JOURNAL OF VIROLOGY, Feb. 1994, p. 1179–1185
0022-538X/94/$04.00+0

Vol. 68, No. 2

Fusion from Without Directed by Human Immunodeficiency Virus Particles

FRANÇOIS CLAVEL* AND PIERRE CHARNEAU

Unité d'Oncologie Virale, Département SIDA et Rétrovirus, Institut Pasteur, 75724 Paris Cedex 15, France

Received 14 July 1993/Accepted 9 October 1993

Fusion from without is the process through which particles of some enveloped viruses can direct fusion of target cells in the absence of viral replication. We demonstrate here that human immunodeficiency virus (HIV) particles can efficiently promote fusion from without. Using HeLa-CD4 cells carrying a Tat-inducible *lacZ* gene, we observed syncytia as early as 6 h after exposure to HIV particles, before HIV gene expression could be detected. Efficient syncytium formation could be obtained when cells were treated with zidovudine, which prevented HIV replication and expression but not cell-cell fusion. Fusion was also observed when cells were exposed to particles of a replication-defective HIV integrase mutant. Fusion from without by HIV particles could be blocked by a monoclonal antibody specific for the V3 loop of the HIV-1 envelope glycoprotein and by soluble CD4. This mechanism of cytopathicity, which can involve cells that do not actively replicate HIV and can be directed by replication-defective particles, could participate in the pathogenicity of the CD4 cell depletion that characterizes HIV infection.

Entry of enveloped viruses into their target cells requires fusion of the viral membrane with the cell membrane. Fusion of the human immunodeficiency virus (HIV) membrane with that of CD4-positive target cells is preceded by attachment the viral envelope glycoprotein gp120 to the CD4 molecule expressed at the cell surface (7, 14, 17, 20) and is mediated by the other component of the envelope glycoprotein complex, gp41 (15, 26). Expression of the viral envelope glycoprotein at the surface of infected cells also allows their fusion with neighboring CD4-expressing cells, leading to the formation of syncytia, a hallmark of the cytopathic effect induced by HIV (16, 25). Therefore, induction of cytopathic effect by HIV is believed to be essentially dependent on expression of the gp120-gp41 complex on infected cells. Here we asked the question whether fusion of CD4-positive cells could also be directly induced by viral particles alone, in the absence of viral gene expression. Such direct induction of cell-cell fusion by cell-free viral particle preparations has been widely documented for several classes of enveloped viruses, especially paramyxoviruses. Among retroviruses, visna virus, an ovine lentivirus, has been reported as being able to produce particle-directed cell-cell fusion (6). This process was coined fusion "from without," as opposed to conventional fusion "from within," which requires expression of the viral envelope at the surface of one of the fusion partners (2, 12).

To easily assess both syncytium formation and viral gene expression in the same cells, we used a line of HIV-infectible HeLa-CD4 cells carrying the bacterial *lacZ* gene under control of the HIV-1 long terminal repeat (LTR). This cell line, termed P4, was derived from HeLa-LTR-*lacZ* clone Z24 (4), in which HIV-1 LTR-driven transcription of the *lacZ* gene and cytoplasmic accumulation of β-galactosidase are strictly dependent on the presence of the HIV transactivator Tat. Expression of the CD4 molecule in Z24 cells was obtained after infection by the murine amphotropic retroviral vector pMCD4 (a gift from O. Schwartz, Institut Pasteur) carrying the CD4 cDNA as an *Eco*RI-*Bam*HI 1.7-kb fragment from pT4B (18) under the control of the mouse phosphoglycerate kinase promoter. One individual cell clone (P4) expressing high levels of CD4 was sorted by flow cytometry. Monolayers of P4 cells were infected in a final volume of 1 ml in 1.5-cm^2 wells, in the presence of 20 μg of DEAE-dextran per ml, as described for similar assays (13, 24). The virus used in these experiments was obtained from filtered (pore size, 0.45 μm) supernatants of cultures of HeLa cells harvested 48 h after transfection by an infectious molecular clone of the HIV-1_{lai} isolate, pBRU3. The P4 cultures were kept exposed to the particle suspension for 24 h, washed in phosphate-buffered saline (PBS), fixed for 5 min in 0.5% glutaraldehyde, and stained with a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution for 4 h as described previously (1). In the absence of viral particles, no β-galactosidase-expressing cells could be detected. Similarly, prolonged incubation of the P4 cells in medium containing DEAE-dextran only did not result in β-galactosidase expression and did not provoke the formation of syncytia (Fig. 1a, panel 1). When P4 cells were exposed to cell-free HIV particles at low concentrations (less than 50 ng of total HIV p24 in the inoculum, as determined by the DuPont p24 core enzyme-linked immunosorbent assay), β-galactosidase expression was easily detected in scattered single cells or in cell doublets at 24 h after infection but there was no evidence of syncytium formation (Fig. 1a, panel 2). At this low multiplicity of infection, fusion resulting from HIV gene expression (fusion from within) can usually be seen around 48 h after infection, and all of the observed syncytia express high levels of β-galactosidase (data not shown). In contrast, when the P4 cells were exposed to higher concentrations of HIV particles (more than 100 ng of p24 in the inoculum), the formation of syncytia occurred much earlier. Figure 1b shows the result of a time course experiment following exposure of P4 cells to HIV particles at a concentration of 150 ng of HIV-1 p24 per ml. No syncytia can be seen after 3 h of contact (Fig. 1b, panel 1). At 6 h, syncytia were readily detectable, but these syncytia did not stain blue at this early time point (Fig. 1b, panel 2). With time (Fig. 1b, panels 3 to 5), more and more cells became recruited in syncytium formation, a process that was paralleled by accumulation of cytoplasmic β-galactosidase. After 24 h, in

* Corresponding author. Mailing address: Unité d'Oncologie Virale, Département SIDA et Rétrovirus, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France. Phone: (33) 1 45 68 89 02. Fax: (33) 1 45 68 88 85. Electronic mail address: fclavel@pasteur.fr.

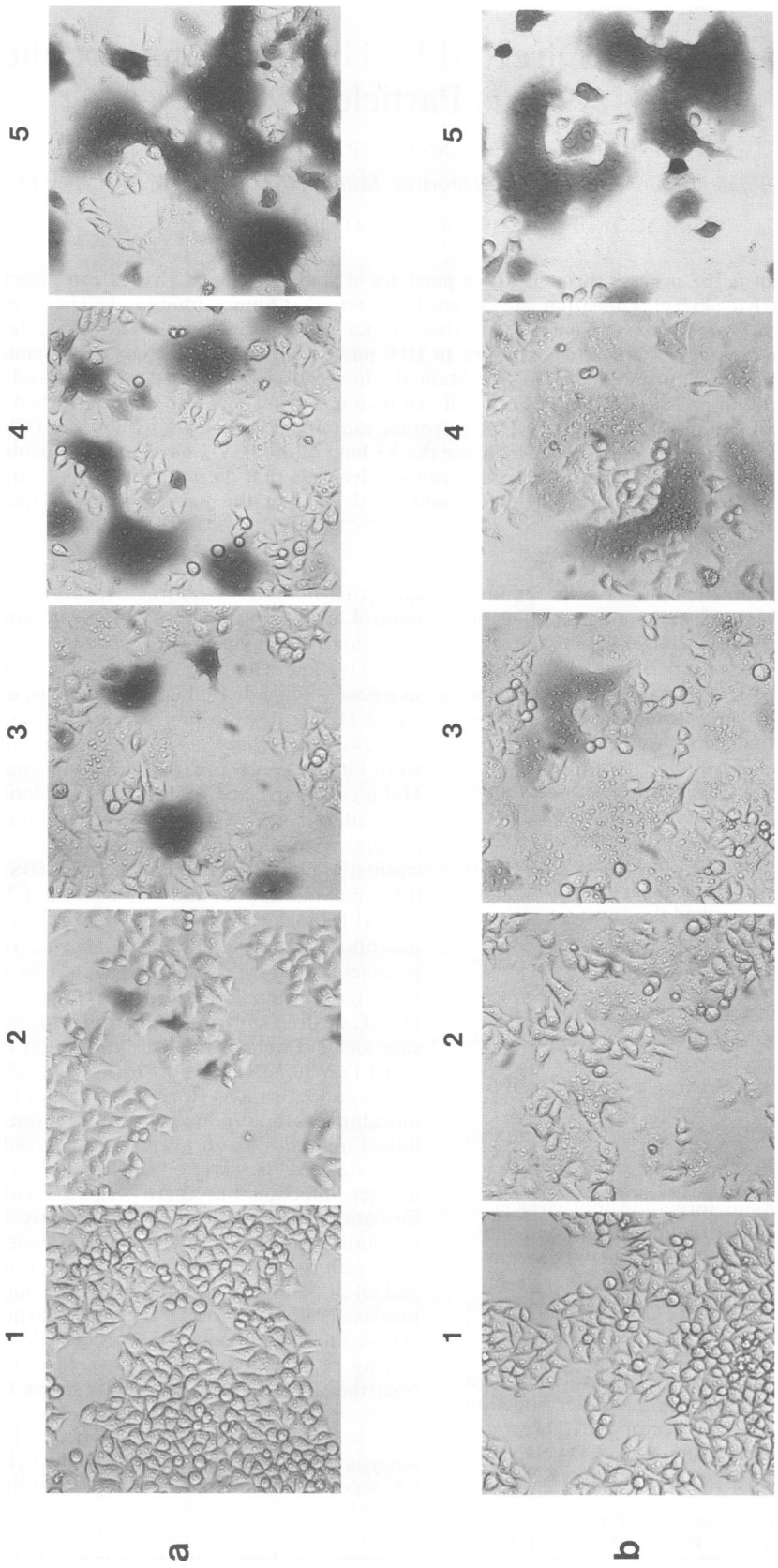

FIG. 1. Fusion from without of $CD4^+$ LTR-*lacZ* HeLa cells (P4 cells) induced by replication-competent HIV-1 particles. (a) Fusion induced by increasing numbers of HIV-1 particles. P4 cells were plated in the wells of 24-well plates. Half-confluent P4 cells were then exposed to increasing concentrations of HIV-1 particles produced by transfected HeLa cells. Panels: 1, no particles; 2, concentration of particles corresponding to 25 ng of HIV-1 p24 per ml; 3, 50 ng of HIV-1 p24 per ml; 4, 100 ng of HIV-1 p24 per ml; 5, 200 ng of HIV-1 p24 per ml. Cells were exposed to virus for 24 h, fixed with 0.5% glutaraldehyde in PBS, and stained with X-Gal. (b) Time course of fusion from without directed by HIV-1 particles. P4 cells were exposed to HIV-1 particles corresponding to 150 ng of HIV-1 p24 per ml, fixed at various time points, and stained. Panels: 1, 3 h of exposure; 2, 6 h; 3, 12 h; 4, 18 h; 5, 24 h.

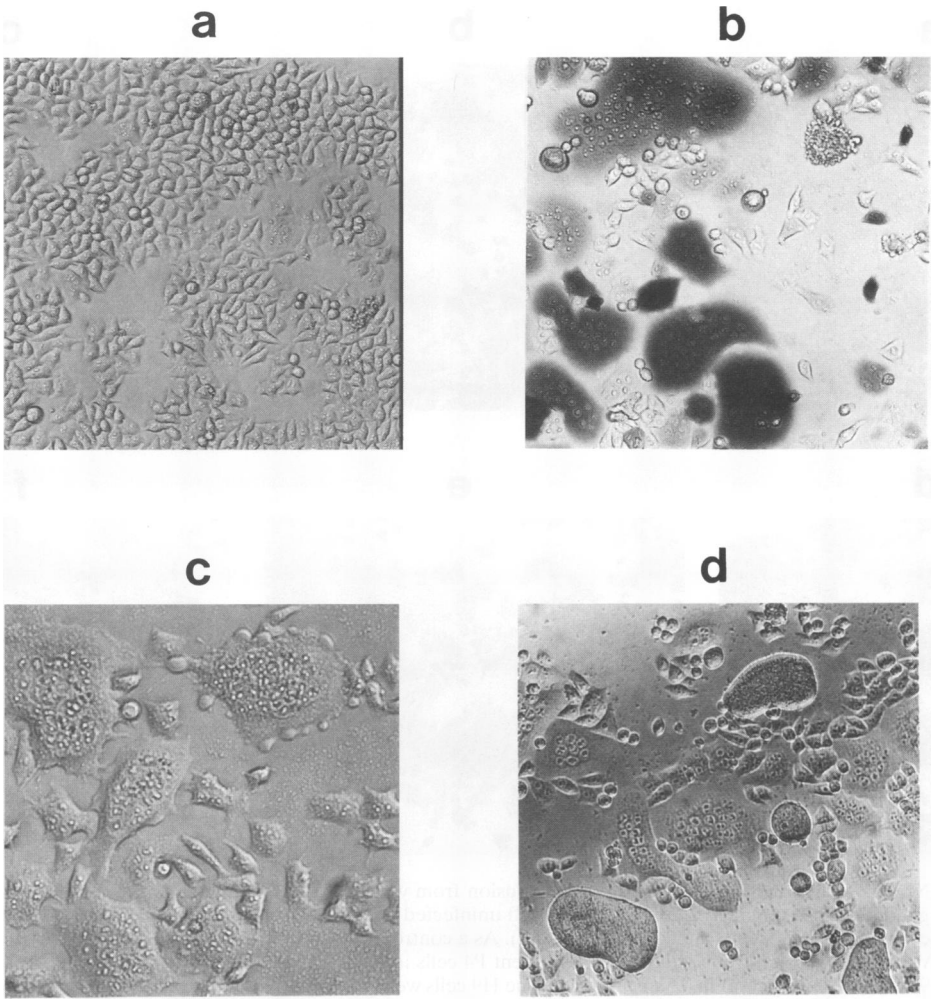

FIG. 2. HIV fusion from without is induced by pelleted particles and can occur in the absence of viral replication. (a) Absence of fusion following exposure of P4 cells to a particle-depleted culture medium from transfected HeLa cells by ultracentrifugation at 100,000 × *g* for 10 min. (b) Fusion induced by particles resuspended from the ultracentrifugation pellet. (c) Fusion induced by HIV particles on P4 cells treated with 5 μM AZT. P4 cells were treated with AZT for 2 h before virus inoculation. (d) Fusion induced by particles of a replication-defective HIV-1 integrase mutant. Half-confluent P4 cells were exposed to HIV particles for 24 h, then glutaraldehyde fixed, and stained with X-Gal.

typical experiments, most of the cells in the monolayer were associated with large blue syncytia (Fig. 1b, panel 5).

Since single HIV-infected cells exposed to a low concentration of virus did not produce syncytia even 24 h after infection, and since syncytia were readily produced after contact with high concentrations of HIV particles as early as 6 h after infection in the absence of detectable HIV gene expression, we hypothesized that the formation of these syncytia was directly induced by the HIV particles, i.e., that HIV particles at high concentrations were able to produce fusion from without. To test this hypothesis, P4 cells were exposed to HIV at a concentration equivalent to 150 ng of p24 per ml, in medium containing 5 μM zidovudine (AZT). At this concentration, AZT has been shown to fully prevent HIV DNA synthesis and further viral gene expression with the HIV-1 isolate used in our experiments. Nonetheless, AZT treatment did not affect the fusion process; after incubation for 24 h, almost all cells were involved in syncytium formation but no HIV expression could be detected in these syncytia, which remained white after X-Gal staining (Fig. 2d). This result demonstrates that even in the absence of HIV replication and gene expression, HIV particles are able to produce syncytia. We therefore hypothesized that replication-defective HIV-1 particles should also be able to direct cell-cell fusion. P4 cells were therefore incubated with particles produced following transfection of HeLa cells with an HIV-1 proviral molecular clone carrying a single-amino-acid change at position 186 in the integrase coding sequence. This mutant produces normal amounts of viral particles, but the produced virions are defective for replication. Exposure of P4 cells with these particles yielded numerous white syncytia, as shown in Fig. 2c, readily demonstrating fusion from without by replication-defective HIV particles.

To ascertain whether syncytium formation was the result of interaction with virions and not of other components of the culture supernatants used in our experiments, viral particles produced by transfected HeLa cells were pelletted by centrifugation at 100,000 × *g* for 10 min, resuspended in culture medium, and used to infect P4 cells. Only cells exposed to the resuspended particles exhibited syncytia (Fig. 2b), whereas no syncytia could be observed in cells incubated with the ultra-

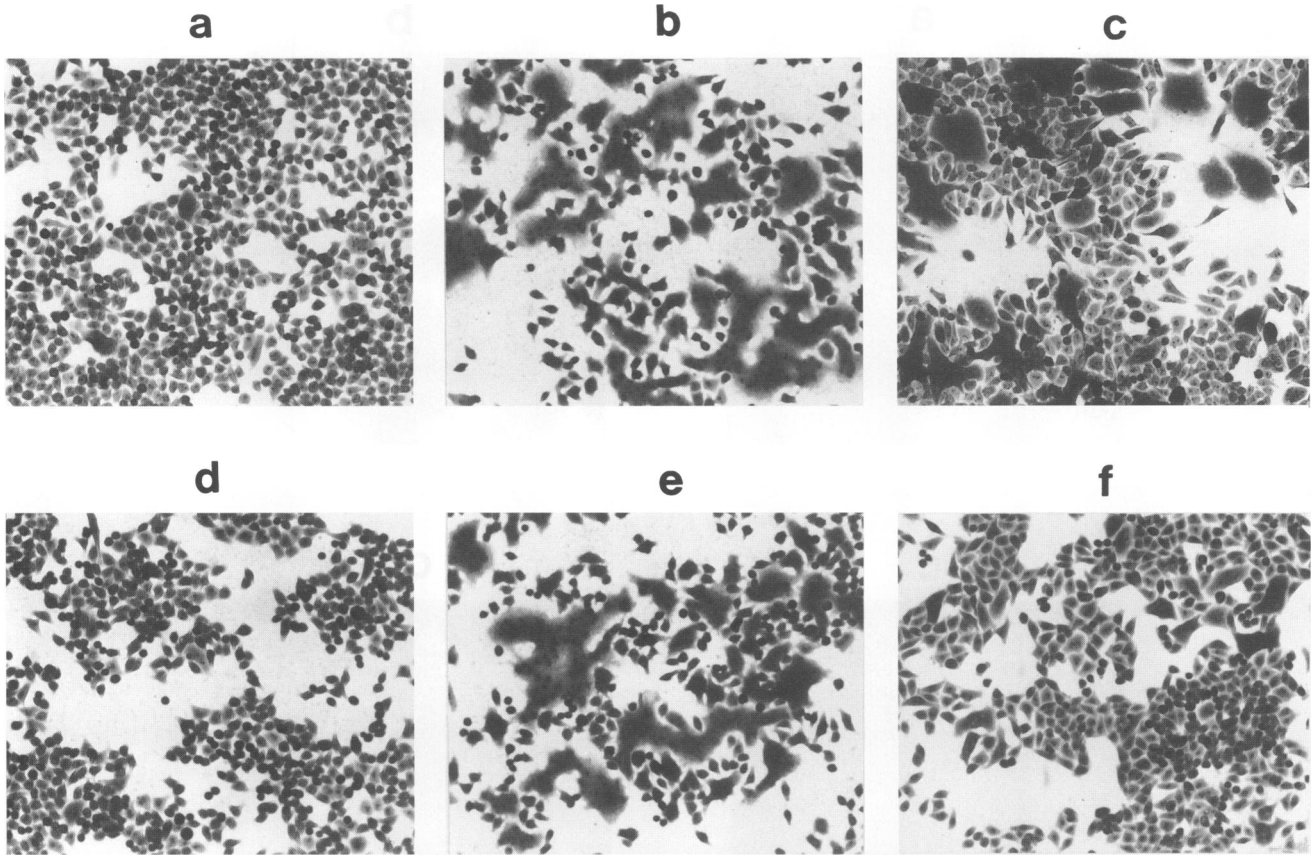

FIG. 3. Effect of DNM, an inhibitor of glucosidase I, on HIV fusion from without. Half-confluent P4 cells were exposed to HIV-1 particles corresponding to 150 ng of HIV-1 p24 per ml for 12 h (b and e) or left uninfected (a and d). At the time of infection, the P4 cells were either treated with 3 mM DNM (panels d and e) or left untreated (panels a and b). As a control for the ability of DNM to inhibit syncytium formation resulting from expression of HIV envelope (fusion from within), half-confluent P4 cells in 48-well plates were cocultured with chronically HIV-1-infected, envelope-expressing H9 cells (10^6 cells per ml in 0.5 ml) for 12 h. The H9 cells were either untreated (c) or treated with 3 mM DNM for 6 h before the coculture as well as during the coculture (f). P4 cells were exposed to HIV particles for 24 h, then fixed with glutaraldehyde, and stained with Giemsa.

centrifugation supernatant (Fig. 2a). This result demonstrates that the fusion phenomenon observed in our previous observations was mediated by viral particles and not by a soluble component of the culture supernatant.

To confirm that the fusion of P4 cells induced by these particles occurred in the absence of de novo synthesis of HIV envelope, we have examined the effect of treatment of the P4 cells with an inhibitor of glucosidase I, desoxynojirimycin (DNM). This drug prevents full maturation of the carbohydrate moiety of the HIV envelope glycoprotein and has been previously shown to block fusion of HIV envelope-expressing cells with $CD4^+$ target cells (9, 21). As shown in Fig. 3b and e, DNM treatment did not affect particle-directed fusion. As a control of drug activity, pretreatment of chronically HIV-infected, envelope-expressing H9 cells with DNM markedly inhibited their fusion with P4 cells (Fig. 3c and f). This shows that blocking the fusion-inducing capacity of newly synthesized HIV envelope in the P4 cells did not impair particle-induced fusion and readily demonstrates that it is indeed fusion from without.

The importance of HIV envelope glycoprotein in the process of fusion from without was further assessed by exposing P4 cells to HIV particles in the presence of increasing concentrations of monoclonal antibody 0.5β, specific for an epitope located within the V3 loop of HIV-1 gp120 (19). This antibody has been reported to completely neutralize fusion of HIV-infected cells with uninfected cells at a concentration of 50 μg/ml and to neutralize cell-free virus infectivity at a concentration of 0.1 μg/ml (19). In our assay, serial 10-fold dilutions of the 0.5β antibody were added to the cultures simultaneously with addition of virus. Under these conditions, 0.5β antibody at 50 μg/ml almost completely neutralized HIV-induced fusion of P4 cells (Fig. 4a). Similarly, the ability of soluble CD4 to block fusion of the P4 cells was examined by using recombinant soluble CD4 produced by CHO cells. A complete inhibition of fusion from without by HIV particles was obtained at a concentration of soluble CD4 of 40 μg/ml (Fig. 4b), which slightly exceeds the concentrations usually required to block cell-cell fusion resulting from expression of HIV envelope (11). In line with this finding, no fusion was observed when CD4-negative HeLa cells were substituted for the P4 cells in our assay. These results establish that both HIV gp120 and CD4 are involved in the HIV fusion-from-without process.

The mechanism by which fusion of receptor-bearing cells can be directly induced by viral particles (fusion from without) is not well understood. Newcastle disease virus mutants that retain one or the other of the two modes of fusion have been described. These studies have suggested that the ability of

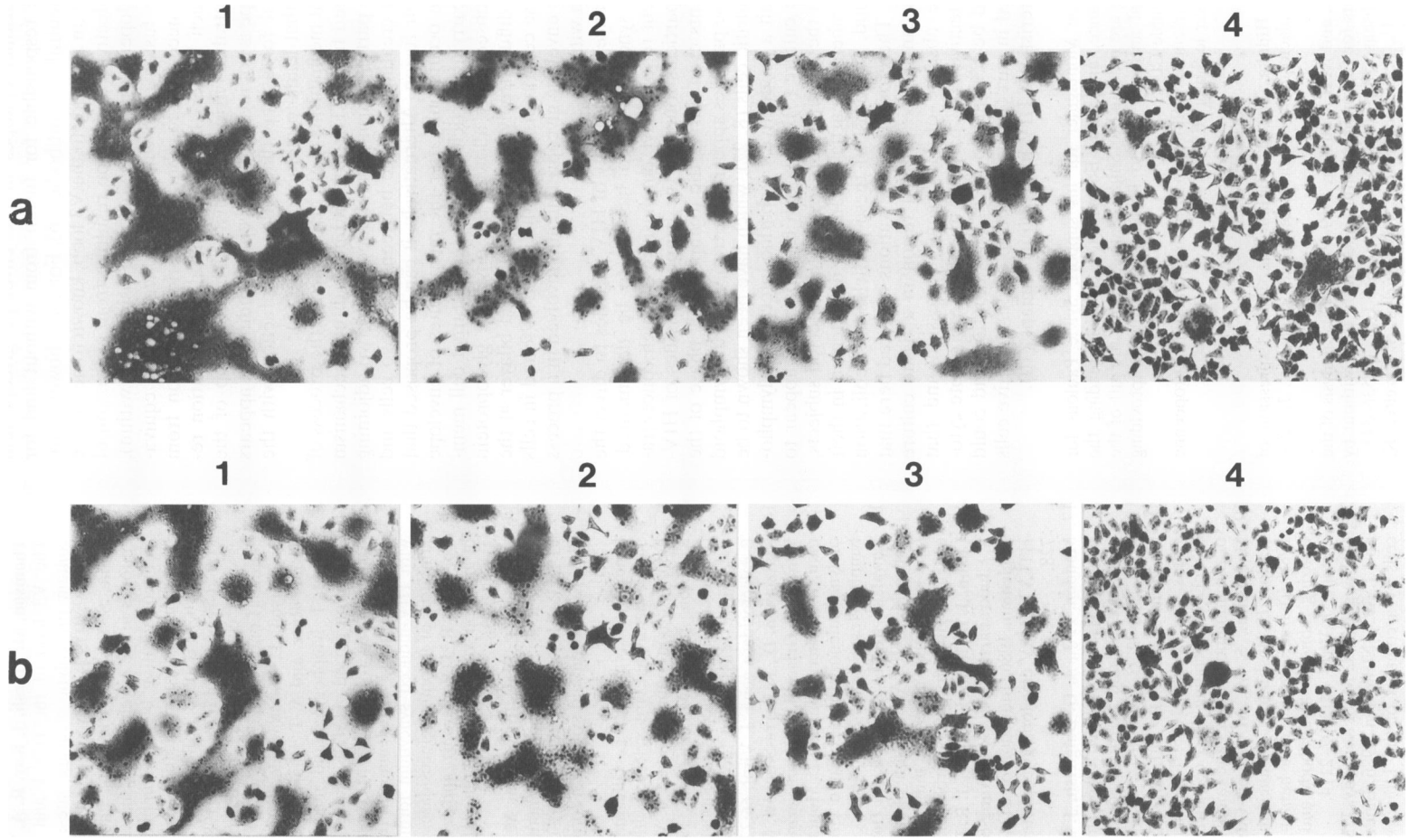

FIG. 4. Neutralization of HIV fusion from without by anti-V3 loop monoclonal antibody and by recombinant soluble CD4. (a) Effect of V3 loop-specific monoclonal antibody 0.5β on HIV fusion from without. P4 cells were exposed to HIV-1 particles corresponding to 150 ng of p24 per ml in the presence of increasing concentrations of purified monoclonal antibody 0.5β. Panels: 1, no antibody; 2, 0.5 μg/ml; 3, 5 μg/ml; 4, 50 μg/ml. (b) Effect of recombinant soluble CD4 on HIV fusion from without. P4 cells were exposed to HIV-1 particles corresponding to 150 ng of p24 per ml in the presence of increasing concentrations of soluble CD4. Panels: 1, no soluble CD4; 2, 0.4 μg/ml; 3, 4 μg/ml; 4, 40 μg/ml. Following incubation with the virus, all cells were fixed with glutaraldehyde and stained with hematoxylin.

virions to induce fusion from without correlates with increased affinity for the receptors and with low neuraminidase activity of the hemagglutinin-neuraminidase envelope protein. With visna virus, distinct epitopes in the envelope glycoprotein appear to be responsible for fusion from without and for induction of neutralizing antibodies (6). For HIV, however, we show here that an antibody to the V3 loop, known to neutralize both virion infectivity and fusion resulting from expression of the HIV envelope at the surface of cells (fusion from within), can neutralize fusion from without at concentrations equivalent to those necessary to obtain inhibition of fusion from within but much higher than the minimal concentration required to neutralize virion infectivity. The V3 loop of the HIV-1 envelope is not involved in receptor binding but appears to be essential for a step that follows interaction with the receptor and that leads to membrane fusion (5).

Two types of mechanisms could be invoked in the process of particle-directed fusion. The first and more direct mechanism would involve particles fusing simultaneously to two adjoining cells, in effect creating a bridge between these two cells and promoting their fusion. Alternatively, it can be proposed that cell-cell fusion occurs only after the membranes of envelope glycoprotein-carrying particles have fused to target cell membranes and subsequently allowed the envelope glycoprotein complexes to diffuse freely in the phospholipid bilayer at the surface of these cells, reproducing the situation found in cells expressing the envelope glycoprotein and involved in a process of fusion from within.

The importance of the ability of HIV particles to direct the formation of syncytia in vivo has yet to be documented. However, recent reports have emphasized the high concentration of HIV particles found in the lymphoid tissues of HIV-infected patients even during the asymptomatic phase of the infection. These particles appear to be trapped in the lymphoid organs by dendritic cells (8, 22), which have been shown to be able to transmit a productive, cytopathic infection to lymphocytes in tissue culture (3). Although HIV particles appear to concentrate in the lymphoid organs of HIV-infected subjects, fairly large numbers of particles can also be detected in their peripheral blood, and most of these particles are replication defective (23). Therefore, the observations reported here that HIV particles can induce cell-cell fusion in a tissue culture system in the absence of HIV gene expression and that replication-defective HIV particles are able to promote cytopathicity could be relevant to an in vivo situation and could account at least in part for the depletion of CD4-positive cells that is characteristic of HIV infection in humans (10).

We thank Luc Montagnier for his support, Sylvie Paulous for technical assistance, and Andy Borman for critically reading the manuscript. We are indebted to Shuzo Matsushita for the gift of the 0.5β monoclonal antibody and to David Klatzmann for providing recombinant soluble CD4.

P.C. is the recipient of a fellowship from the Fondation Européenne de Recherches sur le Sida (FERS).

REFERENCES

1. **An, G., K. Hidaka, and L. Siminovitch.** 1982. Expression of bacterial β-galactosidase in animal cells. Mol. Cell. Biol. **2**:1628–1632.
2. **Bratt, M. A., and W. R. Gallaher.** 1969. Preliminary analysis of the requirements for fusion from within and fusion from without by Newcastle disease virus. Proc. Natl. Acad. Sci. USA **64**:536–543.
3. **Cameron, P. U., P. S. Freudenthal, J. M. Barker, S. Gezelter, K. Inaba, and R. M. Steinman.** 1992. Dendritic cells exposed to human immunodeficiency virus type-1 transmit a vigorous cytopathic infection to CD4+ T cells. Science **257**:383–387.
4. **Charneau, P., M. Alizon, and F. Clavel.** 1992. A second origin of plus-strand synthesis is required for optimal human immunodeficiency virus replication. J. Virol. **66**:2814–2820.
5. **Clements, G. J., M. J. Price-Jones, P. E. Stephens, C. Sutton, T. F. Schulz, P. R. Clapham, J. A. McKeating, M. O. McClure, S. Thomson, M. Marsh, J. Kay, R. A. Weiss, and J. P. Moore.** 1991. The V3 loop of the HIV-1 and HIV-2 surface glycoproteins contain proteolytic cleavage sites: a possible function in viral fusion? AIDS Res. Hum. Retroviruses **7**:3–16.
6. **Crane, S. E., J. E. Clements, and O. Narayan.** 1988. Separate epitopes in the envelope of visna virus are responsible for fusion and neutralization: biological implications for anti-fusion antibodies in limiting virus replication. J. Virol. **62**:2680–2685.
7. **Dalgleish, A. G., P. C. L. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312**:763–766.
8. **Embretson, J., M. Zupancic, J. L. Ribas, A. Burke, P. Racz, K. Tenner-Racz, and A. T. Haase.** 1993. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. Nature (London) **362**:359–362.
9. **Gruters, R. A., J. J. Neefjes, M. Tersmette, R. E. de Goede, A. Tulp, H. G. Huisman, F. Miedema, and H. L. Ploegh.** 1987. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature (London) **330**:74–77.
10. **Ho, D. D., R. J. Pomerantz, and J. C. Kaplan.** 1987. Pathogenesis of infection with human immunodeficiency virus. N. Engl. J. Med. **317**:278–286.
11. **Hussey, R. E., N. E. Richardson, M. Kowalski, N. R. Brown, H. C. Chang, R. F. Siliciano, T. Dorfman, B. Walker, J. Sodroski, and E. L. Reinherz.** 1988. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature (London) **331**: 78–81.
12. **Iorio, R. M., and R. L. Glickman.** 1992. Fusion mutants of Newcastle disease virus selected with monoclonal antibodies to the hemagglutinin- neuraminidase. J. Virol. **66**:6626–6633.
13. **Kimpton, J., and M. Emerman.** 1992. Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated β-galactosidase gene. J. Virol. **66**:2232–2239.
14. **Klatzmann, D., F. Barre-Sinoussi, M. T. Nugeyre, C. Dauguet, E. Vilmer, C. Griscelli, F. Brun-Vezinet, C. Rouzioux, J. C. Gluckman, J. C. Chermann, and L. Montagnier.** 1984. Selective tropism of lymphadenopathy-associated virus (LAV) for helper-inducer lymphocytes. Science **225**:59–63.
15. **Kowalski, M., L. Bergeron, T. Dorfman, W. Haseltine, and J. Sodroski.** 1991. Attenuation of human immunodeficiency virus type 1 cytopathic effect by a mutation affecting the transmembrane envelope glycoprotein. J. Virol. **65**:281–291.
16. **Lifson, J. D., M. B. Feinberg, J. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman.** 1986. Induction of CD4-dependent cell fusion by the HTLVIII/LAV envelope glycoprotein. Nature (London) **323**:725–728.
17. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **42**:93–104.
18. **Maddon, P. J., D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, and R. Axel.** 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. Cell **42**:93–104.
19. **Matsushita, S. M., M. Robert-Guroff, J. Rusche, A. Koito, T. Hattori, H. Hoshino, K. Javaherian, K. Takatsuki, and S. Putney.** 1988. Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. J. Virol. **62**:2107–2114.
20. **McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson.** 1986. Binding of HTLV III/LAV to T4+ cells by a complex of the 110 k protein with the T4 molecule. Science **231**:382–385.
21. **Montefiori, D. C., W. E. Robinson, and W. M. Mitchell.** 1988. Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **85**:9248–9252.

22. **Pantaleo, G., C. Graziosi, J. F. Demarest, L. Butini, M. Montroni, C. H. Fox, J. M. Orenstein, D. P. Kotler, and A. S. Fauci.** 1993. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature (London) **362:**355–358.
23. **Platak, M., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K.-C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson.** 1993. High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR. Science **259:**1749–1754.
24. **Rocancourt, D., C. Bonnerot, H. Jouin, M. Emerman, and J.-F. Nicolas.** 1990. Activation of a β-galactosidase recombinant provirus: application to titration of HIV and HIV-infected cells. J. Virol. **64:**2660–2668.
25. **Sodroski, J. G., W. C. Goh, C. A. Rosen, K. Campbell, and W. A. Haseltine.** 1986. Role of HTLV III/LAV envelope in syncytia formation and cytopathicity. Nature (London) **322:**470–474.
26. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.