

Human Immunodeficiency Virus Envelope V1 and V2 Regions Influence Replication Efficiency in Macrophages by Affecting Virus Spread

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The V3 hypervariable region of the HIV-1 envelope protein is a major determinant of viral tropism for macrophages. However, the replication of macrophage-tropic HIV-1 strains varies considerably in macrophages, and this variability has been linked to the V1 and V2 envelope regions. In the present study, recombinant HIV clones were generated by inserting V1 and V2 sequences from the Ba-L HIV isolate, which has a high macrophage replication level, into the genomic background of a macrophage-tropic clone with a low macrophage replication level. Infection of macrophages with varying multiplicities of infection and direct detection of the number of infected macrophages per culture showed that the Ba-L V1 and V2 envelope sequences enhanced the ability of virus to spread in the cultures. In contrast, macrophage-tropic clones with low replication efficiency infected macrophages initially but showed no evidence of spread to additional cells during the culture period. This effect on virus spread appeared to be macrophage-specific as it was not observed in cultures of T lymphocytes. Comparison of recombinant clones containing V1, V2, and V3 envelope sequences from high-efficiency Ba-L and JR-FL strains indicated that markedly different V1 and V2 sequences could impart the same rapidly spreading phenotype in macrophages. © 1995 Academic Press, Inc.

INTRODUCTION

The envelope protein of human immunodeficiency virus (HIV) is important to the early steps in virus–cell interactions leading to successful infection. Specific regions of envelope are known to bind to CD4, the main cell surface receptor for the virus. All HIV strains appear to infect CD4-positive T lymphocytes from peripheral blood or lymphoid organs; however, some strains can also infect macrophages (Gartner *et al.*, 1986; Koyanagi *et al.*, 1987; Meltzer *et al.*, 1990). Non-macrophage-tropic HIV strains usually predominate late in the course of AIDS (Schuitemaker *et al.*, 1992). *In vitro* these viruses infect CD4-positive HeLa cells (Chesebro *et al.*, 1992) and MT2 cells (Koot *et al.*, 1992; Karlsson *et al.*, 1994) and usually induce syncytia after infection of CD4-positive blood lymphocytes (Cheng-Mayer *et al.*, 1988; Tersmette *et al.*, 1989). Their V3 envelope variable regions show extensive sequence diversity, which appears to be generated independently in each infected individual during virus replication (Simmonds *et al.*, 1991; McNearney *et al.*, 1992; Delwart *et al.*, 1994). These viruses may be involved in the destruction of CD4-positive T lymphocytes, leading to severe immunosuppression (Cheng-

Mayer *et al.*, 1988; Tersmette *et al.*, 1989). In contrast, macrophage-tropic HIV strains do not infect CD4-positive HeLa cells (Chesebro *et al.*, 1992) or MT2 cells (Koot *et al.*, 1992; Karlsson *et al.*, 1994) *in vitro* and do not induce syncytia after infection of CD4-positive blood lymphocytes (Schuitemaker *et al.*, 1991; Tersmette *et al.*, 1989). Macrophage-tropic strains are predominant in patients early after infection by sexual transmission or by transfusion with contaminated factor VIII (McNearney *et al.*, 1992; Zhang *et al.*, 1993; Zhu *et al.*, 1993), suggesting that these strains may be primarily involved in these modes of transmission. In addition, macrophage-tropic strains are detected throughout the entire disease course and may be important as a reservoir for persistent infection (Schuitemaker *et al.*, 1991). Macrophage-tropic strains show markedly reduced sequence heterogeneity in the hypervariable V3 region of the *env* gene (McNearney *et al.*, 1992; Zhang *et al.*, 1993; Chesebro *et al.*, 1992; Fouchier *et al.*, 1992; Zhu *et al.*, 1993). Because these HIV strains share extensive homology in antigenically relevant regions such as V3, they might be appropriate targets for an HIV vaccine.

Previous experiments indicate that the V3 region of HIV envelope is involved in determining macrophage tropism (Shioda *et al.*, 1991; O'Brien *et al.*, 1990; Hwang *et al.*, 1991; Chesebro *et al.*, 1992). The V3 effect on tropism is known to act at the level of viral entry into cells (Cann *et al.*, 1990; Chesebro *et al.*, 1991a), but the mechanism

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by which macrophages distinguish viruses with varying V3 regions is unclear. However, V3 is not the only envelope region influencing lentivirus infection of macrophages. For example, with simian immunodeficiency virus several env regions other than V3 can also influence macrophage tropism (Banapour *et al.*, 1991; Mori *et al.*, 1992), and with HIV the V1 and V2 env sequences upstream from V3 can affect the efficiency of virus replication in macrophages (Shioda *et al.*, 1991; Westervelt *et al.*, 1992; Koito *et al.*, 1994). HIV strains with high-efficiency replication in macrophages produce culture supernatants containing higher p24 or reverse transcriptase levels than macrophage-tropic strains with low-efficiency replication. However, the mechanism of the effect on replication efficiency is not known. One possible explanation is that high-efficiency virus strains are more effective at spreading through macrophage cultures, thus infecting more cells than lower efficiency virus strains. Alternatively, high-efficiency strains might be more responsive to activation of virus transcription induced by cytokines such as IL-6 and TNF- α (Poli *et al.*, 1990; Mellors *et al.*, 1991). This might result in higher levels of viral proteins without necessarily increasing the number of infected cells, as has been observed in macrophages and monocytes under various conditions (Walsh *et al.*, 1991). In addition, such cytokine-mediated effects might be associated only with certain virus strains or cell types (Le Naour *et al.*, 1994).

The present experiments utilized infectious recombinant HIV molecular clones differing at envelope V1 and V2 regions to begin to determine how these envelope regions influence HIV replication efficiency in macrophages. Clones with V1 and V2 regions from a high-efficiency macrophage-tropic strain infected macrophages and spread rapidly to all cells in the culture even after infection with a low-input multiplicity. In contrast, macrophage-tropic clones containing V1 and V2 regions from a low-efficiency strain infected macrophages initially but failed to spread throughout the culture on subsequent days. Thus, high-efficiency replication mediated by certain V1 and V2 env sequences appeared to be mainly the result of increased virus spread in macrophages.

MATERIALS AND METHODS

Virus stocks

Macrophage-tropic HIV isolates Ba-L (Gartner *et al.*, 1986) and JR-FL (Koyanagi *et al.*, 1987) and HIV molecular clones JR-CSF (Koyanagi *et al.*, 1987) and NL4-3 (Adachi *et al.*, 1986) were described previously. Plasmid DNA from molecular clones was used to transfect clone 1022 CD4-positive HeLa cells, and these cells were cocultivated with phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) to produce virus

stocks as described previously (Chesebro *et al.*, 1990, 1991a). HIV isolates were grown directly in PHA-stimulated PBMC. The Ba-L HIV isolate was also grown on macrophages to obtain cDNA for cloning. Virus stocks were titered by endpoint dilution in 96-well trays with PHA-stimulated PBMC. Cultures were followed for 3 weeks, and titers were expressed as tissue culture ID₅₀ per volume inoculated (Chesebro *et al.*, 1991b).

Construction of recombinant HIV clones

Clone 49-5 containing a 0.4-kb *Stu*I–*Nhe*I fragment from the Ba-L HIV isolate in the NL4-3 background was described previously (Chesebro *et al.*, 1992). Clone 107 (Fig. 1) was derived from clone 49-5 by first replacing the 5' envelope sequences from *Eco*RI to *Stu*I in 49-5 with an oligonucleotide polylinker. The polylinker oligonucleotides containing *Eco*RI, *Sal*I, *Not*I, and *Stu*I sites were: 735, 5' AAT-TCG-TCG-ACG-CGG-CCG-CAG-G 3', and 736, 5' CCT-GCG-GCC-GCG-TCG-ACG 3'. The linkers were annealed, phosphorylated with kinase, and cloned into the 49-5 clone using standard techniques. Subsequently HIV DNA amplified by PCR from Ba-L-infected macrophages was inserted in-frame at these sites. Ba-L-infected macrophages were harvested when the reverse transcriptase levels were elevated, and mRNA was prepared using a Fast Track kit (Invitrogen). cDNA was then made using the cDNA Cycle kit (Invitrogen), and HIV envelope sequences were amplified by PCR. The cDNA reaction was primed with oligonucleotide 734: 5' TGC-TGC-TGG-CTC-AGC-TCG-TGG-CT 3'. The *Eco*RI–*Stu*I fragment for clone 107 was amplified by PCR using oligonucleotides: 279, 5' CTG-GCG-GCC-GCA-GCC-ATA-ATA-AGA-ATT-CTG-C 3', and 6833C, 5' CCT-TTG-GAC-AGG-CCT-GTG-TAA-3'. PCR amplification began with incubation for 5 min at 94°, followed by 39 cycles of 1 min at 94°, 1 min 15 sec at 53°, and 3 min at 72°, and 1 cycle identical to the above except the 72° extension time was increased to 10 min. Successful PCR amplification products were cloned into the PCR II vector (Invitrogen), excised by digestion with *Eco*RI and *Stu*I, and inserted into the polylinker site created in clone 49-5. Clone 19A (Fig. 1) was generated by amplifying and cloning a *Stu*I to *Bam*HI fragment from Ba-L-infected macrophage cDNA with oligonucleotides 6813 and 8472C into clone NL4-3 as previously described (Chesebro *et al.*, 1991a). Clone 53 (Fig. 1) was constructed by inserting the *Eco*RI to *Stu*I polylinker into clone 19A and then replacing the polylinker with the *Eco*RI to *Stu*I fragment from clone 107. Clone 81A was generated by inserting the 0.25-kb *Dra*III–*Stu*I fragment from clone 107 into a plasmid containing sequences of NL4-3 from *Eco*RI to *Dra*III, followed by a polylinker with restriction sites including *Dra*III, *Stu*I, and *Bam*HI. The 1.1-kb *Eco*RI–*Stu*I fragment excised from this plasmid was then cloned into clone 49-5.

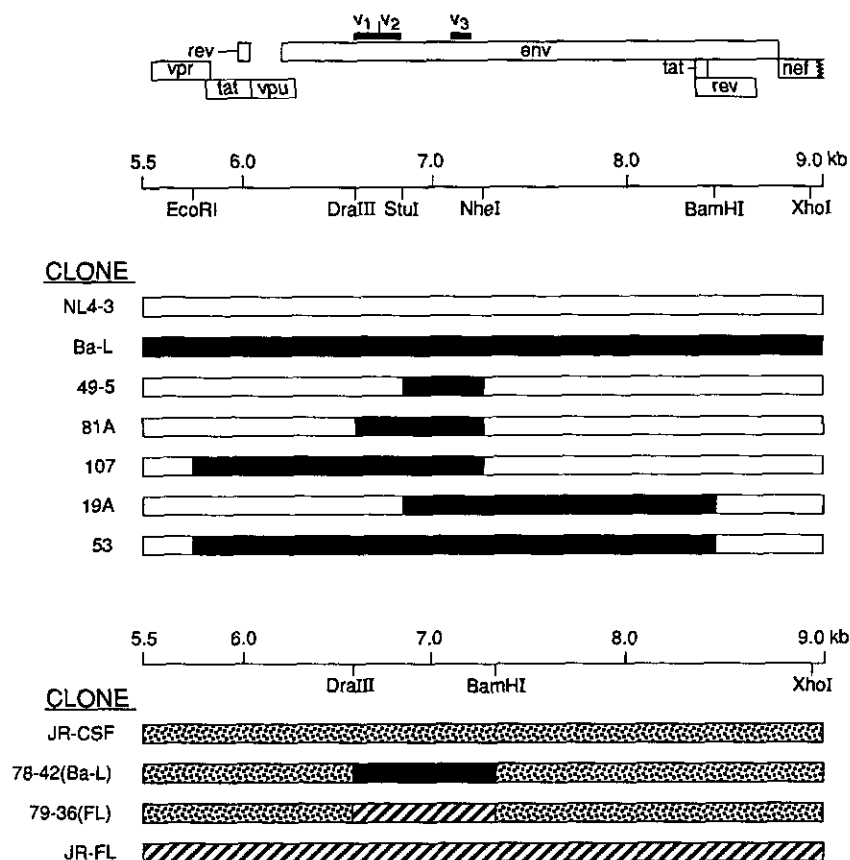


FIG. 1. 3' portion of genomes of recombinant HIV clones. In the NL4-3 genome, the *EcoRI* site used for cloning is at position 5722, which is 500 bp upstream from the envelope start, *DraIII* is at 6591, *StuI* is at 6822, *NheI* is at 7250, and *BamHI* is at 8465. In the JR-CSF genome, the *DraIII* site is at position 6609 and *BamHI* is at 7325. The *DraIII* sites of NL4-3 and JR-CSF are homologous.

Recombinant HIV clones were also made using the genomic background of the infectious molecular clone JR-CSF (Koyanagi *et al.*, 1987). pYKJRCFSF, obtained from Dr. I. Chen, Department of Microbiology, University of California, Los Angeles, School of Medicine, was re-cloned to reduce the amount of flanking sequence outside the provirus by digesting with *EcoRI* and *StuI*, blunting the ends with Klenow enzyme, and religating into pUC19 at the *PvuII* site to create clone 7-23. Approximately 0.7 kb of the *env* gene between *DraIII* and *BamHI* sites including the V1, V2, and V3 regions was removed from clone 7-23 and replaced by a polylinker using oligonucleotides 745, 5' GTG-AGG-CCT-CTA-GAC-GCG-TGC-TAG-CCT-CAG 3', and 746, 5' GAT-CCT-GAG-GCT-AGC-ACG-CGT-CTA-GAG-GCC-TCA-CAG 3', to create clone 12-34. HIV envelope DNA from PHA-stimulated PBMC cultures infected with Ba-L or JR-FL isolates was amplified by PCR using oligonucleotides 294, 5' GTA-AAA-TTA-ACC-CCA-CTC-TGT-GT 3', and 335, 5' CAA-TTT-CTG-GAT-CCC-CTC-C-3', using 4 min at 94°, followed by 30 cycles of 94° for 1 min, 40° for 1 min, and 72° for 2 min. On the final cycle the 72° incubation was for 10 min. Amplified DNA was then digested with *DraIII* and *BamHI*

and ligated into p12-34 to create two full-length recombinant HIV infectious molecular clones: 78-42 with a Ba-L insert and 79-36 with a JR-FL insert (Fig. 1).

Macrophage infectivity assay

Blood-derived monocytes were prepared as described by Freundlich and Aldovic (1983). Briefly, PBMC were obtained by differential centrifugation on Ficoll-Hypaque Lymphocyte Separation Medium (Organon Teknika Corp., West Chester, PA). Adherence and elution steps using gelatin-coated tissue culture flasks were used to separate monocytes from lymphocytes. The monocytes were plated in 0.6 to 1.0 ml RPMI 1640 with 10% heat-inactivated human serum at a density of 5×10^5 cells per well in a 48-well tissue culture plate (Costar). Human serum was selected to give optimum HIV replication using clone JR-CSF or 49-5. Medium was changed on Days 4 and 7, and cells were infected on Day 7 by adding 0.1 to 0.2 ml diluted virus stock to the fresh culture medium. Two days postinfection cultures were washed three times with serum-free medium and then refed. Media was changed every 4 days thereafter, and supernatants were frozen at -20° in order to determine p24 levels at

a later date. On the appropriate day after infection, cells were fixed with 95% ethanol and 3% H₂O₂ for 15 min, rinsed, and stained with anti-p24 monoclonal antibody (clone 183-H12-5C) to quantitate antigen-positive cells as previously described (Chesebro *et al.*, 1992).

Determination of p24 values was done by a p24 capture ELISA developed in this laboratory. The anti-p24 monoclonal antibody 183-H12-5C (NIAID, AIDS Repository, Catalog 1513) was affinity purified on sepharose-Protein G, adjusted to a concentration of 1.0 mg/ml, diluted 1:150 in Dulbecco's PBS without calcium and magnesium ions, and 0.1 ml was added to each well of Immulon II plates (Dynatech Labs, Chantilly, VA) and incubated overnight at 37°. Other protein binding sites on wells were then blocked by incubation with 0.25 ml PBS with 5% FCS for 1 hr at 37°. Plates were washed 6 times with PBS with 0.2% Tween 20. Culture supernatants were mixed with 1/10th volume of PBS with 10% Triton X-100. Dilutions of supernatants and subsequent antibodies were made in PBS-10% FCS-0.5% Triton X-100. One-tenth milliliter of culture supernatants diluted to an appropriate concentration was added to the wells, and trays were incubated at 37° overnight. The plates were then washed six times with PBS-Tween 20, and 100 μ l of a 1:5000 dilution of human anti-HIV antibody (NIAID, AIDS Research and Reference Program, catalog 192), biotinylated by standard procedures, was applied to each well. The plate was incubated for 1 hr at 37° and washed six times with PBS-Tween 20. One-tenth milliliter of peroxidase-conjugated avidin (Organon Teknika Corp., West Chester, PA) at a 1:1000 dilution was added to each well, incubated for 30 min at 37°, and the plates were washed again as previously described. One-tenth milliliter of peroxidase substrate (10 ml 0.1 M sodium acetate, pH 5.0, 0.3 ml of 4 mg/ml tetramethylbenzidine in DMSO, and 5 μ l of 30% H₂O₂) was added and incubated at room temperature for 20 min. The reaction was stopped with 50 μ l 2 M H₂SO₄ and optical density was read at 450 nm. Values were compared to a standard curve made using p24 (NIAID, AIDS Repository, Catalog 382) at concentrations from 25 to 1000 pg/ml.

Sequence and analysis

Sequence was obtained by dideoxy sequencing using the Sequenase version 2.0 DNA Sequencing kit (USB). In some cases the sequence was confirmed using the dsDNA Cycle Sequencing System (BRL-Life Technologies, Inc.). Analysis was done with the Microgenie program. Alignments were done by the ALIGN Plus program developed by Scientific and Educational Software.

RESULTS

Characterization of macrophage-tropic HIV with chimeric env genes

In order to study the effect of envelope V1 and V2 sequences on replication in macrophages, recombinant

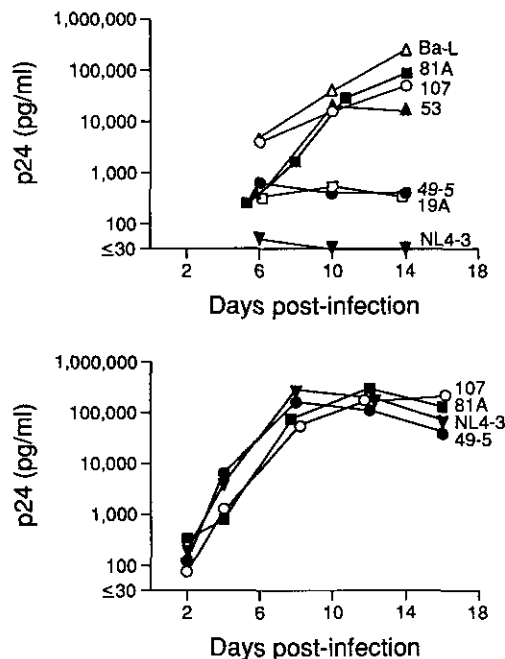


FIG. 2. Influence of HIV envelope sequences on kinetics of p24 production by macrophages (top) and PBMC (bottom). Ba-L (Δ), clone 81A ($\blacksquare$), clone 107 ($\circ$), and clone 53 ($\blacktriangle$) show high p24 levels in macrophages, whereas clones 49-5 ($\bullet$) and 19A ($\square$) show low p24 levels. Non-macrophage-tropic clone NL4-3 ($\blacktriangledown$) showed no detectable p24. In PBMC no significant difference in kinetics of p24 production was seen in the clones studied. Cultures were infected with 10^2 to $10^{2.5}$ TCID₅₀ of each virus stock.

molecular clones were constructed using NL4-3 background genes together with the V3 region of the macrophage-tropic Ba-L isolate plus additional Ba-L env sequences upstream or downstream of V3 (Fig. 1). Macrophage infection experiments with clone 49-5 containing only the V3 region from Ba-L were in agreement with previous results (Chesebro *et al.*, 1992), showing significant macrophage infection compared to the non-macrophage-tropic parental clone NL4-3 (Fig. 2, top panel). Clone 19A containing the Ba-L V3 region plus 1.2 kb of Ba-L envelope sequences downstream of V3 gave results similar to those of clone 49-5. In contrast, clones 81A and 107 containing the Ba-L V3 region plus 0.23 to 1.1 kb of Ba-L sequences upstream of V3 gave much higher p24 levels in macrophages. Clone 53 which contained Ba-L sequences both upstream and downstream of V3 gave a pattern of p24 production in macrophages nearly identical to clones 107 and 81A. Results with clones 81A, 107, and 53 were very similar to those with the uncloned Ba-L biological isolate. Thus, although Ba-L V3 sequences alone conferred macrophage tropism, addition of Ba-L V1 and V2 env sequences upstream of V3 increased p24 production by clone 81A in macrophages. These results were in agreement with those using recombinants containing 5' env sequences of two other HIV strains (Westervelt *et al.*, 1992; Koito *et al.*, 1994).

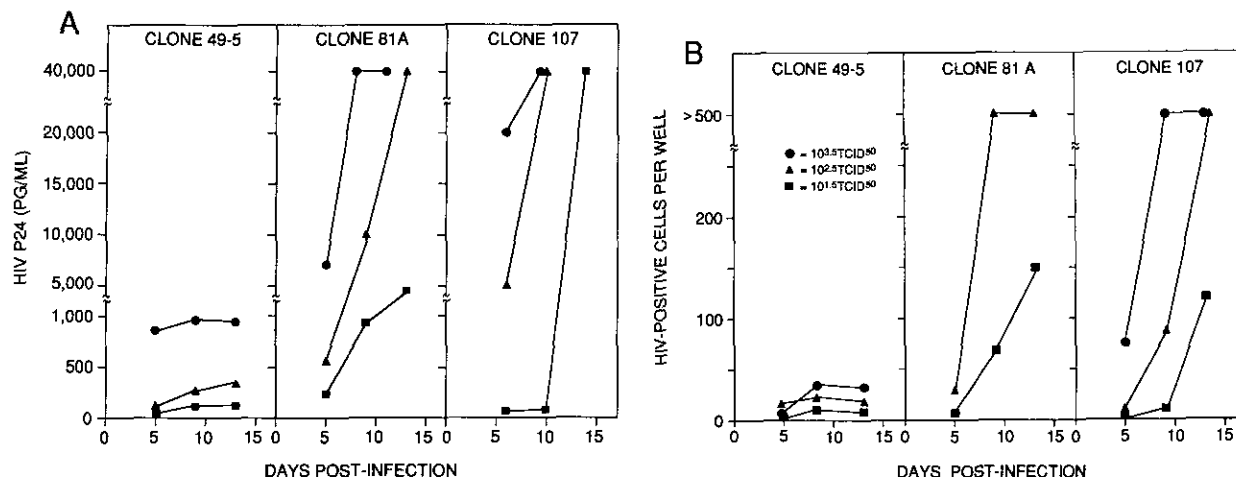


FIG. 3. (A) Influence of input titer on p24 levels in macrophage cultures. Clones 107 and 81A showed rising p24 levels with increasing time postinfection, and the time of initial p24 elevation was related to the original input of infectivity. In contrast, clone 49-5 showed no increase in p24 with time postinfection, and the plateau p24 level was related to the input of infectivity titer. Input titers: (●) $10^{3.5}$ TCID₅₀, (▲) $10^{2.5}$ TCID₅₀, and (■) $10^{1.5}$ TCID₅₀. (B) Quantitation of HIV-positive macrophages at various times after infection. Ba-L V1/V2 envelope sequences in clones 81A and 107 were associated with virus spread in macrophages, whereas NL4-3 V1 and V2 sequences in clone 49-5 were associated with lack of viral spread in macrophages. Cells were infected with varying amounts of each virus, and cultures were fixed and stained with anti-p24 antibody on the days indicated.

To determine whether these strain differences in HIV replication were unique to macrophages, PHA-stimulated PBMC were infected with several of these HIV clones. Clones differing at various *env* regions including V1, V2, or V3 showed no difference in kinetics of replication in PBMC (Fig. 2, bottom panel). Thus, V1 and V2 regions of macrophage-tropic HIV had a specific effect on replication efficiency in macrophages, but not in PHA-stimulated T lymphocytes.

Virus spread in macrophages

In most previous studies and in the above experiments, macrophages were infected with high amounts of infectious HIV. From this work it was unclear whether or not all of the cells were infected initially and whether higher p24 levels were due to increased viral protein production by high-efficiency HIV strains compared to low-efficiency strains. Therefore, to study possible differences in viral spread in macrophages, in the present experiments three different multiplicities of infection (m.o.i.) were used to compare HIV clones 49-5, 81A, and 107. Macrophages infected with clones 107 and 81A gave rising p24 values with increasing time, and the initial p24 expression correlated with the amount of infectious virus used to infect the culture (Fig. 3A). In contrast, after infection with clone 49-5, macrophage cultures showed no increase of p24 values with time, and the level of the p24 detected was related only to the amount of virus originally used to infect the cultures (Fig. 3A). These results indicated that the high-efficiency clones 107 and 81A were somehow amplified by the macro-

phage cultures, whereas the low-efficiency clone 49-5 was not.

In order to determine whether amplification of HIV expression after macrophage infection by clones 81A and 107 was due to increasing viral protein expression per infected cell or to increasing numbers of infected cells during the culture period, cells expressing HIV antigen were quantitated by immunocytochemistry. Cultures were fixed at various days after infection, and virus-infected cells were stained using anti-p24 antibodies. Infection with clones 107 or 81A resulted in increasing numbers of HIV p24-positive cells with time (Fig. 3B). When cultures were incubated for longer times (18–21 days), 90–100% of cells were infected by these clones even after inoculation of the lowest input m.o.i. In contrast, clones 49-5 and 19A showed no consistent increase in the number of infected cells over the course of the experiment (Fig. 3B). Furthermore, the intensity of staining for HIV antigens in individual positive cells appeared to be similar for clones with differing replication efficiencies (Fig. 4). Thus, the differences in replication efficiency of these clones and isolates correlated with their ability to spread to uninfected macrophages over the course of the experiment. Differences in the envelope sequence within or near the V1 and V2 regions of clones 81A and 49-5 appeared to be sufficient to account for these changes. Clones 81A and 49-5 differed at a total of 26 envelope amino acids. Of these, 16 residues were in the V1 region and 8 residues were in the V2 region (Fig. 5A). At this time it is unclear which of these changes are involved in the differences in spread of infection in macrophages.

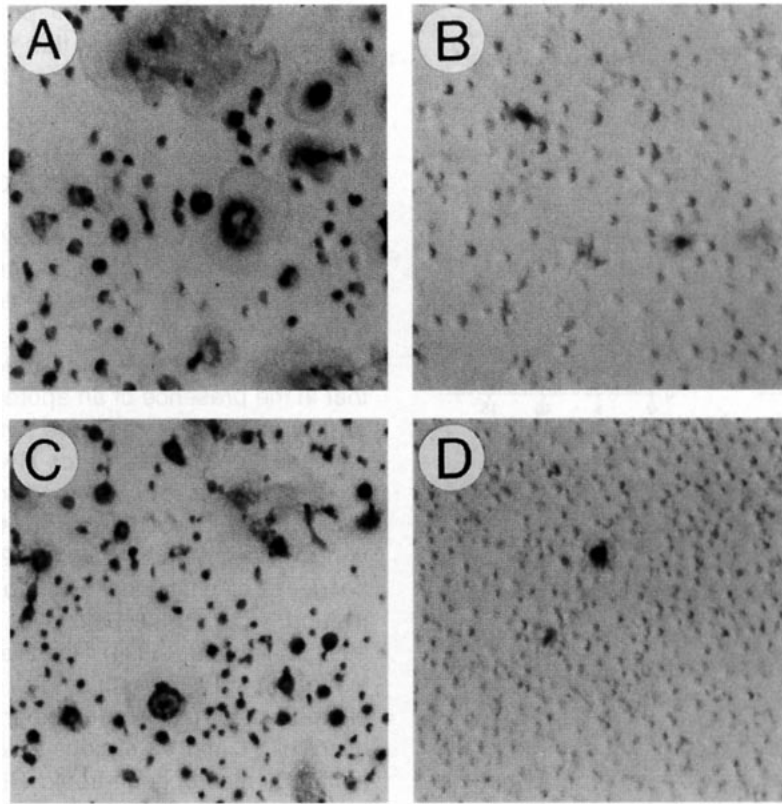


FIG. 4. Immunocytochemical detection of HIV-positive macrophages. On Day 18 postinfection with 10^2 TCID₅₀ of each HIV strain, macrophages were fixed and stained for p24 using the indirect immunoperoxidase technique described under Materials and Methods. (A) Ba-L HIV isolate; (B) clone 49-5; (C) clone 107; (D) clone 19A. In A and C most of the cells are p24-positive and many syncytia can be seen. In contrast, in B and D only occasional p24-positive cells per field are present. However, no differences in the intensity of staining of individual positive cells were noted among different HIV strains studied.

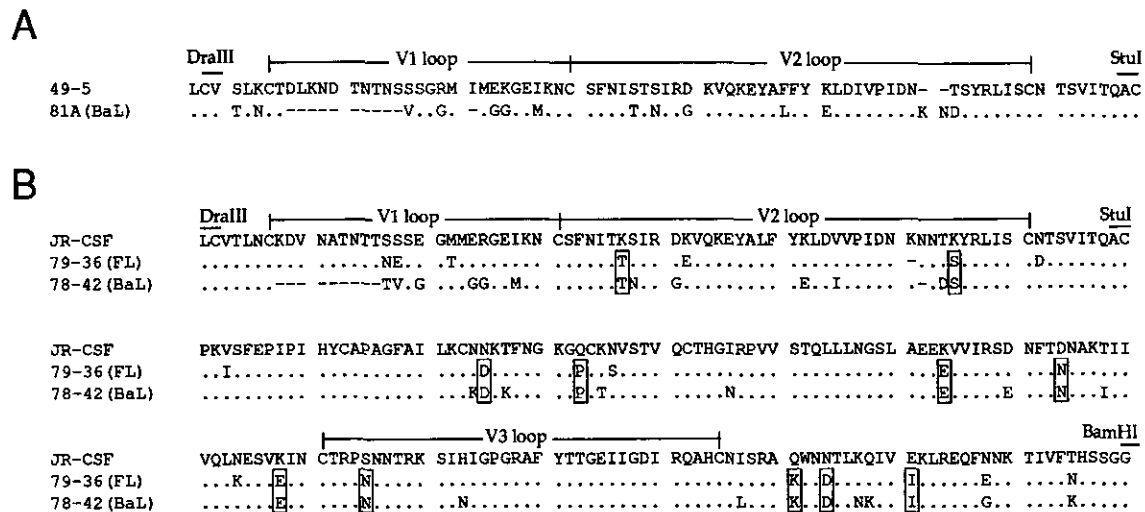


FIG. 5. (A) Comparison of envelope amino acid sequences for the region (Dralll–Stul) which differed between the nonspreading clone, 49-5, and the spreading clone, 81A (Ba-L). This region included V1 and V2 loop variable regions. Dots indicate identical residues and dashes indicate deleted residues. (B) Comparison of 5' HIV envelope sequences from macrophage-tropic clones differing in ability to spread in macrophage cultures. The nonspreading clone, JR-CSF, is compared to the two spreading clones, 78-42 (Ba-L) and 79-36 (FL), which differ from JR-CSF only between Dralll and BamHI sites. The amino acids enclosed in boxes are identical in clones 78-42 and 79-36 and different from clone JR-CSF. The sequences of the two Ba-L-derived clones, 81A and 78-42, are identical.

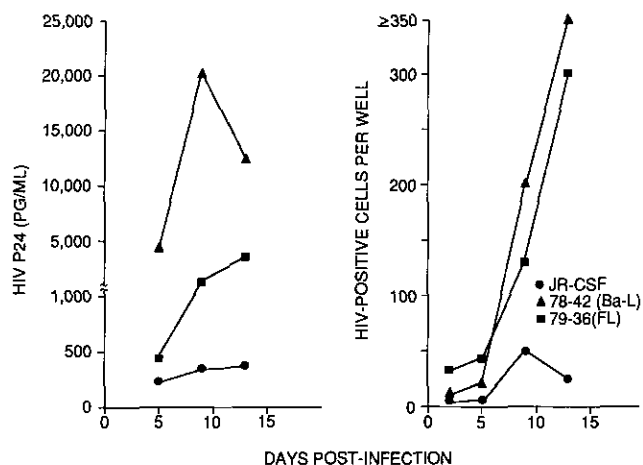


FIG. 6. Comparison of kinetics of macrophage infection by strains JR-CSF (●), 78-42 (Ba-L) (▲), and 79-36 (FL) (■). JR-CSF showed no significant rise in supernatant p24 level or number of HIV-positive cells with time postinfection. In contrast, clones 78-42 and 79-36 showed both rising p24 levels and increasing numbers of HIV-positive cells.

Effect of 5' env sequences of macrophage-tropic HIV strains JR-FL and JR-CSF

Previous studies comparing infectivity of various HIV strains showed that there was no 5' envelope consensus sequence associated with high-efficiency replication in macrophages (Westervelt *et al.*, 1992). Thus, it was possible that different 5' envelope sequences caused high replication efficiency in macrophages by different biological mechanisms. Therefore, a recombinant clone with 5' envelope sequences from Ba-L was compared directly to a homologous recombinant clone containing markedly different sequences from isolate JR-FL, also known to have high replication efficiency in macrophages (Koyanagi *et al.*, 1987; Sperduto *et al.*, 1993). Both of these recombinants were made in the background genes of clone JR-CSF (Fig. 1), which is macrophage-tropic but has low replication efficiency in macrophages (Koyanagi *et al.*, 1987; Sperduto *et al.*, 1993). The results indicated that clones 78-42 and 79-36 containing V1, V2, and V3 env sequences of Ba-L or JR-FL, respectively, could spread in macrophage cultures, whereas the parental clone, JR-CSF, could not (Fig. 6). In spite of the similar ability of clones 78-42 and 79-36 to spread in macrophages, these clones differed at 41 amino acid residues in the inserted envelope fragment, and 24 of these differences were in the V1 or V2 loops (Fig. 5B). There were 11 residues where clones 78-42 and 79-36 differed from JR-CSF but were identical to each other; however, only 2 of these residues were in the V1 or V2 regions (Fig. 5B). These results indicated that clones with markedly differing 5' envelope sequences had a similar ability to spread in macrophage cultures to produce high p24 levels. Furthermore, the similarity in macrophage infection by clones 81A and 78-42, which shared V1, V2, and V3 env se-

quences from Ba-L, but differed in other background genes (Fig. 1), suggested that the background genes of the parental clones NL4-3 and JR-CSF did not influence these results.

DISCUSSION

The V3 region of the HIV envelope protein has been shown to have a profound influence on the differences in the ability of HIV strains to infect macrophages, T cell leukemia lines, CD4-positive T lymphocytes, and CD4-positive HeLa cells. Several studies have also suggested that in the presence of an appropriate V3 region, V1 and V2 env sequences can have a marked influence on the efficiency or magnitude of HIV replication in macrophages (Shioda *et al.*, 1991; Koito *et al.*, 1994; Westervelt *et al.*, 1992). Previous experiments indicated that, in addition to the envelope V1 and V2 regions, *vpr*, *vpu*, and *nef* genes also influence HIV replication in macrophages (Hattori *et al.*, 1990; Balotta *et al.*, 1993; Balliet *et al.*, 1994; Miller *et al.*, 1994). However, these genes were not a variable in the current studies due to the recombinant clones used. The present experiments showed that the effect of V1 and V2 regions is to promote or inhibit the ability of macrophage-tropic HIV to spread in macrophage cultures. This finding was detected by both directly analyzing the number of infected macrophages at various times after infection and comparing the kinetics of p24 production in the supernatant fluid. Both of the methods showed that without the appropriate V1 and V2 sequences, macrophage-tropic HIV clones failed to spread after initial infection, resulting in a plateau level of both p24 production and number of HIV-positive cells. Thus, when nonspreading macrophage-tropic HIV strains were tested, the level of p24 detected was highly dependent on the titer of input virus used to infect the macrophages. Nonspreading macrophage-tropic clones, such as 49-5 or JR-CSF, sometimes appeared to be non-macrophage-tropic if tested with a low-input titer (Chesebro *et al.*, 1992; Willey *et al.*, 1994). In contrast, even low titers of macrophage-tropic HIV strains with the spreading phenotype, such as Ba-L or JR-FL, were able to completely infect a macrophage monolayer during a 14- to 21-day period. Thus, regardless of amount of infectivity used for infection such strains were easy to type as macrophage-tropic.

The present experiments do not distinguish whether the V1/V2 sequence effects on virus spreading occur at the level of the viral RNA or protein sequence. Also, it is not yet clear at what time in the viral life cycle such effects occur. However, the viral envelope protein is known to play an important role in the cell entry, and the V1 and V2 envelope regions have been shown to interact with other envelope regions, including V3, V4, V5, and C4, to influence syncytia formation in T lymphocytes, cell

tropism, and antigenic epitopes (Groenink *et al.*, 1993; Freed and Martin, 1994; Andeweg *et al.*, 1993; Fung *et al.*, 1992; McKeating *et al.*, 1993; Moore *et al.*, 1993; Sullivan *et al.*, 1993; Kang *et al.*, 1994; Koito *et al.*, 1995). Thus it seems likely that similar interactions between envelope protein subregions might be responsible for the V1/V2 effect on virus spread in macrophages. Effects on virus-mediated cell fusion might be restricted to certain cell types, such as macrophages, due to the effects of V1, V2, and V3 on cell tropism. This could explain why the V1/V2 effect on virus spread was observed in macrophages, but not in PBMC (Fig. 2). It is also possible that the V1/V2 region might affect other aspects of the viral life cycle such as transcription, translation, or virus assembly. The present results failed to detect higher intracellular levels of viral protein per infected cell in spreading versus nonspreading clones, so an effect on transcription or translation resulting in higher viral protein production seems unlikely. However, further experiments with more quantitative methods will be required to exclude this possibility. The envelope protein is also involved in virus assembly. In macrophages HIV particles have been observed to bud both at the plasma membrane and into intracellular vesicles (Orenstein *et al.*, 1988). Particles found at the plasma membrane might be much more effective at spreading to uninfected macrophages. By influencing envelope protein trafficking, the V1 and V2 envelope sequences might influence the sites of production and release of infectious viruses by macrophages (Owens *et al.*, 1991). This, in turn, could have a marked influence on virus spread in macrophage cultures. Further characterization of the virions produced by infected macrophages will be required to analyze this aspect of HIV spread in macrophages.

Previous data indicated that several virus strains with differing V1/V2 sequences all had similar high-replication efficiency in macrophages (Westervelt *et al.*, 1992). It is possible that these V1/V2 sequences might influence replication efficiency by additional mechanisms other than spread. However, our results comparing recombinants with V1, V2, and V3 sequences from Ba-L and JR-FL strains indicated that both of these sequences acted to increase spread in macrophages. Thus there appeared to be no V1/V2 sequence associated with the clones which spread well in macrophages. Similarly, the two nonspreading clones, 49-5 and JR-CSF, differed at eight amino acids in V1 and at eight amino acids in V2. The fact that such different sequences produced the same biological phenotype suggests that, if these effects are mediated by the envelope protein, the envelope proteins of clones with a common phenotype might have a similar tertiary structure (Ebenbichler *et al.*, 1993).

The discovery of differences in the ability of HIV strains to spread in macrophage cultures *in vitro* raises the question of the relevance of such differences to HIV

pathogenesis *in vivo*. One would expect that these different phenotypes might have quite different effects *in vivo*. For example, nonspreading macrophage-tropic viruses might be involved in transmission or latency, whereas spreading macrophage-tropic viruses might be more acutely pathogenic and could be involved in immunosuppression, wasting disease, or dementia associated with AIDS, depending on the location of the infected macrophages (Kodama *et al.*, 1993; Power *et al.*, 1994; Cheng-Mayer, 1993; Sperduto *et al.*, 1993; Weiss, 1993; Levy, 1993). In a recent study, macrophage-tropic virus isolates with a slow-replication phenotype were isolated immediately after seroconversion, whereas more rapidly replicating macrophage-tropic viruses were isolated somewhat later (Connor and Ho, 1994). It is possible that these slow- and fast-replicating isolates are similar to the nonspreading and spreading strains studied in the present report. If the slow-replicating nonspreading strains are consistently the earliest ones found after seroconversion following sexual transmission, they may also be the strains involved in this mode of transmission of HIV (Chesebro *et al.*, 1992; McNearney *et al.*, 1992; Zhu *et al.*, 1993).

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REFERENCES

- Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A., and Martin, M. (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and non-human cells transfected with an infectious molecular clone. *J. Virol.* **59**, 284–291.
- Andeweg, A. C., Leeftang, P., Osterhaus, A. D., and Bosch, M. L. (1993). Both the V2 and V3 regions of the human immunodeficiency virus type 1 surface glycoprotein functionally interact with other envelope regions in syncytium formation. *J. Virol.* **67**, 3232–3239.
- Balliet, J. W., Kolson, D. L., Eiger, G., Kim, F. M., McGann, K. A., Srinivasan, A., and Collman, R. (1994). Distinct effects in primary macrophages and lymphocytes of the human immunodeficiency virus type 1 accessory genes *vpr*, *vpu*, and *nef*: Mutational analysis of a primary HIV-1 isolate. *Virology* **200**, 623–631.
- Balotta, C., Lusso, P., Crowley, R., Gallo, R. C., and Franchini, G. (1993). Antisense phosphorothioate oligodeoxynucleotides targeted to the *vpr* gene inhibit human immunodeficiency virus type 1 replication in primary human macrophages. *J. Virol.* **67**, 4409–4414.
- Banapour, B., Marthas, M. L., Ramos, R. A., Lohman, B. L., Unger, R. E., Gardner, M. B., Pedersen, N. C., and Luciw, P. A. (1991). Identification of viral determinants of macrophage tropism for simian immunodeficiency virus SIVmac. *J. Virol.* **65**, 5798–5805.
- Cann, A. J., Zack, J. A., Go, A. S., Arrigo, S. J., Koyanagi, Y., Green, P. L., Koyanagi, Y., Pang, S., and Chen, I. S. Y. (1990). Human immunodeficiency virus type 1 T-cell tropism is determined by events prior to provirus formation. *J. Virol.* **64**, 4735–4742.
- Cheng-Mayer, C., Seto, D., Tateno, M., and Levy, J. A. (1988). Biologic features of HIV-1 that correlate with virulence in the host. *Science* **240**, 80–82.

- Cheng-Mayer, C. (1993). HIV-1 variation: Consequences for disease progression and vaccine strategies. *Trends Microbiol.* **1**, 353–355.
- Chesebro, B., Wehrly, K., and Maury, W. (1990). Differential expression in human and mouse cells of human immunodeficiency virus pseudotyped by murine retroviruses. *J. Virol.* **64**, 4553–4557.
- Chesebro, B., Nishio, J., Perryman, S., Cann, A., O'Brien, W., Chen, I. S. Y., and Wehrly, K. (1991a). Identification of human immunodeficiency virus envelope gene sequences influencing viral entry into CD4-positive HeLa cells, T-leukemia cells, and macrophages. *J. Virol.* **65**, 5782–5789.
- Chesebro, B., Wehrly, K., Metcalf, J., and Griffin, D. E. (1991b). Use of a new CD4-positive HeLa cell clone for direct quantitation of infectious human immunodeficiency virus from blood cells of AIDS patients. *J. Infect. Dis.* **163**, 64–70.
- Chesebro, B., Wehrly, K., Nishio, J., and Perryman, S. (1992). Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 envelope sequence homogeneity in comparison with T-cell-tropic isolates: Definition of critical amino acids involved in cell tropism. *J. Virol.* **66**, 6547–6554.
- Connor, R. I., and Ho, D. D. (1994). Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. *J. Virol.* **68**, 4400–4408.
- Delwart, E. L., Sheppard, H. W., Walker, B. D., Goudsmit, J., and Mullins, J. I. (1994). Human immunodeficiency virus type 1 evolution in vivo tracked by DNA heteroduplex mobility assays. *J. Virol.* **68**, 6672–6683.
- Ebenbichler, C., Westervelt, P., Carrillo, A., Henkel, T., Johnson, D., and Ratner, L. (1993). Structure-function relationships of the HIV-1 envelope V3 loop tropism determinant: Evidence for two distinct conformations. *AIDS* **7**, 639–646.
- Fouchier, R. A. M., Groenink, M., Kootstra, N. A., Tersmette, M., Huisman, H. G., Miedema, F., and Schuitemaker, H. (1992). Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J. Virol.* **66**, 3183–3187.
- Freed, E. O., and Martin, M. A. (1994). Evidence for a functional interaction between the V1/V2 and C4 domains of human immunodeficiency virus type 1 envelope glycoprotein gp120. *J. Virol.* **68**, 2503–2512.
- Freundlich, B., and Avdalovic, N. (1983). Use of gelatin/plasma coated flasks for isolating human peripheral blood monocytes. *J. Immunol. Methods.* **62**, 31–37.
- Fung, M. S., Sun, C. R., Gordon, W. L., Liou, R. S., Chang, T. W., Sun, W. N., Daar, E. S., and Ho, D. D. (1992). Identification and characterization of a neutralization site within the second variable region of human immunodeficiency virus type 1 gp120. *J. Virol.* **66**, 848–856.
- Gartner, S., Markovits, P., Markovitz, D. M., Kaplan, M. H., Gallo, R. C., and Popovic, M. (1986). The role of mononuclear phagocytes in HTLV-III/LAV infection. *Science* **233**, 215–219.
- Groenink, M., Fouchier, R. A. M., Broersen, S., Baker, C. H., Koot, M., van't Wout, A. B., Huisman, H. G., Miedema, F., Tersmette, M., and Schuitemaker, H. (1993). Relation of phenotype evolution of HIV-1 to envelope V2 configuration. *Science* **260**, 1513–1516.
- Hattori, N., Michaels, F., Fargnoli, K., Marcon, L., Gallo, R. C., and Franchini, G. (1990). The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages. *Proc. Natl. Acad. Sci. USA* **87**, 8080–8084.
- Hwang, S. S., Boyle, T. J., Lyster, H. K., and Cullen, B. R. (1991). Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**, 71–74.
- Kang, C. Y., Hariharan, K., Nara, P. L., Sodroski, J., and Moore, J. P. (1994). Immunization with a soluble CD4–gp120 complex preferentially induces neutralizing anti-human immunodeficiency virus type 1 antibodies directed to conformation-dependent epitopes of gp120. *J. Virol.* **68**, 5854–5862.
- Karlsson, A., Parsmyr, K., Sandström, E., Fenyő, E. M., and Albert, J. (1994). MT-2 cell tropism as prognostic marker for disease progression in human immunodeficiency virus type 1 infection. *J. Clin. Microbiol.* **32**, 364–370.
- Kodama, T., Mori, K., Kawahara, T., Ringler, D. J., and Desrosiers, R. C. (1993). Analysis of simian immunodeficiency virus sequence variation in tissues of rhesus macaques with simian AIDS. *J. Virol.* **67**, 6522–6534.
- Koito, A., Harrowe, G., Levy, J. A., and Cheng-Mayer, C. (1994). Functional role of the V1/V2 region of human immunodeficiency virus type 1 envelope glycoprotein gp120 in infection of primary macrophages and soluble CD4 neutralization. *J. Virol.* **68**, 2253–2259.
- Koito, A., Stamatatos, L., and Cheng-Mayer, C. (1995). Small amino acid sequence changes within the V2 domain can affect the function of a T-cell line-tropic human immunodeficiency virus type 1 envelope gp120. *Virology* **206**, 878–884.
- Koot, M., Vos, A. H., Keet, R. P., de Goede, R. E., Dercksen, M. W., Terpstra, F. G., Coutinho, R. A., Miedema, F., and Tersmette, M. (1992). HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay. *AIDS* **6**, 49–54.
- Koyanagi, Y., Miles, S., Mitsuyasu, R. T., Merrill, J. E., Vinters, H. V., and Chen, I. S. Y. (1987). Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. *Science* **236**, 819–822.
- Le Naour, R., Clayette, P., Henin, Y., Mabondzo, A., Raoul, H., Bousseau, A., and Dormont, D. (1994). Infection of human macrophages with an endogenous tumour necrosis factor- α (TNF- α)-independent human immunodeficiency virus type 1 isolate is unresponsive to the TNF- α synthesis inhibitor RP 55778. *J. Gen. Virol.* **75**, 1379–1388.
- Levy, J. A. (1993). Pathogenesis of human immunodeficiency virus infection. *Microbiol. Rev.* **57**, 183–289.
- McKeating, J. A., Shotton, C., Cordell, J., Graham, S., Balfe, P., Sullivan, N., Charles, M., Page, M., Bolmstedt, A., Olofsson, S., Kayman, S. C., Wu, Z., Pinter, A., Dean, C., Sodroski, J., and Weiss, R. A. (1993). Characterization of neutralizing monoclonal antibodies to linear and conformation-dependent epitopes within the first and second variable domains of human immunodeficiency virus type 1 gp120. *J. Virol.* **67**, 4932–4944.
- McNearney, T., Hornickova, Z., Markham, R., Birdwell, A., Arens, M., Saah, A., and Ratner, L. (1992). Relationship of human immunodeficiency virus type 1 sequence heterogeneity to stage of disease. *Proc. Natl. Acad. Sci. USA* **89**, 10247–10251.
- Mellors, J. W., Griffith, B. P., Ortiz, M. A., Landry, M. L., and Ryan, J. L. (1991). Tumor necrosis factor- α /cachectin enhances human immunodeficiency virus type 1 replication in primary macrophages. *J. Infect. Dis.* **163**, 78–82.
- Meltzer, M. S., Skillman, D. R., Gomatos, P. J., Kalter, D. C., and Gendelman, H. E. (1990). Role of mononuclear phagocytes in the pathogenesis of human immunodeficiency virus infection. *Annu. Rev. Immunol.* **8**, 169–194.
- Miller, M. D., Warmerdam, M. T., Gaston, I., Greene, W. C., and Feinberg, M. B. (1994). The human immunodeficiency virus-1 nef gene product: A positive factor for viral infection and replication in primary lymphocytes and macrophages. *J. Exp. Med.* **179**, 101–113.
- Moore, J. P., Sattentau, Q. J., Yoshiyama, H., Thali, M., Charles, M., Sullivan, N., Poon, S. W., Fung, M. S., Traincard, F., Pinkus, M., Robey, G., Robinson, J. E., Ho, D. D., and Sodroski, J. (1993). Probing the structure of the V2 domain of human immunodeficiency virus type 1 surface glycoprotein gp120 with a panel of eight monoclonal antibodies: human immune response to the V1 and V2 domains. *J. Virol.* **67**, 6136–6151.
- Mori, K., Ringler, D. J., Kodama, T., and Desrosiers, R. C. (1992). Complex determinants of macrophage tropism in env of simian immunodeficiency virus. *J. Virol.* **66**, 2067–2075.
- O'Brien, W. A., Koyanagi, Y., Namazie, A., Zhao, J.-Q., Diagne, A., Idler, K., Zack, J. A., and Chen, I. S. Y. (1990). HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. *Nature* **348**, 69–73.

- Orenstein, J. M., Meltzer, M. S., Phipps, T., and Gendelman, H. E. (1988). Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: An ultrastructural study. *J. Virol.* **62**, 2578–2586.
- Owens, R. J., Dubay, J. W., Hunter, E., and Compans, R. W. (1991). Human immunodeficiency virus envelope protein determines the site of virus release in polarized epithelial cells. *Proc. Natl. Acad. Sci. USA* **88**, 3987–3991.
- Poli, G., Bressler, P., Kinter, A., Duh, E., Timmer, W. C., Rabson, A., Justement, J. S., Stanley, S., and Fauci, A. S. (1990). Interleukin 6 induces human immunodeficiency virus expression in infected monocytic cells alone and in synergy with tumor necrosis factor alpha by transcriptional and post-transcriptional mechanisms. *J. Exp. Med.* **172**, 151–158.
- Power, C., McArthur, J. C., Johnson, R. T., Griffin, D. E., Glass, J. D., Perryman, S., and Chesebro, B. (1994). Demented and non-demented patients with AIDS differ in brain-derived human immunodeficiency virus type 1 envelope sequences. *J. Virol.* **68**, 4643–4649.
- Schuitemaker, H., Kootstra, N. A., de Goede, R. E. Y., de Wolf, F., Miedema, F., and Tersmette, M. (1991). Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture. *J. Virol.* **65**, 356–363.
- Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., de Goede, R. E., van Steenwijk, R. P., Lange, J. M., Schattenkerk, J. K., Miedema, F., and Tersmette, M. (1992). Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: Progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus populations. *J. Virol.* **66**, 1354–1360.
- Shioda, T., Levy, J. A., and Cheng-Mayer, C. (1991). Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. *Nature* **349**, 167–169.
- Simmonds, P., Zhang, L. Q., McOmish, F., Balfe, P., Ludlam, C. A., and Brown, A. J. L. (1991). Discontinuous sequence change of human immunodeficiency virus (HIV) type 1 *env* sequences in plasma viral and lymphocyte-associated proviral populations in vivo: Implications for models of HIV pathogenesis. *J. Virol.* **65**, 6266–6276.
- Sperduto, A. R., Bryson, Y. J., and Chen, I. S. (1993). Increased susceptibility of neonatal monocyte/macrophages to HIV-1 infection. *AIDS Res. Hum. Retroviruses* **9**, 1277–1285.
- Sullivan, N., Thali, M., Furman, C., Ho, D. D., and Sodroski, J. (1993). Effect of amino acid changes in the V1/V2 region of the human immunodeficiency virus type 1 gp120 glycoprotein on subunit association, syncytium formation, and recognition by a neutralizing antibody. *J. Virol.* **67**, 3674–3679.
- Tersmette, M., Gruters, R. A., de Wolf, F., de Goede, R. E. Y., Lange, J. M. A., Schellekens, P. T. A., Goudsmit, J., Huisman, H. G., and Miedema, F. (1989). Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: Studies on sequential HIV isolates. *J. Virol.* **63**, 2118–2125.
- Walsh, D. G., Horvath, C. J., Hansen-Moosa, A., MacKey, J. J., Sehgal, P. K., Daniel, M. D., Desrosiers, R. C., and Ringler, D. J. (1991). Cytokine influence on simian immunodeficiency virus replication within primary macrophages. TNF-alpha, but not GM-CSF, enhances viral replication on a per-cell basis. *Am. J. Pathol.* **139**, 877–887.
- Weiss, R. A. (1993). How does HIV cause AIDS? *Science* **260**, 1273–1279.
- Westervelt, P., Trowbridge, D. B., Epstein, L. G., Blumberg, B. M., Li, Y., Hahn, B. H., Shaw, G. M., Price, R. W., and Ratner, L. (1992). Macrophage tropism determinants of human immunodeficiency virus type 1 in vivo. *J. Virol.* **66**, 2577–2582.
- Wiley, R. L., Theodore, T. S., and Martin, M. A. (1994). Amino acid substitutions in the human immunodeficiency virus type 1 gp120 V3 loop that change viral tropism also alter physical and functional properties of the virion envelope. *J. Virol.* **68**, 4409–4419.
- Zhang, L. Q., MacKenzie, P., Cleland, A., Holmes, E. C., Brown, A. J. L., and Simmonds, P. (1993). Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection. *J. Virol.* **67**, 3345–3356.
- Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A., and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261**, 1179–1181.