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CXCR4 utilization is sufficient to trigger CD4+ T cell depletion in HIV-1-infected human lymphoid tissue

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ABSTRACT **The human chemokine receptors CCR5 and CXCR4 have emerged as the predominant cofactors, along with CD4, for cellular entry of HIV-1 *in vivo* whereas the contribution of other chemokine receptors to HIV disease has not been yet determined. CCR5-specific (R5) viruses predominate during primary HIV-1 infection whereas viruses with specificity for CXCR4 (R5/X4 or X4 viruses) often emerge in late stages of HIV disease. The evolution of X4 viruses is associated with a rapid decline in CD4+ T cells, although a causative relationship between viral tropism and CD4+ T cell depletion has not yet been proven. To rigorously test this relationship, we assessed CD4+ T cell depletion in suspensions of human peripheral blood mononuclear cells and in explants of human lymphoid tissue on exposure to paired viruses that are genetically identical (isogenic) except for select envelope determinants specifying reciprocal tropism for CXCR4 or CCR5. In both systems, X4 HIV-1 massively depleted CD4+ lymphocytes whereas matched R5 viruses depleted such cells only mildly despite comparable viral replication kinetics. These findings demonstrate that the coreceptor specificities of HIV-1 are a causal factor in CD4+ T cell depletion *ex vivo* and strongly support the hypothesis that the evolution of viral envelope leading to usage of CXCR4 *in vivo* accelerates loss of CD4+ T cells, causing immunodeficiency.**

HIV-1, the causative agent of AIDS, represents a large number of diverse variants that differ genetically and phenotypically from one another. Until recently, HIV-1 isolates were classified according to their ability to infect and induce syncytia in various cells lines or monocyte-derived macrophages in culture. Although all isolates are capable of infecting cultures of human peripheral blood mononuclear cells (PBMCs), some isolates also can infect cultured macrophages but fail to infect and induce syncytia in established T-cell lines [referred to as non-syncytium-inducing (non-SI) or M-tropic strains]. In contrast, other variants do not infect macrophages but are capable of infecting T-cell lines and inducing syncytia in these cultures (referred to as SI or T-tropic). These properties are determined largely by the envelope gene of HIV-1 (reviewed in ref. 1).

The molecular mechanism(s) underlying differences in both cellular tropism and the SI/non-SI phenotype among different strains of HIV-1 became apparent with the discovery of the human chemokine receptors CCR5 and CXCR4 (2–6) as coreceptors along with human CD4 for the cellular entry of most strains of HIV-1. Expression studies of these receptors revealed that both CXCR4 and CCR5 are expressed by PBMC (5, 7–10), but most T-cell lines express only CXCR4 (7). Monocyte-derived macrophages express high levels of CCR5 but much lower levels of CXCR4 (11, 12). In fact, all reported SI and non-SI viruses segregate according to CXCR4 (designated X4 viruses) or CCR5 (designated R5 viruses) utilization, respectively (13–16). Although many other chemokine receptors demonstrate some degree of HIV-1 coreceptor activity *in vitro*, their importance to viral spread and pathogenesis *in vivo* remains to be elucidated.

The biological importance of the HIV-1 coreceptor classification scheme (17) is highlighted by changes in the phenotypes of HIV-1 strains that predominate *in vivo* during progression of HIV-1 disease to AIDS. HIV-1 transmission appears to be mediated by non-SI/R5 viruses (18, 19), which predominate throughout early stages of HIV-1 disease (13, 14, 20, 21). Later in the course of HIV disease, viruses that use CXCR4—either exclusively or in addition to CCR5—often evolve and become dominant (13, 14). The evolution of SI (X4 or R5X4) variants is associated with disease acceleration, in particular with a rapid drop in T cell counts (22, 23). Such a switch of viral phenotype and coreceptor tropism occurs typically in rapid progressors and in some slow progressors (13, 14, 20–23) whereas non-SI (R5) viruses remain dominant in long-term nonprogressors (22, 24, 25).

Thus, both *in vitro* and *in vivo* studies might suggest that CXCR4 utilization *per se* is linked to the stronger cytopathic phenotype and that the evolution toward CXCR4 use by HIV-1 *in vivo* directly contributes to disease acceleration. However, earlier studies have not fully excluded potential contributions of other viral characteristics because neither the basis for the switch in coreceptor usage nor a causal relationship between the switch and disease progression were established (26, 27). To rigorously test the hypothesis that CXCR4 utilization is sufficient to cause T cell depletion, we infected human PBMC and lymphoid tissue *ex vivo* with pairs of isogenic viruses differing only in their coreceptor utilization profiles. A direct comparison of the extent of CD4+ T cell depletion caused by infection of these systems with X4 viruses and R5 viruses demonstrated a causal role for CXCR4-dependent envelopes in augmenting virus pathogenicity.

MATERIALS AND METHODS

Cells and Transfections. PBMC were isolated from whole blood buffy coats (Stanford Blood Bank, Palo Alto, CA) by Ficoll-Hypaque density gradient centrifugation (Histopaque 1077, Sigma), were cultured for 48 h in medium (RPMI 1640) (Mediatech, Washington, DC) supplemented with 10% fetal calf serum (FCS, Gemini Biological Products, Calabasas, CA) and 2 μg/ml phytohemagglutinin (Sigma), and were stored at −80°C. Before their use in experiments, thawed cells were cultured for 2 days in RPMI medium 1640 with 10% FCS and

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Abbreviations: PBMC, peripheral blood mononuclear cells; SI, syncytium-inducing; FCS, fetal calf serum.

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5 units/ml recombinant human interleukin-2 (courtesy of Chiron). COS-7 cells (American Type Culture Collection) were maintained and transfected as described (28) with combinations of expression vectors encoding human CD4 (pCD4Neo) (29), CCR5 (pCMVFCCR5) (30), or CXCR4 (pCMVFCXCR4, provided by K. Neote, Pfeizer, Inc.).

Preparation of Viral Stocks. Except where noted otherwise, all proviral plasmids, envelope expression constructs, and viruses were from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The primary isolate BaL was prepared by culturing on monocyte-derived macrophages derived from human PBMC by plastic adherence. The molecular clones 49–5, 61–21, and JR-CSF were generous gifts from B. Chesebro (Rocky Mountains Laboratories, National Institute of Allergy and Infectious Diseases). To prepare viruses for infections, proviral plasmids were transfected into 293T cells by using the $CaPO_4$ method (Promega) as described (30), and viral stocks were harvested after both 36- and 60-h incubations. The p24 Gag concentration (ng/ml) was assessed by ELISA (New England Nuclear). To prepare pseudotype virus carrying the luciferase gene, 2 μg of pNL-Luc-E-R- (5) and 2 μg of the NL4–3 envelope expression vector pDOLenv or 2 μg of 49–5 proviral plasmid were cotransfected into 293T cells growing as described (30), and supernatants were harvested.

Coreceptor Utilization Assay for Single-Round Infection. COS-7 cells transfected with pCD4neo alone or pCD4neo in combination with pCMVFCCR5 or pCMVFCXCR4 were infected with 200 μl of pseudotype viruses. After 48 h, luciferase expression was assessed by enzymatic activity (relative light units) as described (5) according to the manufacturer's instructions (Analytical Luminescence Laboratory, San Diego).

PBMC Infections. To titrate viruses to achieve similar viral replication kinetics among different viruses for depletion studies, varying amounts of each were added to 500,000 PBMCs and were incubated overnight at 37°C. Cells then were washed extensively with PBS and were cultured in medium (RPMI 1640), 10% FCS and interleukin-2 as above. Supernatant (20 μl) was harvested every 2 days for p24 determinations and was replenished with an equal amount of RPMI medium 1640, 10% FCS, and interleukin-2 medium. For depletion studies, cultures infected with virus doses selected to achieve similar viral production during the assay were harvested 10 days after infection and were analyzed.

mAbs, Immunofluorescence Staining, and Flow Cytometry. PBMC were washed in 4 ml of PBS and 3% FCS before immunostaining. Cells were incubated on ice for 30 min in 100 μl of 1:20 dilutions of various fluorochrome-conjugated anti-CD4 (PE-conjugated), anti-CD8 (PerCP-conjugated), and anti-CD3 (FITC-conjugated) mAbs (clones SK3, SK1, and SK7, respectively) or the Tritest kit combining all three antibodies (Becton Dickinson). Cells were washed again with 4 ml of PBS and 3% FCS serum and were analyzed with a FACScan (Becton Dickinson). Lymphocytes (20,000) were counted by forward/side scatter characteristics, and the data were analyzed with CELLQUEST software (Becton Dickinson). CD4+ lymphocytes were sorted by positively selecting CD4+/CD8− lymphocytes using a FACS Vantage (Becton Dickinson).

Infection of Human Lymphoid Tissue *ex Vivo*. Human tonsil tissue removed during routine tonsillectomy and not required for clinical purposes was received within 5 h of excision and was sectioned into 2- to 3-mm blocks. These tissue blocks were placed onto collagen sponge gels in the culture medium at the air-liquid interface and were infected as described (31–33). In a typical experiment, equal amounts of various isolates (0.5 ng of p24 per block) were applied as 3–5 μl of clarified virus-containing media to the top of tissue blocks from the same donor. Productive HIV-1 infection was assessed by measuring p24 in the culture medium by using an HIV-1 p24 antigen ELISA (Cellular Products and AIDS Vaccine Program, National Cancer Institute, Frederick, MD): specifically, the concentration of p24 accumulated in 3 ml of culture medium bathing six tissue blocks during the 3 days between the successive medium changes was used as a measure of virus replication. Similar methods were used with human spleen samples derived at autopsy (provided by National Diabetes Research Interchange, Philadelphia).

Flow cytometry was performed as described above on cells mechanically isolated from control and infected tissue, and CD4+ T cell depletion is expressed as a ratio of CD4+ to CD8+ T cells blocks as described earlier (31). For characterization of productively HIV-infected cells, the following cell surface markers were used in combination with anti-p24 RD1 (Coulter); anti-CD3 PerCP (Becton Dickinson), anti-HLA-DR APC, anti-CD68 FITC, and anti CD25 APC (Caltag, Burlingame, CA); and anti-CD64 Cy5 and anti-CD69 FITC (PharMingen). Cells first were stained for the cell surface antigens, were fixed and permeabilized by using Cytofix-Cytoperm (PharMingen), and were stained for intracellular p24.

RESULTS AND DISCUSSION

To test the hypothesis that CXCR4 tropism alone is sufficient to produce HIV-1-induced pathogenesis, we used pairs of isogenic viruses differing exclusively in envelope determinants that control preferences for the coreceptors CXCR4 and CCR5 leading to distinct cellular tropisms. NL4–3 (34) is a well known, molecularly cloned strain of HIV-1 that exhibits strict CXCR4-tropism (35, 36). 49–5 (37) is a recombinant virus based on NL4–3 that contains the envelope V3 loop sequence derived from that of BaL (38), a molecularly cloned primary isolate that exhibits selectivity for CCR5 (5, 6). Because the V3 loop has been shown to be the predominant (4, 35, 39–43) though not exclusive (44, 45) determinant of HIV coreceptor specificity, 49–5 was expected to be CCR5-restricted. This phenotype was verified by transiently transfecting COS-7 cells with plasmids encoding human CD4 along with human CXCR4 or CCR5 and infecting them with pseudotype HIV-1 preparations, 49–5 and NL4–3, that encoded the firefly luciferase gene. As expected, NL4–3 utilized CXCR4 and not CCR5 as a coreceptor whereas 49–5 used CCR5 and not CXCR4 (Fig. 1). This distinction provides a basis for examining the relationship between coreceptor utilization by HIV-1 and CD4+ T cell depletion while holding constant the remainder of the viral genome.

We therefore compared CD4+ T cell depletion in phytohemagglutinin-blasted PBMCs cultured with interleukin-2 and infected with NL4–3 or 49–5. Viral replication was monitored by measuring the HIV-1 capsid protein (p24) in the culture supernatants over time, which revealed very similar replication kinetics for NL4–3 and 49–5 (Fig. 2 *Inset*). Cells were harvested 10 days after infection, were immunostained with anti-CD4 and anti-CD8 antibodies, and were analyzed by fluorescence-based flow cytometry. In three independent experiments, NL4–3 severely depressed the CD4/CD8 ratios in these cultures whereas 49–5 exerted significantly less effect (Fig. 2). The differences in CD4+/CD8+ ratios indicate that infection with a CXCR4-dependent virus induces significantly greater CD4+ T lymphocyte depletion in PBMC suspension cultures than does the isogenic CCR5-tropic strain, despite comparable viral replication kinetics.

In vivo viral replication occurs in whole tissues rather than in isolated cell populations. Furthermore, general cell activation itself can modulate coreceptor expression (8) and also can contribute to cell death (46). We therefore extended our study of CD4+ T cell depletion to integral human tonsillar tissue infected *ex vivo* by using a system that has been shown to

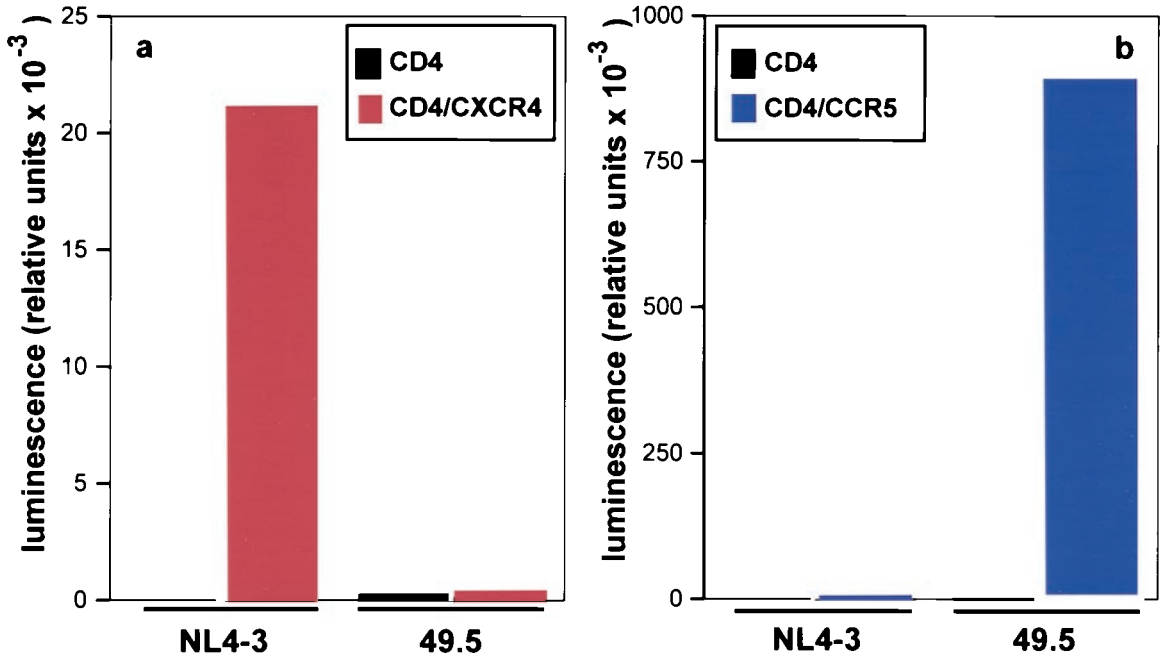

FIG. 1. Reciprocal utilization of CXCR4 and CCR5 by paired, isogenic HIV-1 strains differing in envelope determinants. COS-7 cells were transfected with plasmids encoding CD4 alone or CD4 in combination with CCR5 or CXCR4. Cells subsequently were infected with pseudotype NL4–3 or 49–5. After 48 h, luciferase expression was assessed by enzymatic activity (relative light units). (*a*) Transfection with CD4 (black bars) alone or with CD4/CXCR4 (red bars). (*b*) Transfection with CD4 (black bars) alone or with CD4/CCR5 (blue bars).

preserve tissue cytoarchitecture and to support productive infection by all HIV-1 isolates without exogenous stimulation (31, 32). We tested NL4–3, BaL, and the chimeric virus 49–5 for immunodepletion in these three-dimensional cultures. All three viruses replicated well in the tonsillar histocultures as evaluated by the increase of p24 in the culture medium (Fig. 3). Replication of each became evident, starting approximately from day 6 after infection, and continued to increase during the course of experiment. The absolute level of replication varied 2- to 3-fold from tissue donor to donor. In experiments with tissue from five donors, there were no consistent differences among different viral variants in the rate of replication or in the amount of total virus produced during the 12-day experiments (Fig. 3).

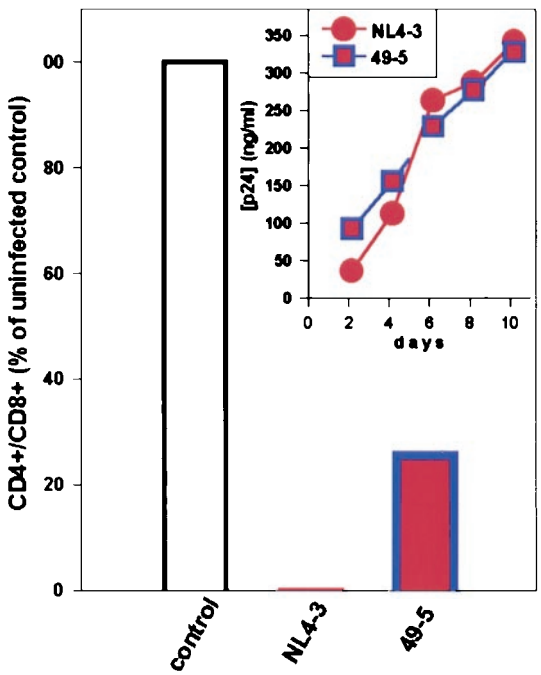

FIG. 2. CD4+ T lymphocyte depletion in PBMC cultures by paired HIV-1 strains. Shown are CD4+/CD8+ ratios in PBMC infected with NL4–3 (red bars) or 49–5 (red/blue bars) after a 10-day infection. The ratios are expressed as a percentage of that in a control (uninfected) PBMC culture. (*Inset*) The concentration of p24 in culture medium of PBMC infected with NL4–3 (red circles) or 49–5 (red/blue squares). These experiments are representative of three independent experiments using PBMC isolated from different donors. Color code: An individual color was assigned to each viral isolate as shown under the bar graph. The bar colors for isogenic chimeras correspond to the colors of parental viruses whereas the color of the edges correspond to the sources of Env sequences.

Despite comparable replication properties, marked differences in the abilities of different viruses to deplete CD4+ T cells were observed. NL4–3 severely depleted the tonsil tissues of CD4+ T cells (Fig. 3*a*) as assessed by the CD4+/CD8+ ratio (31). In contrast, BaL and the chimeric virus 49–5 containing the V3 loop derived from BaL exhibited only minimal depletion effects on CD4+ T lymphocytes (Fig. 3*b*).

To verify that the genomic environment of the envelope sequences does not affect CD4+ T cell depletion in this system, we performed experiments with a second, reciprocal set of matched viruses. JR-CSF (47) represents a molecularly cloned, CCR5-dependent primary isolate, and chimeric virus 61–21 is its isogenic partner containing the backbone of JR-CSF and the V1-V3 loop segments derived from NL4–3 that have been shown previously to exhibit strict CXCR4-dependence (35). In these experiments, NL4–3, JR-CSF, and 61–21 replicated with kinetics similar to those of other tested viruses (Fig. 3). Despite comparability in replication properties, only the CXCR4-dependent viruses (NL4–3 and 61–21) caused prominent depletion of CD4+ T cells (Fig. 3). In these and in the earlier experiments, the decline of CD3+/CD4+ cells by depleting viruses was not accompanied by a relative increase of CD3+/CD8−/CD4− cells. Thus, viral infections in these cultures trigger CD4+ T cell loss rather than down-regulation of cell surface CD4 expression accounting for these changes.

We also tested these viruses for effects on CD4+ T cells in noninflammatory human spleen tissue. Because spleen tissue

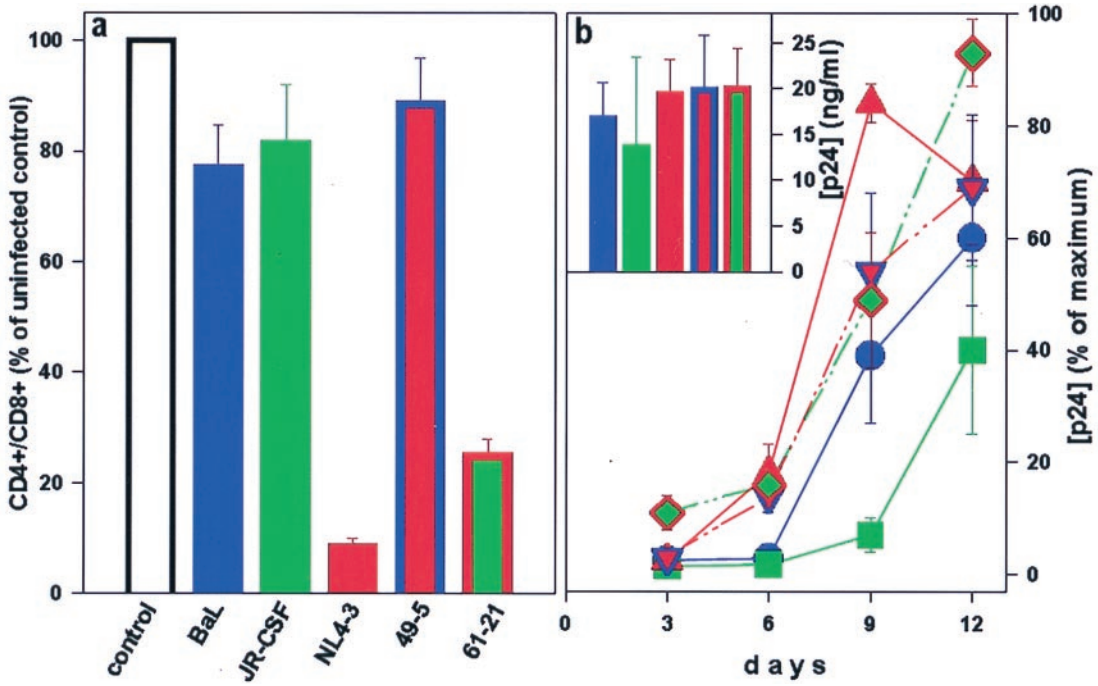

FIG. 3. CD4+ T cell depletion and viral replication in human tonsillar tissue infected *ex vivo* by matched HIV-1 strains. (*a*) CD4+ T cell depletion, as assessed by CD4+/CD8+ ratio on day 12 after infection. For each measurement, cells were isolated from 6–10 tissue blocks and were analyzed by flow cytometry. Mean values (± SEM) are shown from experiments with tissues from three to six different donors infected in parallel with a panel of tested viruses. (*b*) Viral replication, as assessed by p24 values in the histoculture supernatants. Tissues from three to five different donors were infected as indicated, and viral replication was monitored. To compare replication kinetics in tissues from different donors, the absolute value for viral replication for a given donor tissue was normalized by the maximum value of viral replication for the entire tested panel. Mean values ± SEM are shown for experiments with tissues from three to six different donors. (*Inset*) Absolute value of viral replication of the same histocultures, as assessed by measurements of p24 concentration in the culture medium measured on day 12 after infection. For each measurement, medium bathing six blocks of tissue was pooled. Presented are mean values ± SEM for experiments with tissues from three to six different donors. The same tissue blocks were used for measurements presented in *a* and *b*. The color code is the same as in Fig. 2.

persists longer as viable histocultures than does tonsillar tissue, use of this system further allowed us to determine whether R5 viruses cause CD4+ T cell depletion over greater lengths of time. After prolonged infection of the spleen samples, the CCR5-tropic virus 49–5 had only modest effects on CD4+ T cell survival whereas parental NL4–3 in the same time frame massively depleted the samples of CD4+ T cells (Fig. 4). As in the earlier experiments, the difference in CD4+ T cell depletion could not be attributed to differences in viral replication rates (Fig. 4 *Inset*). Indeed, the total amount of virus produced in tissue infected with the depleting NL4–3 was even lower than that produced in tissue infected with its nondepleting 49–5 derivative.

Therefore, these reciprocal experiments collectively demonstrate that the preference for CXCR4 is a major causative factor in accelerating depletion of CD4+ T cells. One hypothesis to account for these results is that differences in cellular tropism among X4 and R5 viruses cause different pathologic outcomes. However, both X4 and R5 viruses were found to infect T cells in these tissues. This fact was established by flow cytometry of cells mechanically isolated from infected tissues and immunostained for CD3 and p24. The frequency of CD3+/p24+ cells varied between 0.4 and 2.8% of all T cells regardless of coreceptor properties, which is in accordance with the data reported for tissues from asymptomatic HIV-infected patients (48, 49). These findings indicate that the less pathogenic behavior of R5 variants is not attributable to their inability to infect T cells.

To confirm that other cells are not required for differential CD4+ T cell depletion, we compared the effects of infection and cell depletion by NL4–3 and its CCR5-dependent derivative 49–5 in a sorted population (98% purity) of peripheral blood CD4+ T cells. In agreement with the results presented above, CXCR4-dependent NL4–3 nearly obliterated the sorted CD4+ T cell cultures whereas the CCR5-dependent derivative 49–5 depleted only mildly. Counts of viable (trypan blue-excluding) cells demonstrated that NL4–3 depleted to 0.6% of the uninfected control whereas 49–5 depleted to 61% of the control (Fig. 5). The replication of both viruses in the cultures was comparable (Fig. 5 *Inset*), again excluding replication rates as the basis for these differences in CD4+ T cell depletion.

Other attempts have been made to establish a correlation between cellular tropism and CD4+ T cell depletion. Studies using human PBMC transplanted into immunodeficient mice have found complex relationships among viral replication rates, cellular tropism, and CD4+ T cell depletion (27, 50, 51). Recent work comparing X4/R5 and R5 strains was highly suggestive regarding the possible contribution of CXCR4, although it did not fully distinguish between the pathogenic effects of CXCR4 itself versus the combined effects of CCR5 with CXCR4 (27). Other experiments with human lymphoid tissue *ex vivo* also have demonstrated a consistent relationship between T-cell tropism and CD4+ T cell depletion (31, 32), but, as in natural human infections, diverse genomic differences among the tested viruses obscured the ability to assign a singular causative role to the coreceptor preferences. Thus, the present recombinant strategy utilizing pairs of viruses that differ only in these select envelope sequences and replicate to similar levels provides definitive evidence that viral use of CXCR4 is sufficient to produce severe CD4+ T lymphocyte depletion in multiple human lymphoid culture systems.

R5 viruses also cause some degree of CD4+ T cell depletion in these experimental systems and *in vivo*, but the more aggressive behavior of X4 strains in the present studies appears to recapitulate their clinical properties in human infection. An

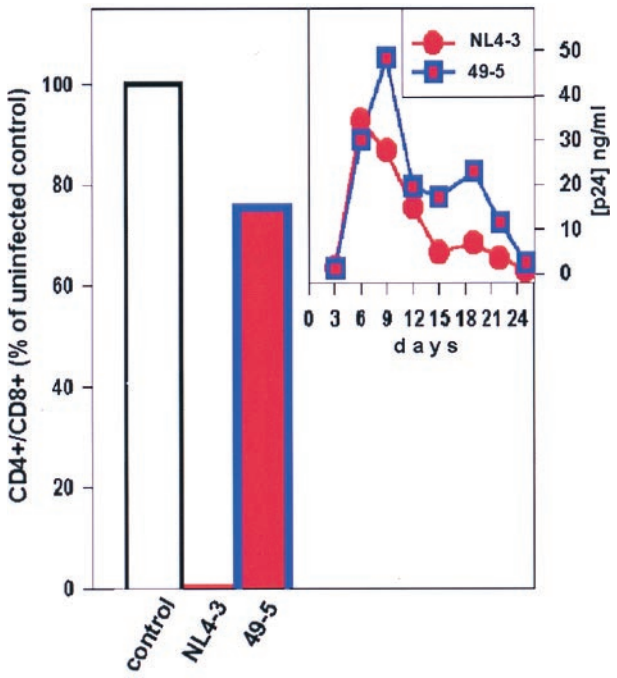

FIG. 4. CD4+ T lymphocyte depletion in human spleen histocultures by paired HIV-1 strains. Mean CD4+/CD8+ ratios from duplicate samples are shown from an experiment performed with human spleen histocultures infected with NL4–3 or 49–5. The p24 concentrations of these viruses in the culture medium over time are indicated as in Fig. 3 (*Inset*). The color code is the same as in Fig. 2.

interesting question regards the mechanism of virus-specific lymphocyte depletion. One possibility is that the ranges of

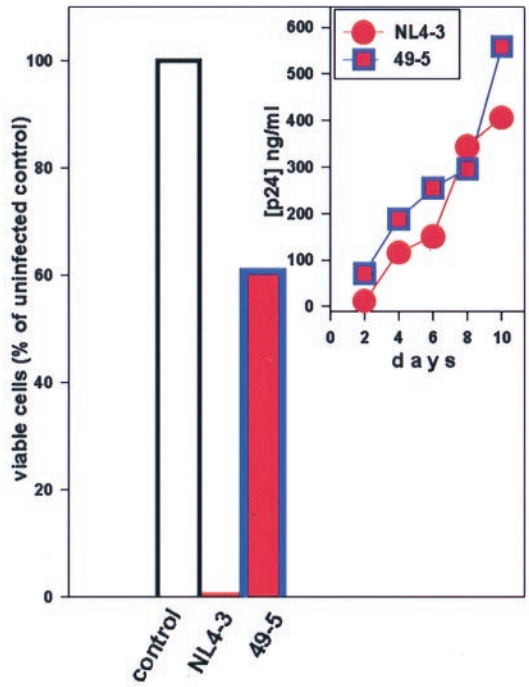

FIG. 5. Viability of purified CD4+ T lymphocyte cultures after infection by paired HIV-1 strains. CD4+ T lymphocyte cultures were infected with NL4–3 or 49–5. Viability of the cultures also was assessed 10 days after infection by counting cells that excluded trypan blue, and results are expressed as a percentage of the viable cell counts in the uninfected control cultures; this experiment was performed in duplicate, and mean values are shown. (*Inset*) The p24 concentrations of these viruses in the culture medium over time. The color code is the same as in Fig. 2.

specific targets among the T cells differ for X4 and R5 strains, which may lead to distinct consequences for the lymphocyte population overall. An intriguing alternative hypothesis is that viral usage of a particular coreceptor is a direct factor in the fate of an infected cell. Further investigation will be needed to define the relevant mechanisms. In any event, as patients harboring X4 viruses progress to AIDS more rapidly than do similar cohorts harboring exclusively R5 viruses (13, 14, 20–22, 24), these findings strongly support the postulate that the emergence of HIV-1 strains that utilize CXCR4 is a causative factor in disease acceleration.

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