JOURNAL OF VIROLOGY, Sept. 1989, p. 4074–4078
0022-538X/89/094074-05$02.00/0

Vol. 63, No. 9

Cell Surface Expression of Several Species of Human Immunodeficiency Virus Type 1 Major Core Protein

ANNE G. LAURENT, BERNARD KRUST, MARIE-ANNE REY, LUC MONTAGNIER, AND ARA G. HOVANESSIAN*

Unité d'Oncologie Virale (URA 153 Centre National de la Recherche Scientifique), Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris Cédex 15, France

Received 30 January 1989/Accepted 26 May 1989

The major core protein (p25) of the human immunodeficiency virus type 1 (HIV-1) was characterized by two-dimensional-gel isoelectric focusing. The p25 detectable in HIV-1-infected cells is composed of four species with related isoelectric points. This is due in part to the phosphorylated state of p25. The four species of p25 are expressed on the cell surfaces of infected cells, but only the two most basic species are incorporated into the HIV-1 virion. These findings emphasize the importance of p25 in understanding infection with HIV and might have implications for the development of vaccines.

The human immunodeficiency virus type 1 (HIV-1) *gag* gene codes for a 55,000-molecular-weight precursor polyprotein (p55) which is processed by a protease coded for by the *pol* gene to give internal structural proteins p18, p25, and p15 (also referred to as p17, p24, and p14, respectively [2, 5, 6, 7, 14]). p18 is the matrix protein derived from the N-terminal portion of the p55 precursor. It is N myristylated and phosphorylated. p25 is the major core protein of HIV-1 derived from the middle fragment of p55 and, like p18, is phosphorylated (7, 10, 17). p15 is derived from the C-terminal side of p55. It is cleaved to give the nucleocapsid proteins p9 and p7 (10, 17). In cultured cells infected with HIV, large quantities of p25 are produced and become incorporated into HIV virions. Similarly, large quantities of p25 are probably produced in individuals infected with HIV, since p25 becomes detectable very soon after contamination and much before the development of p25-specific cellular and humoral responses (9). Accordingly, detailed characterization of p25 is essential for understanding infection with HIV. Here we provide evidence to show that p25 produced by HIV-1-infected cells is composed of several related species, some of which become integrated into the HIV-1 virion, whereas others become expressed on the cell surfaces of infected cells or become secreted. The cell surface expression and secretion of p25 might account for the *gag*-specific cellular immune responses observed in patients with acquired immunodeficiency syndrome (13, 18) and might also explain the presence of circulating p25 in early and late stages of the disease (9).

HIV-1 BRU isolate (1) was used in this study to infect CEM clone 13 cells (12). Four days after infection with this HIV-1 isolate, about 80 to 90% of the cells produce viral particles and can be recognized by a cytopathic effect corresponding to vacuolization of cells and appearance of small syncytia. During this period, large quantities of p25 are produced. Figure 1A shows two-dimensional-gel isoelectric-focusing analysis of [^{35}S]methionine-labeled proteins synthesized in HIV-1-infected cells. The p25 was resolved as four species (designated species a, b, c, and d) with isoelectric points of 6.8, 6.6, 6.5, and 6.3, respectively. In addition, species c and d had electrophoretic mobilities in polyacrylamide gels slightly slower than those of species a and b. The identities of these four species were confirmed by immunoprecipitation with a monoclonal antibody specific for p25 of HIV-1 (Fig. 1B). All four species of *gag* p25 are also detectable in the culture medium of HIV-1-infected cells. In order to determine which p25 species are incorporated into the HIV-1 virions, culture medium was centrifuged at 100,000 × *g* for 2 h to pellet the virus. Only species a and b were found to be associated with the virus pellet, whereas species c and d along with a proportion of species a and b remained in soluble form in the supernatant after centrifugation at 100,000 × *g* (Fig. 1C). Therefore, HIV-1-infected cells synthesize four species (a, b, c, and d) of p25, but only a proportion of species a and b is incorporated into HIV-1 virions, whereas the rest of p25 is probably secreted. The presence of soluble forms of p25 in the culture medium is not likely to be because of lysis of infected cells, since intracellular precursors of the envelope and *gag* genes (gp160 and p55) were not detectable in the culture medium (data not shown). The presence of a small proportion of species a and b in the 100,000 × *g* supernatant might also reflect the presence of some defective virus particles which do not sediment with the infectious virus. Whatever the case, it is interesting to note that species c and d are not precipitable even after a prolonged (4- to 6-h) centrifugation at 100,000 × *g*.

The differences in the pI values of the four species of p25 might be due to posttranslational modifications, such as phosphorylation. Indeed, several reports have provided evidence that p25 is phosphorylated at the residues serine and threonine (7, 10, 17). Figure 2A shows two-dimensional gel isoelectric-focusing analysis of p25 phosphorylated in vivo in HIV-1-infected CEM cells. Although these cells produced all four species (a, b, c, and d) of p25, only species b and d were found to be labeled with $^{32}PO_4$. Another approach to show the phosphorylation of p25 is by dephosphorylation using bacterial alkaline phosphatase (BAP). For this experiment, [^{35}S]methionine-labeled p25 was purified by immunoprecipitation with extracts from the infected cells (to purify species a, b, c, and d). Portions of such samples were then digested by the BAP before analysis by two-dimensional gel isoelectric focusing. Digestion of the four species of p25 with BAP led to a modification in the pattern corresponding to these four spots (Fig. 2B). The levels of [^{35}S]methionine-labeled species b and d were decreased, whereas those of species a

* Corresponding author.

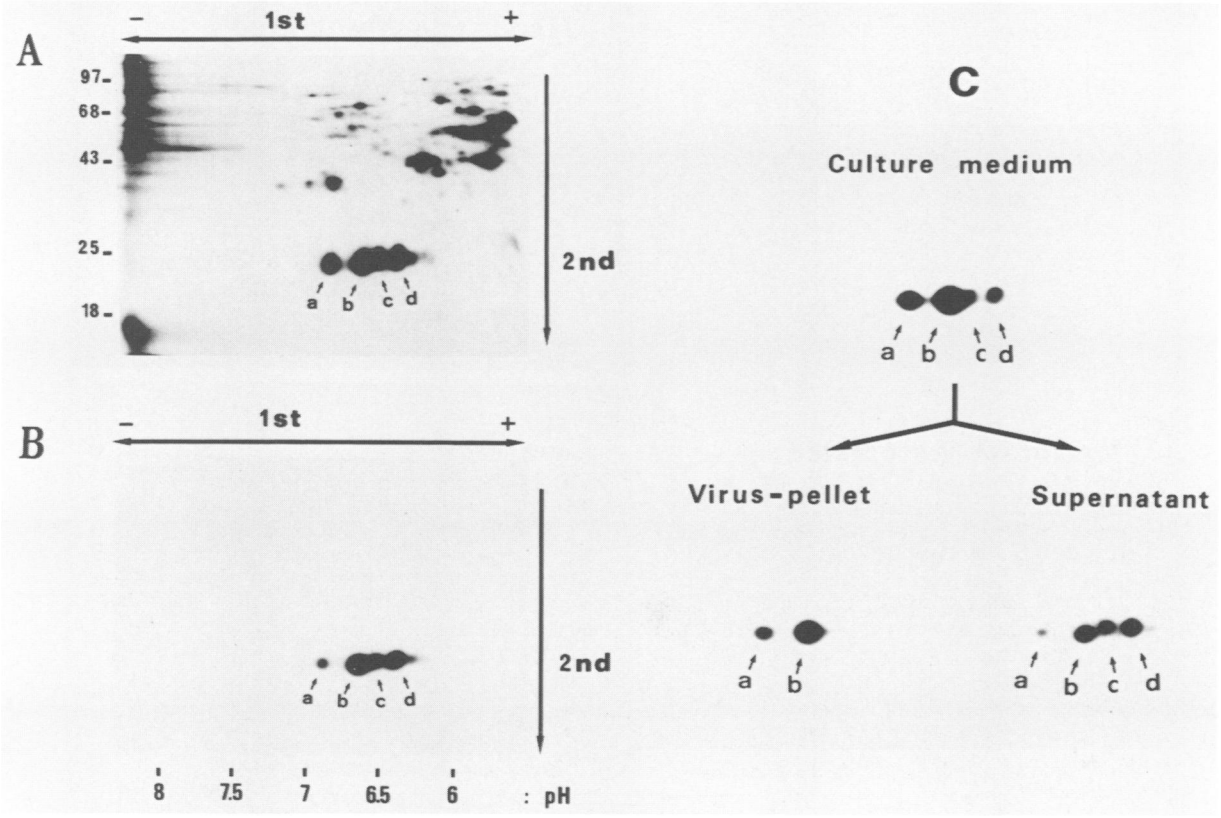

FIG. 1. Analysis of [^{35}S]methionine-labeled p25 by two-dimensional-gel isoelectric focusing. (A) Labeled extracts from HIV-1-infected cells (10^6 cells). (B) Species of p25 immunoprecipitated from crude cell extracts by using a monoclonal antibody specific for p25 (MAb 25/3; Genetic Systems). (C) Species of p25 immunoprecipitated by MAb 25/3 by using extracts from the culture medium of labeled cells (shown in section A), virus pellet (obtained by centrifugation at 100,000 × *g* for 30 min), and 100,000 × *g* supernatant. HIV-1 BRU-infected human CEM clone 13 cells (12) were cultured in suspension in RPMI 1640 medium containing 10% fetal calf serum and 2 μg of Polybrene (Sigma) per ml. On day 4, infected cells were labeled (16 h, 37°C) in culture medium without L-methionine and serum but supplemented with 200 μCi of [^{35}S]methionine (Amersham, 1,000 Ci/mmol) per ml. Cell and viral extracts were prepared in lysis buffer containing 10 mM Tris hydrochloride (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 100 U of aprotinin (Iniprol) per ml. For the culture medium, 10-fold-concentrated lysis buffer was added to the clarified supernatant from infected cells. Immunoprecipitation in the presence of protein A-Sepharose was carried out in binding buffer containing 20 mM Tris hydrochloride (pH 7.6), 50 mM KCl, 150 mM NaCl, 1 mM EDTA, 1% (vol/vol) Triton X-100, 20% (vol/vol) glycerol, 7 mM β-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, and 100 U of aprotinin per ml (12). Two-dimensional-gel isoelectric focusing was performed as described previously (11). The pH gradient obtained was from 5.0 to 8.5. The proteins were resolved in the second dimension on a 12.5% polyacrylamide–sodium dodecyl sulfate gel containing 0.1 instead of 0.2% (wt/vol) bisacrylamide. Fluorographs of the gels are presented. On the left are the positions of molecular weight protein markers (in thousands).

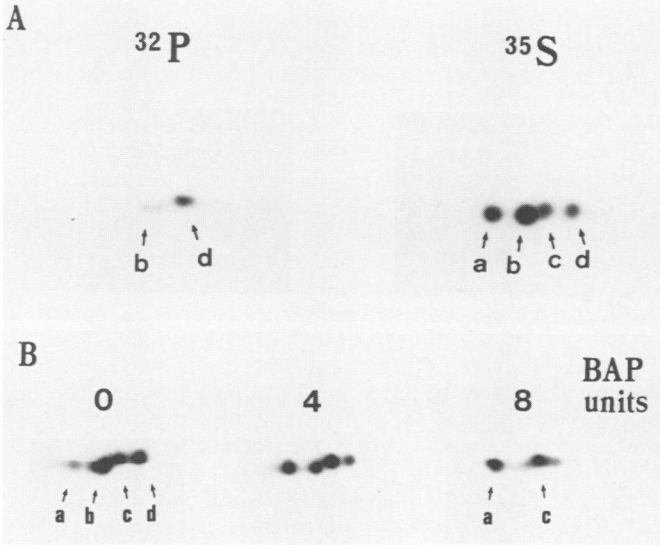

FIG. 2. Species b and d of p25 are the phosphorylated forms of species a and c. (A) HIV-1-infected CEM cells were incubated in phosphate-free medium containing 200 μCi of ^{32}P for 4 h. Cell extracts were then immunoprecipitated with MAb 25/3, and the purified p25 was analyzed by two-dimensional-gel isoelectric focusing (as described in the legend to Fig. 1). An autoradiogram is shown. A sample of [^{35}S]methionine-labeled p25 was also analyzed in parallel for the determination of the position of the four spots. (B) Dephosphorylation of p25 with bacterial alkaline phosphatase. HIV-1-infected cells were labeled (16 h) with [^{35}S]methionine (200 μCi/ml). The different species of p25 were purified by immunoprecipitation with MAb 25/3. Equal portions of the immunoprecipitated p25 were suspended in 50 μl of buffer (20 mM Tris hydrochloride [pH 7.6], 50 mM NaCl, 5 mM $MgCl_2$, 3 mM $MnCl_2$, 100 U of aprotinin per ml) and incubated as such (0) or with 4 and 8 U of BAP for 90 min at 37°C. All the samples were analyzed by two-dimensional-gel isoelectric focusing. Fluorographs are shown.

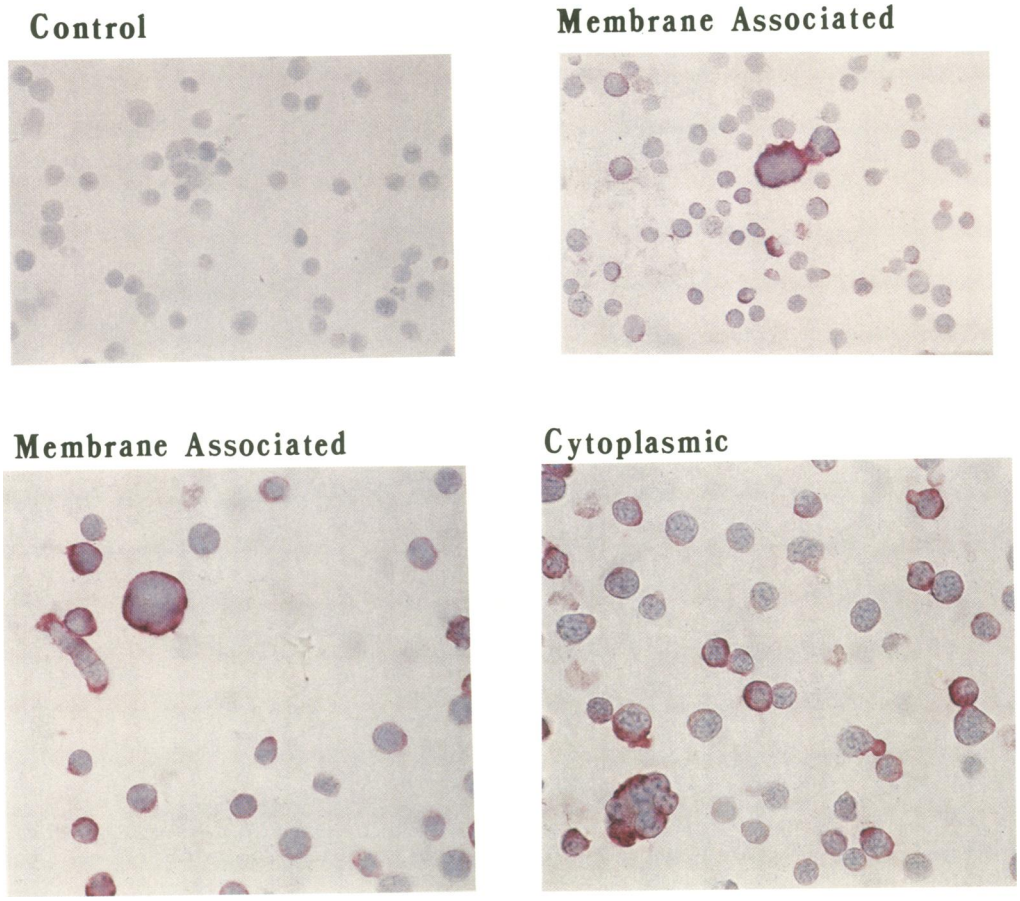

FIG. 3. Reactivity of a monoclonal antibody specific for p25 on the cell surface and in the cytoplasm of HIV-1-infected cells. HIV-1-infected cells (day 4 of infection) were washed with phosphate-buffered saline before fixation in either 0.3% formaldehyde or acetone. The presence of p25 was then assayed by using MAb 25/3. These antibodies were revealed first by rabbit anti-mouse immunoglobulins and then by alkaline phosphatase anti-alkaline phosphatase. The color was developed with a solution containing 10 mg of Naphthol AS-MS (Sigma Chemical Co.), 1 or 2 drops of dimethylformamide, 10 ml of 50 mM Tris hydrochloride (pH 9), and 10 mg of fast red (Sigma). After 10 to 15 min in this solution, the slides were washed in water and the nuclei were stained with hematoxylin. Control uninfected cells showed no reactivity with MAb 25/3, whereas infected cells were positive either for membrane or cytoplasmic antigen.

and c were increased. This shift was accentuated by using higher doses of BAP (Fig. 2B). Under these conditions, species d and b were completely dephosphorylated to species c and a, respectively; however, species c was not converted to species b. The presence of only two phosphorylated species of p25 is in agreement with the pattern of phosphorylated species obtained by in vivo phosphate labeling of infected cells (Fig. 2A). It might be possible, therefore, to differentiate the four species of p25 into two main categories: (i) species a and b and (ii) species c and d, species b and d being the phosphorylated forms of a and c, respectively. The difference between species a and c might be due to another posttranslational modification of p25.

In a recent article by Mervis et al. (10), the presence of different forms of p25 in HIV-1-infected A3.01T lymphocytes was discussed. However, the characterization of these forms was not shown by these authors. The different forms of p25 described by Mervis et al. probably correspond to the species a, b, c, and d that we have described here. Although the four species of p25 are detectable at the same time as cleavage products of the *gag* precursor p55 (data not shown), there might be different processing pathways for the production of species a and b and species c and d. Two forms of *gag* p40 (partial digestion product of p55) species have been described (10), one resulting from the C-terminal processing of p55 and the other originating either from N-terminal processing of p55 or from de novo synthesis. These two forms of p40 might then generate the different species of p25, probably by distinct pathways to give species a and b and species c and d, respectively.

The expression of p25 was investigated in HIV-1-infected CEM cells either fixed with formaldehyde (to localize membrane-associated antigens) or fixed with acetone (to localize cytoplasmic antigens). In these experiments we used a monoclonal antibody specific for p25 in an immunoenzymatic-staining assay. Uninfected cells manifested no reactivity with the monoclonal antibody, whereas infected cells were positive for either cytoplasmic or membrane antigens (Fig. 3). Cell surface expression of p25 was confirmed by lactoperoxidase catalyzed iodination of the cell surface proteins (8) present on the surface of HIV-1-infected cells. The presence of viral proteins in these infected cells was monitored by prelabeling a small proportion of such cells with [^{35}S]methionine for 6 h. Extracts of the latter were prepared immediately before the iodination experiment. [^{35}S]methionine- and ^{125}I-labeled extracts were purified by immunoprecipitation with monoclonal antibodies specific for p25 or with HIV-1-positive serum (to purify *gag* and enve-

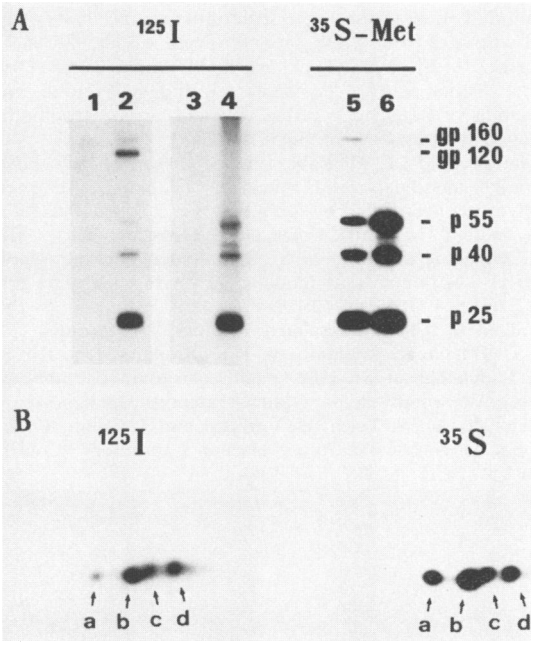

FIG. 4. Expression of p25 on the surface of HIV-1-infected CEM cells. (A) ^{125}I labeling of cell surface proteins present on uninfected control (lanes 1 and 3) and HIV-1-infected (lanes 2 and 4) CEM cells was carried out as described previously (8). Briefly, control cells (uninfected) and HIV-1-infected cells (day 4 of infection; 2×10^7 CEM cells) were washed with phosphate-buffered saline (2×25 ml), and the pellet was suspended in 2 ml of phosphate-buffered saline containing 10 mM D-glucose and 2 mCi of ^{125}I (100 mCi/ml; Amersham Corp.), 2 U of lactoperoxidase, and 2 U of glucose oxidase (Calbiochem-Behring). After 10 min of incubation at 22°C, the cells were washed in phosphate-buffered saline (2×25 ml) and extracted in the lysis buffer (as described in the legend to Fig. 1). In lanes 5 and 6, HIV-1-infected cells were labeled for 6 h with [^{35}S]methionine (200 Ci/ml) and extracted at the same time as in the ^{125}I labeling. Extracts were immunoprecipitated either with an HIV-1-positive serum (lanes 1, 2, and 5) or with MAb 25/3 (lanes 3, 4, and 6). (B) ^{125}I-labeled p25 is composed of four species. Two-dimensional-gel isoelectric focusing of ^{125}I-labeled and [^{35}S]methionine-labeled p25. These samples correspond to the ones shown in lanes 2 and 5.

lope products). The monoclonal antibodies can also immunoprecipitate p55 and p40, which correspond to the *gag* precursor polyprotein and its partially cleaved product. Accordingly, p55, p40, and p25 were present in these [^{35}S]methionine-labeled HIV-1-infected cells in addition to the envelope glycoprotein precursor gp160 and the extracellular envelope glycoprotein gp120 (Fig. 4A, lanes 5 and 6). In contrast, cell surface iodination experiments indicated that the major ^{125}I-labeled proteins were gp120 and p25 (Fig. 4A, lanes 2 and 4). These results, therefore, show that a proportion of p25 synthesized in HIV-1-infected cells becomes exposed on the cell surface along with gp120. A comparison of ^{125}I-labeled proteins on the cell surface with [^{35}S]methionine-labeled ones synthesized in infected cells indicated that surface labeling was specific, since precursors of *gag* and envelope (gp55/40 and gp160, respectively) were only slightly labeled with ^{125}I, and their detection required a very long exposure of the autoradiogram. The ^{125}I-labeled p25 was further analyzed by two-dimensional gel isoelectric focusing along with the [^{35}S]methionine-labeled species (Fig. 4B). The ^{125}I-labeled p25 exposed on the cell surface was found to be composed of four species, a, b, c, and d. These data rule out the possibility that cell surface iodination of p25 might be due to labeling of the core protein in the budding virus, since species c and d, which are not associated with the virus particles, are labeled. These results (Fig. 3 and 4) show for the first time cell surface expression of one of the core proteins of a retrovirus (19). Previously, several workers have reported the cell surface expression of glycosylated forms of *gag* polyproteins in cells infected with murine leukemia virus (3, 4, 15, 16). However, none of the structural proteins comprising the core of murine leukemia viruses was shown to be expressed on the cell surface (19).

The data discussed here provide evidence for the first time for the secretion and cell surface expression of HIV-1 core protein p25. The secretion of some forms of p25 without incorporation into HIV-1 particles might account, at least in part, for the appearance of this antigen in the sera of patients with acquired immunodeficiency syndrome. In analogy with our results, the detection of circulating p25 might reflect a high level of virus replication in the late stages of the disease. Finally, the proportion of p25 that becomes exposed on the cell surfaces of infected cells might account for the triggering of *gag*-specific cellular immune responses, such as cytotoxic T-lymphocyte activation (13, 18), observed in HIV-seropositive individuals. Such observations emphasize the importance of p25 as a reasonable candidate along with the envelope glycoproteins in the development of an efficient vaccine against HIV.

This work was supported by grants from the French National Program on AIDS Research.

LITERATURE CITED

1. **Barré-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Brun-Vézinet, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:** 868–871.
2. **Chassagne, J., P. Verrelle, C. Dionet, F. Clavel, F. Barré-Sinoussi, J. C. Chermann, L. Montagnier, J. C. Gluckman, and D. Klatzmann.** 1986. A monoclonal antibody against LAV *gag* precursor: use for viral protein analysis and antigenic expression in infected cells. J. Immunol. **136:**1442–1445.
3. **Edwards, S. A., and H. Fan.** 1979. *gag*-related polyproteins of Moloney murine leukemia virus: evidence for independent synthesis of glycosylated and unglycosylated forms. J. Virol. **30:**551–563.
4. **Edwards, S. A., and H. Fan.** 1980. Sequence relationship of glycosylated and unglycosylated *gag* polyproteins of Moloney murine leukemia virus. J. Virol. **35:**41–51.
5. **Essex, M., J. S. Allan, P. J. Kanki, M. F. McLane, G. Malone, L. Kitchen, and T. H. Lee.** 1985. Antigens of human T-lymphotropic virus type III/lymphadenopathy-associated virus. Ann. Intern. Med. **103:**700–703.
6. **Ferns, R. B., R. S. Tedder, and R. A. Weiss.** 1987. Characterization of monoclonal antibodies against the human immunodeficiency virus (HIV) *gag* products and their use in monitoring HIV isolate variation. J. Gen. Virol. **68:**1543–1551.
7. **Henderson, L. E., T. D. Copeland, R. C. Sowder, A. M. Schultz, and S. Oroszlan.** 1988. Analysis of proteins and peptides purified from sucrose gradient banded HTLV-III. UCLA Symp. Mol. Cell. Biol. **71:**135–147.
8. **Hynes, R. O.** 1973. Alterations of cell surface proteins by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. USA **70:**3170–3174.
9. **Lange, J. M. A., D. A. Paul, H. G. Huisman, F. de Wolf, H. Van den Berg, R. A. Coutinho, A. Danner, J. Van der Noordaa, and J. Goudsmit.** 1986. Persistent HIV antigenaemia and decline of HIV core antibodies associated with transition to AIDS. Br. Med. J. **293:**1459–1462.

10. **Mervis, R. J., N. Ahmad, E. P. Lillehoj, M. G. Raum, F. H. Chan Rick Salazar, and S. Venkatesan.** 1988. The *gag* gene products of human immunodeficiency virus type 1: alignment within the *gag* open reading frame, identification of posttranslational modifications, and evidence for alternative *gag* precursors. J. Virol. **62**:3993–4002.
11. **O'Farrel, P. H.** 1975. High resolution two dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
12. **Rey, M. A., B. Krust, A. G. Laurent, L. Montagnier, and A. G. Hovanessian.** 1989. Characterization of human immunodeficiency virus type 2 envelope glycoproteins: dimerization of the glycoprotein precursor during processing. J. Virol. **63**:647–658.
13. **Riviere, Y., F. Tanneau-Salvadori, A. Regnault, O. Lopez, P. Sansonetti, B. Guy, M. P. Kieny, J. J. Fournel, and L. Montagnier.** 1988. Human immunodeficiency virus-specific cytotoxic responses of seropositive individuals: distinct types of effector cells mediate killing of targets expressing *gag* and *env* proteins. J. Virol. **63**:2270–2277.
14. **Robey, W. G., B. Safaiand, and S. Oroszlan.** 1985. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. Science **228**:593–595.
15. **Schultz, A. M., S. M. Lockhart, E. M. Rabin, and S. Oroszlan.** 1981. Structure of glycosylated and unglycosylated *gag* polyproteins of Rauscher murine leukemia virus: carbohydrate attachment sites. J. Virol. **38**:581–592.
16. **Schultz, A. M., E. H. Rabin, and S. Oroszlan.** 1979. Posttranslational modification of Rauscher leukemia virus precursor polyproteins encoded by the *gag* gene. J. Virol. **30**:255–266.
17. **Veronese, F. M., T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1988. Biochemical and immunological analysis of human immunodeficiency virus *gag* gene products p17 and p24. J. Virol. **62**:795–801.
18. **Walker, B. D., L. Chakrabarti, B. Moss, T. J. Paradis, T. Flynn, A. G. Durno, R. S. Blumberg, J. C. Kaplan, M. S. Hirsch, and R. T. Schooley.** 1987. HIV-specific cytotoxic T-lymphocytes in seropositive individuals. Nature (London) **328**:345–348.
19. **Weiss, R. A., N. Teich, H. Varmus, and J. Coffin.** 1984. RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.