Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5932–5936, June 1994
Medical Sciences

Combined intra- and extracellular immunization against human immunodeficiency virus type 1 infection with a human anti-gp120 antibody

(human immunodeficiency virus 1 envelope protein/intracellular antibody and immunization/AIDS/gene therapy/CD4+ T cells)

SI-YI CHEN*, YOUSEF KHOURI*, JESSAMYN BAGLEY*, AND WAYNE A. MARASCO†‡

Departments of †Medicine and *Pathology, Division of Human Retrovirology, Dana–Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

Communicated by Bernard Fields, January 24, 1994

ABSTRACT **In this study, a human CD4+ T lymphocyte line was transduced to secrete Fab fragments of a broadly neutralizing human monoclonal antibody F105 that reacts with the CD4-binding site of human immunodeficiency virus type 1 (HIV-1) envelope protein. In the transduced cells infected with HIV-1, the nascent Fab fragments bind intracellularly to the HIV-1 envelope protein and inhibit HIV-1 production. The secreted Fab fragments are able to neutralize cell-free HIV-1. In addition, the nascent Fab fragments can inhibit HIV-1 production by binding intracellularly to envelope mutants that escape neutralization by extracellular F105 antibody. The combined intra- and extracellular binding activities of the expressed Fab fragments result in the efficient blocking of cytopathic syncytium formation and infectious virus production. Thus, these antibody-producing T lymphocytes are not only resistant to HIV-1 infection but also can protect surrounding lymphocytes by secreting neutralizing antibodies. This novel strategy of combining intracellular and extracellular immunization may be useful for gene therapy of AIDS and other diseases.**

The human immunodeficiency virus type 1 (HIV-1) envelope protein has been implicated in both the viral cytopathic effects and the immune disregulation associated with HIV-1 infection (1). The HIV-1 envelope protein is synthesized as a glycoprotein precursor, gp160, which is cleaved into mature gp120/gp41 proteins in the Golgi complex (2). Following transport to the plasma membrane, gp120/gp41 glycoproteins are incorporated into virions that infect CD4+ cells (3–5). In addition, gp120/gp41 on the HIV-1-infected cell surface can interact with the CD4 molecules on uninfected cells to form syncytia (3–5). Therefore, blockage of HIV-1 envelope protein transport and surface expression may be an excellent therapeutic target.

In previous studies, we demonstrated that an anti-gp120 single-chain antibody that is retained in the endoplasmic reticulum (ER) can block transport of the envelope glycoprotein and production of HIV-1 virus (6, 7). In this study, an anti-HIV-1 approach that uses the combined intra- and extracellular binding activities of a neutralizing antibody was tested. A human CD4+ lymphocyte line was transduced to stably express the Fab fragments of the human monoclonal antibody F105, which reacts with the CD4-binding site on the HIV-1 gp120 and has broadly neutralizing activity (8). These Fab105-expressing lymphocytes are immunized against HIV-1 infection by the intracellular binding activity of the nascent Fab fragments and also protect surrounding HIV susceptible cells from virus infection.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

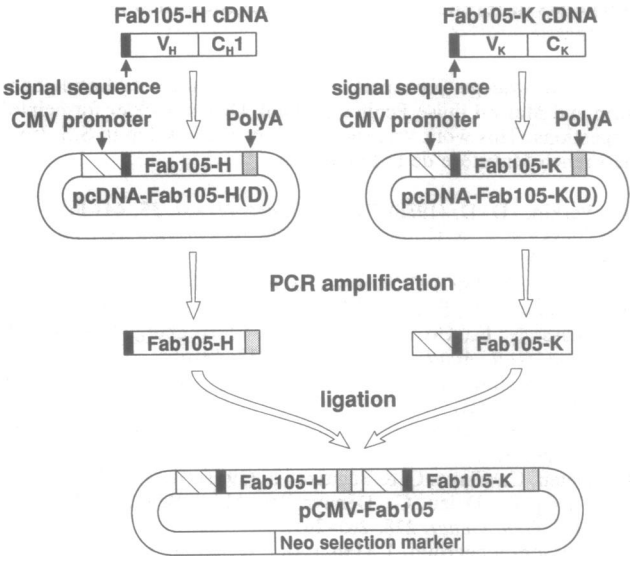

FIG. 1. Schematic representation of Fab105 expression vector.

MATERIALS AND METHODS

Construction of Expression Vectors. The Fd or κ chain cDNA fragments were amplified from the F105 hybridoma (8) by polymerase chain reaction (PCR) and were cloned into an expression vector pcDNA (pcDNA-Fab105H and pcDNA-Fab105K, respectively; Fig. 1). To construct a bicistronic expression vector, the Fd gene and polyadenylylation sequences from pcDNA-Fab105H(D) were amplified by PCR by using the primers 5′-TTTGAATTCAAGCTTACCATGGAACATCTGTGGTTC-3′ and 5′-CCGATATCTTAATTAAGGCGCGCCGCGTTGATACCATGGCGGG-3′ (9). To amplify the cytomegalovirus (CMV) promoter and κ chain gene sequences from pcDNA-Fab105K(D), the primers 5′-TTTGATATCTTAATTAAGGCGCGCCCTTCGCGATGTACGGGCCAG-3′ and 5′-TATTTTCTAGATTAACACTCTCCCCTGTTGAA-3′ were used. The PCR reactions were performed as described (10). The Fd–poly(A) DNA fragment and CMV promoter–κ chain DNA fragment were cloned into pRc/CMV vector (Invitrogen). The resulting construct (pCMV-Fab105) was confirmed by DNA sequencing.

Establishment of Continuous Cell Lines and Detection of Protein Expression. To establish continuous lymphocyte lines, 1×10^6 SupT-1 cells were transfected by using lipofectin (BRL) and were selected in RPMI 1640 medium

Abbreviations: HIV-1, human immunodeficiency virus type 1; ER, endoplasmic reticulum; CMV, cytomegalovirus; $TCID_{50}$, tissue culture 50% infective dose; CAT, chloramphenicol acetyltransferase.

‡To whom reprint requests should be addressed.

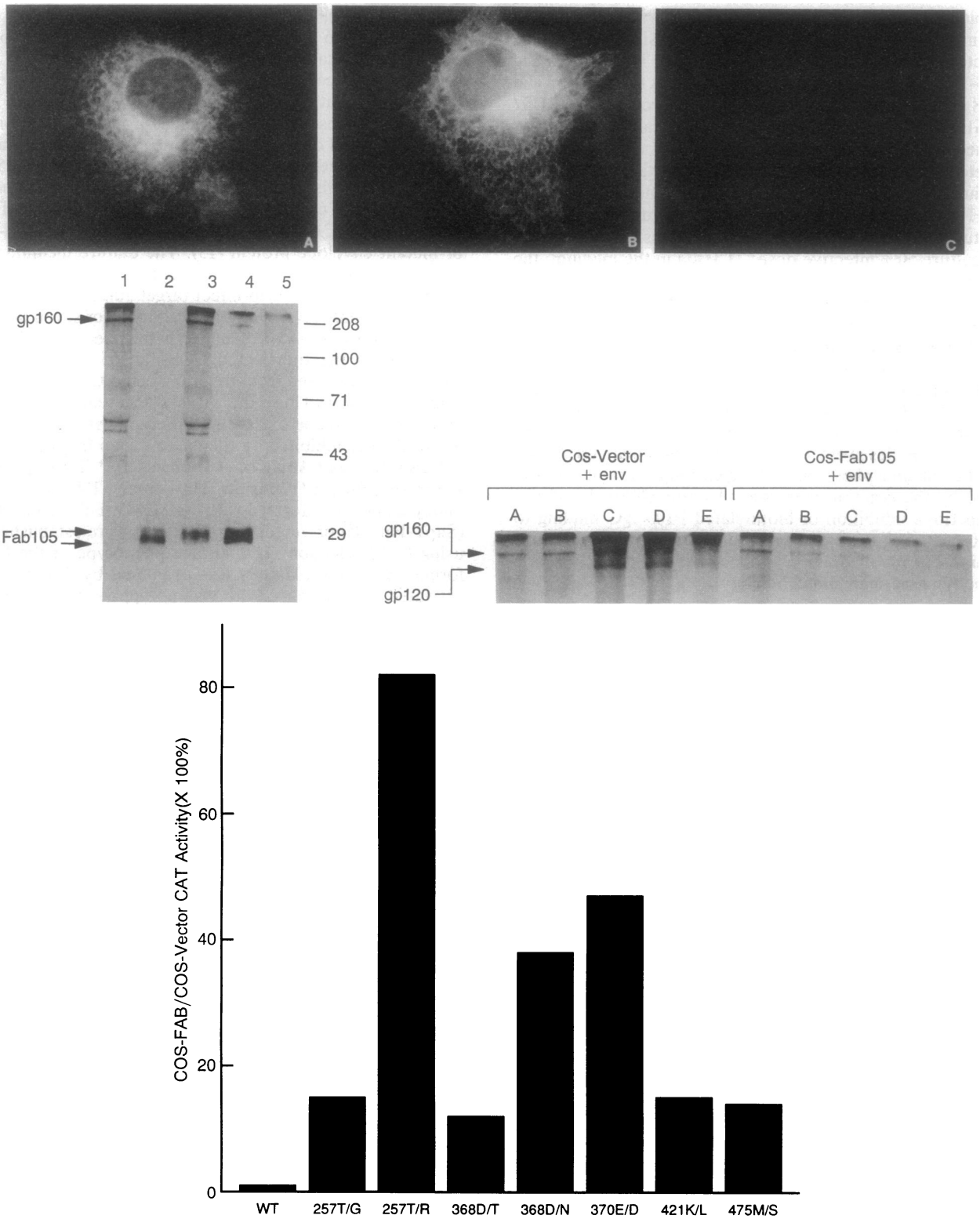

FIG. 2. (*Top*) Immunofluorescent staining of COS-Fab105 cells. COS-Fab105 or COS-vector cells grown on coverslips were fixed and stained with rabbit anti-human κ or heavy chain (a gift from G. Silverman, Univ. of California, San Diego) antibody followed by fluorescein isothiocyanate-labeled anti-rabbit IgG; COS-Fab105 stained with anti-κ (*A*) or anti-heavy (*B*) chain antibody and COS-vector stained with anti-κ chain (*C*). (×1800.) (*Middle Left*) Coprecipitation of the envelope protein with Fab105 fragments. COS-Fab105 and COS-vector cells were transfected with 5 μg of pSVIIIenv, respectively, and then radiolabeled 60 hr after transfection for 30 min. The cell lysates were precipitated with sheep anti-gp120 (from the AIDS Research and Reference Reagent Program) or anti-human κ chain, and the precipitates were analyzed by SDS/PAGE under reducing conditions. Lanes: 1, COS-vector transfected with pSVIIIenv and precipitated with anti-gp120; 2, COS-Fab105 mock-transfected and precipitated with anti-κ chain; 3 and 4, COS-Fab105 transfected with pSVIIIenv and precipitated with anti-gp120 (lane 3) or anti-κ chain (lane 4); 5, COS-vector mock-transfected and precipitated with anti-gp120 and κ chain. (*Middle Right*) Processing of the HIV-1 envelope proteins in COS-Fab105. COS-Fab105 and COS-vector cells were transfected with 5 μg of pSVIIIenv DNA and then pulse-labeled for 30 min and chased for the indicated times 60 hr after transfection. The cell lysates were then precipitated with sheep anti-gp120 and analyzed by SDS/PAGE under reducing conditions. Lanes: A, 0-min chase; B, 30-min chase; C, 2-hr chase; D, 4-hr chase; and E, 6-hr chase. (*Bottom*)

supplemented with 10% (vol/vol) fetal calf serum and G418 at 1 mg/ml on 24-well plates. The G418-resistant cells were selected and subcloned by limited dilutions. The transformed COS-1 cell lines were established after DNA transfection and G418 selection. Radiolabeling, immunoprecipitation, SDS/PAGE, and indirect immunofluorescent staining were performed as described (6).

Detection of Virus Infection. Viral particles in culture medium were measured by using a radioimmunoassay kit for the HIV-1 p24 Gag antigen (DuPont) according to the manufacturer's instructions. To determine virus infectivity (tissue culture 50% infective dose; $TCID_{50}$) in the medium, the supernatants were titrated on H9 cells as described by Johnson and Byington (11). A transcomplementation assay was performed as described by Helseth *et al.* (12).

Detection of Binding Activity of Expressed Fab105. Microplates (Dynatech, Chantilly, VA) were coated with recombinant gp120 of the HIV-1_{IIIB} strain (American Biotechnology, Cambridge, MA) at 100 ng per well. SupT-Fab105 and SupT-vector culture media were added to wells, and the bound antibodies were detected by anti-human IgG (Fab-specific) alkaline phosphatase-labeled conjugate (Sigma), followed by the reaction with the substrate pNPP (Bio-Rad). Competitive inhibition of biotinylated F105 IgG binding to gp120-coated plates was measured by adding serial dilutions of SupT-Fab105 medium or monoclonal anti-gp120 V3 loop antibody (American Biotechnology).

RESULTS

Construction and Expression of Fab Fragments of Antibody F105. A bicistronic F105 expression vector (pCMV-Fab105), which contains both the F105 Fd and κ chain genes under the control of independent CMV promoters was made (Fig. 1). Subsequently, transformed COS-1 cells (COS-Fab105) were generated with G418 selection. Two protein bands of approximately 28 (κ chain) and 30 (Fd) kDa were detected in the lysates and culture medium of COS-Fab105, while no corresponding bands were detected in COS-vector control cells after radiolabeling and immunoprecipitation against human IgG. Immunofluorescent staining with either anti-heavy or anti-κ chain antibody was observed predominantly in the cytoplasmic vesicles of the ER and Golgi complex, a pattern typical of secretory proteins (Fig. 2 *Top*).

Intracellular and Extracellular Binding Activity of Expressed Fab105 Fragments. To test whether the expressed Fab105 fragments are able to bind gp160 within cells, COS-Fab105 and COS-vector cells were transfected with the plasmid pSVIIIenv DNA, which expresses the envelope protein of HIV-1_{IIIB} (12). The transfected cells were radiolabeled and immunoprecipitated with either anti-gp120 or anti-κ chain antibody. Both the Fab105 and gp160 were detected on the gel when the cell lysate was precipitated with anti-gp120 or anti-κ chain antibody (Fig. 2 *Middle Left*). To examine the maturation of gp120 in COS-Fab105 cells, COS-Fab105 and COS-vector cells were transfected with pSVIIIenv, pulse-labeled, and chased for various times. In the COS-vector cells, the envelope protein was gradually processed from gp160 to gp120 during a 6-hr chase, while in the COS-Fab105 cells, an almost undetectable amount of gp120 was observed on the gel during the same chase period (Fig. 2 *Middle Right*). Thus, the nascent Fab105 fragments bind intracellularly to gp160 and interfere with its maturation.

We further examined whether the expressed Fab105 fragments can intracellularly interfere with the function of envelope mutants that escape neutralization by extracellular F105 antibody. A transcomplementation assay (13) was performed by using a panel of envelope mutants that escape neutralization from extracellular F105 antibody. The COS-Fab105 and COS-vector cells were cotransfected with *env*-defective HIV-1 provirus carrying the bacterial chloramphenicol acetyltransferase (CAT) gene and DNA encoding the wild-type or mutant envelope protein (13). The culture medium containing released viruses with the capacity for a single round of infectivity was used to infect target Jurkat lymphocytes. All of the mutants were inhibited, and four of seven mutants examined showed >85% reduction in the release of infectious viruses from the transfected COS-Fab105 cells compared with viruses released from the transfected COS-vector cells (Fig. 2 *Bottom*). Thus, the viruses that contain envelope mutants that escape extracellular neutralization can still be intracellularly inhibited by the nascent Fab fragments.

Generation and Analyses of Human $CD4^+$ T Lymphocytes Expressing Fab105 Fragments. The human $CD4^+$-SupT1 lymphocyte lines transformed with the pCMV-Fab105 construct (SupT-Fab105) or vector (SupT-vector) were established by using G418 selection. The surface phenotype of the transformed SupT-Fab105 cells was examined by fluorescence-activated cell sorting. There was no apparent difference in fluorescence intensity between SupT-vector and SupT-Fab105 cells for the surface markers of CD3, CD4, CD5, CD7, CD8, and β_2-microglobulin. Microscopic examination revealed no morphologic difference between the SupT-vector and SupT-Fab105 cells. In both of the cell lines, there was >10-fold increase in [^{3}H]thymidine incorporation after incubation with phytohemagglutinin (0.8 μg/ml) and [^{3}H]thymidine for 6 hr.

A high level of expression of both the Fd and κ chains was detected from the lysates and medium of SupT-Fab105 cells (Fig. 3 *Top*). Either the anti-κ or anti-heavy chain (data not shown) antibody coprecipitated both the Fd and κ chains. When the samples were analyzed under nonreducing conditions, a predominantly higher molecular weight band appeared on the gel (Fig. 3 *Top*), which corresponds to a disulfide-linked Fab fragment.

Binding and Neutralization Activity of Expressed Fab105 from SupT-Fab105 Cells. The binding activity of secreted Fab105 fragments from SupT-Fab105 cells corresponding to approximately 0.5 μg per 24 hr per 10^6 cells was detected, while no binding activity was detected in the medium of SupT-vector cells by ELISA. The binding specificity was further confirmed by a competitive inhibition ELISA in which a dose-dependent inhibition of biotinylated-F105 IgG binding was observed in the wells incubated with SupT-Fab105 medium (Fig. 3 *Middle*). To examine the neutralizing activity of the expressed Fab105, the medium from SupT-Fab105 or SupT-vector cells (72 hr incubation at about 1 × 10^6 cells per ml) was incubated with a HIV-1_{IIIB} virus, and the virus infectivity in the mixtures was determined. After incubation with the SupT-Fab105 medium (1:1 ratio), virus infectivity was decreased 87%, while no inhibition was observed after incubation with the SupT-vector medium (Fig. 3 *Bottom*).

Inhibitory effects of expressed Fab105 fragments on escaping gp120 mutants. Recombinant *env*-defective viruses containing the wild-type or mutant envelope proteins and carrying the bacterial CAT gene were produced in COS-Fab105 or COS-vector cells (13). The virions in the culture media were incubated with Jurkat lymphocytes. The percentage of the CAT activity observed in the Jurkat cells for each mutant from COS-Fab105 cells relative to the CAT activity observed from COS-vector cells is shown. These experiments were performed in duplicate. The mutant envelope proteins used are listed as follows with amino acids in single-letter code: 257T/G, 257T/R, 368D/T, 368D/N, 370E/D, 421K/L, 475M/S, and wild-type (WT) envelope protein control (13).

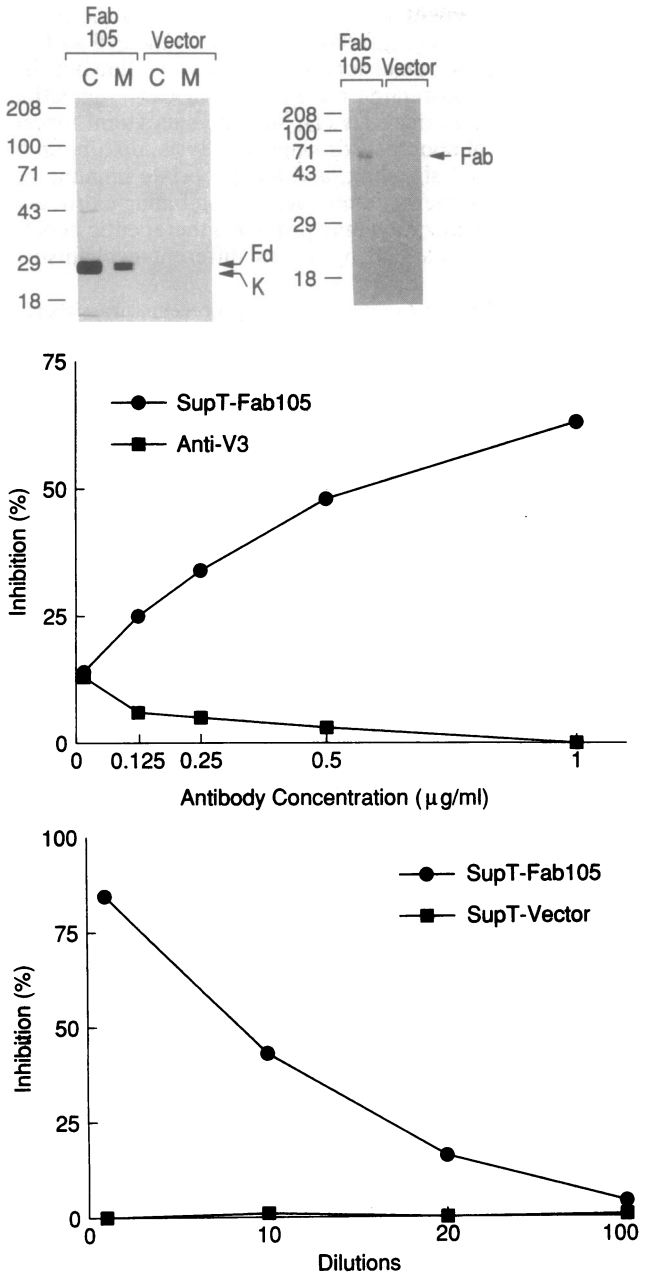

FIG. 3. (*Top*) Fab105 expression in the transduced SupT-Fab105 lymphocytes. SupT-Fab105 cells were pulse-labeled with [^{35}S]cysteine for 30 min and chased for 4 hr. The cell lysates (C) and media (M) were immunoprecipitated with anti-human κ chain antibody and analyzed by SDS/PAGE under reducing conditions (*Top Left*). Media were also analyzed under nonreducing conditions (*Top Right*). Sizes are shown in kDa. (*Middle*) Competitive inhibition of expressed Fab105 with parental F105 antibody. Microplates coated with gp120 were incubated with biotinylated F105 IgG (100 ng/ml) containing serial dilutions of either anti-V3 loop antibodies in the RPMI 1640 medium or culture medium from SupT-Fab105 cells overnight at 4°C. Bound antibodies were detected by the addition of alkaline phosphatase-labeled avidin (Vector Laboratories) and reaction with substrate pNPP (Bio-Rad). Data represent mean of duplicate determinations. (*Bottom*) Neutralizing activity of Fab105. Serial dilutions of the culture medium from the SupT-Fab105 or SupT-vector cells (72 hr incubation of about 1×10^6 cells per ml) were incubated with HIV-1_{IIIB} virus (10,000 cpm reverse transcriptase activity) for 1 hr at 37°C, and the mixtures were subject to virus titration on H9 cells. Percentages of reduction of virus infectivity from duplicate determinations are shown.

Marked Reduction of Virus Production from SupT-Fab105 Cells. To examine the infectability of SupT-Fab105 cells, the transcomplementation assay was used (12). Comparable amounts of CAT activity were observed from the SupT-Fab105 and SupT-vector cells infected with the supernatants containing single-round infectivity viruses, indicating that the SupT-Fab105 cells are susceptible to HIV-1 infection (Fig. 4 *Upper Left*).

Next, the cells were washed with fresh medium to remove the secreted Fab105 and infected with the HIV-1_{IIIB} virus. After 4 days of incubation, the infectious HIV-1 in the culture medium was titrated. As shown in Fig. 4 *Upper Right*, 4.6×10^3 $TCID_{50}$ infectious viruses were detected in the medium of the infected SupT-vector cells, while a significantly lower level of infectious viruses (7 $TCID_{50}$) was detected in the medium of the infected SupT-Fab105 cells, indicating a significant reduction (>99.9%) of infectious virus production from SupT-Fab105 cells.

Since the neutralizing activity of the secreted Fab105 fragments could have contributed to the reduction of infectious viruses, the 4-day culture medium from uninfected SupT-Fab105 cells was mixed 1:1 (vol/vol) with the medium from the HIV-1-infected SupT-vector cells and was titrated after 2 hr. A virus titer of 1×10^3 $TCID_{50}$ (Fig. 4 *Upper Right*, bar C) was observed, which represents only a 78% reduction of virus infectivity. Since virus production from SupT-Fab105 cells showed >99.9% of reduction compared with that from SupT-vector cells, it is evident that the reduction of infectious HIV-1 viruses in the medium of SupT-Fab105 cells is largely due to the release of less infectious virus from the infected SupT-Fab105 cells.

Block of HIV-1_{IIIB} Virus Infection in SupT-Fab105 Cell Cultures. To examine HIV-1 infection in SupT-Fab105 cultures, SupT-Fab105 and SupT-vector cells in parallel were washed with fresh culture medium and then infected with the HIV-1_{IIIB} virus. Large numbers of syncytia were observed in the SupT-vector cells, reaching a peak at days 9–12, then gradually decreasing, and disappearing after day 18 (Fig. 4 *Lower*). Correspondingly, a significant increase of p24 levels was observed in infected SupT-vector cell cultures and were maintained at a significant level throughout the 43-day period (Fig. 4 *Lower*). In contrast, only small numbers of syncytia were observed in the SupT-Fab105 culture 12–15 days after infection. Furthermore, only a transient, low level of p24 was observed in the medium of infected SupT-Fab105. After 27 days of infection, p24 levels in the medium returned to a background level (Fig. 4 *Lower*). Thus, the spread of HIV-1_{IIIB} virus infection was blocked in the SupT-Fab105 cell culture.

DISCUSSION

Several approaches using intracellular immunization (14) to inhibit HIV-1 infection have been described (15–20). However, the strategy proposed here has the unique advantage of combined intra- and extracellular immunization. The intracellular Fab105 fragments can bind to gp160 or gp120 throughout the secretory pathway rather than only in the ER as described (6, 7). This may prolong the interaction between antibodies and envelope proteins, thus allowing antibody binding to any gp120 that has been transported beyond the ER. The Fab105 fragments were shown to maintain intracellularly inhibitory activity to the mutants that escape from the neutralization of extracellular F105, probably because of the high concentration of antibodies in the secretary vesicles (21).

Recent studies demonstrate that human lymphoid organs are the major sites of HIV-1 infection in HIV-1-infected patients (20, 22). In a gene therapy application, the transduced Fab-producing T lymphocytes may repopulate *in vivo* the T-cell dependent areas in lymphoid tissues, and, subsequently, the local concentration of secreted antibodies may reach a high enough level to block virus infectivity and cytopathic effects. The Fab fragments may also have an advantage over the whole antibodies by excluding the Fc-

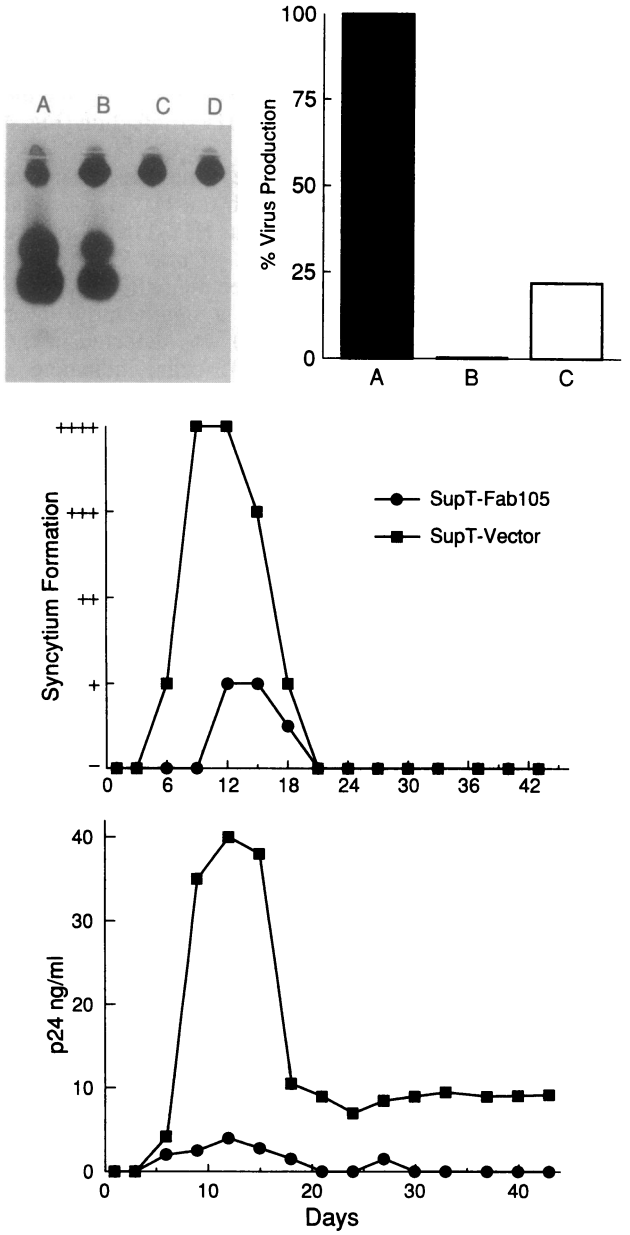

FIG. 4. (*Upper Left*) Transcomplementation assay of SupT-Fab105 and SupT-vector cells. Lanes: A and B, SupT-vector (lane A) or SupT-Fab105 (lane B) cells infected with the culture medium from the cells cotransfected with 10 μg of pHXBΔenvCAT and 10 μg of pSVIIIenv plasmid; C and D, SupT-vector (lane C) or SupT-Fab105 (lane D) cells infected with the medium from the cells only transfected with 10 μg of pHXBΔenvCAT plasmid. (*Upper Right*) Reduction of virus production from SupT-Fab105 cells. SupT-Fab105 or SupT-vector (1×10^6 cells) were infected with HIV-1_{IIIB} virus (10,000 cpm reverse transcriptase activity), and the media from the virus-infected cell culture 4 days after infection were harvested and then titrated on H9 cells. The virus titer from the mock-infected SupT-vector cells was undetectable. Bars: A, SupT-vector cells infected with HIV-1_{IIIB} virus; B, SupT-Fab105 cells infected with HIV-1_{IIIB} virus; C, medium from the uninfected SupT-Fab105 culture incubated with the medium from the infected SupT-vector [1:1 (vol/vol) ratio] for 2 hr. Viruses in the mixture were then titrated on H9 cells. (*Lower*) Virus production and syncytium formation in SupT-Fab105 cell culture. SupT-Fab105 or SupT-vector cells (1×10^6) were washed with RPMI 1640 medium, infected with HIV-1_{IIIB} virus (10,000 cpm reverse transcriptase activity) overnight, and then replaced with RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. The syncytium formation in the cell culture was scored (6). Half of the cell culture was harvested to examine p24 level in the medium with radioimmunoassay kits from DuPont, and fresh culture medium was added to the other half of culture every 3 or 4 days.

mediated enhancement of HIV-1 infection (23). Furthermore, since the interaction of gp120-CD4 either on the cell surface or intracellularly is crucial not only for virus infection but also for many of the postulated mechanisms of $CD4^+$-depletion and functional abnormalities (1), such as syncytium formation, induction of apoptosis or single-cell lysis, disruption of CD4-mediated cell signaling, and inappropriate immune reactions, this gene therapy approach of combining extra- and intracellular immunization may provide a therapeutic benefit for HIV-1-infected patients by several different mechanisms.

We thank Ms. Christy Zani and Edna Teng for technical assistance. We thank Drs. Marshall Posner and Lisa Cavacini for providing the biotinylated F105 antibody. We thank Drs. Joseph Sodroski and Markus Thali for providing the envelope mutants. This work was supported by National Institutes of Health Grants P30AI28691 (Center for AIDS Research), P30CA06516 (Cancer Center Grant), AI28785, AI33802, and AI31783; Claudia Adams Barr Innovative Basic Science Program; Virus Research Institute, Inc.; and a gift from the G. Harold and Leila Y. Mathers Charitable Foundation.

1. Pantaleo, G., Graziosi, C. & Fauci, A. (1993) *N. Engl. J. Med.* **328**, 327–335.
2. Earl, P. L., Doms, R. W. & Moss, B. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 648–652.
3. Maddon, P. J., Dalgleish, A. G., McDougal, J. S., Clapham, P. R., Weiss, R. A. & Axel, R. (1986) *Cell* **47**, 333–348.
4. Sodroski, J., Goh, W. C., Rosen, C., Campbell, K. & Haseltine, W. A. (1986) *Nature (London)* **322**, 470–474.
5. Lifson, J. D., Feinberg, M. B., Reyes, G. R., Rabin, L., Banapour, B., Chakrabarti, S., Moss, B., Wong-Staal, F., Steimer, K. S. & Engleman, E. G. (1986) *Nature (London)* **323**, 725–728.
6. Marasco, W. A., Haseltine, W. A. & Chen, S.-Y. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7889–7893.
7. Chen, S.-Y., Bagley, J. & Marasco, W. A. (1994) *Hum. Gene Ther.* **5**, 595–601.
8. Posner, M. R., Hideshima, T., Cannon, T., Mukherjee, M., Mayer, K. H. & Byrn, R. A. (1991) *J. Immunol.* **146**, 4325–4332.
9. Kabat, E. A., Wu, T. T., Reid-Miller, M., Perry, H. M. & Gottseman, K. S. (1987) *Sequences of Proteins of Immunological Interest* (U.S. Dept. of Health and Human Services, Government Printing Office, Washington, DC).
10. Marasco, W. A., Bagley, J., Zani, C., Posner, M., Cavacini, L., Haseltine, W. A. & Sodroski, J. (1992) *J. Clin. Invest.* **90**, 1467–1478.
11. Johnson, V. A. & Byington, R. E. (1990) in *Techniques in HIV Research*, eds. Aldovini, A. & Walker, B. D. (Stockton, New York), pp. 71–76.
12. Helseth, E. M., Kowalski, M., Gabuzda, D., Olshevsky, U., Haseltine, W. A. & Sodroski, J. (1990) *J. Virol.* **64**, 2416–2420.
13. Thali, M., Furman, C., Ho, D. D., Robinson, J., Tilley, S., Pinter, A. & Sodroski, J. (1992) *J. Virol.* **66**, 5635–5641.
14. Baltimore, D. (1988) *Nature (London)* **335**, 395–396.
15. Malim, M. H., Bohnlein, S., Hauber, J. & Cullen, B. R. (1989) *Cell* **58**, 205–214.
16. Trono, D., Feinberg, M. B. & Baltimore, D. (1989) *Cell* **59**, 113–120.
17. Sullenger, B., Gallardo, H. F., Ungers, G. E. & Giboa, E. (1990) *Cell* **63**, 601–608.
18. Green, G., Ishino, M. & Loewenstein, P. M. (1989) *Cell* **58**, 215–223.
19. Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, P. A., Stephens, D. A. & Rossi, J. J. (1990) *Science* **247**, 1222–1225.
20. Pantaleo, G., Graziosi, C., Demarest, J. F., Butini, L., Montroni, M., Fox, C. H., Orenstein, J. M., Kotler, D. P. & Fauci, A. S. (1993) *Nature (London)* **362**, 355–358.
21. Buonocore, L. & Rose, J. K. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2695–2699.
22. Embrelson, J., Zupanic, M., Ribas, J. L., Burke, A., Racz, P., Tenner-Racz, K. & Haase, A. T. (1993) *Nature (London)* **362**, 359–362.
23. Takeda, A., Tuazon, C. U. & Ennis, F. A. (1988) *Science* **242**, 580–583.