JOURNAL OF VIROLOGY, Sept. 1991, p. 5096–5101
0022-538X/91/095096-06$02.00/0

Vol. 65, No. 9

Cytopathic Variants of an Attenuated Isolate of Human Immunodeficiency Virus Type 2 Exhibit Increased Affinity for CD4

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Received 5 February 1991/Accepted 31 May 1991

Naturally occurring isolates of human immunodeficiency virus (HIV) have been described which are deficient in their ability to fuse with and kill CD4$^+$ target cells. Although the molecular basis for their attenuation has not yet been defined, several lines of evidence point toward the viral envelope gene as a key determinant of viral pathogenicity. In the present article, we report the biological characterization of two highly cytopathic variants derived by repeated cell-free passage of an attenuated isolate of HIV type 2 (HIV-2), termed HIV-2/ST. Unlike the parental virus, the cytopathic variants were found to infect Sup-T1 cells with great efficiency and to induce both cell fusion and profound killing in these cultures. To determine whether changes in the viral envelope gene were responsible for the observed phenotypic differences, we examined the CD4 binding affinity of these viruses using a novel assay designed to quantitate the binding of fluoresceinated CD4 to viral envelope in its native configuration on the cell surface. The results demonstrated that the affinity of parental HIV-2/ST envelope for CD4 was 2 orders of magnitude reduced, while the cytopathic variants exhibited a high CD4 binding affinity, comparable to that of cytopathic HIV-1 and HIV-2 isolates. From these data, we conclude that the cytopathic potential of HIV depends, at least in part, on its receptor-binding affinity. In addition, our study documents strong selection pressures for viruses with increased CD4 affinity during propagation in immortalized T-cell lines, thus emphasizing the need to study HIV envelope biology in natural target cells.

Isolates of human and simian immunodeficiency viruses (HIV and SIV) are known to differ in their host range for CD4$^+$ (13–15, 26, 32, 33, 46, 50) and CD4$^-$ (10, 19, 27, 39, 57) cells, their replication kinetics (1, 8, 50, 59), and their cytopathic potential (7, 8, 15–17, 35, 36, 58). Although a number of viral gene products including *vpu*, *vpx*, *vif*, *vpr*, and *nef* may contribute to infectivity and replication kinetics, several studies have strongly implicated the viral envelope as a key determinant for both cell tropism and cytopathicity (34, 40, 47, 52, 54, 56, 61, 65). The mechanisms, however, by which differences in the viral envelope contribute to the biological heterogeneity of HIVs are poorly understood.

We have previously reported the isolation (35) and molecular cloning (37) of an attenuated isolate of HIV type 2 (HIV-2), termed HIV-2/ST, which does not induce cell fusion or killing during infection of CD4$^+$ cell lines or peripheral blood T cells. Initial characterization demonstrated that HIV-2/ST replicated to high titers but with markedly delayed kinetics, indicating a defect at the level of cell entry (35, 37). This defect was subsequently mapped to the viral envelope gene by recombinant vaccinia virus expression studies which documented a failure of the HIV-2/ST envelope glycoproteins to mediate cell fusion (45). Recent studies of *Drosophila*-expressed HIV-2/ST gp120 revealed a markedly decreased (greater than 280-fold) CD4 binding affinity compared with that of prototypic HIV type 1 (HIV-1) (28). To examine a possible link between CD4 binding affinity and viral pathogenicity, we analyzed two highly cytopathic variants of HIV-2/ST, which were derived from the parental strain by repeated cell-free transmission. Using a novel assay in which the affinity of cell-associated envelope for recombinant CD4 was measured, we found that the CD4 binding affinity of the cytopathic HIV-2/ST variants was markedly increased compared with that of the noncytopathic parental virus. These data thus provide the first direct evidence for an association between envelope-CD4 binding affinity and HIV cytopathicity in vitro.

Limiting dilution of cells infected by the original HIV-2/ST isolate resulted in the identification of cellular clones which produced viruses that differed in the length of their transmembrane envelope glycoproteins (35). One biological clone, termed ST/24, produced virus with a short transmembrane molecule of 30 kDa, while virions from four other lines designated ST/23, ST/9, ST/17, and B12 contained a larger transmembrane protein of 41 kDa (35, 37). The short transmembrane molecule of ST/24 has been shown to result from an in-frame stop codon 166 amino acids from the carboxy terminus of the envelope open reading frame (18). In an effort to evaluate the stability of the noncytopathic phenotype of HIV-2/ST viruses with long and short transmembrane molecules, cell-free supernatants from biologically cloned cell lines were serially passaged onto Sup-T1 cells and cultures were monitored for cytopathic effects. Interestingly, after two passages, both syncytium formation and cell death were noted in two independent cultures inoculated with virus from the ST/24 cell line (Fig. 1). Viruses derived from cells which survived these cytopathic infections, termed ST/24.1C and ST/24.2C, remained highly cytopathic during subsequent passages in Sup-T1 cells. In contrast, no cytopathic effects were observed during as many as 10 serial passages of HIV-2/ST viruses with long transmembrane molecules including virus derived from the molecular clone JSP4-27, which does not contain a stop codon in the envelope gene (35).

The ST/24.1C and ST/24.2C variants of HIV-2/ST were

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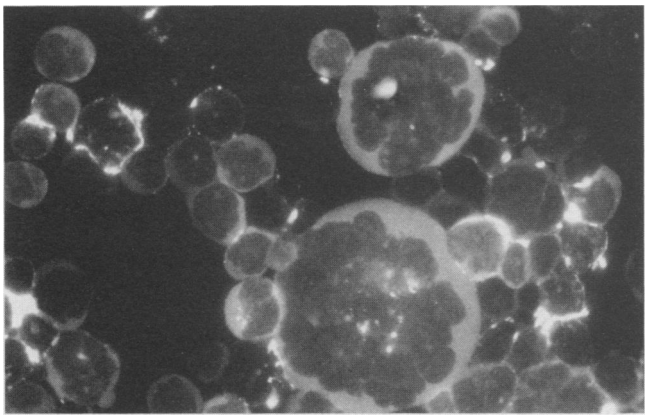

FIG. 1. Cell fusion induced by cytopathic variants of HIV-2/ST. Sup-T1 cells infected with ST/24.1C were stained with monoclonal antibody 3A8 (specific for HIV-2 and SIV $p25^{gag}$) and analyzed by indirect immunofluorescence. Prominent cytopathic effects were observed as demonstrated by the formation of large syncytia in the infected culture.

formally compared with the noncytopathic parental virus, ST/24, with respect to their replication kinetics and cytopathic potential in Sup-T1 cells. A total of 100 50% tissue culture infective dose units were used to inoculate Sup-T1 cells, and cultures were subsequently monitored for cell number, viability, reverse transcriptase activity, and the percentage of cells expressing viral antigens. Similar to the original HIV-2/ST isolate, infection by the parental noncytopathic ST/24 virus remained completely noncytopathic and occurred without a reduction in cell viability or growth rate and without syncytium formation (Fig. 2). Viral spread in this culture was also slow, with more than 4 weeks required for >80% of cells to express viral antigens. In contrast, cultures infected by either ST/24.1C or ST/24.2C exhibited a dramatic reduction in cell number and viability coincident with the appearance of large syncytia (Fig. 1). Moreover, the kinetics of infection were markedly accelerated, with 100% of cells expressing viral antigens within 1 to 2 weeks. Reverse transcriptase activity also increased more rapidly in cultures infected by the two cytopathic variants, although this activity eventually reached comparable levels for both cytopathic and noncytopathic viruses (Fig. 2D).

As described above, recent studies have suggested that the attenuated biological phenotype of HIV-2/ST relates, at least in part, to a markedly reduced affinity in its envelope glycoprotein for CD4 (28). To further evaluate this possibility and to determine whether the striking biological differences observed between HIV-2/ST and its cytopathic variants reflected changes in this affinity, we developed an assay in which the binding of recombinant CD4 to cell-associated viral envelope could be quantified. Recombinant CD4 was fluoresceinated (F-CD4), as described previously for labeling

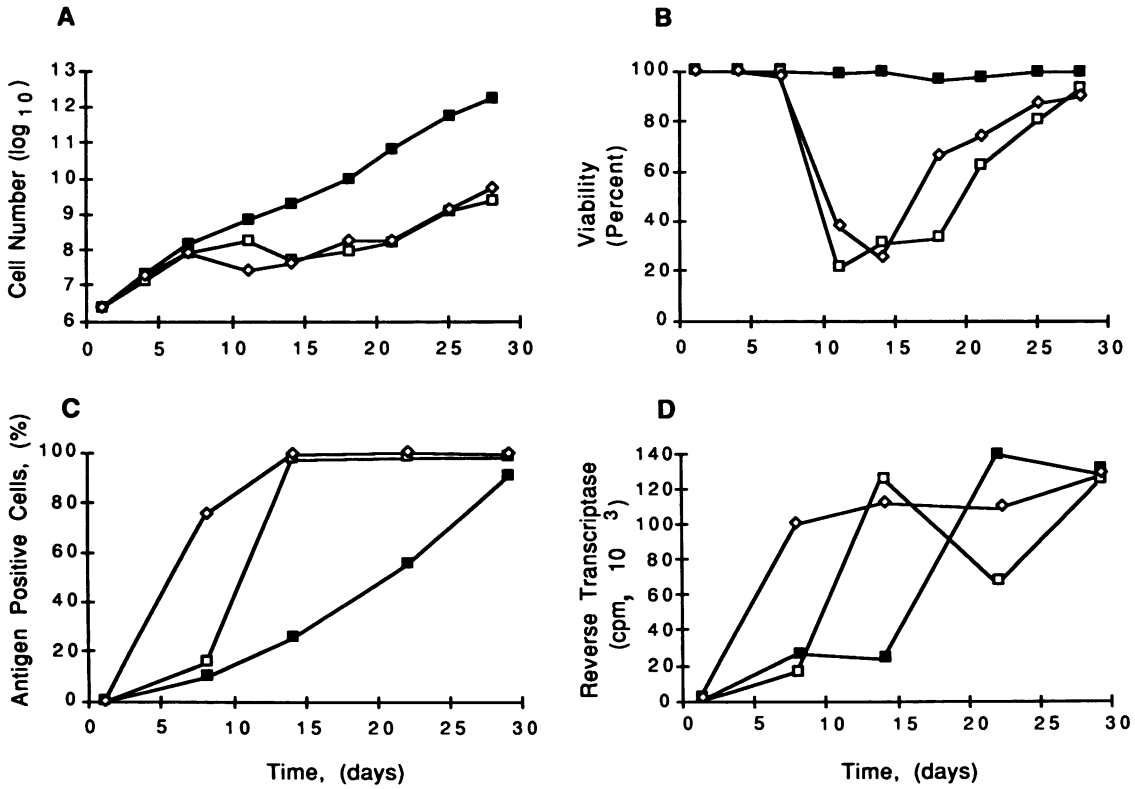

FIG. 2. Kinetics of Sup-T1 cell infection by noncytopathic and cytopathic HIV-2/ST viruses. Sup-T1 cells were inoculated with cell-free supernatant containing ST/24 (■) or the cytopathic variant viruses ST/24.1C (□) or ST/24.2C (◇). Cultures were subsequently monitored for cell number (A); viability, as determined by trypan blue dye exclusion (B); the percentage of cells expressing viral antigens, determined by immunofluorescence microscopy (25) with an HIV-2 and SIV *gag*-specific monoclonal antibody (C); and reverse transcriptase activity (25) (D). The calculation for total cell number was adjusted for cell dilutions that occurred during repetitive passaging of cell cultures during the experiment.

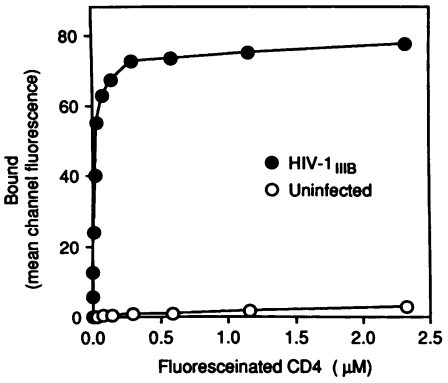

FIG. 3. Binding of F-CD4 to HIV-1/IIIB-infected Sup-T1 cells. Recombinant CD4 was fluoresceinated as described previously (51). Briefly, 0.5 mg of CD4 in 0.5 ml of phosphate-buffered saline (pH 7.4) was mixed with 50 μl of 5% sodium carbonate buffer (pH 8.5) followed by the addition of 0.5 mg of fluorescein isothiocyanate-Celite (Calbiochem-Behring, La Jolla, Calif.) for 5 min at 25°C. F-CD4 was separated from the reaction mixture on a PD-10 column (Pharmacia, Piscataway, N.J.). Optical densities at 280 and 493 nm were determined, and the fluorescein-to-protein (F/P) ratio was calculated by using an extinction coefficient for CD4 of 2.87. F/P ratios for 20 preparations of F-CD4 (± SEM) were 5.2 ± 0.4. F-CD4 was added in various amounts at 37°C to HIV-1/IIIB-infected and uninfected Sup-T1 cells as described in the text, and fluorescence intensity was quantitated with a FACScan flow cytometer. The mean channel of fluorescence intensity, as an indicator of F-CD4 binding, is shown on infected and uninfected Sup-T1 cells at each concentration of F-CD4. The estimated K_d for F-CD4 binding to the HIV-1/IIIB-infected cells in the experiment shown was 24 nM.

of monoclonal antibodies (51) (see the legend to Fig. 3). Cells (2.5×10^5) were aliquoted in 50 μl of staining buffer (phosphate-buffered saline, 0.2% bovine serum albumin, 0.05% sodium azide, pH 7.4), preincubated for 5 min with 10 μl of fetal calf serum, and incubated for 60 min at 37°C with various dilutions of F-CD4 in a final volume of 85 μl. Cells were washed twice in staining buffer and fixed in 4% paraformaldehyde, and fluorescence intensity was determined with a FACScan flow cytometer (Becton Dickinson and Co., San Jose, Calif.). Background fluorescence was determined for cells incubated without F-CD4 and was subtracted from the mean channel of fluorescence intensity for each sample incubated with F-CD4. For Scatchard analysis, fluorescence intensity units were used as a measure of bound F-CD4 and the total micrograms of F-CD4 added to each sample were used as an approximation of unbound CD4. Dissociation constants for F-CD4 binding were determined by using SCATCH, a program which allows ligand binding data to be evaluated for one or more classes of saturable and nonsaturable binding sites (4).

To validate this binding assay with HIV strains of known binding affinity, we first examined the binding of F-CD4 to Sup-T1 cells chronically infected with the IIIB isolate of HIV-1 and to uninfected Sup-T1 cells. As shown in Fig. 3, saturable binding of F-CD4 was observed on infected but not on uninfected Sup-T1 cells, indicating that F-CD4 binding in this assay was specific. Maximal F-CD4 binding was achieved at 45 min and showed no significant decrease for up to 2 h at 37°C (23). Under the conditions of this assay, all cell-associated CD4 remained bound to the cell surface and was not internalized, as determined by fluorescence microscopy (data not shown). Scatchard analysis of F-CD4 binding to HIV-1-infected cells was consistent with a single class of saturable binding sites, although the units of fluorescence intensity used as an indication of bound CD4 did not permit a determination of the actual number of binding sites on each cell. Interestingly, in six experiments with three different preparations of F-CD4, the K_d for F-CD4 binding to HIV-1/IIIB-infected cells (± standard error of the mean [SEM]) was 21 ± 1 nM, indicating that the affinity of CD4 for cell-associated envelope was roughly fivefold lower than the binding of recombinant CD4 to cell-free HIV-1 gp120 (2, 53). These differences were not due to an alteration in CD4 induced by the fluoresceination reaction since, in competition experiments, the binding of F-CD4 to infected cells was inhibited by equimolar amounts of nonfluoresceinated CD4 (23). Similar results were also seen when unlabeled recombinant CD4 was used in binding assays and detected with a fluoresceinated anti-CD4 monoclonal antibody, OKT4 (data not shown).

We next sought to determine whether differences could be detected in envelope-CD4 affinity between HIV-1/IIIB, HIV-2/ST, and the cytopathic variant, ST/24.2C. For these experiments, Sup-T1 cells were used which were chronically infected with an early passage of a noncytopathic parental HIV-2/ST virus that expressed a long transmembrane molecule. As shown in Fig. 4 and Table 1, striking differences in CD4 binding were apparent with HIV-2/ST-infected cells compared with HIV-1/IIIB-infected cells. In six experiments in which F-CD4 binding affinity was determined, the estimated K_d (± SEM) on HIV-1/IIIB-infected cells was 21 ± 1 nM. In contrast, the estimated K_d for F-CD4 binding to the HIV-2/ST-infected cells was 1,800 ± 200 nM. Since the binding of F-CD4 to the HIV-2/ST-infected cells was not fully saturated even at the highest F-CD4 concentrations, the estimated K_d is a lower limit of this affinity. In contrast, the K_d for F-CD4 binding to ST/24.2C-infected cells was 31 ± 4 nM, similar to the value for the HIV-1/IIIB-infected cells (Table 1). The affinity of F-CD4 for the ST/24.C envelope was also found to be similar to that of the cytopathic HIV-2 isolate HIV-2/ROD (26 ± 2 nM) when CD4 binding was evaluated on Sup-T1 cells chronically infected with this virus. Binding assays could not be performed on cell lines infected with virus derived from the JSP4-27 molecular clone of HIV-2/ST because of insufficient amounts of envelope glycoproteins expressed on the cell surface (data not shown). Taken together, these findings indicated that the increased cytopathicity of the ST/24.2C virus for Sup-T1 cells was associated with a >60-fold increase in the affinity of its envelope for CD4.

Differences in affinity of HIV and SIV envelope glycoproteins for CD4 have recently been demonstrated by using viral (42) and recombinant (28) envelope glycoproteins, and these studies have indicated that HIV-1/BaL (28), HIV-2/ROD (42), and SIV_{mac} (28) bind recombinant soluble CD4 with 6, 25, and 70 times lower affinity than HIV-1/IIIB gp120. Moreover, the K_d for CD4 binding of HIV-2/ST gp120 produced by a *Drosophila* cell line was estimated at >1,400 nM (28), a result similar to our own determination of >1,800 nM for the binding of F-CD4 to HIV-2/ST envelope expressed in its native conformation on the surface of infected cells. The biological signficance of these differences in affinity has been unclear, although they may contribute to variation in the amount of recombinant CD4 needed to inhibit particular isolates in vitro (5, 10). Our findings for HIV-2/ST and its cytopathic variants demonstrate for the first time that repeated passage of an attenuated virus in $CD4^+$ cells selects for variants with an increased affinity for

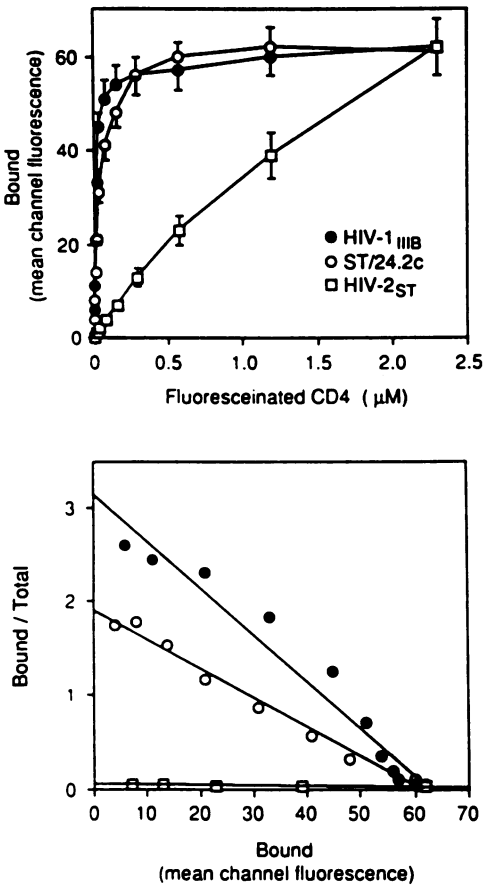

FIG. 4. Binding of F-CD4 to HIV-1/IIIB- and HIV-2/ST-infected cells. F-CD4 binding was determined as shown in Fig. 3 on Sup-T1 cells infected by HIV-1/IIIB; an early passage, noncytopathic HIV-2/ST; and the cytopathic variant of HIV-2/ST, ST/24.2C. The top panel shows the mean channel of fluorescence intensity as an indication of total F-CD4 bound. Each point represents the mean ± SEM from six experiments. The bottom panel shows a Scatchard analysis of the same data in which points represent the average values for the six experiments. The values for K_ds derived from these data are summarized in Table 1.

CD4 and that this difference may contribute to an increased cytopathic potential. The cytopathic variants may have been present in the initial isolate or may have been generated by mutations during in vitro culture. Regardless of the mechanism, these results suggest that variants with an increased CD4 binding affinity have an in vitro growth advantage and exhibit increased cytopathic effects during infection.

Aside from the observed increase in CD4 binding affinity, additional studies from our laboratory have indicated that other factors must be involved to fully explain the biological properties of the cytopathic HIV-2/ST variants. Parental HIV-2/ST as well as its molecular clone exhibit a restricted host range and are unable to infect a number of $CD4^+$ cell lines, including CEM, Jurkat, and Molt-4, that are highly susceptible to the cytopathic isolates HIV-1/IIIB and HIV-2/ROD (24). Despite an increase in affinity for CD4 to levels comparable to that of HIV-2/ROD, the cytopathic variants of HIV-2/ST remained unable to infect these cell types. This restricted host range must therefore be the result of other factors which mediate cell entry including conformational changes in envelope or CD4 (38, 43, 60), shedding of gp120 molecules (20, 31, 43, 44), and/or proteolytic cleavage of gp120 by cell surface proteases (11, 29) that may be necessary to expose hydrophobic regions of the transmembrane envelope molecule required for subsequent fusion with the cell membrane. In addition, an interaction with cellular molecules besides CD4 has been implicated for both HIV-1 (3, 9, 10, 21, 22, 41) and SIV_{mac} (26, 33) entry, and it is possible that such molecules could also play a role in determining the range of cells susceptible to infection by cytopathic and noncytopathic HIV-2/ST viruses.

TABLE 1. Affinity of F-CD4 binding to HIV-infected cells[a]

Virus	K_d (nM)	P^b
HIV-1/IIIB	21 ± 1	
ST/24.2C	31 ± 2	0.004
HIV-2/ST	1,800 ± 200	<0.001

[a] Affinity of F-CD4 binding to infected Sup-T1 cells was determined as described in the text and the legend to Fig. 3. The results are the mean ± SEM from six experiments.

[b] P values indicate comparisons with HIV-1/IIIB, as calculated by the paired t test.

It is of interest that cytopathic viruses were derived only from an HIV-2/ST strain which expressed a truncated transmembrane envelope molecule. Remarkably, after as many as 10 serial passages of HIV-2/ST viruses with long transmembrane molecules, the spontaneous appearance of cytopathic viruses has not been observed. Desrosiers and coworkers (32) have described truncations in transmembrane molecules for SIV_{mac} which appeared when these viruses were grown in human but not macaque lymphocytes and proposed that this truncation resulted from propagation of these simian viruses in unnatural (i.e., human) host cells. In this regard, HIV-2/ST is of particular interest as a human virus from which distinct subtypes with long and short transmembrane molecules have been derived. Interestingly, for SIV_{mac} the appearance of transmembrane truncations correlated with increased viral replication in human cells (32). Mutagenesis of the SIV_{mac} envelope has also suggested that, at least on some cell types, a shortened transmembrane molecule increases the replication potential of this virus (6). Because the original ST/24 virus was neither fusogenic nor cytolytic despite having a truncated transmembrane molecule, it is apparent that this change is not sufficient to produce the increased cytopathic effects observed for the ST/24.1C and ST/24.2C viruses. Nonetheless, it is possible that transmembrane truncations enable mutations in other regions of the envelope to have biologically significant consequences, possibly by influencing one or more steps involved in envelope processing, assembly, or stability that could facilitate viral entry and increase cytopathic effects during replication. Sequence analysis and comparison of cytopathic and noncytopathic molecular clones of HIV-2/ST should provide insight into the role of transmembrane truncations as a contributing factor to viral cytopathicity.

The identification of pathogenic determinants for HIV-1 and HIV-2 has been complicated by the extraordinary degree of molecular diversity among different isolates, particularly for envelope genes (30, 37, 49, 55, 63), as well as findings that even single amino acid changes in the envelope can have profound biological effects (12, 48, 62, 64). Ongoing efforts in our laboratory to characterize cytopathic and noncytopathic isolates of HIV-2/ST at a molecular level should lead to a better understanding of the structural and functional relationships for these viruses and allow us to

identify specific determinants which contribute to their diverse biological properties.

We thank S. Shattil for assistance with F-CD4 binding assays, R. C. Gallo for providing HIV-1/IIIB, and L. Montagnier for providing HIV-2/ROD. We also gratefully acknowledge J. McClure (Oncogen, Seattle, Wash.) for providing the HIV-2- and SIV-specific monoclonal antibodies and R. Sweet (SmithKline Beecham, King of Prussia, Pa.) for generously providing recombinant CD4 and for helpful discussions.

This work was supported by Public Health Service grants AI28208, AI25380, AI27290, and AI25291 from the National Institute of Allergy and Infectious Diseases and by a grant from Carter Wallace Inc. J. A. Hoxie is a scholar of the Leukemia Society of America.

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