J. Biochem. **138**, 571–582 (2005)
doi:10.1093/jb/mvi165

Suppression of Multiclade R5 and X4 Human Immunodeficiency Virus Type-1 Infections by a Coreceptor-Based Anti-HIV Strategy

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Received July 6, 2005; accepted August 10, 2005

A cyclic chimeric dodecapeptide (cCD) mimicking the conformation-specific domains of CCR5 and CXCR4 was prepared in which Gly-Asp links the amino and carboxyl termini of two combined pentapeptides (S169–G173 of CCR5; E179-R183 of CXCR4) derived from human immunodeficiency virus type-1 (HIV-1) coreceptors. The immunization of Balb/c mice with cCD conjugated with a multiple-antigen peptide (cCD-MAP) induced seven cCD-specific monoclonal antibodies (mAbs, CPMAb-I to -VII) that reacted with native CCR5 and CXCR4. Among the tested mAbs, CPMAb-I and -II potently inhibited the infection of both the R5 and X4 laboratory strains. CPMAb-III and -VI were effective against only R5 laboratory strains, and also against some X4 and R5 primary isolates. CPMAb-IV and -V had potent antiviral activities against the R5 and X4 primary isolates. In particular, CPMAb-VII was protective against not only R5 and X4 laboratory strains, but also most of the R5 and X4 primary isolates. Moreover, cCD-MAP immunization also induced antibodies that were effective against R5 and X4 multiclade HIV-1 isolates *in vitro* in two of three cynomolgus monkeys. Taken together, the results suggest that cCD-MAP is a candidate multiclade immunogen that can be used to block multiclade R5 and X4 HIV-1 infections.

Key words: AIDS, autoantibody, chemokine receptor, HIV, vaccine.

Abbreviations: AIDS, acquired immune deficiency syndrome; cCD, cyclic chimeric dodecapeptide; CPMAb; CTLs, cytotoxic T lymphocytes; ECL-2, extracellular loop-2; FITC, fluorescein isothiocyanate; HIV-1, human immunodeficiency virus type 1; mAb, monoclonal antibody; MAP, multiple-antigen peptide; UPA, undecapeptidyl arch; SHIV, simian/human immunodeficiency viruses.

HIV-1 strains are classified into R5, X4 and R5X4 on the basis of their coreceptor usage (*1*, *2*). R5 HIV-1 dominates the early stage of HIV-1 infection, whereas X4 HIV-1 often arises in late-stage infection and is associated with the rapid decrease in $CD4^+$ cell count and the progression of acquired immune deficiency syndrome (AIDS) (*3–5*). Some studies have demonstrated that humoral and cellular immunity represents an important anti-HIV vaccine strategy, while one of the major obstacles is the emergence of escape mutants from neutralizing antibodies (Abs) or cytotoxic T lymphocytes (CTLs) against HIV-1 (*6–8*) caused by highly error-prone reverse transcription. There is thus the necessity to develop next-generation vaccines targeting HIV-1 Env, such as those developed on the basis of the consensus or ancestor sequences selected to minimize genetic differences between vaccine strains and contemporary isolates (*9*, *10*), and alternative HIV vaccines targeting host proteins such as CCR5 and CXCR4. Therefore, we examined the immunogenicity of cyclic peptides mimicking the HIV-1 coreceptor CCR5 or CXCR4 and the anti-HIV activities of induced Abs and antisera (*11–13*).

The results of some studies support our strategy of targeting CCR5 and CXCR4. Regarding CCR5, (i) individuals homozygous for a 32-bp deletion in CCR5 (CCR5Δ32) are resistant to HIV-1 infection (*14*); (ii) CCR5-specific autoantibodies with anti-R5 HIV activity have been found in HIV-seronegative individuals at risk of HIV-1 infection (*15*); and (iii) despite exposure to anti-CCR5 Abs, HIV seronegative individuals are healthy and have no immune system abnormalities (*16*). On the other hand, regarding CXCR4, (i) CXCR4 is critical for growth and development (*17*), but is probably dispensable in adults because the safety of small-molecule CXCR4 inhibitors such as ALX40-4C and AMD3100 in humans has been reported (*18*, *19*); and ii) the blockade of CXCR4 may provide an advantage in not only inhibiting X4 virus entry, but also in blocking AIDS progression (*20*). Furthermore, Rusconi *et al*. reported that the blockade of CCR5 and CXCR4 is necessary to attain marked HIV-1 inhibition since an HIV-1–infected individual may carry the R5 and X4 viruses, and there is often an R5-to-X4 shift of coreceptor usage during the clinical deterioration to AIDS (*21*). Hence, we focused on the undecapeptidyl arch (UPA; R_{168} to C_{178} in CCR5 and N_{176} to C_{186} in CXCR4) of extracellular loop-2 (ECL-2), which is a critical domain for HIV-1 entry (*22*, *23*), in designing a novel cyclic chimeric immunogen targeting HIV-1 coreceptors. We evaluated whether the immune

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response that results in the production of specific Abs simultaneously recognizing CCR5 and CXCR4 is capable of inhibiting clade B and non-clade B HIV-1 infections.

In this study, the immunization of mice with cCD-MAP induced seven mAbs with anti-R5 and -X4 HIV-1 activities. In addition, antisera from two of three cynomolgus monkeys immunized with cCD-MAP effectively inhibited infection by R5 and X4 HIV-1 isolates, from not only clade B but also non-clade B, and pathogenic R5 and X4 simian/human immunodeficiency viruses (SHIVs). These results suggest that cCD-MAP may be useful in overcoming obstacles to the development of treatment strategies, drugs, or vaccines for HIV-1/AIDS.

MATERIALS AND METHODS

HIV-1 and SHIV Strains—Clade B laboratory strains, HIV-1_{JRFL} and HIV-1_{LAV-1}, and $SHIV_{SF162P3}$ and $SHIV_{KU-1}$, which are chimeric forms of HIV-1 and the simian immunodeficiency virus (SIV) for use in the HIV-1/AIDS monkey model (*24*, *25*), and non-clade B strains, HIV-$1_{93RW004}$, HIV-$1_{92TH009}$, HIV-1_{MJ4}, HIV-$1_{92UG029}$, HIV-1_{CMU08}, and HIV-$1_{98IN017}$ (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD), were used.

Preparation of cCD-MAP and cCD-Multi-Pin Block, and Biotinylated cCD—cCD-MAP (as antigen) and cCD-Multi-Pin Block for ELISA were prepared as described previously (*11*). Biotinylated cCD was prepared by substituting *N*-ε-biotinyl-Lys for Asp in the spacer arm, and used to confirm the specific binding of antibodies to cCD as a BIAcore biosensor.

Preparation of Anti-cCD-MAP mAbs and ELISA—Monoclonal Abs against cCD-MAP were prepared as previously described (*11*). In the screening, supernatants were tested for reactivity against the cCD-Multi-Pin Block, and hybridomas were then cloned by the limiting dilution method. Finally, the activities of monoclonal Abs and antisera against cCD-MAP were determined by ELISA using the cCD-Multi-Pin Block according to our standard method (*11*). Antibody titers in antisera against the cCD antigen were also determined by the same ELISA, performing doubling dilutions of antisera from cCD-MAP– or MAP-immunized monkeys, and presented as reciprocals before and after each immunization.

Flow Cytometry—The Abs and antisera used were as follows: mAbs to cCD-MAP and antisera diluted 32-fold in PBS(–) at predetermined concentrations from cynomolgus monkeys at preimmunization (Pre) or 6 weeks after the initial immunization, an isotype-matched control antibody (Sigma Chemical Co., St. Louis, Mo), fluorescein isothiocyanate (FITC)–conjugated anti-mouse IgM, and FITC-conjugated anti-monkey IgG.

NP2/CD4, NP2/CD4/CXCR4 and NP2/CD4/CCR5 (*26*) and MAGIC-5 (*27*) cells were incubated with one of the above-mentioned antibodies as the primary antibody at 4°C, washed with washing buffer [PBS containing 2% fetal calf serum (FCS) and 0.02% NaN_3], and resuspended in the same washing buffer containing FITC-conjugated anti-mouse IgM [for monkey antisera, FITC-conjugated goat anti-monkey IgG (BETHYL Laboratories, Inc., Montgomery, TX)]. After 30 min of incubation at 4°C, the cells were washed three times, and then analyzed using an EPICS XL flow cytometer (Beckman Coulter). For competition assays, MAGIC-5 cells were incubated with CPMAb-I or antisera for 30 min on ice with or without FITC-conjugated 12G5 (10 μg/ml), FITC-conjugated 2D7(10 μg/ml), or both, washed, and then analysed using a flow cytometer as described above.

Chemotaxis Assay—The migration of Molt4#8 and CEM-CCR5 cells induced by MIP-1β and SDF-1α, respectively, was assayed in 24-well cell-culture chambers using an insert with 5.0-μm pore membranes (Corning, Corning, NY) according to the protocol of Gosling *et al*. (*28*). The cells (5×10^6) were pretreated with or without CPMAb-I to -VII for 30 min and placed in the upper chamber. Chemotaxis assays were conducted in the presence of 4 nM SDF-1α and 8 nM MIP-1β (placed in the lower chamber). After incubation for 3 h at 37°C, the cells that migrated from the upper chamber to the lower chamber were quantified by trypan blue exclusion. Control experiments were carried out with or without MIP-1β or SDF-1α under identical conditions using an isotype mouse IgM antibody instead of CPMAb-I to -VII.

Antiviral Activities—The antiviral activities of the monoclonal Abs and sera taken before and after immunization with cCD-MAP were determined using MAGIC-5 cells as described previously (*27*). The MAGIC-5 cells were plated at 0.75×10^4 cells per well (96-well plates) and incubated overnight in culture medium (50 μl); the medium was then replaced with fresh medium containing the monoclonal antibody diluted with PBS(–), or 32-fold diluted antiserum with PBS(–). Diluted virus stocks that produced 100 to 300 cells stained blue were used. The number of cells stained blue was expressed as a percentage (%) relative to the number of cells stained blue in the control. All data represent the means ± SD obtained from three separate experiments. Control experiments were carried out under identical conditions, except for the omission of antibody. Student's *t*-test was used for the statistical analysis of these data.

Real-Time Biomolecular Interaction Analysis Using Surface Plasmon Resonance—The recognition of the cyclic structure of cCD by antiserum from a cCD-MAP–immunized or a MAP-immunized monkey was analyzed by the surface plasmon response technique using BIAcore 2000, as described previously (*12*, *13*). Biotinylated cCD was immobilized onto streptavidin-coated sensor chips. In order to detect anti-cCD Abs in antisera derived from the immunized cynomolgus monkeys, the antisera were diluted tenfold in PBS(–).

Immunization Schedule—All cynomolgus monkeys were housed in individual cages and maintained according to the rules and guidelines of the National Institute for Infectious Diseases (NIID) for experimental animal welfare. Three cynomolgus monkeys, 4–6 years old, were immunized intraperitoneally at 0 and 1 week with 300 μg of cCD-MAP in complete Freund's adjuvant, and booster immunizations consisting of 300 μg of cCD-MAP in incomplete Freund's adjuvant were given subcutaneously at 6 weeks. Another three cynomolgus monkeys were immunized with MAP following the same immunization schedule. Blood samples were obtained before and 0, 1, 2, 4, 6 and

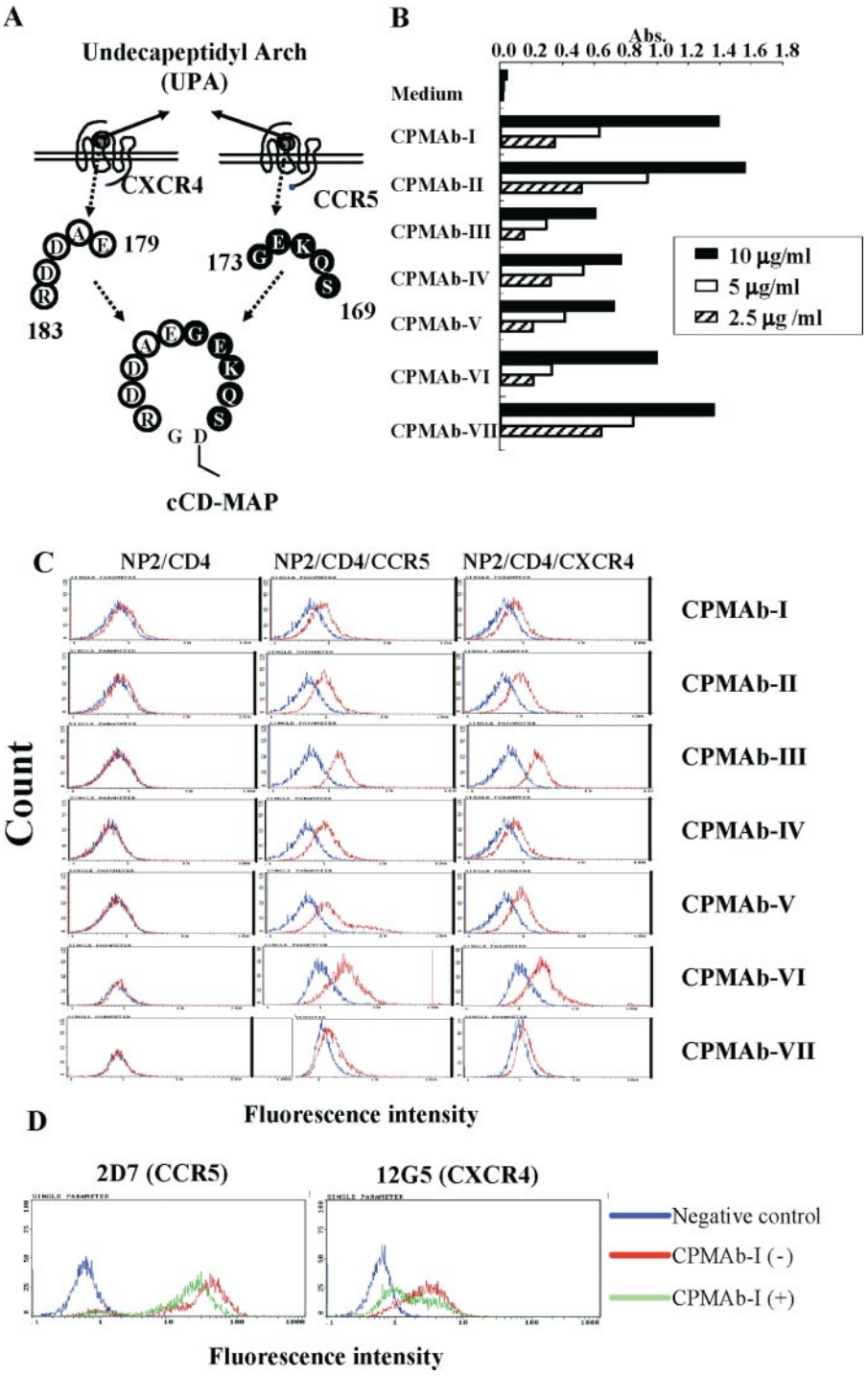

Fig. 1. **Binding of CPMAb-I to -VII to cCD antigen and native cell surface chemokine receptors (CCR5 and CXCR4).** Overview of the preparation of cyclic chimeric peptide (A). The binding of CPMAb-I to -VII to the cCD-Multi-Pin Block and chemokine receptors (CCR5 and CXCR4) was determined by Multi-Pin ELISA (B) and FACS (C), respectively. B, Briefly, the cCD-Multi-Pin-Block was separately incubated with the indicated concentrations of CPMAb-I to -VII, and then bound Abs were detected using HRP-conjugated mouse Abs as described in "MATERIALS AND METHODS." C, NP2/CD4, NP2/CD4/CCR5 and NP2/CD4/CXCR4 cells were separately incubated with CPMAb-I to -VII (1 μg, red line), or isotype-matched IgM (1 μg, blue line for control) as the primary antibody at 4°C, and detected using the FITC-conjugated anti-mouse IgM antibody. D, Competition for the binding of well-characterized anti-CXCR4 and anti-CCR5 Abs by the anti-cCD antibody (CPMAb-I). The competition assay was carried out as described in "MATERIALS AND METHODS." MAGIC-5 cells were incubated with (green line) or without (red line) CPMAb-I in the presence of 2D7 or 12G5.

8 weeks after immunization, and these were subjected to BIAcore analysis and MAGIC-5 assay.

Determination of MIP-1α, MIP-1β, RANTES and SDF-1α Levels in Antisera—Antisera were subjected to a specific antigen–captured enzyme-linked assay using Quantikine ELISA kits to determine the levels of MIP-1α, MIP-1β, RANTES and SDF-1α (R&D System, Inc., MN, USA) according to the manufacturer's instructions. Control experiments were carried out under the same conditions except for the omission of antiserum.

RESULTS

cCD-MAP was constructed with a spacer-armed Gly-Asp dipeptide and two pentapeptides (S_{169}-Q_{170}-K_{171}-E_{172}-G_{173} of CCR5 and E_{179}-A_{180}-D_{181}-D_{182}-R_{183} of CXCR4) of UPA in ECL-2 (Fig. 1A), and used to immunize female BALB/c mice at 1-week intervals by intravenous administration in Freund's adjuvant, with the final immunization taking place 3 days prior to splenectomy. Of the novel mAbs that have been cloned recently, six in addition to CPMAb-I

described previously (*11*), CPMAb-II to -VII (IgM), were obtained. These mAbs showed dose-dependent activities against the cCD-Multi-Pin Block as determined by ELISA (Fig. 1B), and also recognized native CCR5- or CXCR4-expressing NP2/CD4 cells, but not non–CCR5- and –CXCR4-expressing cells as determined by FACS (Fig. 1C). To elucidate their specificity further, MAGIC-5 cells expressing CCR5 and CXCR4 were separately incubated with these mAbs at relatively high concentrations (10 μg/ml) in the presence of a CCR5-specific mAb (2D7) or CXCR4-specific mAb (12G5). The results demonstrate that these mAbs specifically recognize both CCR5 and CXCR4. Representative data (in the case of CPMAb-I) are shown in Fig. 1D.

The anti-HIV activities of CPMAb-I to -VII were determined using MAGIC-5 cells (*27*). The cells were separately inoculated with HIV-1_{JRFL} (R5, clade B laboratory strain) (Fig. 2, A–G) and HIV-1_{LAV-1} (X4, clade B laboratory strain) (Fig. 2, H–N) with or without CPMAb-I to -VII at the indicated concentration. CPMAb-I and -II inhibited the infections by HIV-1_{JRFL} (Fig. 2, A and B) and HIV-1_{LAV-1} (Fig. 2, H and I) in a dose-dependent manner, whereas CPMAb-IV (Fig. 2, D and K) and -V (Fig. 2, E and L) did not. CPMAb-III (Fig. 2, C and J) and -VI (Fig. 2, F and M) were effective against R5 (JRFL) alone. CPMAb-VII was effective against both R5 and X4 at relatively high concentrations (1.5–6.0 μg/ml) (Fig. 2, G and N).

The inhibitory effects of these mAbs against non-clade B primary isolates were subsequently investigated, and the results are presented in Fig. 3. CPMAb-I (Fig. 3A) and -II (Fig. 3B) potently inhibited the infection by both the R5 and X4 laboratory strains of clade B (JRFL and LAV-1), rather than the primary isolates used in this study. On the other hand, CPMAb-IV (Fig. 3D) and -V (Fig. 3E) were more effective against the primary isolates (CPMAb-IV: R5 of clades A, C, and E; CPMAb-V: R5 of clades A and E, X4 of clade E) rather than the R5 and X4 laboratory strains of clade B. Although both CPMAb-III (Fig. 3C) and -VI (Fig. 3F) were effective against only the R5 laboratory strain of clade B, CPMAb-III (Fig. 3C) was also effective against the X4 primary isolate of only clade C, whereas CPMAb-VI (Fig. 3F) potently inhibited infection by the X4 primary isolates of clades A, C, and E as well as the R5 of clades A and C. Furthermore, CPMAb-VII provided significant protection against not only the R5 and X4 laboratory strains of clade B but also against the R5 (clades A and E) and X4 (clades A, C and E) primary isolates (Fig. 3G).

The effects of CPMAb-I to -VII binding on chemokine receptor activity were investigated by a chemotaxis assay using MIP-1β and SDF-1α as chemoattractants. CPMAb-II inhibited both MIP-1β-and SDF-1α-induced chemotaxis at low concentrations with anti–HIV-1 activity against R5 and X4 laboratory strains, and the other mAbs had various effects on chemotaxis (Fig. 4). In particular, CPMAb-IV and -V significantly enhanced SDF-1α–induced chemotaxis ($P < 0.02$), and CPMAb-VII strongly enhanced MIP-1β–induced chemotaxis ($P < 0.02$), whereas CPMAb-VI inhibited MIP-1β– and SDF-1α–induced chemotaxis at high concentrations (10–20 μg/ml and 20 μg/ml, respectively, $P < 0.02$) (Fig. 4).

To evaluate the immunogenicity and usefulness of cCD-MAP in nonhuman primates, three cynomolgus monkeys (SS1, SS2, and SS3) were immunized with cCD-MAP

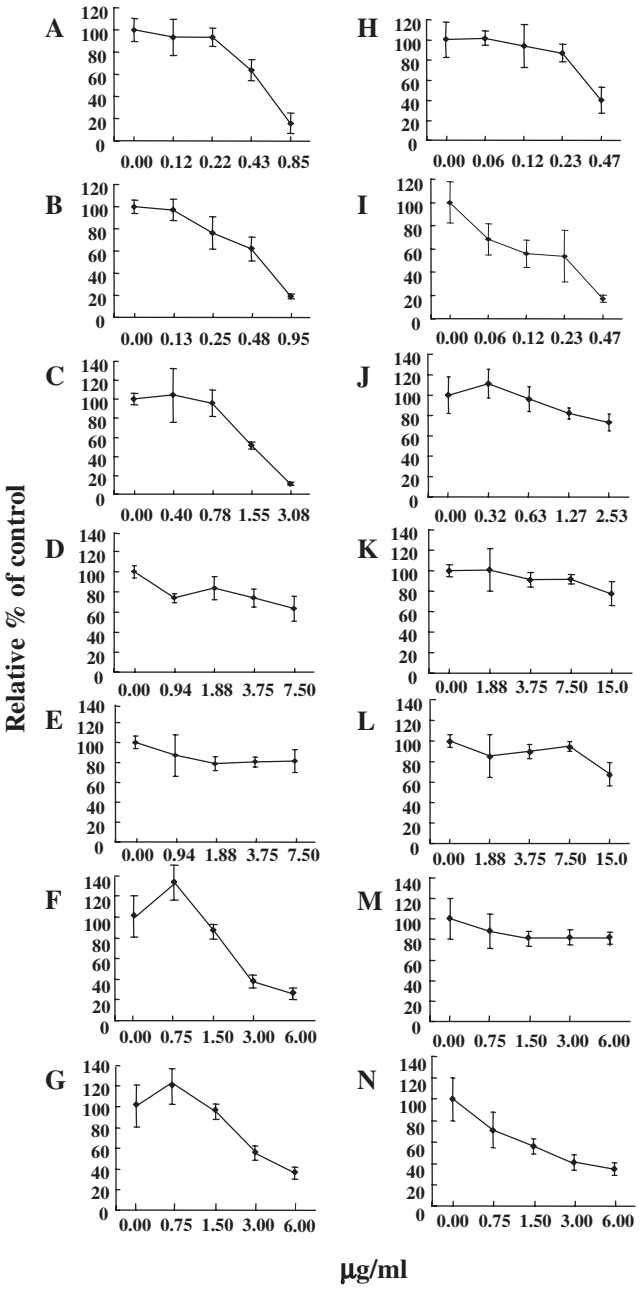

Fig. 2. **Effect of CPMAb-I to -VII on infection by HIV-1 R5 and X4 laboratory strains.** The antiviral activities of CPMAb-I to -VII were determined by an infection assay using MAGIC-5 cells (*26*). MAGIC-5 cells were inoculated with HIV-1_{JRFL} (A–G) and HIV-1_{LAV-1} (H–N). Infection was conducted in the presence of DEAE dextran and various concentrations of antibodies (CPMAb-I, A and H; CPMAb-II, B and I; CPMAb-III, C and J; CPMAb-IV, D and K; CPMAb-V, E and L; CPMAb-VI, F and M; or CPMAb-VII, G and N). After incubation for 48 h, the cells were fixed and stained with X-gal. The number of cells stained blue is expressed as a percentage (%) relative to the number of cells stained blue in the control. All data represent means ± SD obtained in three separate experiments. No significant cytotoxicity of CPMAb-I to -VII was observed.

according to the immunization schedule shown in Fig. 5A. Three other cynomolgus monkeys (SS4, SS5 and SS6) were immunized with MAP as a control using the same immunization schedule. Immune responses were

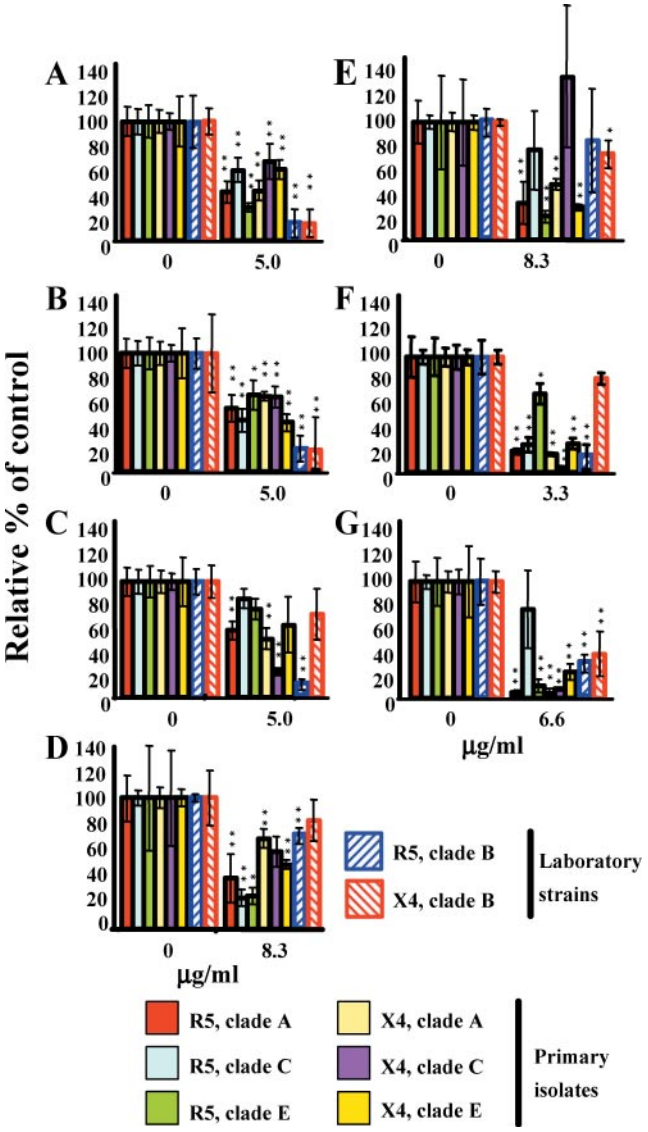

Fig. 3. **Activity of CPMAb-I to -VII against R5 and X4 HIV-1 primary isolates.** The antiviral activities of CPMAb-I to -VII were determined by an infection assay using MAGIC-5 cells. The primary isolates used were 93RW004 (R5 of clade A, shown in red), MJ4 (R5 of clade C, shown in light blue), 92TH009 (R5 of clade E, shown in green), 92UG029 (X4 of clade A, shown in light yellow), 98IN017 (X4 of clade C, shown in light red) and CMU08 (X4 of clade E, shown in yellow). For comparison, JRFL (R5, hatched blue) and LAV-1 (X4, hatched red) were used as clade B laboratory strains. A: CPMAb-I, B: CPMAb-II, C: CPMAb-III, D: CPMAb-IV, E: CPMAb-V, F: CPMAb-VI, G: CPMAb-VII. Statistical significances are indicated as asterisks (**$P < 0.02$, *$P < 0.05$).

determined quantitively in a serum dilution range of 10–400 fold by ELISA and BIAcore analysis. The antisera sampled from SS1, SS2 and SS3 6 weeks after immunization showed a positive response to the cCD-immobilized BIAcore sensor chip (Fig. 5, B–D), and also recognized CCR5- and CXCR4-expressing MAGIC-5 cells as determined by FACS (Fig. 5, E–G). On the other hand, the control antisera sampled from SS4, SS5 and SS6 before and after immunization did not show significant immunoreactivity as determined by BIAcore analysis (data not shown) and FACS (Fig. 5, H–J). The immunoreactivity of the antisera sampled from the cCD-MAP– or MAP-immunized monkeys 6 weeks after immunization to the cCD antigen was also determined by ELISA as described above, and the resultant Abs to the cCD antigen were detected only in the cCD-MAP–immunized monkeys, at titers of 1:10–1:400 (1:10: SS1; 1:100: SS2; 1:400; SS3, data not shown). To characterize the specificity of the antisera, FITC-conjugated 12G5 and 2D7, well-characterized anti-CXCR4 and -CCR5 Abs, were used for flow cytometric analysis. The antisera obtained 6 weeks after immunization inhibited the binding of these FITC-labeled Abs to CCR5 and CXCR4 (Fig. 5K). These results indicate that cCD-MAP can significantly induce Abs against CCR5 and CXCR4 not only in BALB/c mice, but also in cynomolgus monkeys.

The anti–HIV-1 activity of antisera from the cCD-MAP–immunized monkeys was determined using MAGIC-5 cells. The cells were separately inoculated with HIV-1_{JRFL} and HIV-1_{LAV-1} with or without 32-fold diluted antisera taken before and after immunization with cCD-MAP. In cynomolgus monkey SS1, the antisera sampled 4 and 6 weeks after immunization showed increased protection with increased postimmunization time against HIV-1_{JRFL} infection ($P < 0.02$), but the serum sampled 8 weeks after immunization showed weak protection ($P < 0.05$). On the other hand, the antisera sampled 1 week after immunization had potent anti–HIV-1 activity against HIV-1_{LAV-1} infection and showed sustained inhibitory activity for 8 weeks ($P < 0.02$, Fig. 6A). In contrast, in SS2, the anti–HIV-1 activity against HIV-1_{JRFL} infection was significantly induced 1 week after immunization ($P < 0.02$) (Fig. 6B). In SS3, the anti–HIV-1 activities of the antisera against HIV-1_{JRFL} and HIV-1_{LAV-1} infections increased strongly over the 8-week period from the first immunization, and almost completely inhibited HIV-1_{LAV-1} infection (Fig. 6C). None of the antisera from the MAP-immunized monkeys (control) significantly inhibited HIV-1 infection (Fig. 6, D–F). The results indicate that cCD-MAP immunization can induce Abs with potent activities against R5 and X4 HIV-1, although some differences in these activities among the individual cynomolgus monkeys were observed.

To determine whether the differences in anti–HIV-1 activity among the cCD-MAP–immunized monkeys were due to a significant generation of natural chemokine ligands to CCR5 and CXCR4, the levels of CC chemokines (RANTES, MIP-1α and MIP-1β) and CXC chemokine (SDF-1α) in the antisera from the cCD-MAP– or MAP-immunized monkeys were assayed using specific immunoassay Quantikine kits. Although the levels of SDF-1α in SS5, RANTES in SS4 and SS5, and MIP-1β in SS2 increased with increasing postimmunization time, the results suggest that the differences in anti–HIV-1 activity among the cCD-MAP immunized monkeys were due to individual differences in the generation of Abs against both receptors and/or either coreceptor without the generation of CC and CXC chemokines. (Fig. 7)

We next examined whether antisera from cCD-MAP–immunized monkeys can inhibit infection by CCR5-tropic $SHIV_{SF162P3}$ and CXCR4-tropic $SHIV_{KU1}$ bulk isolates, which are known for their pathogenicity in nonhuman primates, and HIV-1 primary isolates. The

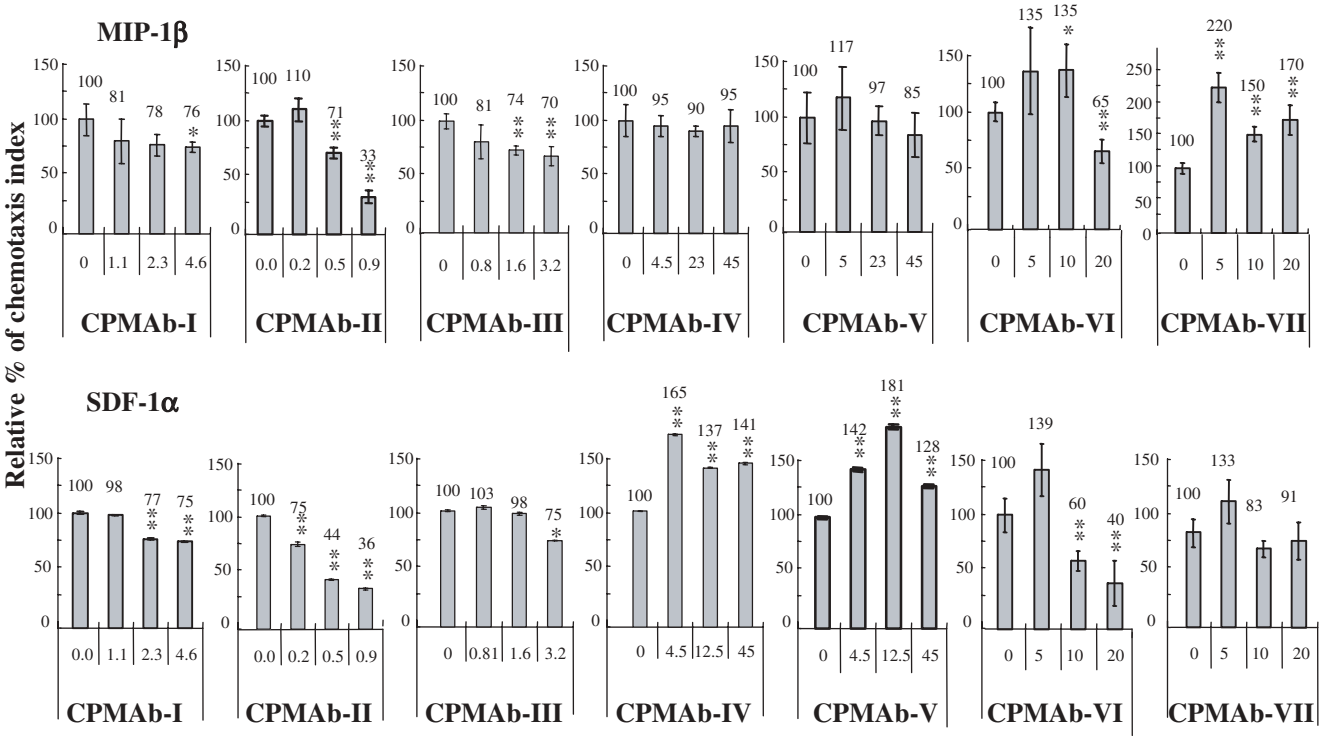

Fig. 4. **Effects of CPMAb-I to -VII on MIP-1β– and SDF-1α–induced chemotaxis.** CEM-CCR5 cells and Molt4#8 cells were separately pretreated with or without CPMAb-I to -VII for 30 min, and placed in the upper chamber of 5.0-μm pore membranes (Corning). Chemotaxis was induced with or without 8 nM MIP-1β or 4 nM SDF-1α (placed in the lower chamber). After incubation for 3 h at 37°C, the cells migrating from the upper chamber to the lower chamber were quantified by trypan blue dye exclusion. Data are expressed as relative percentages of the chemotaxis index, which is defined as the percentage of migrating cells (*28*). Statistical significance of differences between groups was established using unpaired Student's *t*-tests with matched controls, and represented as asterisks (**$P < 0.02$, *$P < 0.05$).

antisera sampled from the cCD-MAP–immunized monkeys 6 weeks after immunization inhibited $SHIV_{SF162P3}$ and $SHIV_{KU1}$ infections (Fig. 8, A–C). In addition, antisera from SS2 and SS3 sampled 6 weeks after immunization significantly inhibited infections by five (R5, clade C; R5, clade E; X4, clade A; X4, clade C; X4, clade E in Fig. 8E) and all six (R5, clade A; R5, clade C; R5, clade E; X4, clade A; X4, clade C; X4, clade E in Fig. 8F) primary HIV-1 isolates, respectively, while that from SS1 partially inhibited the infections by only two isolates (R5, clade A; X4, clade A in Fig. 8D) among the primary isolates. On the other hand, the antisera from control cynomolgus monkeys failed to show significant inhibition of infections by SHIVs and the non–clade B HIV-1 isolates (data not shown). These results suggest that antibodies raised against cCD-MAP in cynomolgus monkeys can also cross-inhibit not only clade B HIV-1 infections, but also non–clade B HIV-1 infections.

DISCUSSION

Since the discovery of HIV/AIDS 20 years ago, despite continuous efforts to develop effective preventive vaccines, no candidate vaccines have passed the phase-III clinical trial stage. We evaluated whether the immune response resulting from the induction of specific Abs against the main common receptors CCR5 and CXCR4, which are involved during HIV-1 transmission and evolution, is capable of inhibiting R5 and X4 multiclade HIV-1 infections.

The results of some studies have supported our strategy as follows. In humans, CCR5-specific Abs that inhibit R5 HIV-1 infection in vitro have been reported in seronegative women at risk of HIV-1 infection (*15*), subjects with Δ32 homozygous CCR5 exposed to CCR5-expressing blood cells through sexual activity (*29*), normal human IgG (i.v. Ig) used in therapy for patients with autoimmune diseases (*30*), alloimmune HLA typing sera collected from multiparous women, and sera from women alloimmunized with PBMCs from their partners as a therapeutic measure for recurrent spontaneous abortion (RSA) (*31*). Furthermore, in animal models, some attempts to generate specific antibodies against CCR5 or CXCR4 have also been made (*12*, *13*, *32–36*). Regarding CCR5, we have previously induced CCR5-specific Abs with anti-R5 HIV-1 activity by inoculating cDDR5-MAP from the UPA (Arg_{168} to Cys_{178}) of ECL-2 in CCR5 into BALB/c mice (*12*). As for CXCR4, we have recently induced CXCR4-specific Abs with anti–X4 HIV-1 activity by inoculating cDDX4-MAP from the UPA (Arg_{168} to Cys_{178}) of ECL-2 in CXCR4 into BALB/c mice and cynomolgus monkeys (*13*).

Immunogenicity of cCD-MAP in mice—In this study, cCD-MAP was constructed with two UPA and used to immunize BALB/c mice. Seven monoclonal Abs, CPMAb-I to -VII, were obtained, and all reacted with both CCR5 and CXCR4 with some differences in affinity. The seven

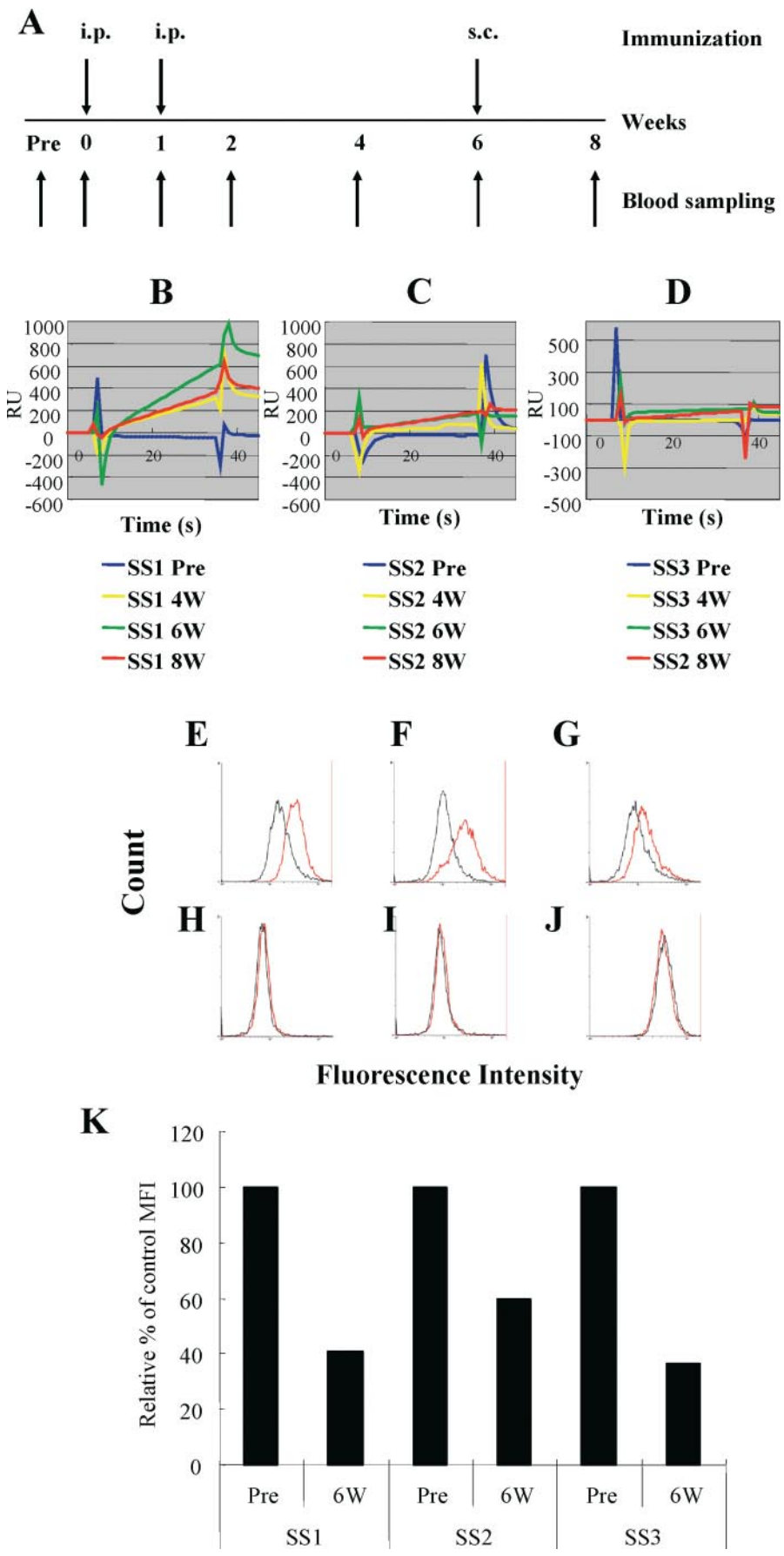

Fig. 5. **Immunization schedule and antiserum-antibody response to cCD-immobilized sensor chip and native chemokine receptors (CCR5 and CXCR4)**. (A), Immunization schedule for cynomolgus monkeys. Three cynomolgus monkeys (SS1, SS2, and SS3) were immunized intraperitoneally (i.p.) with cCD-MAP at 0 and 1 week, and given a subcutaneous (s.c.) booster immunization of cCD-MAP at 6 weeks. Another three cynomolgus monkeys (SS4, SS5 and SS6) were immunized with MAP using the same schedule. Blood samples were collected before, and 0, 1, 4, 6 and 8 weeks after immunization. (B–G), Detection of anti-cCD Abs in sera taken from immunized cynomolgus monkeys. Serum samples obtained before (blue), and 4 (yellow), 6 (dark green) and 8 (red) weeks after immunization from the monkeys (B; SS1, C; SS2, D; SS3) were diluted tenfold with PBS(–) and analyzed with a BIAcore2000 using a cCD-immobilized sensor chip as described in "MATERIALS AND METHODS." (E–J), Immunoreactivity of the antisera obtained before or 6 weeks after immunization against native CCR5 and CXCR4. CCR5- and CXCR4-coexpressing MAGIC-5 cells were incubated separately with the antisera sampled before (black) and 6 weeks after (red) immunization of the cCD-MAP–immunized monkeys (SS1:E, SS2:F and SS3:G) and MAP-immunized monkeys (SS4:H, SS5:I and SS6:J). Bound antibodies were then detected with the FITC-conjugated anti–monkey IgG antibody using an EPICS XL flow cytometer. K, Specificity of antisera obtained 6 weeks after immunization. The antisera sampled before and 6 weeks after immunization were incubated with MAGIC-5 cells with or without FITC-conjugated-CCR5 (2D7) and -CXCR4 (12G5) Abs. Binding of these FITC-conjugated Abs in the presence of antisera sampled before and after immunization is represented on the *y*-axis as a relative percentage (%) of control MFI (mean fluorescence intensity).

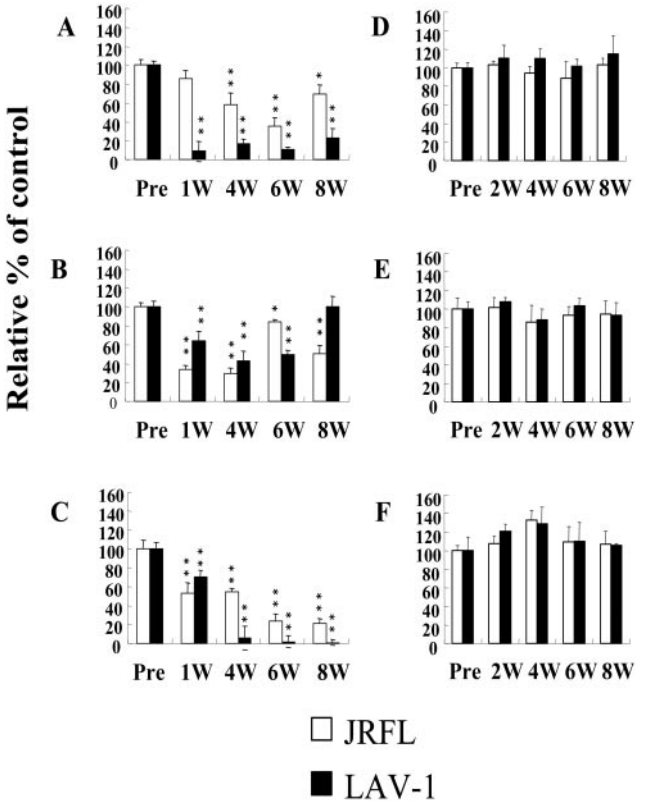

Fig. 6. **Effects of antisera from cCD-MAP–immunized monkeys on clade B HIV-1 (LAV-1 and JRFL) infection.** MAGIC-5 cells were inoculated with HIV-1_{LAV-1} and HIV-1_{JRFL} in the presence of antisera diluted 32-fold with PBS(–). For HIV-1_{JRFL} (open columns) and HIV-1_{LAV-1} (closed columns) infections, antiserum obtained before and 1, 4, 6 and 8 weeks after immunization of the cCD-MAP–immunized monkeys (A, SS1; B, SS2; C, SS3), or before and 2, 4, 6, and 8 weeks after immunization of the MAP-immunized monkeys (D, SS4; E, SS5; F, SS6) was used. The number of cells stained blue is expressed as a percentage (%) relative to the number of cells stained blue in the serum sampled before immunization for each monkey. Statistical significances are indicated as asterisks (**$P < 0.02$, *$P < 0.05$).

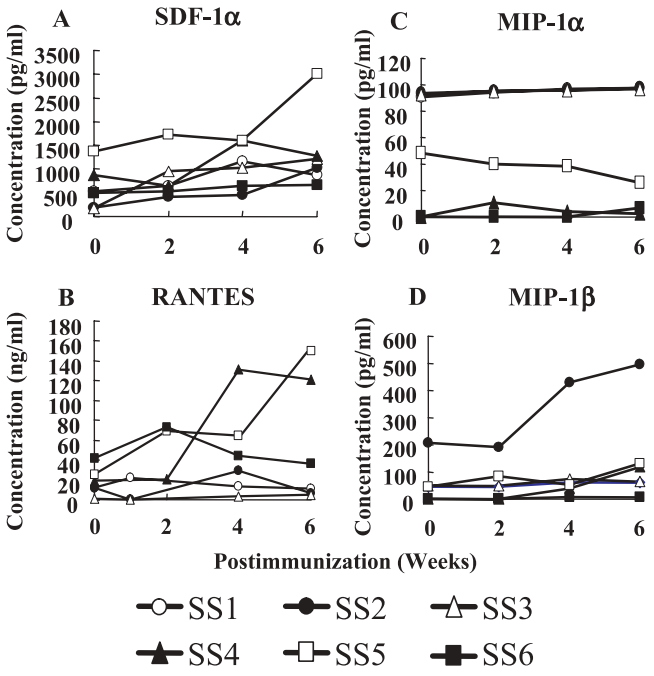

Fig. 7. **Determination of MIP-1α, MIP-1β, RANTES and SDF-1α levels in antisera.** The levels of MIP-1α, MIP-1β, RANTES and SDF-1α in antiserum samples obtained before, and 2, 4 and 6 weeks after immunization of the cCD-MAP– (SS1: open circles, SS2: solid circles and SS3: open triangles) or MAP-immunized monkeys (SS4: solid triangles, SS5: open squares and SS6: solid squares) were determined using specific antigen–captured enzyme-linked assay Quantikine kits.

mAbs prepared using the cCD-Multi-PIN Block in hybridoma screening were divided into four classes on the basis of their anti–HIV-1 activities (Figs. 2 and 3). Class 1 CPMAb-I and -II had potent anti–HIV-1 activity against the R5 and X4 (clade B) laboratory strains, rather than against the R5 and X4 (non–clade B) primary isolates. Class 2 CPMAb-III and -VI were effective against only the R5 (the clade B) laboratory strain and also against some (non–clade B) primary isolates. In contrast, Class 3 CPMAb-IV and -V had potent antiviral activities against the R5 and X4 primary isolates rather than the R5 and X4 laboratory strains. Class 4 CPMAb-VII was highly effective against most of the R5 and X4 HIV-1 primary isolates (R5 of clades A and E, X4 of clades A, C, and E) and clade B laboratory strains used in this study. These mAbs provided unique protection against infection by the clade B laboratory strains and non–clade B primary isolates. These unique protective properties were likewise observed when the unbound Abs were completely washed away before the inoculation of the virus in the MAGIC-5 assay. Moreover, the addition of mAbs to the cell culture 2 h post-infection had little effect on the viral infections. Therefore, we concluded that these mAbs concomitantly recognize both CCR5 and CXCR4. It should be noted that Abs raised against cCD-MAP have a potent and broad-spectrum inhibitory activity against primary isolates. These results suggest that polyclonal antibodies against cCD *in vivo* prevent not only infection by clade B but also infections by clades A, C and E regardless of the receptors (CCR5 or CXCR4) they use. The activities of anti–cCD-MAP Abs are summarized in Table 1. The differences in anti–HIV-1 activities to multiclade HIV-1 among the Abs may be dependent on delicate conformational wobbling motions induced by protein–protein interactions in receptor-antibody binding affinity, and on the differences in the conformations recognized by the antibodies, because some recent studies have shown that CCR5 and CXCR4 on the cell surface exist in various conformations or active forms (*37*, *38*).

Of all the mAbs tested for their effects on chemotaxis through CCR5 and CXCR4, CPMAb-II inhibited chemotaxis mediated by both these receptors in a dose-dependent manner within a low concentration range, while the other mAbs showed no or only mild inhibition of chemotaxis. Our results show that most mAbs against CCR5 and CXCR4 induced by cCD-MAP immunization preserve the chemotaxis mediated by these receptors. Moreover, we confirmed that some mAbs we tested did not inhibit chemokine/receptor interactions (data not shown).

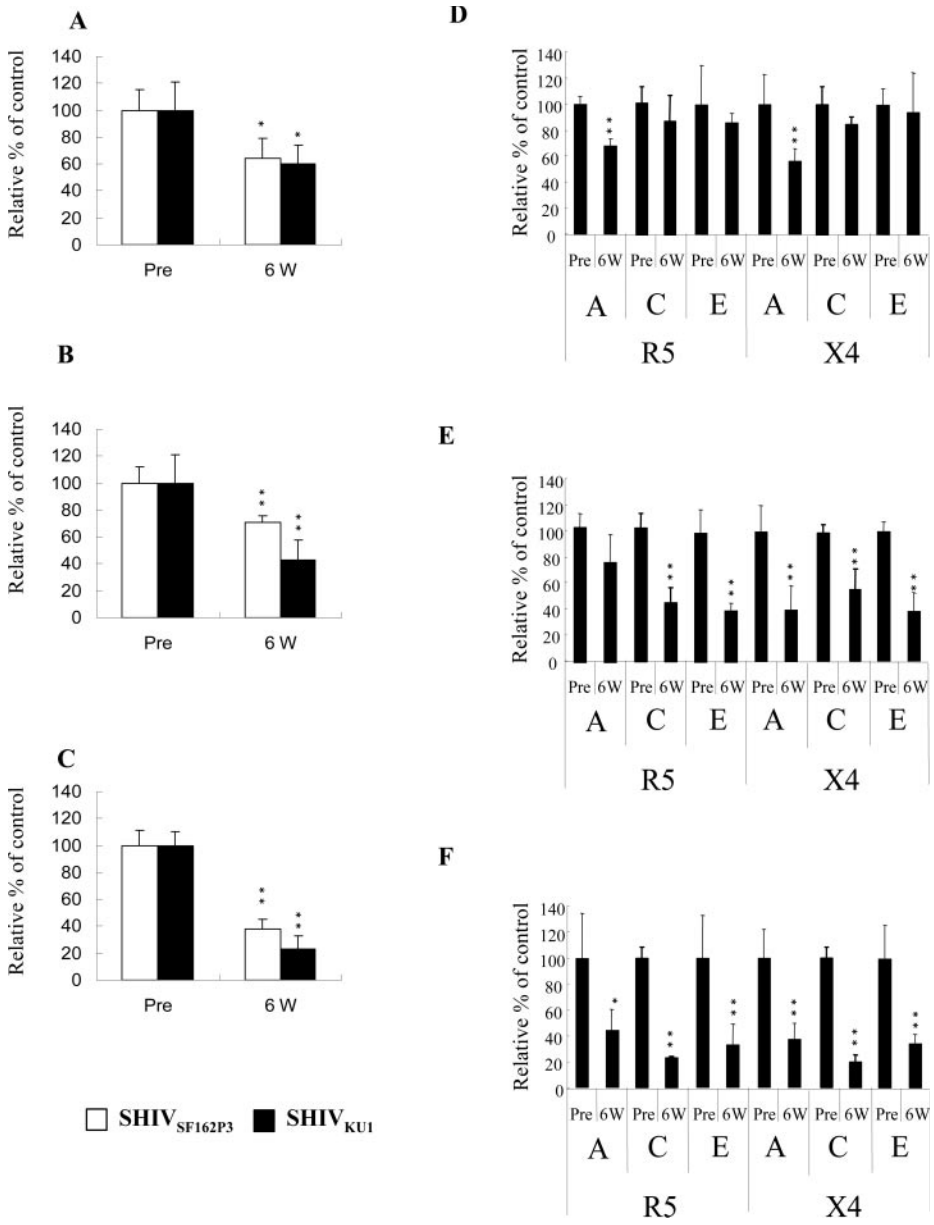

Fig. 8. **Effects of antisera from cCD-MAP–immunized monkeys before and 6 weeks after immunization on SHIV or non–clade B HIV-1 infection.** MAGIC-5 cells were inoculated with SHIV (A–C) or non–clade B (D–F) HIV-1 in the presence of antisera diluted 32-fold with PBS(–) from the monkeys (SS1; A and D, SS2; B and E, SS3; C and F). For $SHIV_{SF162P3}$ (open columns) and $SHIV_{KU1}$ (closed columns) infections, the serum samples obtained before and 6 weeks after immunization of the monkeys were used. For non–clade B HIV-1 infection, HIV-$1_{93RW004}$ (R5, clade A), HIV-1_{MJ4} (R5, clade C), HIV-$1_{92TH009}$ (R5, clade E), HIV-$1_{92UG029}$ (X4, clade A), HIV-$1_{98IN017}$ (X4, clade C) and HIV-1_{CMU08} (X4, clade E) were used. The number of cells stained blue is expressed as a percentage (%) relative to the number of cells stained blue in the serum obtained before immunization for each monkey. Statistical significances are indicated as asterisks (**$P < 0.02$, *$P < 0.05$).

cCD-MAP Immunogenicity in Cynomolgus Monkeys—The immunization of cynomolgus monkeys induced cCD-specific Abs within at most 6 weeks after immunization, as shown by the results of the BIAcore analysis, although some differences in cCD-specific antibody induction were observed among the monkeys. The antisera sampled from SS1, SS2 and SS3 6 weeks after immunization specifically recognized CCR5- and CXCR4-expressing MAGIC-5 cells, and inhibited HIV-1 infections by the R5 and X4 laboratory strains of clade B (Fig. 6, A–C). Interestingly, the antiserum sampled from SS3 6 weeks after immunization did not sufficiently immunoreact with the BIAcore sensor chip (Fig. 5D), but it immunoreacted satisfactorily with native cell surface chemokine receptors, and inhibited HIV-1_{JRFL} and HIV-1_{LAV-1} infections (Fig. 6C). We next examined whether antisera can broadly inhibit infection by two SHIV bulk isolates and primary HIV-1 isolates. The antisera sampled 6 weeks after immunization also inhibited R5 and X4 SHIV (SF162P3 and KU1) infections. In addition, the antisera from SS2 and SS3 also broadly and potently inhibited infections by five and all six HIV-1 isolates of non-clade B, respectively (Fig. 8, E and F), while that from SS1 partially inhibited infections by only two HIV-1 isolates of non-clade B with a weak antiviral activity (Fig. 8D). The antiviral activities against multiclade R5 and X4 HIV-1 isolates used here correlated with the induction of anti-cCD antibodies, but not with the strength of the response of those antibodies to cCD antigens as determined by BIAcore analysis. We failed to resolve this discrepancy directly, but some critical points in determining the induction of cCD-specific Abs may be involved. One is that the titer of Abs from SS1 determined by ELISA 6 weeks after immunization (1:10) was lower than the titers from SS2 and SS3 (1:100 and 1:400, respectively). Moreover, the flexibility of the cCD moiety corresponding to the antibody recognition site that was immobilized on the BIAcore

Table 1. **Classification of CPMAb-I-VII based on antiviral activities against clade B and non-clade B HIV-1.**

Class	CPMAb	Anti-HIV-1 activity[a]				Chemotaxis[b]	
		Clade B (laboratory strain)		Non-clade B (primary isolate)			
		R5	X4	R5	X4	MIP-1β	SDF-1α
1	I	+++	+++	+ (E)	–	→	→
	II	+++	+++	–	–	↓	↓
2	III	+++	–	–	++ (C)	→	→
	VI	+++	–	+++ (A), ++ (C)	+++ (A, C), ++ (E)	↑	↑
3	IV	–	–	++ (C, E), + (A)	–	→	↑
	V	–	–	+++ (E), + (A)	++ (E)	→	↑
4	VII	++	+	+++ (A, E)	+++ (A, C), ++ (E)	↑	↑

[a]Anti–HIV-1 activity was determined by the MAGIC-5 assay as described in "MATERIALS AND METHODS," and is represented as the average of duplicate experiments. +++: 80–90%, ++: 70–80%; +: 60–70%; ±: 50–60%, –: ∼50%. The clades of primary isolates affected are shown in parentheses, [b]Chemotaxis was determined as described in "MATERIALS AND METHODS," and relative % of chemotaxis index is presented by arrows: →, no effect; ↓, effective; ↑, enhanced chemotaxis at 5 μg/ml.

sensor chip would have been restricted. Therefore, the titers of Abs induced by a cCD-MAP–derived antigen with more flexible cCD moiety *in vivo* could be underestimated by the BIAcore sensor chip. Further analyses based on the molecular structure of the native chemokine receptors CCR5 and CXCR4, and the cCD antigen *in vivo* may also provides further classification of the similarities between these molecules. Taken together, our results suggest that not only Abs fitted against the cCD antigen immobilized on the sensor chip, but also multiple cCD-MAP–induced Abs with higher anti–HIV-1 and receptor-binding activities or either activity are induced in cynomolgus monkeys. Furthermore, James *et al*. (*39*) have recently demonstrated that an identical antibody existing in two conformations can bind to two structurally distinct antigens. Therefore, cCD-MAP may induce Abs capable of recognizing both CCR5 and CXCR4, and/or either one of them without causing immunological tolerance. In addition, recent report by Thompson *et al*. (*40*) showed that, in contrast to clade B isolates, a cluster of residues in the second extracellular loop of CCR5 significantly affected the fusion and entry of all non–clade B isolates they tested. This report also supports our present results.

In this study, we demonstrate that Abs against cCD-MAP cross-inhibit infection by the intraclade and interclade R5 and X4 HIV-1 strains *in vitro*. These results suggest that the induction of polyclonal Abs against the cCD antigen *in vivo* contributes to the prevention of multiclade HIV-1 infection. Therefore, cCD-MAP immunization may result in the sufficient induction of antibodies against CCR5 and CXCR4 for cross-clade HIV-1 inhibition, and that cCD-MAP immunization may be a useful self-defense strategy for overcoming the worldwide AIDS epidemic.

We thank Dr. S. Harada and Dr. Y. Maeda (Kumamoto University) for providing the NP2 cell lines. We also thank Dr. M. Tatsumi (National Institute of Infectious Diseases, Tokyo, Japan) for providing the MAGIC-5 cells. BIAcore analysis was supported by the Center for AIDS Research, Kumamoto University. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Health, Labour and Welfare of Japan, Japan Health Science Foundation, and the Ministry of Education, Culture, Sports, Science and Technology of Japan. The following strains were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: non-clade B strains; HIV-$1_{93RW004}$, HIV-$1_{92TH009}$, HIV-1_{MJ4}, HIV-$1_{92UG029}$, HIV-1_{CMU08}, and HIV-$1_{98IN017}$, and SHIVs; SHIV-$1_{SF162P3}$ and $SHIV_{KU1}$.

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