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# Chimeric Influenza Virus Induces Neutralizing Antibodies and Cytotoxic T Cells against Human Immunodeficiency Virus Type 1

SHENGQIANG LI,[1] VICTORIA POLONIS,[2] HIDECKI ISOBE,[1] HABIB ZAGHOUANI,[1] ROSARIO GUINEA,[1] THOMAS MORAN,[1] CONSTANTIN BONA,[1] AND PETER PALESE[1]*

*Department of Microbiology, Mount Sinai School of Medicine, New York, New York 10029,[1] and Department of Retroviral Research, Walter Reed Army Institute of Research, Washington, D.C. 20307-5010[2]*

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**Expression vectors based on DNA or plus-stranded RNA viruses are being developed as vaccine carriers directed against various pathogens. Less is known about the use of negative-stranded RNA viruses, whose genomes have been refractory to direct genetic manipulation. Using a recently described reverse genetics method, we investigated whether influenza virus is able to present antigenic structures from other infectious agents. We engineered a chimeric influenza virus which expresses a 12-amino-acid peptide derived from the V3 loop of gp120 of human immunodeficiency virus type 1 (HIV-1) MN. This peptide was inserted into the loop of antigenic site B of the influenza A/WSN/33 virus hemagglutinin (HA). The resulting chimeric virus was recognized by specific anti-V3 peptide antibodies and a human anti-gp120 monoclonal antibody in both hemagglutination inhibition and neutralization assays. Mice immunized with the chimeric influenza virus produced anti-HIV antibodies which were able to bind to synthetic V3 peptide, to precipitate gp120, and to neutralize MN virus in human T-cell culture system. In addition, the chimeric virus was also capable of inducing cytotoxic T cells which specifically recognize the HIV sequence. These results suggest that influenza virus can be used as an expression vector for inducing both B- and T-cell-mediated immunity against other infectious agents.**

Chimeric viruses expressing foreign antigens or antigenic structures have been described for several DNA and plus-stranded RNA viruses (1, 4, 27). In contrast, negative-stranded RNA viruses such as influenza viruses were resistant to genetic manipulation and therefore could not be used as vectors for the expression of foreign epitopes. Recently, a reverse genetics approach which allows the rescue of RNA transcribed from plasmid DNA by infectious influenza virus particles has been undertaken (9, 23). This method has been successfully used to study the *cis* elements required for transcription and replication of the influenza virus genome (20, 22), and it has also been used to generate attenuated influenza viruses (28). In a previous study, we engineered an influenza virus transfectant which expresses on its surface B-cell epitopes from both H1 and H3 subtype hemagglutinins (HA) (19). This influenza virus transfectant was capable of inducing neutralizing immune responses against both H1 and H3 subtype influenza viruses. These results led us to investigate whether antigenic structures from other infectious agents can be expressed on the HA, a surface glycoprotein known to be able to induce neutralizing antibodies and cytotoxic T lymphocytes (CTLs) against influenza virus (21, 26).

The envelope glycoprotein gp120 and its precursor gp160 of human immunodeficiency virus (HIV) have been targets for the development of subunit vaccines against HIV (3, 33). The third variable region of gp120 forms a loop (V3 loop) with a disulfide bridge between two cysteine residues at positions 303 and 338. Studies in humans and experimental animals suggest that the V3 loop induces the major neutralizing antibodies against the virus (12, 25, 31, 34). In addition, the V3 loop has been shown to stimulate both $CD4^+$ and $CD8^+$ T-cell responses in mice as well as in humans (6, 7, 36–38).

Thus, we inserted a 12-amino-acid peptide which was derived from the V3 loop of gp120 of HIV-1/MN into the loop of antigenic site B of the HA of influenza A/WSN/33 virus. In this paper, we report the antigenicity and immunogenicity of this peptide in the chimeric influenza virus. We show for the first time that a chimeric influenza virus is able to elicit both a humoral and a cell-mediated immune response against HIV-1.

## MATERIALS AND METHODS

**Viruses and cells.** Influenza A/WSN/33 (H1N1) and HK-WSN (H3N1) viruses were grown in Madin-Darby bovine kidney (MDBK) cells. HIV-1/MN was grown in H9 cells. HK-WSN is a reassortant virus which derives its HA gene from influenza A/Hong Kong/68 virus and its remaining genes from WSN virus (10). MDBK cells were used for the generation and characterization of the chimeric influenza virus. CEMss and H9 cells were used for the in vitro neutralization assay of HIV-1/MN. P815 cells were used for the cytotoxic assay.

**Construction of plasmid.** By introducing silent mutations, a unique *Pst*I restriction enzyme site was generated in the previously described plasmid pT3/WSN-HAm (19), at nucleotide position 489 of the WSN HA (15). The resulting plasmid was designated pT3/WSN-HAm1. Plasmid pWSN-MN was constructed by replacing the *Pst*I-*Hin*dIII fragment of pT3/WSN-HAm1 with a polymerase chain reaction product which was obtained by using pT3/WSN-HAm1 as the template and the primer pair 5′-GCGCCTGCAGCCATAGGGGA-3′ and 5′-GCGCAAGCTTTGGGTATGAATCTGTTGTATAGAA TGCCCTTCCTGGTCCTATATGTATCCCCTTCTTCGTC AGC-3′. The latter primer contains nucleotides complemen-

* Corresponding author.

tary to the cRNA of the WSN HA from positions 563 to 546 and 545 to 530 and a 36-nucleotide insertion between positions 545 and 546. The complementary sequence of those 36 nucleotides encodes a 12-amino-acid peptide, IHIGPGRAFYTT, corresponding to the V3 loop of gp120 of HIV-1/MN at positions 312 to 323. The numbering for gp120 of the MN virus is according to reference 29.

**Generation of infectious chimeric virus.** Transfection of RNA derived from plasmid pWSN-MN into MDBK cells and selection of infectious chimeric virus were done as previously described (19). Briefly, 0.5 μg of *Ksp*632I-digested plasmid DNA, 10 μl of purified influenza virus nucleoprotein (NP) and the three polymerase proteins (1 μg of total protein), and 2 μl of T3 RNA polymerase (50 U/μl; Stratagene) were incubated at 37°C for 20 min in the presence of 0.5 mM each of the nucleoside triphosphates. The resulting ribonucleoprotein complex was transfected into HK-WSN virus-infected MDBK cells by using the DEAE-dextran transfection protocol (10). At 18 h posttransfection, the supernatant was collected and used at different dilutions to infect fresh MDBK cells in the presence of 0.05% anti-HK HA antiserum; 48 h later, the supernatant was used for plaque assays in the presence of the same antiserum at a final concentration of 0.01%. The rescued virus was plaque purified three times before being characterized.

**Peptides.** Peptide C11-29 (RKSIHIGPGRAFYTTGEII) and peptide W11-28 (RRSLSIGPGRAFRTREII), corresponding to the consensus V3 loop or the V3 loop of the WMJ2 isolate (18), were obtained from IAF Biochem (Quebec, Quebec, Canada). Peptide MN314-323 (IGPGRAFYTT), corresponding to the V3 loop of HIV-1/MN, and peptide NP147-161 (TYQRTRALVRTGMDP), corresponding to the influenza A/WSN/33 virus NP, were synthesized in the Department of Pharmacology, Mount Sinai Medical Center (New York, N.Y.). The purity of the peptides was tested by high-pressure liquid chromatography.

**Antipeptide antibodies.** Antibodies specific for peptides C11-29 and W11-28 were generated by immunizing rabbits with 500 μg of peptide coupled with keyhole limpet hemocyanin in Freund's complete adjuvant and boosting 2 weeks later with the same dose of peptide. The rabbits were bled weekly beginning 11 days after the boosting. The antipeptide antibodies were purified over a peptide-bovine serum albumin (BSA)-Sepharose 4B column as previously described (42).

**HI and virus neutralization assays.** Hemagglutination inhibition (HI) assays were done as previously described (32). Anti-WSN HA monoclonal antibodies 2G9, 4B2, 2F10, and 25-5 were prepared by standard procedures. A human anti-gp120 monoclonal antibody, MedI 11, was kindly provided by J. Young and S. Koenig, Medimmune Inc., Gaithersburg, Md. Ascites fluid containing anti-WSN HA monoclonal antibodies was treated with receptor-destroying enzyme as previously described (32).

For virus neutralization assays, MDBK cells in 30-mm-diameter dishes were infected with approximately 100 PFU of virus. After a 1-h adsorption, agar overlay containing antibody at different dilutions was added. The cell monolayer was stained with 0.1% crystal violet at 72 h postinfection.

**Immunization.** Six-week-old BALB/c mice were either infected via the aerosol route with 500 PFU of virus or immunized intraperitoneally (i.p.) with 10 μg of purified virus. For all booster immunizations, 10 μg of purified virus was administered i.p. Sera were collected 7 days after each immunization.

**Radioimmunoassay.** The radioimmunoassay was performed as previously described (42). Briefly, microtiter plates were coated with 5 μg of peptide-BSA conjugate per ml, saturated with 2% BSA in phosphate-buffered saline (PBS), and incubated with various dilutions of serum. Bound antibodies were revealed by using a $^{125}$I-labeled anti-mouse kappa monoclonal antibody.

**Radioimmunoprecipitation.** The human T-cell line H9 was acutely infected with HIV-1/MN. Four days postinfection, $5 \times 10^7$ infected cells were labeled with [$^{35}$S]cysteine, [$^{35}$S]methionine, and [$^3$H]isoleucine at $2 \times 10^6$/ml in medium containing 100 μCi of each isotope per ml. After 20 h of metabolic labeling, the radioactive virions were pelleted by centrifugation for 1 h at 45,000 rpm. The pellet was resuspended in 1.0 ml of lysis buffer containing 1% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride. Twenty microliters of serum or 0.5 μg of monoclonal antibody (in 20 μl of PBS) and 175 μl of virion lysate were incubated overnight at 4°C in 0.5 ml of immunoprecipitation buffer containing 0.5% sodium dodecyl sulfate, (SDS), 1 mg of BSA per ml, 2% Triton X-100, and 50 mM sodium phosphate (pH 7.4). The antigen-antibody complexes were bound to protein A-Sepharose beads and analyzed by electrophoresis on an SDS–10% polyacrylamide gel.

**HIV-1 neutralization assays.** The in vitro neutralization assay was done as described previously (30). Briefly, serial twofold dilutions of heat-inactivated serum were incubated for 1 h at room temperature with 150 to 200 syncytium-forming units of HIV-1/MN produced in H9 cells. The virus-serum mixture was incubated for 1 h at 37°C with 50,000 DEAE-dextran-treated CEMss cells (adhered to microplate dishes by using poly-L-lysine) or 50,000 H9 suspension cells. After virus adsorption, the unbound virus was removed and 200 μl of medium was added to each well. Four days postinfection, 50 μl of supernatant medium was removed for viral $p24^{gag}$ protein quantitation (Coulter Source, Inc.). The total number of syncytia in CEMss cells was counted 5 days postinfection. The neutralization titers were calculated by comparison with control wells of virus only and are expressed as the reciprocal of the highest serum dilution that reduced the number of syncytia by more than 50% or inhibited p24 synthesis by more than 50%.

**Induction of CTL response.** BALB/c mice were immunized with 0.2 ml of viral suspension containing $10^7$ PFU of WSN or chimeric WSN-MN virus. Seven days later, spleen cells were obtained and restimulated in vitro for 5 days with irradiated spleen cells, alone or coated with peptide MN314-323 or NP147-161, in the presence of 10% concanavalin A in the supernatant as previously described (43).

**Cytolysis assay.** The P815 target cells coated with peptide MN314-323 or NP147-161 were labeled with $Na^{51}CrO_4$ (100 μCi/$10^6$ cells) for 1 h at 37°C. After being washed twice, the cells were transferred to V-bottom 96-well plates, the effector cells were added, and the mixture was incubated at 37°C in 7% $CO_2$. Four hours later, the supernatant was harvested and counted. The maximum chromium release was determined by incubating the cells with 1% Nonidet P-40. The percentage of specific lysis is calculated according to the following formula: [(cpm samples − cpm spontaneous release)/cpm maximum release − cpm spontaneous release)] × 100.

## RESULTS

**Generation of a chimeric influenza virus expressing a V3 loop peptide of HIV.** The antigenic site B of the influenza virus HA is located at the top of the molecule (5, 40). We have demonstrated previously that the loop of antigenic site B of the WSN virus HA (H1 subtype) can be replaced with the corresponding sequences of the A/Japan/57 virus HA (H2 subtype) or the A/Hong Kong/68 virus HA (H3 subtype) (19). However,

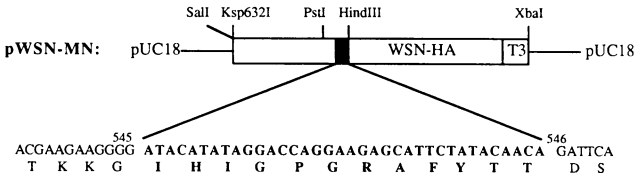


FIG. 1. Plasmid pWSN-MN, expressing the V3 peptide. pUC18-derived plasmid pWSN-MN contains the complete nucleotide sequence of the HA gene of influenza A/WSN/33 virus, the truncated bacteriophage T3 RNA polymerase promoter, and a *Ksp*632I restriction enzyme site. In addition, it contains a 36-nucleotide insertion in the HA gene between positions 545 and 546, as indicated by the black box (not to scale). The nucleotide sequence and encoded amino acids forming the loop of site B of the mutated HA gene are shown. The inserted nucleotides and encoded amino acids are indicated in boldface letters. The 12-amino-acid peptide is derived from the V3 loop of HIV-1/MN gp120, at positions 312 to 323.

TABLE 1. Analysis of the antigenicity of the chimeric WSN-MN virus by using anti-WSN and anti-HIV antibodies

| Antibody | Titer of antibody to virus | | | |
|---|---|---|---|---|
| | WSN | | WSN-MN | |
| | HI[a] | NT[b] | HI | NT |
| Anti-WSN | | | | |
| 2G9 | 1,280 | ND[c] | 1,280 | ND |
| 4B2 | 5,120 | ND | 5,120 | ND |
| 2F10 | 2,560 | ND | <40 | ND |
| 25-5 | 5,120 | ND | <40 | ND |
| Anti-HIV | | | | |
| Anti-C11-29 | <4 | <100 | 1,024 | 3,200 |
| Anti-W11-28 | <4 | <100 | 256 | 400 |
| MedI 11 | <4 | <100 | 65,536 | 120,000 |

[a] Expressed as the reciprocal of the highest antibody dilution which inhibited hemagglutination.
[b] Neutralization titer (NT) is expressed as the reciprocal of the highest antibody dilution which reduced the plaque number in MDBK cells by 50%.
[c] ND, not determined.

replacing the loop with unrelated (nonhomologous) sequences abolished the viability of the chimeric viruses (18a). We then developed a different mutational strategy, in which the foreign sequence was inserted into the loop between the glycine and aspartic acid residues. This approach allowed for the generation of viable chimeric viruses carrying foreign sequences.

The 12-amino-acid peptide IHIGPGRAFYTT, derived from the V3 loop of HIV-1/MN, was selected for expression in site B of the WSN virus HA. Previously described results suggested that this peptide may contain overlapping B-cell and $CD8^+$ T-cell epitopes (16, 38). We initially constructed plasmid pWSN-MN, in which 36 nucleotides encoding the V3 peptide were inserted into the HA gene between positions 545 and 546 (Fig. 1). RNA derived from plasmid pWSN-MN was transfected into HK-WSN virus-infected MDBK cells. When supernatant from the transfected cells was used to infect fresh MDBK cells in the presence of anti-HK HA antiserum, a cytopathic effect was detected. The rescued virus, designated WSN-MN virus, was plaque purified three times. Sequencing of the RNA derived from purified WSN-MN virus confirmed the insertion in frame of 36 nucleotides encoding the V3 peptide (data not shown). The chimeric WSN-MN virus replicated in tissue culture to a level similar to that of wild-type (wt) WSN virus (data not shown). This finding suggests that the insertion of 12 amino acids affected neither the function of the HA nor the ability of the chimeric virus to replicate in mammalian cells.

**Antigenicity of the chimeric WSN-MN virus.** The antigenic properties of the chimeric WSN-MN virus were investigated in HI and neutralization assays using anti-WSN and anti-HIV antibodies. As shown in Table 1, the anti-WSN HA monoclonal antibodies 2G9 and 4B2 reacted with the chimeric WSN-MN virus at HI titers identical to those of wt WSN virus, but monoclonal antibody 2F10, specific for the loop of site B of WSN virus HA, had no reactivity to WSN-MN virus. Interestingly, monoclonal antibody 25-5, specific for an epitope located outside the loop of site B, as shown by HI analysis using the chimeric W(H1)-H3 virus (19), also lost reactivity to WSN-MN virus. These data suggest that although the insertion of the 12-amino-acid peptide did not affect the function of the HA, it may have caused a conformational change near the loop which resulted in altered antigenic properties.

The antigenicity of the inserted V3 peptide was analyzed in HI and neutralization assays by using two rabbit antipeptide antibodies and a human anti-gp120 monoclonal antibody. Peptide C11-29, which corresponds to the consensus V3 loop sequence (18), contains the identical 12 amino acids which are expressed on the chimeric WSN-MN virus. Peptide W11-28, which corresponds to the V3 loop of the WMJ2 isolate, has eight amino acid residues identical to those expressed on the WSN-MN virus (see Materials and Methods). Rabbit antibodies raised against both peptides reacted with the chimeric WSN-MN virus in the HI assay, exhibiting titers of 1,024 and 256, respectively (Table 1). In contrast, neither antibody reacted with wt WSN virus. The human monoclonal antibody MedI 11, which recognizes GPGR residues on the V3 loop of gp120, reacted with the chimeric WSN-MN virus at an HI titer of 65,536 and did not react with wt WSN virus. Similarly, these antibodies were able to neutralize the chimeric WSN-MN virus in the plaque neutralization assay (Table 1). The neutralization titers were 256 and 1,024 for the antipeptide antibodies and 120,000 for the monoclonal antibody.

**Induction of antibody response against HIV gp120.** To investigate whether the chimeric WSN-MN virus is able to elicit antibodies against HIV, BALB/c mice were immunized with the chimeric virus, and mice in the control group were inoculated with wt WSN virus. Immunization was done either by aerosol infection with 500 PFU of virus or by i.p. administration of 10 μg of purified virus. All subsequent booster immunizations were then given i.p. The presence of specific anti-HIV gp120 antibodies was first determined by radioimmunoassay using the synthetic peptides C11-29 and W11-28. The results obtained from mice immunized by aerosol and boosted twice with WSN-MN virus or wt WSN virus are shown in Fig. 2. Sera from all 10 mice immunized with the chimeric WSN-MN virus showed binding to peptide C11-29 (Fig. 2A), although individual differences among the 10 mice were noted. In contrast, sera from mice immunized with wt WSN virus did not specifically bind to peptide C11-29 (Fig. 2C). The sera from mice immunized with WSN-MN virus also bound specifically to peptide W11-28 (Fig. 2B). Immunizations by the i.p. route gave similar results (data not shown). Analysis of isotypes of the V3 peptide-specific antibodies showed that they are mainly of immunoglobulin G2a (IgG2a) and IgG2b isotypes. We also detected an IgA isotype specific for the C11-29 peptide (data not shown).

The specificity of the WSN-MN virus-induced antibodies against HIV gp120 was further confirmed by a radioimmunoprecipitation assay. As shown in Fig. 3, serum samples from mice immunized with the chimeric WSN-MN virus were

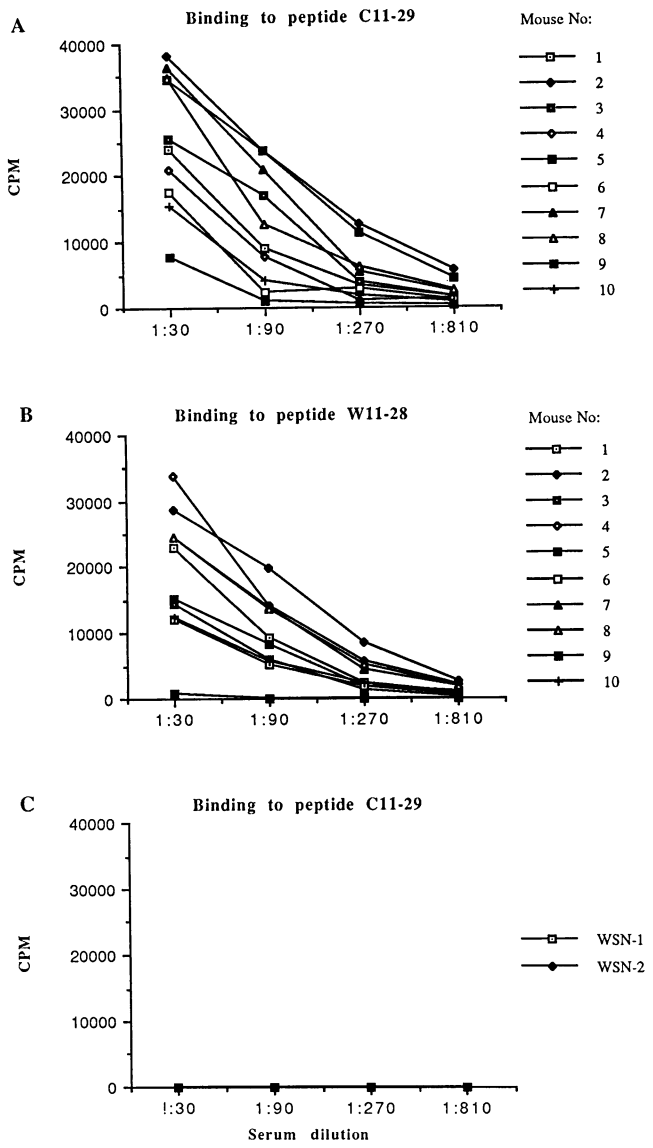


FIG. 2. Reactivities of mouse sera to the synthetic V3 peptide as determined by radioimmunoassay. (A) Binding of sera from 10 mice immunized with WSN-MN virus to peptide C11-29; (B) binding of the same sera to peptide W11-28; (C) binding of sera from mice immunized with wt WSN virus to peptide C11-29. All sera were derived from mice immunized via aerosol and twice boosted with WSN-MN or wt WSN virus.

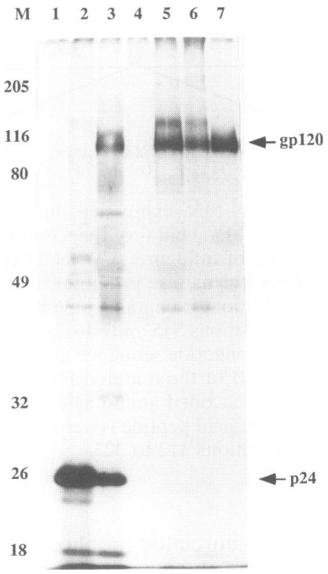


FIG. 3. Immunoprecipitation of gp120 of HIV-1/MN by sera from mice immunized with chimeric WSN-MN virus. M, molecular weight markers (in kilodaltons). Lanes: 1, normal human serum; 2, rabbit anti-p24$^{gag}$ antiserum; 3, HIV-positive human serum; 4, serum from a mouse immunized with wt WSN virus: 5, pooled sera from WSN-MN-immunized mice 7, 9, and 10; 6, serum from WSN-MN immunized mouse 8; 7, anti-MN virus gp120 monoclonal antibody 50.1. Positions of the bands of immunoprecipitated gp120 and p24 of HIV-1/MN are indicated by arrows. Labeled MN virus was prepared from acutely infected H9 cells. The anti-WSN-MN sera were tested at a 1:25 dilution.

capable of precipitating gp120 of HIV-1/MN. These data demonstrate that the WSN-MN virus-induced antibodies were able to recognize the native envelope protein of HIV-1/MN. It should be noted that there is a nonspecific precipitation band of a protein of 150 kDa (Fig. 3, lanes 5 and 6).

We then determined the neutralizing properties of the antibodies induced by chimeric WSN-MN virus. Several in vitro inhibition assays were performed: inhibition of p24 synthesis in CEMss and H9 cells infected with HIV-1/MN, as well as inhibition of syncytium formation in infected CEMss cells. Pooled sera from mice obtained after three immunizations were used for the neutralization assays because only limited amounts of sera from individual mice were available for these assays. As shown in Table 2, all of the sera showed neutralization activity as assessed by measuring p24 synthesis from HIV-1/MN-infected CEMss and H9 cells. Sera 2, 3, and 4 also showed inhibition of syncytium formation. In addition, sera obtained after four immunizations showed neutralization titers of 320 to 640, which were based on a 90% inhibition level of p24 synthesis in MN virus-infected CEMss cells. It should be noted that one of the three normal sera used in this study showed inhibitory activity in the p24 assay, but no syncytium inhibition activity was detected (data not included in Table 2).

**Induction of V3 loop-specific CTLs.** The decapeptide IG PGRAFYTT, corresponding to the V3 loop of MN virus at positions 314 to 323, has been shown to represent a CTL epitope which is recognized by CD8$^+$ T cells in association with class I major histocompatility complex molecules (3a). To examine the ability of chimeric WSN-MN virus to induce specific CTLs, BALB/c mice were immunized with the chimeric WSN-MN virus. Nonimmunized mice or mice immunized with wt WSN virus were used as controls. After 7 days, spleen cells were stimulated in vitro with irradiated spleen cells alone or with cells coated with peptide MN314-323 or NP147-161. Peptide NP147-161 contains a K$^d$-restricted CTL epitope specific for the WSN virus NP (39). The cytolytic activities of CTLs against P815 target cells coated with peptide MN314-323 or NP147-161 were studied. CTLs specific for peptide MN314-323 or NP147-161 were not generated from nonimmunized mice (Fig. 4A and B). CTLs derived from mice immunized with WSN-MN virus and restimulated in vitro with peptide MN314-323 specifically lysed P815 cells coated with peptide MN314-323 (Fig. 4C). This lysis was inhibited by monoclonal antibody HB75 (anti-$D^d$) and not by monoclonal antibody

TABLE 2. Neutralization of HIV-1/MN infectivity by sera of mice immunized with the chimeric WSN-MN virus

| Serum | Neutralization titer | | |
|---|---|---|---|
| | P24 synthesis[a] | | Syncytium formation[b] |
| | CEMss cells | H9 cells | |
| Normal | <8 | <10 | <8 |
| | <8 | <10 | <8 |
| Anti-WSN | | | |
| Pool 1 | <8 | <10 | <8 |
| Pool 2 | <8 | <10 | <8 |
| Anti-WSN-MN | | | |
| Pool 1 | 16 | 40 | <8 |
| Pool 2 | 16 | 80 | 16 |
| Pool 3 | 64 | 80 | 16 |
| Pool 4 | 64 | 80 | 16 |
| MAb 50.1[c] | 4,096 | ND[d] | ND |

[a] Expressed as the reciprocal of the highest serum dilution which reduced p24 synthesis by more than 50%. The actual p24 level of the supernatant samples in the absence of antibody was 76.8 ng/ml. Each of the pooled anti-WSN sera is from three mice immunized with WSN virus. Sources of anti-WSN-MN sera are as follows: pool 1, mice 1, 2, and 3; pool 2, mice 4, 5, and 6; pool 3, mice 7 and 8; and pool 4, mice 9 and and 10. All sera were obtained after immunizing mice three times with WSN-MN or wt WSN virus.

[b] Expressed as the reciprocal of the highest serum dilution which reduced the number of syncytia in CEMss cells by more than 50%. The number of syncytia in the absence of antibody was 154.

[c] A monoclonal antibody (MAb) directed against the V3 loop of MN virus gp120.

[d] ND, not determined.

HB77 (anti-$K^d$) (data not shown). The same CTL population did not lyse P815 cells which were not sensitized, EL-4 (H-$2^b$) cells which were sensitized with peptide MN314-323 (data not shown), or P815 cells coated with peptide NP147-161 (Fig. 4D). No cytolytic activity against P815 cells coated with peptide MN314-323 was detected with CTLs derived from mice immunized with WSN-MN virus and restimulated with spleen cells alone or coated with NP147-161 (Fig. 4C). The chimeric WSN-MN virus was able to induce NP-specific CTLs (Fig. 4D), as expected. The wt WSN virus induced NP-specific CTLs; however, no CTLs specific against the HIV T-cell epitope were detected (Fig. 4E and F). Our results clearly demonstrate that the chimeric WSN-MN virus was able to prime in vivo CTL precursors specific for a peptide located in the V3 loop of HIV-1/MN.

## DISCUSSION

Chimeric viruses expressing foreign proteins or epitopes have been used to induce specific immunity against various pathogens or to study antigen processing and presentation. Large DNA viruses like vaccinia viruses are being developed as live vectors for the expression of foreign proteins (2, 17, 27). Recently, the possibility of using positive-stranded RNA viruses for presentation of foreign antigenic structures has been studied. For example, HIV-1 immunogenic epitopes have been expressed in chimeric polioviruses (8, 11), and a vector based on Sindbis virus has been used to express peptides containing CTL epitopes derived from the HA of influenza virus (13, 14). Thus, chimeric viruses expressing foreign epitopes may represent a new approach to develop polyvalent vaccines and also could provide a new tool with which to investigate the generation and structures of peptides recognized by T cells in association with major histocompatibility complex molecules. The study described in this paper demonstrates that influenza virus, a negative-stranded RNA virus, can also be used as an expression vector.

We report the antigenicity and immunogenicity of an engineered chimeric influenza virus bearing a 12-amino-acid peptide derived from the V3 loop of gp120 of HIV-1/MN. Previous studies suggested that this peptide contained overlapping B- and T-cell epitopes (16, 38). Insertion of this peptide into the loop of antigenic site B of the WSN virus HA did not affect viral replication in mammalian cells, nor did it alter the function of the HA protein, despite the fact that the receptor binding site of the HA is located near the loop (41). However, a change in conformation of the HA next to the loop was observed, as shown by an HI analysis using a panel of anti-WSN HA monoclonal antibodies.

The 12-amino-acid peptide is exposed on the WSN-MN virus surface because it is recognized by an anti-gp120 monoclonal antibody as well as by specific antipeptide antibodies. Since these antibodies inhibit the hemagglutinating activity of the chimeric virus and efficiently neutralize the chimeric virus, it is likely that the structure of the peptide in the HA context is similar to that in gp120. It has been postulated that the GPGR residues form the beta turn in the V3 loop of gp120 (12). It is possible that these residues also form a beta turn in the WSN-MN virus HA, therefore maintaining the original structure and stabilizing the extended loop in the chimeric protein.

The chimeric WSN-MN virus was capable of inducing anti-HIV antibodies. The induced antibodies were able to bind to peptide C11-29 as well as to peptide W11-28. Peptide W11-28 shares a stretch of seven residues (IGPGRAF) with the V3 peptide expressed on the WSN-MN virus HA. Thus, the V3 loop-specific antibodies induced by the chimeric virus may mainly recognize these seven residues. Furthermore, our data support the previous finding that the synthetic peptide GPGRAF is able to elicit neutralizing antibody against HIV (16). Sera from mice immunized with WSN-MN virus were able to precipite gp120 of HIV-1/MN, indicating that the antibodies induced by the chimeric virus recognize native gp120. The most striking observation is the ability of these antibodies to neutralize HIV-1. This property would be very important for the potential use of such chimeric viruses as vaccines.

Furthermore, the chimeric WSN-MN virus was able to induce CTLs specifically recognizing an HIV sequence. $CD8^+$ T cells recognize the decamer IGPGRAFYTT, which is contained in the 12-amino-acid peptide expressed in the chimeric virus. In vivo induction of HIV-specific CTLs by the chimeric virus suggests that the different flanking sequences of the CTL epitope, at least to the C-terminal site, do not abrogate the generation of specific CTLs. How the flanking sequence might influence the level of induction of CTLs will have to be determined by further mutational analysis. It should be mentioned that a chimeric virus expressing a T-cell epitope of *Plasmodium yoelii* circumsporozoite protein in a different antigenic site of the WSN virus HA is also able to prime CTLs in vivo (18b). These results suggest that chimeric influenza viruses may represent an excellent tool with which to study antigen processing and presentation in vivo as well as in vitro. Together with other expression systems such as vaccinia virus or Sindbis virus, the influenza virus system may help in mapping and analyzing the structures of T-cell epitopes.

The results described above clearly demonstrate that influenza virus can be used as an expression vector to induce both B- and T-cell-mediated immunity against other infectious agents. Influenza virus is known to induce IgA-mediated mucosal immunity as well as serum antibody responses. Inac-

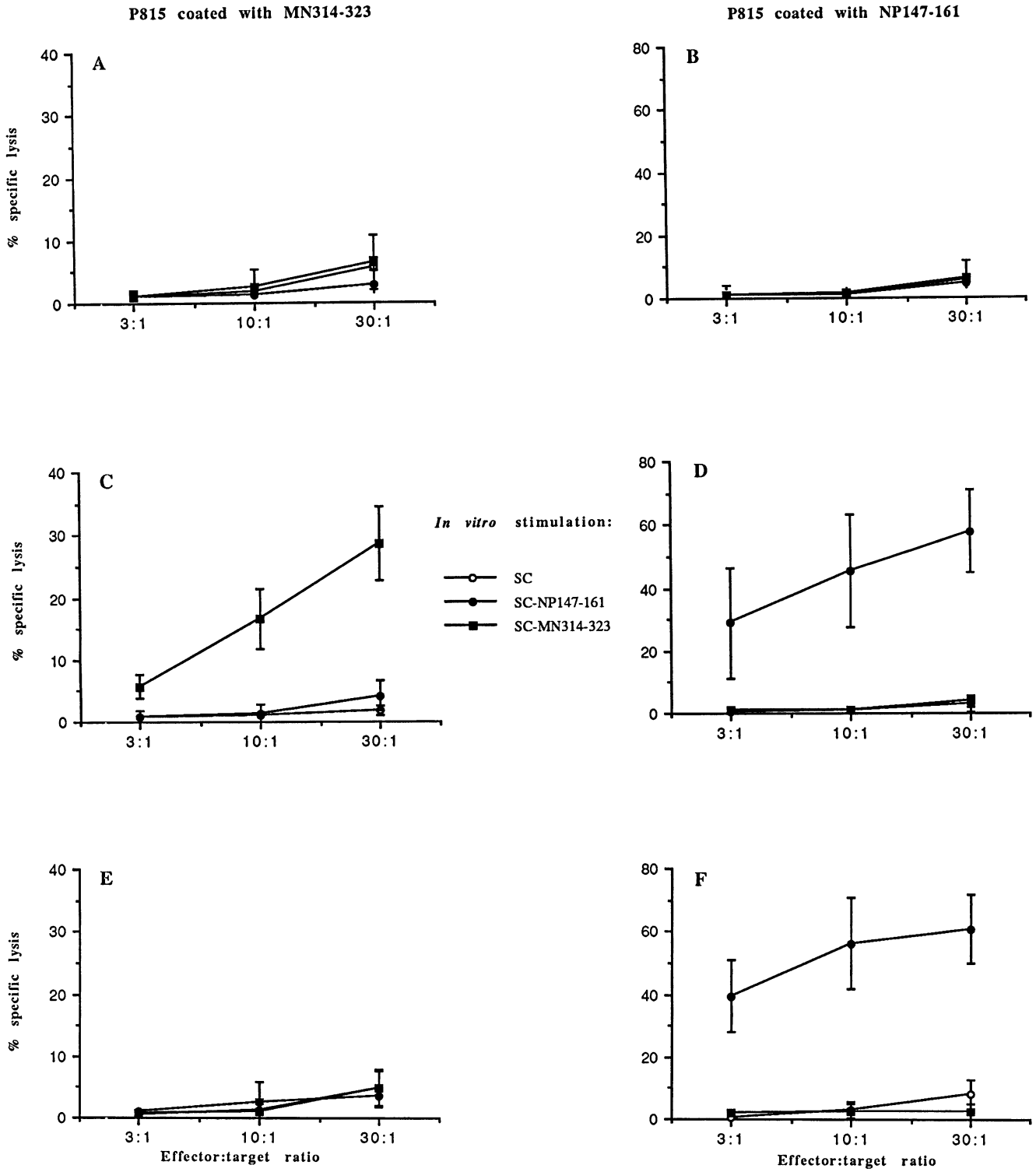


FIG. 4. Induction of CTLs recognizing an HIV sequence. Spleen cells derived from normal mice (A and B), mice immunized with chimeric WSN-MN virus (C and D), or mice immunized with wt WSN virus (E and F) were restimulated in vitro in the presence of irradiated spleen cells (SC) alone (○), spleen cells coated with peptide NP147-161 (●), or spleen cells coated with peptide MN314-323 (■). The cytolytic activity of the resulting CTLs was measured by using P815 target cells coated with peptide MN314-323 (A, C, and E) or target cells coated with peptide NP147-161 (B, D, and F). Each point represents the average of percent specific lysis of CTLs derived from three mice ± standard error.

tivated influenza virus vaccines have been widely used. In addition, live attenuated vaccines have been extensively studied in human clinical trials (24). Both types of influenza virus vaccines have been shown to be safe. Thus, chimeric influenza viruses expressing foreign antigenic structures may represent a novel type of live virus vaccine against pathogens such as HIV, since protection against this virus may depend mainly on a strong mucosal immunity. Influenza virus undergoes frequent antigenic changes, so influenza viruses with different HAs can be easily generated and may be repeatedly used as vaccine vectors. The development of attenuated influenza viruses, either by using cold adaptation (24) or by introducing attenuation markers (28), may allow influenza virus to be used as a vaccine vector against a variety of diseases.

Our results provide further evidence indicating that live viral vectors carrying HIV epitopes can elicit a specific immune response against a retrovirus. One may ask whether influenza virus has advantages over other live vectors. Recombinant vaccinia viruses expressing envelope proteins of HIV-1 could elicit both humoral and cellular-mediated immune responses. However, the expression of an entire HIV-1 envelope protein does not preclude the immunosuppressive effect of some gp120 sequence (35), nor is it possible to use vaccinia virus to boost the immune response. In contrast, influenza viruses with different HA proteins could be used for repeated immunization protocols. Also, the available data on use of live poliovirus as a vector for bearing an HIV-1 epitope showed only the induction of neutralizing antibodies (8, 11). In contrast, the chimeric influenza virus described above showed, for the first time, the ability to induce neutralizing antibodies and to prime the CTL precursors. In addition, immunization with influenza virus vectors would be able to confer immunity against both influenza virus and HIV-1 infection with minimal side effects and thus should be beneficial to patients in different risk groups.

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