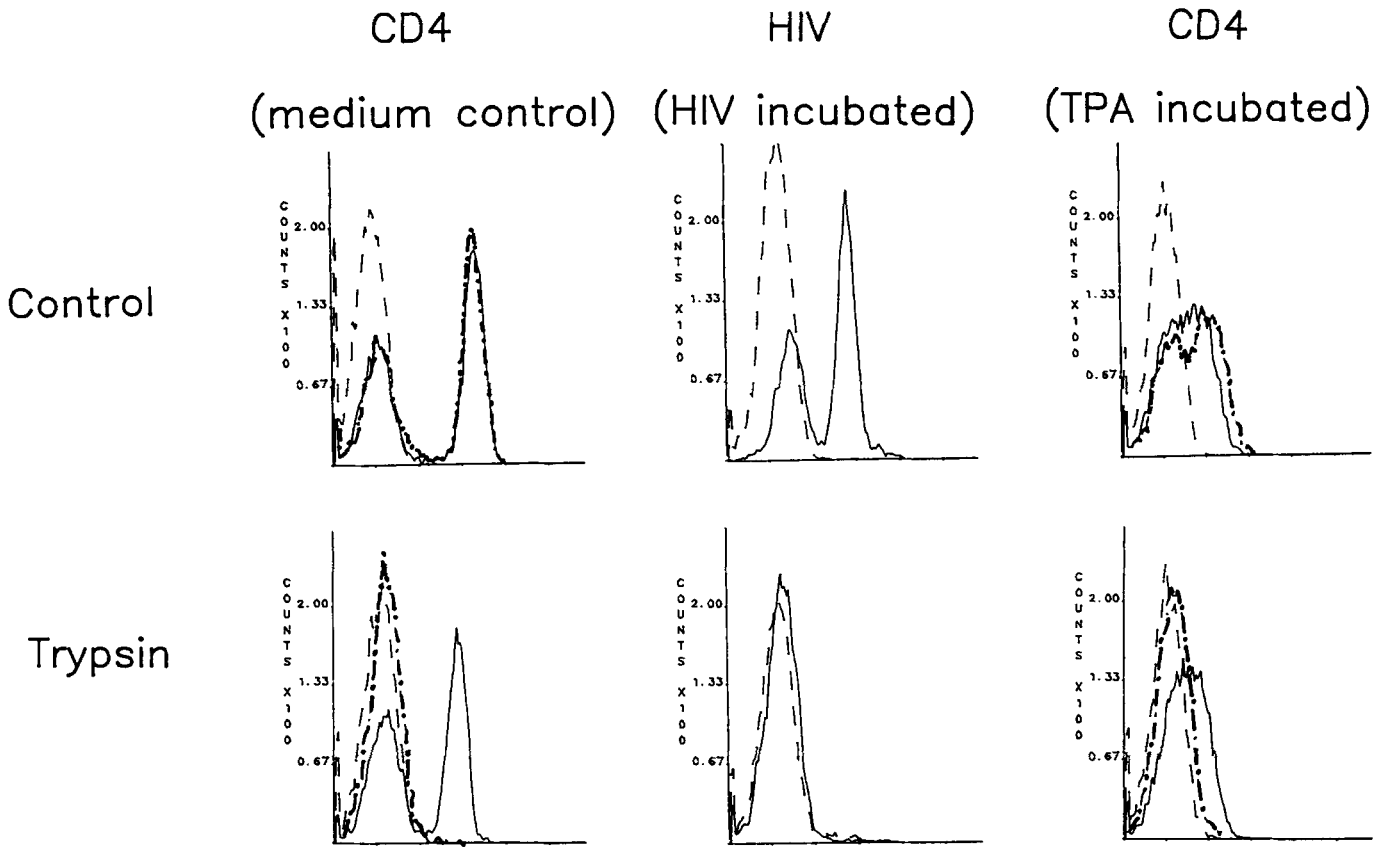

Figure 1. Trypsinization of PBL. PBL were incubated with medium (*left*), with medium containing HIV (*middle*), or with medium containing TPA (*right*) for 2 h at 37°C. The cells were washed and half were treated with trypsin as per text. Cells were stained by indirect immunofluorescence for cell surface CD4 using OKT4 mAb (——) or OKT4a mAb (– ● –) (*left* and *right panels*) and for bound HIV (——) (*middle panels*) and analyzed by cytofluorometry. *Dashed lines* represent background (reagent control) immunofluorescence.

inhibitor H7, in doses sufficient to prevent TPA-induced down modulation of CD4, did not inhibit infection nor did equimolar amounts of HA1004 (a related isoquinoline derivative with relatively weak inhibitory activity for PKC but greater activity for other kinases) (23). No effect on infectivity was found with 8 mM EGTA (a concentration that effectivity chelates extracellular Ca^{2+}), with 3 μg/ml cyclosporin A (a concentration that inhibits PHA-, Con A- and OKT3 mAb-induced proliferation), with pertussis toxin (a concentration resulting in complete ADP-ribosylation), or 10 μg/ml cytochalasin E (a concentration that prevents the mAb-induced shedding of CD4 described below).

Effect of inhibitors on detection of HIV DNA. The earliest event after HIV internalization that can be detected is the reverse transcription of HIV RNA to unintegrated DNA. Accordingly, cells were incubated with HIV for 4 h in the presence or absence of the various inhibitors; total cellular DNA was harvested; and HIV DNA sequences were amplified by PCR and detected by Southern blot hybridization (Fig. 3). An HIV DNA signal was obtained in cells after a 4-h incubation with HIV. It was not obtained in cells that are negative for CD4 (e.g., HSB-2, (24)) nor in cells not exposed to HIV, and the signal was absent or barely detectable in cells pretreated with OKT4a mAb. The HIV inoculum had to be pretreated with DNAse because all sources of HIV we have tested contain some HIV-related DNA and, despite washing the cells, this can contaminate the final cellular DNA preparation.

Cells incubated with HIV in the presence of the inhibitors, H7, HA1004, EGTA, CsA, cytochalasin E, colcemid, NH_4Cl, and pertussis toxin registered a positive signal (Fig. 3). These results are concordant with those obtained with the infectivity assay. We interpret as negative the absence of signal or a signal intensity less than that of OKT4a-treated cells that are included in each run. We have not found the variation in relative intensities of positive signals to be meaningful. Although positivity and negativity have been completely reproducible in the several runs we have performed with each of the inhibitors, the variation in relative intensity of the positive signals is not.

HIV binding and entry do not induce down modulation of CD4 expression. A saturating concentration of HIV was allowed to bind and accumulate on PHA-stimulated PBL at 0°C for 1 h. Sufficient HIV was used to prevent the binding of OKT4a mAb. The temperature was raised to 37°C, and the cell surface distribution of CD4 was monitored over time using the OKT4 mAb (Figs. 4*a* and 5). OKT4 mAb and HIV bind to distinct regions of CD4, thus permitting the detection of CD4 in the presence of HIV (compare Fig. 5, *a* and *c*). The CD4 density remained unchanged in HIV-preincubated cells (Figs. 4*a*, and 5, *c* and *g*). The same results were obtained with fresh PBL or CEM cells and with a high titer HIV culture supernate rather than the concentrated HIV preparation (data not shown).

In cells incubated in the continuous presence of HIV, HIV density remained unchanged (Fig. 4*b*) whereas HIV density diminished in cells that were washed after prein-

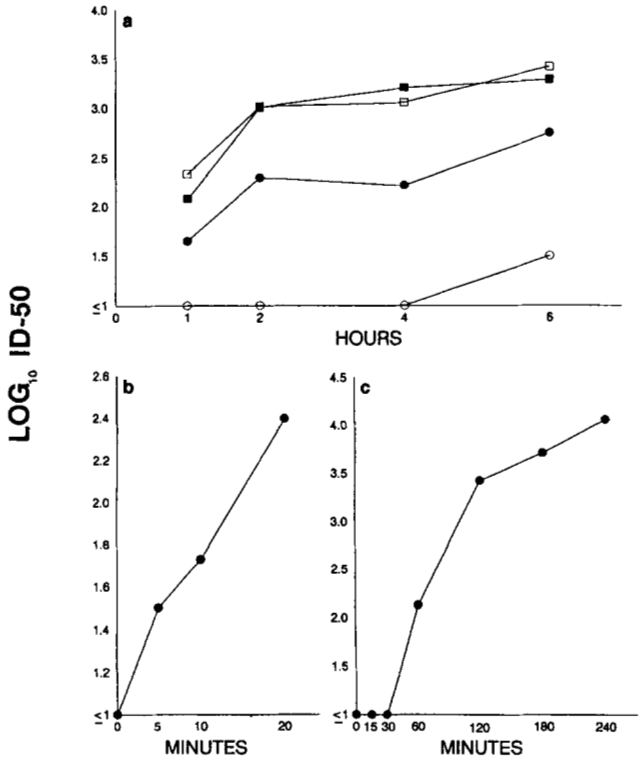

Figure 2. Infectivity assay. ID_{50} titers were determined in PHA-stimulated PBL under different conditions of exposure to an HIV inoculum (10-fold dilutions of HIV; 10 cultures per dilution). *a*, Cells were exposed to HIV at 37 or 0°C. At the indicated times, the cells were washed, either trypsinized or not trypsinized, and plated in microculture; 37°C—no trypsin (■———■); 37°C—trypsin (□———□); 0°C—no trypsin (●———●); 0°C—trypsin (○———○). *b*, Cells were exposed to HIV at 0°C for 4 h. At 0 time, the cells were washed and moved to a 37°C incubator. At the indicated times, the cells were diluted in ice-cold buffer, washed, trypsinized, and plated in microculture. *c*, Cells were initially incubated with HIV at 37°C. At the indicated times, the cells were moved to 0°C and incubation continued for a total of 4 h. The cells were then washed, trypsinized, and plated in microculture.

cubation with HIV (Figs. 4*b*, and 5, *d* and *h*). The diminished density of HIV on the cell surface may reflect shedding of HIV, but a portion of it was due to penetration of HIV. HIV-incubated cells were treated with trypsin such that cell-surface HIV was removed and not detected by cytofluorometry. Intracellular HIV was detected in acid-alcohol fixed cell preparations by immunofluorescence microscopy. We used two types of virus preparations: a highly concentrated particulate viral preparation rendered free of soluble viral proteins by ultracentrifugation and an unmanipulated high-titer, virus-containing culture supernate. Although we cannot determine the proportion of infectious to noninfectious particles in our virus stocks, both preparations result in detectable penetration under the conditions of exposure that we used. We could not detect intracellular CD4 in the same trypsin-stripped cell preparations that contained detectable intracellular HIV (using CD4 mAb that react with trypsin-sensitive epitopes of CD4 and do not interfere with HIV binding, e.g., OKT4E and OKT4C).

Assay for detection of internalized CD4. Because we could not reliably detect internalized CD4 after trypsin stripping and fixation of cells, an alternate assay was developed. This involved prelabelling CD4 with a fluorescence probe and tracking the signal. Total cell associated fluorescence was distinguished from internal fluorescence by removing the cell-surface bound probe. Cells were prestained at 0°C with OKT4 mAb followed by FITC-conjugated antimouse Ig mAb. This combination of reagents does not interfere with HIV binding or infectivity (data not shown). The FITC signal is completely removed by washing the cells once in pH 3.0 citrate-saline buffer. If cells are prestained with these reagents at 0°C and then cultured in media at 37°C, there is a gradual increase in the low pH-resistant signal. These changes do not occur in prestained cells maintained in media at 0°C. These kinetics are greatly accelerated in the presence of TPA (Fig. 6). It would have been simpler to use directly FITC-labeled OKT4 mAb. Unfortunately, FITC-OKT4 mAb bound to cells can not be eluted by low pH buffer (nor is it susceptible to trypsin). Thus the low pH elution of signal we obtain in the indirectly stained cells is due to breaking the FITC-antimouse Ig:OKT4 bond rather than

TABLE I

Effect of inhibitors on HIV infectivity[a]

Cell Type:/Inhibitor Inhibitor/Cells	HIV Titer:Log_{10} ID_{50}		
	Inhibitor present during HIV exposure	Inhibitor present after HIV exposure	No inhibitor present
PHA-stimulated PBL			
H7[b] (50 μM)	4.06	3.98	3.87
HA1004[b] (50 μM)	4.07	4.07	3.87
EGTA (8 mM)	2.91	2.43	3.23
Cyclosporin A (3 μg/ml)	2.90	3.25	3.43
Cytochalasin E (10 μg/ml)	2.63	2.89	3.42
Pertussis toxin[c] (100 ng/ml)	2.71	2.55	2.50
Soluble CD4 (20 μg/ml)	<1.00	3.91	2.89
Unstimulated PBL[d]			
H7[b] (50 μM)	1.37	1.35	1.29
HA1004[b] (50 μM)	1.38	1.40	1.29
EGTA (8 mM)	1.44	2.19	2.27
Cyclosporin A (3 μg/ml)	1.25	1.00	1.00
Cytochalasin E (10 μg/ml)	2.15	2.11	2.38
Pertussis toxin[c] (100 ng/ml)	1.87	1.94	2.01
Soluble CD4 (20 μg/ml)	<1.00	2.10	1.67

[a] See text for details. Cells were incubated for 4 h with serial 10-fold dilution of HIV, washed, trypsinized, incubated 4 h, washed, and plated in microculture. Inhibitor was added to one set of cells before and during the first 4-h incubation. One set of cells was incubated with inhibitor during the second 4-h incubation; and one set was not incubated with inhibitor.

[b] H7 and HA1004 were tested in the same experiment.

[c] For pertussis toxin, the two incubations were for 24 h instead of 4 h.

[d] Cells were stimulated with PHA and IL-2 in microculture after the two 4-h incubations.

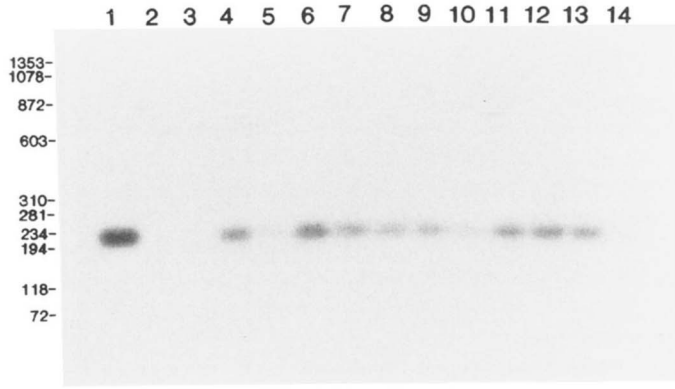

Figure 3. Detection of amplified HIV DNA in cells 4 h after exposure to HIV and various inhibitors. PHA-stimulated PBL (3×10^6) were preincubated with various inhibitors (time and concentration as indicated in text and Table I), and these concentrations were maintained during a 4-h incubation with a DNAse I-treated HIV inoculum (10 ml; ID-50 titer 10^6). The cells were washed and processed for amplification of a 216 base region of HIV DNA by PCR: *lane 1*, amplified HIV plasmid DNA; *lane 2*, buffer control (no cells); *lane 3*, negative control (cells incubated in medium); *lane 4*, positive control (cells incubated with HIV, no inhibitor); *lane 5*, OKT4a mAb pretreated cells; *lane 6*, H7; *lane 7*, HA1004; *lane 8*, EGTA; *lane 9*, CsA; *lane 10*, cytochalasin E; *lane 11*, colcemid; *lane 12*, NH_4Cl; *lane 13*, pertussis toxin; *lane 14*, 10% DMSO.

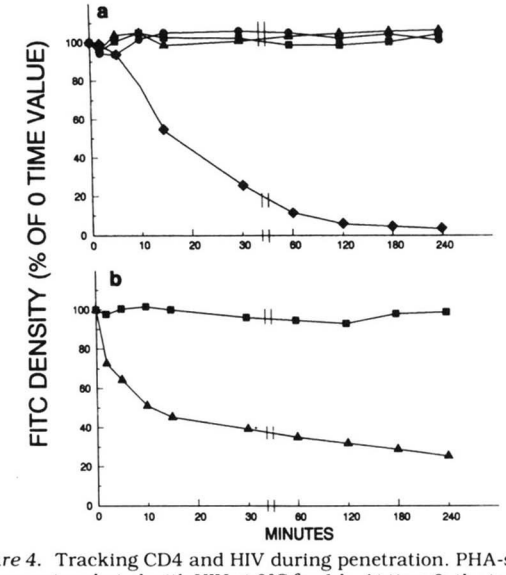

Figure 4. Tracking CD4 and HIV during penetration. PHA-stimulated PBL were preincubated with HIV at 0°C for 1 h. At time 0, the temperature was raised to 37°C, and CD4 density or HIV density was monitored over time by cytofluorometry: media control, (●——●); HIV preincubated cells that were washed free of unbound HIV at time 0, (▲——▲); HIV preincubated cells where HIV was left in media for the duration of the experiment, (■——■); cells incubated with 100 ng/ml TPA, (♦——♦). In *a*, cells were removed at various times to ice-cold buffer and stained for CD4 by indirect immunofluorescence. In *b*, the HIV-preincubated cells from the same experiment as *a* were stained for surface HIV.

the OKT4:CD4 bond. Direct conjugates of Leu 3a mAb (FITC and phycoerythrin) or OKT4A (FITC) are readily eluted by low pH buffer, but could not be used because they block HIV binding. The kinetics of CD4 internalization were identical when we compared the indirect labeling procedure with directly conjugated Leu 3a or OKT4a. Internalization was about twice as rapid in PHA-stimulated PBL as in PBL. The uptake of signal is not inhibited or perturbed in the presence of any of the following reagents: 50 μM H7; 1 μg/ml colcemid; 10 μg/ml cytochalasin E; or 10 mM NH_4Cl.

We considered the possibility that the slow uptake of CD4 with time at 37°C, as measured by cytofluorometric readings of low pH-resistant signal, may be an underestimate of uptake. If the signal coalesces or enters a low pH compartment of the cell, some quenching of signal may occur. We performed parallel readings of fluorescence by cytofluorometry and by fluorometry of cell lysates (cells lysed in PBS containing 1% Triton X-100, pH 8.5). The uptakes determined by both methods were the same.

In cells that were prestained and washed in neutral buffer, there is a dramatic decline in total fluorescence intensity (internal plus cell surface signal) (Fig. 6). This is due to loss of CD4 from the membrane (not just loss of the FITC antimouse Ig signal). Neither restaining the cells nor washing the cells with low pH buffer followed by restaining restored the intensity to that of cells that were held at 0°C or of cells that were cultured at 37°C but not prestained. Fluorometric readings of cell lysates also indicate that this is due to loss of signal from the cells rather than due to quenching of signal. The decline in total fluorescence was inhibited by cytochalasin E (10 μg/ml) but not by H7 (50 μM), colcemid (1 μg/ml), or NH_4Cl (10 mM). Fluorescence microscopic examination of wet mounts of the cells revealed linear surface staining in cells stained at 0°C that became speckled with time at 37°C but polar caps were rarely seen. TPA-treated cells developed three to four clumps of fluorescence per cell.

HIV does not induce internalization of CD4. Cells were prestained with OKT4 mAb and FITC anti-mouse Ig mAb, pretreated with saturating amounts of HIV at 0°C, and the uptake of CD4 (low pH-resistant signal) and total CD4 fluorescence (cells washed in neutral buffer) were monitored at 37°C. Saturating amounts of HIV did not perturb the kinetics of CD4 uptake or the kinetics of CD4 shedding (Fig. 6). Duplicate determinations of the slow internalization of CD4 were performed on 11 pairs of control specimens on different occasions. If uptake is expressed as a percent of total FITC density at time 0, the uptake values rose to 6 to 7% at 2 h. The difference in this value between duplicates was not more than 2% (average difference ~SD = 0.42 ~0.66%). The uptake in HIV-incubated cells did not differ from the uptake in control cells by more than this value (Fig. 6).

DISCUSSION

We used two assays to screen for potential inhibitors of penetration. Neither assay directly detects penetration, but both assays are dependent on penetration. In the PCR assay, a small number of cells are exposed to a large amount of virus and reverse transcribed viral DNA is detected. It is the earliest event that can be detected after penetration, and has the additional advantage that inhibitors affecting later stages of viral replication would not affect the assay. However, the infectivity assay registers productive viral replication, a much later event, resulting from exposure to limiting amounts of virus. Special measures are required to ensure that the opportunity for penetration occurs only during exposure to inhibitor, and controls for residual effects of inhibitor on later steps in replication (delayed addition controls) are necessary. Agents that act at a step proximate to penetration would affect both assays, for instance, agents that inactivate or disrupt the virus or that inhibit virus binding. Separate experiments are required to determine if these steps are involved. The assays should complement each other, and concordant results were obtained with all the agents we

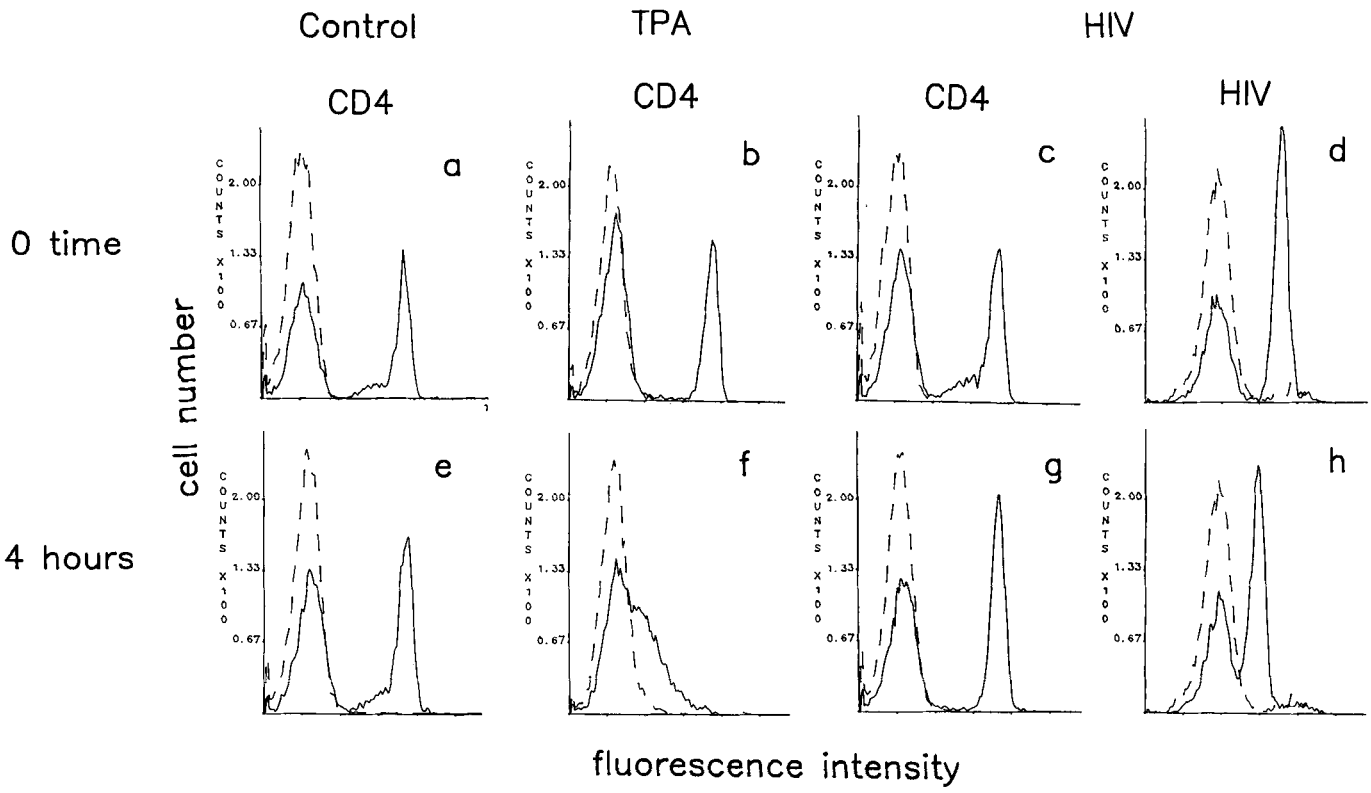

Figure 5. Tracking CD4 and HIV during penetration. Cytofluorographs representative of the 0- and 4-h time points for the experiment described in Figure 4 are shown. Cell-surface CD4 density (*a, b, c, e, f,* and *g*) and HIV density (*d* and *g*) was determined by indirect immunofluorescence on medium-incubated cells (control), TPA-incubated cells, and cells preincubated for 1 h at 0°C with HIV. *Dotted lines* are background (reagent control) immunofluorescence.

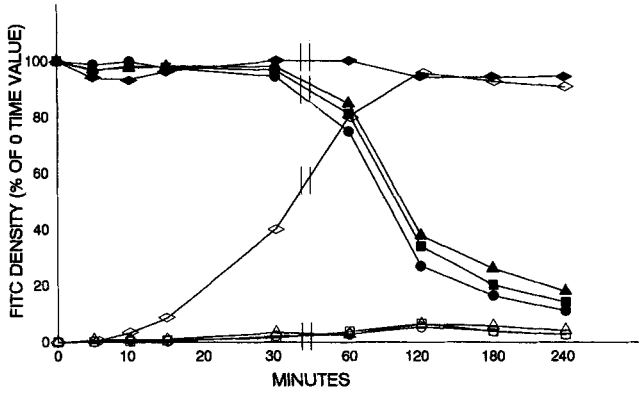

Figure 6. Tracking CD4 during HIV penetration in cells prelabeled with OKT4 mAb. PHA-stimulated PBL were prestained with OKT4 mAb and FITC anti-mouse Ig mAb at 0°C. Portions were then preincubated for 1 h at 0°C with media, with HIV, or with TPA and at 0 time, the temperature was raised to 37°C. At various times, cells were removed and washed in ice-cold neutral buffer (*closed symbols*) or ice-cold low pH buffer (*open symbols*) and analyzed by cytofluorometry: media control (●——●); HIV preincubated cells that were washed free of unbound HIV at time 0, (▲——▲); HIV preincubated cells where HIV was left in media for the duration of the experiment, (■——■); cells incubated with 100 ng/ml TPA, (♦——♦).

tested. If discordant results are obtained, the use of the two assays may give some indication of the mechanism of inhibition. For instance, a class of reagents that inhibit reverse transcription would register inhibition in the PCR assay but would not necessarily affect the infectivity assay, providing the inhibition was reversible. As an example, we have found that 10% DMSO abolishes reverse transcriptase activity in a cell-free assay. DMSO (10%) reproducibly results in a very weak or no PCR signal (Fig. 1) but registered no effect in the infectivity assay (data not shown).

The rationale for the choice of inhibitors was based on observations related to the movement of CD4 and its apparent role in normal immune responses and signal transduction. CD4 is thought to function with the CD3:TCR complex as a coreceptor in associative recognition of class II MHC structures on APC (25–32). There is also evidence for a direct physical association of CD4 with the CD3:TCR complex on the same cell (33) and of CD4 with class II MHC on opposing cells (7, 32). HIV and purified gp120 have been shown to mimic the effect of CD4 mAb on CD4 T cell responses in vitro, but it is unlikely that CD3, the TCR, or class II MHC participate directly in infection because cells lacking these structures can be readily infected, and mAb to these structures do not prevent infection (13, 24, 34). Upon T cell activation, CD4 undergoes internalization, a process that is dependent on PKC activation, requires an intact cytoplasmic segment of CD4, and is associated with phosphorylation of the cytoplasmic segment of CD4 (4–7). HIV or gp120 have been reported to induce PKC-dependent phosphorylation of CD4 by some investigators (8) but not by others (10). Inhibition of PKC activity, sufficient to prevent phorbol ester-induced CD4 internalization, did not prevent penetration in our two assays (Table I; Fig. 3). These results are consistent with previous work by us and others using CD4 constructs that are incapable of PKC-dependent phosphorylation and internalization (6, 11). Although not required for the penetration phase of infection, PKC activity is required for some later step in productive viral infection. The continuous presence of

H7 in culture, in our work, abrogates productive viral infection as previously reported by others (8, 9). Cytochalasin E inhibited the shedding of CD4 induced by CD4 mAb. However, HIV does not induce this type of CD4 mobility (Fig. 4*a*), nor does this inhibitor affect HIV infectivity (Table I; Fig. 3). In some systems, CD4 mAb inhibit the influx of extracellular Ca^{2+} that occurs after stimulation of the TCR (35), and HIV infection of CD4 cells results in diminished CD4 expression and impaired Ca^{2+} influx (36). HIV gp120 has been reported to induce Ca^{2+} influx in CD4 cells either directly (37) or to block Ca^{2+} influx after cross-linking of gp120 with antibody (38). Effective chelation of extracellular Ca^{2+} did not impair penetration nor did CsA (Table I; Fig. 3). This latter compound does not inhibit Ca^{2+} influx per se; rather, CsA reacts with the Ca^{2+}-binding protein calmodulin and inhibits biochemical pathways that are dependent on Ca^{2+} mobilization (39, 40). Although CD4 may be involved in Ca^{2+} mobilization, and this process may be affected by HIV, neither influx of extracellular Ca^{2+} nor events dependent on Ca^{2+} mobilization appear to be involved or required for penetration. Recently, the cytoplasmic segment of CD4 has been shown to be physically associated with a T cell-specific tyrosine-protein kinase denoted lck (41–43). We did not examine involvement of this pathway. Nevertheless, it is unlikely that the CD4:lck enzyme interaction is required for penetration because cells expressing CD4 but lacking lck can be readily infected (24, 43). Two additional inhibitors were examined even though there is no precedent for suspecting involvement of the pathways they inhibit. The deacidifying agent NH_4Cl did not inhibit penetration as shown in more detailed studies by Stein et al. (2) and McClure et al. (3). Pertussis toxin, an inhibitor of signal transduction pathways involving G proteins (17, 18), had no effect on penetration (Table I; Fig. 3).

Gowda et al. (44) detected reverse transcribed viral DNA in mitogen-activated T cells but not in resting T cells after a 6- to 48-h exposure to HIV using a system that detects full length HIV DNA. We obtained evidence of infection in resting PBL by amplification of HIV LTR sequences in the PCR assay (data not shown) and in the infectivity assay (Table I). The cells had to be activated after the penetration phase of the infectivity assay to amplify viral replication. The apparent discrepancy of our results with those of Gowda et al. (44) may have been reconciled by the recent study of Zack et al. (45). They find that penetration and the initial reverse transcription of HIV LTR sequences occurs in nonactivated cells. However, complete transcription of full length viral DNA is much more efficient in activated cells.

At 0°C, HIV accumulates on the surface of CD4 cells but does not penetrate. When the temperature is raised to 37°C, penetration occurs relatively rapidly (Fig. 2). We measured cell-surface CD4 density by quantitative cytofluorometry using reagents that do not interfere with HIV binding or infection. No perturbation in cell surface CD4 was detected in cells loaded with saturating amounts of HIV at 0°C and monitored during 37°C incubations (Figs. 4 to 6). In cells stripped of cell-surface CD4, we could not reliably detect internalized CD4 on fixed and permeabilized cells. This is possibly because CD4 mAbs react poorly with CD4 after fixation as has been reported by others (46). Rather, CD4 was prelabeled with OKT4 mAb and FITC anti-mouse Ig mAb. The signal was followed into the cells and distinguished from cell-surface signal by its resistance to elution in low pH buffer. The necessity of prelabeling CD4 with mAb raises the question of whether the observed movements of CD4 at 37°C are physiologic processes or a consequence of mAb binding. Clearly, the loss of total signal due to shedding of CD4 from the membrane does not occur at 37°C in the absence of mAb (compare Figs. 4*a* and 6). Of interest, CD4 mAb induce shedding of CD4 (Fig. 6), but HIV by itself does not (Fig. 4*a*), nor does HIV binding interfere with shedding induced by CD4 mAb (Fig. 6). Thus, HIV and CD4 mAb binding are fundamentally different with respect to their consequent effects on CD4 anchoring and mobility.

The slow entry of CD4 at 37°C (the low pH-resistant signal) is detected with all CD4 mAb we have tested, and based on the work of others using a somewhat different system, does not require that the mAb be bivalent (47). We do not know if the internalized CD4 is degraded or recycled to the membrane. However, after internalization, the signal does not return to the membrane in a form that can be eluted by low pH buffer. The slow uptake of signal is not inhibited by cytochalasin E or H7 at concentrations that effectively block the shedding of CD4 by mAb or PKC-dependent endocytosis, respectively. Therefore, it would appear to be mechanistically distinct from these modes of CD4 movement. The slow uptake of signal is consistent with turnover of a constituitively expressed membrane protein, but whether this represents detection of a physiologic process that occurs in the absence of CD4 mAb can not be stated with certainty. In any event, the presence of saturating amounts of HIV does not result in increased internalization of CD4, nor does HIV perturb the kinetics of either the uptake or shedding of CD4 in CD4 mAb-prelabeled cells (Fig. 6). It is appropriate to note that CD4 was monitored on T cells and that CD4 internalization could possibly be a more significant mode of entry for other cell types such as monocytes/macrophages.

Although we could not detect an effect of HIV binding and penetration on cell-surface CD4, its trafficking, or its internalization, it remains possible that CD4 does in fact enter cells with HIV. We simply may not have detected it because our techniques are not sensitive enough despite our attempts to saturate the cells and synchronize the penetration event. For instance, only a small proportion of HIV bound to CD4 may actually penetrate, or CD4 might be rapidly recycled or replaced on the cell membrane after entry with HIV. Our data do not definitively rule out these possibilities. However, we would make the following argument that CD4 internalization, if it occurs with HIV, should have been detected in our system. We did not detect internalization of CD4 under conditions where internalization of HIV was readily detected. Although technical problems were encountered in staining CD4 on trypsin-stripped and fixed cells, this was not a problem with the HIV detection reagent. The HIV detection reagent is equivalent to the CD4 detection reagents with respect to intensity of surface staining and, if anything, somewhat inferior in terms of signal:background ratio (Fig. 5). Thus, if the proportion of surface CD4 that enters the cell is similar to the proportion of surface HIV that enters the cell, we should have detected CD4 entry in the protocol where CD4 was prelabeled. Also, if CD4

enters and is rapidly returned to the membrane, the attached probe would also have to recycle, otherwise the event would have been detected. Of course, this is possible, but it would necessarily require a pathway that is distinct from that of two other means of CD4 internalization where the probe does not return to the membrane: the slow uptake of CD4 and TPA-induced internalization of CD4.

In summary, we find no evidence that CD4 is internalized with HIV during penetration or that attachment of HIV to CD4 triggers an activation event that is required for penetration. The sole function of CD4 in viral entry may be to focus HIV in proximity to the host cell membrane. This is not a trivial function. By virtue of its high binding affinity, the gp120:CD4 interaction may overcome repulsive forces that exist between membranes allowing effective secondary interaction for viral and host membrane fusion. The viral mediator of fusion is likely the transmembrane protein gp41 (48–50), but its target or the particular properties of the host membrane required for interaction remain to be defined.

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