Human Immunodeficiency Virus Infection of T Cells and Monocytes Proceeds via Receptor-mediated Endocytosis

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Abstract. The rates of internalization and uncoating of 32p-labelled human immunodeficiency virus (HIV) in the human T lymphoid cell line CEM are consonant with a receptor-mediated endocytosis mechanism of entry. This interpretation was affirmed by electron microscopic observation of virions within endosomes. Virus binding and infectivity were inhibited to the same extent by pretreatment with OKT4A antibody, therefore, the CD4 receptor-dependent pathway of internalization appears to be the infectious route of entry. The pattern of internalization by the human monoblastoid cell line U937 proved to be more complex, involving rapid and efficient CD4-independent internalization. Electron microscopy revealed the presence of large intracellular vesicles, each containing several virions. Antibody against the CD4 receptor for virus efficiently blocked infection, but did not reduce significantly HIV binding or internalization in the U937 cell line. Consequently, U937 cells have a CD4-independent pathway of virus internalization that does not coincide with the route of entry for infectious HIV.

The human immunodeficiency virus (HIV) infects primarily T cells of the helper/inducer class (17) and monocytes (6, 13, 24). To a large extent, this viral tropism is determined by the presence of the CD4 surface antigen on susceptible cells (5, 17, 20). The envelope glycoprotein of HIV (gp120) forms a noncovalent complex with the CD4 molecule and it is this reaction that determines the mechanism for HIV attachment to cells (19). Virus attachment is specific; the OKT4A monoclonal antibody binds the CD4 molecule and also blocks virus attachment and infectivity (14).

Subsequent to the attachment step, all enveloped viruses require fusion between viral and cellular membranes for insertion of their genome into host cells. For paramyxoviruses such as Sendai, measles, and respiratory syncytial virus (reviewed in reference 21) fusion takes place at the plasma membrane and is mediated by a specific viral peptide. Among other enveloped viruses, most enter the cell via endocytosis and fusion occurs between the viral and endosomal membranes once the vesicle has been internalized. The latter process is designated receptor-mediated endocytosis and constitutes the route of entry for murine retroviruses (2).

The unique chemical environment of the endosome facilitates the uncoating reaction. Shortly after internalization, endosomes become acidified (11, 22). Under these acidic conditions, the virus membrane fuses with the endosomal membrane. Acidic conditions initiate membrane fusion and also promote disaggregation of the nucleoprotein core during influenza virus infection (reviewed in reference 21). Other distinctive features of the endosome can also promote uncoating of viruses. The elegant studies of White and Helenius (33) demonstrate that endosomal membranes are specifically enriched in cholesterol and this provides the required target for Semliki Forest virus (SFV) fusion and uncoating. Interestingly enough, the envelopes of both HIV and SFV are enriched in cholesterol (1, 33). The fusion reaction between viral and endosomal membranes is complex and may well require low pH, unusual membrane lipid composition, or even both of these features.

Formation of syncytia among infected cells is still another consequence of viral protein involvement in membrane fusion reactions. In the case of HIV, polykaryon formation among infected T cells is the most evident in vitro cytopathic effect (18) although this reaction has not yet been shown to be a significant factor in vivo. Polykaryon formation does not indicate a priori the mode of virus entry even though cell fusion occurs at neutral pH and is initiated by the presence of a viral protein in the cell membrane. Sendai virus, known to enter cells by direct membrane fusion, can be inactivated, then added to cell cultures, and polykaryons will form nonetheless (26). Vesicular stomatitis virus (VSV), on the other hand, gains entry into cells by receptor-mediated endocytosis yet is still able to induce syncytium formation among infected cells (4, 25). As regards VSV, cell fusion occurs 4–12-h postinfection, but does not occur with inactivated virus (34) and, moreover, requires protein synthesis in the infected cell (25). Consequently, polykaryon formation per se, is not a dependable indicator of the mode for virus entry.
The present work analyzes HIV entry into susceptible T lymphoid (CEM) and monoblastoid (U937) cells. The kinetics of entry and uncoating of radiolabeled virus prove to be consonant with receptor-mediated endocytosis being the predominant mode of penetration rather than virus entry via plasma membrane fusion. This conclusion is affirmed by extensive electron microscopic observation of the frequency of virions within endosomal vesicles. No evidence was obtained that would lend support to the notion of direct fusion between the viral and plasma membranes.

Materials and Methods

Cell and Virus Strains

CEM, a human T lymphoid cell line and U937, a human monoblastoid cell line derived from a histiocytic leukemia (31), were used in these studies. The LA/1-A strain of HIV, provided by Dr. D. Richman (University of California, San Diego), was used in all of these experiments.

Preparation of HIV Stocks

 Cultures of CEM cells growing logarithmically in RPMI-1640 medium plus 10% heat-inactivated FBS, were infected with 0.2 TCI~Dso/cell and then incubated at 37°C in 5% CO2. The volume of the culture was doubled at 2.5-d postinfection by the addition of fresh medium. At 5-d postinfection, cells and debris were removed by centrifugation. Virus was precipitated from the clarified supernatant by the addition of polyethylene glycol-8000 to a final concentration of 10% (28). The virus pellet was resuspended in 0.01 of the original culture volume. Concentrated preparations had titers ranging from 5 × 106 to 1 × 107 TCID~Dso/ml; infectivity was determined by measuring the cytopathic effect on MT-2 cells (10). Virus stocks were stored at −70°C. The reverse transcriptase activity of infected cell supernatants was determined by the method of Popovic et al. (28) with minor modifications.

Preparation of 32P-labeled Virus

Infected cultures were prepared as described. At 3-d postinfection the cells were recovered by centrifugation, and then washed, and resuspended in DME medium lacking phosphate (Gibco Laboratories, Grand Island, NY). Dialyzed FBS was added to a final concentration of 10%. Carrier-free H33-32PO4 (New England Nuclear, Boston, MA) was added to a concentration of 1 mCi/ml of culture; the cell concentration at the time of labeling was 5 × 107/ml and ~80% of the cells stained positively in an indirect immunofluorescence assay for detection of cell-associated viral proteins (not shown). 5 h after the addition of label, the cells were removed by centrifugation. Supernatant virus was concentrated by centrifugation for 2 h at 18,000 rpm, 4°C in a centrifuge (model J2-21; Beckman Instruments Inc., Palo Alto, CA). The pellet was resuspended in 1 ml of NET buffer (16) containing 22% sucrose. The sample was layered atop a sucrose step gradient in which the upper phase was 22% and the lower phase 50% sucrose (16). After 16 h of centrifugation at 16,500 rpm, the purified virus was collected from the interface between the two sucrose solutions.

Characterization of Labeled Virus Preparations

The labeled virus preparations were characterized four separate ways to determine the percentage of radioactivity present as high molecular weight viral RNA. In the initial characterization, the material displayed the expected sedimentation characteristics in the sucrose gradient. Second, all of the counts could be pelleted by centrifugation through buffer at 18,000 rpm for 12 h. Third, the counts could be immunoprecipitated specifically with antibody from HIVinfected individuals. Fourth. 85-90% of the counts remained TCA-precipitable after treatment at 37°C for 1 h with 25 μg/ml RNaseA. Consequently, 90% of the 32P counts were susceptible to nuclease degradation in disrupted virions; this property is consistent with identification of the labeled material as high molecular weight viral RNA. These characteristics were virtually identical in three individual preparations of labeled virus.

Measurement of Virus Binding

In all virus-binding experiments, and in the subsequent determinations of virus uptake and uncoating, mild sonication disrupted viral aggregates before their addition to cell cultures. Dilutions of labeled virus were then added to tubes containing 2 × 106 cells in 1 ml of medium; the cells were cooled to 4°C for 10 min before the addition of virus. The mixtures were then incubated for 1 h at 4°C. Unbound virus particles were removed by spinning the cells through a 0.5-ml cushion of unlabeled bovine serum. Further washing did not decrease the number of counts significantly, thus a single wash was deemed adequate. The virus was inactivated by heating to 65°C for 1 h. Cerenkov radiation was determined by scintillation counting.

Determination of the Kinetics for Virus Uptake and Uncoating

For each time interval in the kinetic experiments, 15,000 cpm of virus were added to 7.5 × 106 cells in 3 ml of medium. The cells were cooled to 4°C before adding the virus; the mixtures were then incubated at 4°C for 15 min. Finally, each sample was divided into three 1-ml aliquots that were processed in parallel.

After preincubation at 4°C, the samples were warmed to 37°C for as long as 90 min. At the appropriate intervals, cells were washed by spinning through a 1-ml cushion of bovine serum to remove the unbound counts; they were then resuspended in 1 ml of RPMI-1640 (without serum). A 0.25-ml sample was removed, inactivated, and set aside for determination of total cell-associated counts.

Then, 0.25 ml of 0.25% trypsin solution were added to each of the remaining 0.75 ml aliquots; the samples remained at room temperature for 5 min. Virus particles bound to the surface of cells were released by the trypsin treatment and by again spinning the samples through a 1-ml serum cushion, a wash that prevented further enzymatic action of trypsin. The resulting cell pellets were suspended in 0.75 ml of medium plus 10% serum, 0.25-ml aliquots removed, and added to 1.0 ml of cold 10% TCA; these samples represent the internalized counts. The remaining cells in suspension were lysed by two cycles of freeze/thawing, alternating between dry ice/ethanol and 37°C water bath. A 0.25-ml aliquot was removed and added to 1.0 ml of cold 10% TCA; this treatment yielded the coated plus uncoated counts. Repeated freeze/thawing did not alter the ratio of internalized to coated plus uncoated counts. The term coated plus uncoated has been introduced to account for the differences between this and the internalized counts value. Since this difference is not an artifact of sample preparation, we must assume that the internalized counts contain a portion of material that becomes TCA soluble subsequent to cell lysis. Accordingly, we presume that internalized, degraded RNA was precipitated when whole cells were added to TCA; when lysed cells were added these degraded counts became TCA soluble. The coated plus uncoated value refers to that portion of internalized counts present as macromolecular RNA. To the remaining 0.25-ml fraction, RNaseA was added to a final concentration of 25 μg/ml and the mixture was incubated at 37°C for 30 min. 1.0 ml of cold 10% TCA was then added.

Table I. The Influence of Multiplicity of Infection on Virus Release by U937 and CEM Cells

| Cell line | Day 1 | Day 4 | Day 4 + OKT4A |
|-----------|-------|-------|---------------|
| U937 0.2  | 2,350 ± 125 | 6,375 ± 360 | 1,592 ± 205 |
| U937 1.0  | 3,235 ± 76  | 10,060 ± 283 | ND            |
| U937 10.0 | 8,065 ± 160 | 20,430 ± 455 | ND            |
| CEM 0.2   | 2,573 ± 65  | 12,570 ± 465 | 4,395 ± 185  |
| CEM 1.0   | 3,860 ± 115 | 22,681 ± 1,050 | ND           |
| CEM 10.0  | 8,145 ± 263 | 49,850 ± 2,230 | ND           |

* Multiplicity of infection represents the TCID50 of HIV per cell

Reverse transcriptase activity was determined by scintillation counting.
TCA precipitation was continued for 15 min at 4°C and the pellets were recovered by centrifugation in a Eppendorf microfuge (Brinkman Instruments, Inc., Westbury, NY) for 2.5 min. The pellets were rinsed with 70% ethanol, dried, and Cerenkov radiation was measured by scintillation counting. The triplicate determinations were averaged and recorded as single data points, standard errors did not exceed 15% of the mean.

**Results**

**Electron Microscopic Analysis of Infected Cells**

Cells in suspension at 4°C were infected with ~30 TCID₅₀/cell and the mixture kept at this temperature for 15 min. The value of 30 TCID₅₀/cell was selected to provide ~10,000 virus particles for each cell in the suspension (2). After the preincubation at 4°C, infected cultures were warmed to 37°C for intervals ranging from 2.5 to 60 min; the cells were then concentrated by centrifugation and resuspended in cold PBS. Immediately thereafter, the cells were fixed by glutaraldehyde and osmium tetroxide (12), dehydrated, embedded in epoxy resin, sectioned and then stained with saturated methanolic uranyl acetate and lead citrate before electron microscopy. Micrographs were recorded in a Philips 300 electron microscope equipped with an eccentric goniometer stage. The microscope was operated at 80 kV with a 50 µm objective aperture.

**CEM and U937 Cells are Permissive for HIV Replication**

The laboratory stocks of the LAI-A isolate of HIV were effective in establishing productive infection in the T lymphoid cell line CEM and the monoblastoid line U937. These results are consistent with previous observations and confirm that our cell lines were susceptible and our virus preparations were infectious. Table I delineates the relationship between multiplicity of infection and the yield of newly synthesized virus on d-1 and -4 postinfection. Cells were also pretreated with the OKT4A antibody (provided by Dr. Gideon Goldstein, Ortho Pharmaceuticals, Raritan, NJ) and then infected at low multiplicity. This antibody pretreatment reduced substantially the level of virus released and indicated that productive infection of CEM and U937 cells requires interaction between HIV and the CD4 molecule. These experiments were performed routinely at an antibody concentration of 5 µg/ml. Complete (>95%) inhibition of infection was obtained at antibody concentrations of >10 µg/ml; the former conditions were used to conserve OKT4A material.

At a multiplicity of infection of 1 TCID₅₀/cell, ~85% of the CEM cells in culture, and 70% of the U937 cells on d-3 postinfection, reacted positively with antibody directed against the viral p24 protein (not shown).

**Binding of Radiolabeled Virus to CEM and U937 Cells**

The binding of radiolabeled HIV to two susceptible cell lines was assayed in an experiment that served as the baseline for further characterization of the labeled virus preparations and selection of conditions for optimal binding of HIV to cells. The capacity for OKT4A antibody to block virus binding was also assessed.

Results of four independent binding experiments (using two independent virus preparations) are shown in Fig. 1. The binding data are presented in the form of a Scatchard plot so that the relative affinity constants may be perceived more clearly. The T cell line, CEM, has a rather typical binding profile for radiolabeled HIV; at low virus concentrations, a steep curve with negative slope indicates that the radiolabeled material binds to cells with high affinity. At higher virus concentrations, the more flattened curve is indicative of low affinity binding. High affinity binding between the virus and the CD4 molecule on the cell surface had been anticipated, but the low affinity binding might be attributed to the presence of broken or degraded virions in the preparation, or to dissociation of some virions after binding to the cell surface, or perhaps even the presence of a second class of lower affinity binding sites.

A different binding profile was found for the U937 cells. Here no steep curve of negative slope was observed even at very low virus concentrations. The low affinity binding curve was similar to that observed for the T cell lines. However it is possible that the lack of high affinity virus receptors on the U937 cells may be more a reflection of the limit of this experimental tool. High affinity receptors might well have been detectable had the radiolabeled virus preparations been of higher specific activity. Accordingly, we conclude only that the U937 cells possess significantly smaller numbers of high affinity receptors than the T cells.

To determine the role of CD4 molecules in the binding reaction, aliquots of CEM and of U937 cells were preincubated with the OKT4A antibody and then interacted with radiolabeled HIV. The open symbols in Fig. 1 show the amount of virus binding to antibody-coated cells. Pretreatment with 5 µg/ml of antibody prevented ~75% of the binding of HIV to CEM cells; this value is consistent with the observed differences between high and low affinity receptors and shows that the high affinity binding was prevented by antibody. However, the same antibody blocked <20% of the binding to U937 cells. These data indicate that most of the virus binding to T cells occurs via the CD4 surface molecule and is of high affinity, whereas the predominant binding event between HIV and U937 cells is of low affinity and does not involve the CD4 molecule.

**Kinetics of HIV Uptake and Uncoating in CEM Versus U937 Cells**

The analysis of HIV penetration into susceptible cells was extended further by measuring the rates of virus internalization and uncoating. The assay relied on the use of virus...
Table II. Distribution* of \( ^{32} \)P-labeled HIV in CEM or U937 Cells at 0- and 30-min Postinfection

| Cell line   | CEM    | CEM    | U937   | U937   |
|-------------|--------|--------|--------|--------|
| \(^{32} \)P of \( ^{32} \)P-labeled HIV/2.5 \( \times 10^6 \) cells | 5,000  | 5,000  | 5,000  | 5,000  |
| Time at 37°C | 0 min  | 30 min | 0 min  | 30 min |
| \(^{1} \)Cell-associated virus | 2,850 ± 405 | 3,650 ± 521 | 1,270 ± 185 | 1,310 ± 176 |
| \(^{2} \)Internalized, trypsin resistant, virus | 185 ± 24  | 2,072 ± 283 | 114 ± 27 | 1,061 ± 102 |
| \(^{3} \)Coated plus uncoated | 150 ± 32  | 1,480 ± 211 | 89 ± 17  | 531 ± 64   |
| \(^{4} \)Coated, internalized virus (RNase resistant) | 135 ± 27  | 962 ± 127  | 64 ± 14  | 459 ± 47   |

* The experimental method is detailed in the text.
† Counts per minute of cerenkov radiation.

preparations in which the viral nucleic acid was the principal radiolabeled component. Indeed, the \( ^{32} \)P-labeled HIV preparations yielded >90% virion counts in the RNA. As in the binding experiments, radiolabeled virus and cells were admixed and incubated at 4°C for 15 min before use. Samples were then warmed to 37°C to initiate virus uptake. It is noteworthy that no uptake was measurable unless the cells had been warmed. At specific intervals subsequent to warming, samples were removed and the distribution of radioactivity into four separate fractions determined. The total number of bound counts was designated as cell-associated virus. Surface-bound viral particles were removed by trypsinization and the total number of counts remaining was labeled internalized virus. After the surface-bound counts had been removed by trypsin treatment, the cells were lysed by freeze/thawing and then, to discriminate between macromolecular and degraded viral RNA, the samples were precipitated with cold TCA. The counts recovered at this point represent the total of coated plus uncoated virus. Finally, the uncoated virus RNA present in the cell extracts was degraded by RNaseA treatment and the residual counts were then recovered by TCA precipitation; this fraction contained the coated virus. The amount of internalized, but degraded, viral material was calculated by subtracting the coated plus uncoated value from the internalized value. Analogously, the amount of uncoated virus was determined by subtracting the value for coated virus from the coated plus uncoated virus value. We have used freeze/thaw lysis in order to avoid procedures involving manual disruption of HIV-infected cells. It must be noted that this procedure disrupts organelle structure thus we cannot discriminate between uncoated viral RNA released to the cytoplasmic matrix and that portion remaining in the vesicle. We presume that the majority of uncoated, macromolecular HIV RNA is present in the cytoplasm.

An example of the results from three experiments is shown in Table II. The samples that were not incubated at 37°C serve as baseline controls; the data demonstrate that trypsin treatment removed the surface-bound virus counts efficiently and no internalization was observed at low temperature.

Fig. 2 illustrates the time course of virus uptake and its uncoating in CEM cells. The experiments were performed with virus concentrations that reflected high affinity binding (Fig. 1); the data are a composite of eight individual experiments involving three different preparations of radiolabeled virus. Virus internalization proved to be a relatively slow and inefficient process in the CEM cells. A maximum of 60% of the total counts had been internalized after 30-min incubation at 37°C. The differences between the curve representing internalized counts and that for coated plus uncoated counts were relatively small, indicating that little degradation of the internalized material had occurred. Virus uncoating, demonstrated by the difference between the coated plus uncoated and the coated curves, was also slow but had reached appreciable levels within 30 min after warming to 37°C. The latter two curves diverged significantly at longer intervals, indicating the presence of stable uncoated viral RNA. The half-life of the uncoated viral RNA was calculated to be >1 h.

The time course of HIV uptake and uncoating in U937 cells was significantly different from that for the \( T \) cell line (Fig. 3). Here, internalization of virus was rapid and efficient. Approximately 90% of the cell-associated material had been internalized within 5 min; rapid changes in the amounts of uncoated and coated virus were perceived as well. The differences between values for internalized and coated plus uncoated were substantial, indicating that the internalized material was being degraded rapidly. At longer intervals, there was but little difference between the coated plus uncoated and coated values, thus the uncoated viral RNA appeared to be rather unstable. No accurate estimate of the half-
life of the viral RNA was made; presumably this value was well under 15 min.

The biochemical analysis of virus penetration had provided strong evidence that internalization proceeded via receptor-mediated endocytosis. The case was particularly clear for CEM cells where RNase-resistant particles were accumulated in the cells; this phenomenon is incompatible with the notion of direct membrane fusion as the mode of entry. Similar particles were accumulated in the U937 cells, although the rates of uptake and uncoating were different from those observed in the T cell line. It is possible that a small percentage of bound virions penetrate the U937 cells in the same manner as observed for the T cell line. However, it was not feasible to measure these slower rates as they would have been obscured by the faster, dominant, mode of entry. Accordingly, the biochemical data alone were inadequate to discriminate between these opposing possibilities. Moreover, the conclusion that HIV entered the cells by receptor-mediated endocytosis had rested on a single, crucial, experimental observation: RNase resistant particles were internalized. That the virus could have entered the cell by direct fusion with the plasma membrane and that the ribonucleoprotein core of the virus remained RNase resistant for some time thereafter remained a real possibility. It was to discern the structural basis for the disparate rates of uptake and uncoating between the two cell lines, and especially to gain independent evidence concerning the mode of virus entry that the time course analysis was repeated using electron microscopy as the preferred means of following directly the structure and distribution of infecting virus particles.

**Structural Analysis of HIV Penetration**

The experimental design was similar to that used in the earlier biochemical analysis of virus entry: cells and virus were preincubated at 4°C and then warmed to 37°C for various intervals. The cells were infected at a multiplicity of 30 TCID50/cell to yield ~10,000 virus particles per cell. Infected cells were collected by centrifugation, resuspended in ice-cold saline, and then fixed with glutaraldehyde and osmium tetroxide. Samples taken from each point in the kinetic experiments were sectioned, stained, and observed via electron microscopy. Representative specimens were photographed and assembled into a series displaying accurately the time course of events.

The pathway of virus entry into the T cell line is clearly delineated in Fig. 4. It is evident that bound virions are enclosed in vesicles and then internalized. Fig. 4, A and B show the bound virus and a single particle within a fold on the cell surface; this appears to be the initial stage of vesicle formation. Fig. 4, C and D reveal both surface-bound virions and intact virus particles enclosed entirely in intracellular vesicles; the vesicles appear to be endosomes. Fig. 4 E depicts a section in which most of the virus particles are within endosomes. Particles within endosomal vesicles are sometimes seen as having fused directly with the endosomal membrane as is evident in Fig. 4 F. Internalized virions have slightly smaller diameters than extracellular particles and lack a clearly defined limiting membrane. The ribonucleoprotein core appears to be disorganized in internalized particles and is spherical as opposed to the characteristic cylindrical core structure of HIV. In most cases, the virus particles appear to interact directly with the endosomal membrane; the area of contact between virus and endosomal membranes is generally greater than the area of contact observed for particles bound to the exterior plasma membrane. To provide additional evidence in support of our contention that fusion occurs between the viral and endosomal membranes, we examined other examples by tilting the samples in an electron microscope equipped with a goniometer stage, and then photographing the sample from three distinct angles; these data are shown in Fig. 5. Close interaction between viral and endosomal membranes was observed from all angles. The examples shown in Fig. 5, and numerous other samples, appear to reveal fusion between the viral and endosomal membrane.

The use of the tilting stage established that the close apposition of viral particles and endosomal membranes was not an artifact of preparation or analysis; however, it has not yet been possible to provide unequivocal evidence for continuity between viral and endosomal membranes. Accordingly, these data indicate that virus particle uncoating can occur in the endosome and are supportive of the contention that HIV penetration proceeds via receptor-mediated endocytosis.

The appearance of intact viral particles within endosomes and the observation of structures consistent with direct fusion between the viral and vesicular membranes constitutes compelling evidence that viral particles do enter the cell via receptor-mediated endocytosis. It should be noted that, in the course of this study, >2,000 surface-bound virus particles were examined. In no instance was there any indication of direct fusion with the surface plasma membrane.

An explanation for the differences in rates of uptake and uncoating between the CEM and U937 cell lines was provided by electron microscopic analysis. Occasionally, there were observed individual virions contained within vesicles similar to those seen in the CEM cells. However, the great majority of virus particles were contained within membrane-bounded vesicle structures; one example is shown in Fig. 6. Accordingly, it seems apparent that virus particles are generally engulfed in phagocytic vesicles; inasmuch as phagocytic vesicles represent a degradative compartment, this observation could account for the enhanced rate of uptake and degradation of HIV in U937 cells. The majority of U937-internalized virus particles are distinct structurally from CEM-internalized virus. In U937 cells, multiple, well-de-
Figure 4. Pathway of HIV entry into CEM cells. Mode of infection and specimen preparation are detailed in the text. The six panels depict the time course of virus penetration. The time postinfection is: (A) 0, (B) 2.5, (C) 5, (D) 7.5, (E) 10, and (F) 20 min. Virus particles are recognized as spheres with dense cores and are ~100 nm in diameter. These particles were never encountered in uninfected cells. Particular attention is directed to the structure indicated in F; here, the virion membrane is fused to the endosomal membrane during uncoating of the virus particle.

Fined virions were observed in single vesicles and most of these particles do not appear to interact with the vesicular membrane. The particles have intact limiting membranes and well-defined ribonucleoprotein cores.

In no instance were vesicles containing more than a single virion observed in CEM cells. Even though the route of virus penetration into U937 cells is more complex than for the T cell line, no evidence for direct fusion between the viral and plasma membranes was obtained; this despite examination of >1,000 virions bound to the surface of the U937 cells.

Discussion

The biochemical and electron microscopy analyses reported here strongly affirm that HIV enters the T cell (line CEM) via receptor-mediated endocytosis and that the CD4 surface antigen is an important component of the viral receptor. These conclusions derive from four experimental observations: (a) virus binding and infectivity were inhibited to the same extent by OKT4A antibody pretreatment of CEM cells, (b) virus particles were internalized within endosomes, (c) fusion between viral and endosomal membranes was observed, and (d) fusion between the viral and plasma membranes was never observed. Accordingly, the dominant route of particle internalization into T cells is identical to the infectious route of entry and is believed to proceed in three stages: (a) binding of HIV to the CD4 surface receptor, (b) internalization of the receptor–virus complex, and (c) fusion between the viral and endosomal membranes.

The pathway of virus penetration into the monoblastoid cell line U937 is more complex than the situation in T cells. Internalization and degradation of HIV were very rapid in these cells, thus, we presume that this represents phagocytosis of virus as opposed to the slower rates of endocytic internalization observed in CEM cells. Pretreatment of U937 cells with CD4-blocking antibody does not inhibit virus binding to any significant extent yet it efficiently prevents infection. Recall that only 20% of virus binding was prevented by pretreatment of cells with 5 µg/ml of OKT4A monoclonal antibody and that ~90% of the cell-associated counts were internalized more rapidly than in CEM cells; these two populations of cell surface-bound virions appear to be functionally distinct. Accordingly, the infectious route of entry into U937 cells does not appear to parallel the dominant pathway for internalization of virus particles. It is conceivable that some viral particles enter the U937 cells by the slower pathway of receptor-mediated endocytosis but thus
far, it has not been feasible to measure these rates directly as they are obscured by the more rapid, phagocytic pathway. Although not yet demonstrated conclusively, it would seem likely that entry of infectious HIV into U937 cells occurs via receptor-mediated endocytosis and that the CD4-independent phagocytic pathway is noninfectious.

The data presented here and the conclusions drawn stand in sharp contrast to those of Stein et al. (30) who reported that HIV penetration into the human T lymphoid cell line VB occurred via direct fusion between the viral and plasma membranes and that endosomes containing virus particles were never encountered. Three reservations to these conclu-
sions can now be entered. First, the authors reported that lysosomotropic agents failed to inhibit virus entry although significant inhibition of HIV provirus accumulation had been observed when the cells were pretreated with either chloroquine or monesin. The decrease in proviral accumulation in the presence of chloroquine, was similar to the observed inhibition of murine retrovirus infection in mouse fibroblasts (2); in the murine experiments these data were interpreted in favor of receptor-mediated endocytosis. Second, experiments concerning the ability of HIV to promote polykaryon formation do not constitute very compelling evidence as regards the route of entry. Indeed the inability of inactivated HIV to initiate syncytium formation is rather suggestive that cells must be infected, and express virus proteins de novo, for fusion to occur. A similar result has been obtained for VSV; VSV penetrates cells by receptor-mediated endocytosis and subsequent formation of syncytia then depends on protein synthesis in the infected cells (34). Finally, the electron microscopic evidence presented by Stein (30) was insufficient to support a firm conclusion. Previous demonstrations of viral penetration by membrane fusion (e.g., Fig. 10 in reference 23) were based on unequivocal visual evidence for continuity between the viral and plasma membranes. Such direct connection between the viral and cellular membranes is not established by the single example presented in Stein et al. (30).

It should also be noted that the experiments of Stein et al. (30) were performed with the VB cell line and the HTLV-IIb viral strain whereas the data in the present report were obtained with CEM and U937 cells and using the LAV-1A strain of HIV. Although it seems unlikely that the virus would use different modes of entry into two different human T cell lines, this possibility is not excluded at present. Rather compelling evidence for HIV penetration by receptor-mediated endocytosis is presented here; these results are consis-

Figure 6. Phagocytosis of HIV by the U937 cell line. Numerous virus particles are evident within a phagocytic vesicle. The lower portion shows a low magnification reproduction of the same cell to provide perspective as regards the size of the vesicle relative to the size of the cell.
tent with a previous conjecture by Maddon et al. (20). The fact that the CD4 surface molecule is internalized upon lymphocyte activation (32) or in consequence of binding a ligand, in this case monoclonal antibody (29), provides additional evidence supporting receptor-mediated endocytosis as the route of HIV penetration.

The findings which emerge from this study encourage the view that antibody responses elicted via vaccination might, after all, be effective in protecting individuals against infection. Studies on the uptake and uncoating of HIV in the U937 cell line are consonant with the view that virus entering these cells via phagocytosis no longer is infectious. Accordingly, it is anticipated that antigen–antibody complexes can be cleared from the circulation without deleterious effect on the host monocyte/macrophage population. It is possible that antibody–dependent enhancement of infectivity, as seen with Flaviviruses (7, 8), may occur in the HIV situation. However, antibody enhances the rate of visna virus uptake by sheep macrophages without increasing the rate of infection (16).

The fact that HIV enters the cell via an endocytotic route also portends the likelihood of novel targets for vaccine development. The studies of Gollins and Porterfield (9) have already revealed that it is possible to generate neutralizing antibody responses against the West Nile Virus, despite failure of these antibodies to block virus binding. Presumably such antibody molecules prevent the interactions between bound virus and a second site on the endosomal membrane where the uncoating process is initiated. In that event, vaccines directed at this second site in the HIV-uncoating machinery could well offer the further advantage of not inducing antibody responses that interfere with the CD4 molecule.

This would be a favorable development as this lymphocyte surface entity has been shown to be intimately involved in the activation of T cells (15).

In the course of our basic studies on the interactions between human immunodeficiency virus and susceptible cells, several important features of the mechanism of virus penetration have been elucidated. It is most likely that the infectious route of entry for HIV into T cells proceeds via receptor-mediated endocytosis. Consequently, cells become susceptible to HIV infection because the CD4 molecule is present at the cell surface and is being internalized actively. It will be important to determine whether the interaction between HIV and the CD4 molecule can promote internalization or, as is the case for transferrin receptor (3, 27), cycling to the interior is independent of ligand binding. It is likely that virus penetration into macrocytes also occurs by the endocytic mechanism despite the fact that phagocytosis of viral particles, which appears to be a noninfectious process, is dominant. Agents that inhibit virus penetration into T cells would thus be protective of macrocytes as well. Accordingly, the present studies provide a fresh experimental model to support development and testing of antiviral compounds that inhibit HIV penetration of susceptible cells.

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