Recombinant p51 as Antigen in an Immune Complex Transfer Enzyme Immunoassay of Immunoglobulin G Antibody to Human Immunodeficiency Virus Type 1

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Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT), a heterodimer of p66 and p51 (p66 devoid of C-terminal amino acids) (6, 28), has been reported to be advantageous over that of rp66 and rRT in an immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 rather long DNA fragment (3,012 bp) of the whole HIV-1 pol gene, and it was impossible to produce fusion proteins of RT for simple purification, since rRT is a heterodimer of p66 and p51. In this study, recombinant HIV-1 p51 and p66 with Ser-Ser at the N termini (Ser-Ser-rp51 and Ser-Ser-rp66) were produced in E. coli as fusion proteins with maltose binding protein containing a factor Xa site between the two proteins and were purified after digestion with factor Xa. Ser-Ser-rp51 was produced in larger amounts and purified in higher yields with less polymerization than Ser-Ser-rp66. Polymerized Ser-Ser-rp66 tended to be precipitated on mercaptoacetylation for conjugation to β-D-galactosidase (used as a label) and showed higher nonspecific and lower specific signals in an immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 than Ser-Ser-rp51. The signals for serum samples of HIV-1-seropositive subjects by immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 using Ser-Ser-rp51 as antigen (Y) were well correlated to those obtained using rRT as antigen (X) (log Y = 0.99 log X + 0.23; r = 0.99). Thus, the use of rp51 as antigen was advantageous over that of rp66 and rRT in an immune complex transfer enzyme immunoassay of antibody IgG to HIV-1.

An ultrasensitive enzyme immunoassay (immune complex transfer enzyme immunoassay) of antibody immunoglobulin G (IgG) to human immunodeficiency virus type 1 (HIV-1) has been developed using recombinant HIV-1 reverse transcriptase (rRT) as antigen. However, some disadvantages were noted in the use of rRT as antigen: rRT was produced only with low efficiency in widely used strains of Escherichia coli using a rather long DNA fragment (3,012 bp) of the whole HIV-1 pol gene, and it was impossible to produce fusion proteins of RT for simple purification, since rRT is a heterodimer of p66 and p51. In this study, recombinant HIV-1 p51 and p66 with Ser-Ser at the N termini (Ser-Ser-rp51 and Ser-Ser-rp66) were produced in E. coli as fusion proteins with maltose binding protein containing a factor Xa site between the two proteins and were purified after digestion with factor Xa. Ser-Ser-rp51 was produced in larger amounts and purified in higher yields with less polymerization than Ser-Ser-rp66. Polymerized Ser-Ser-rp66 tended to be precipitated on mercaptoacetylation for conjugation to β-D-galactosidase (used as a label) and showed higher nonspecific and lower specific signals in an immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 than Ser-Ser-rp51. The signals for serum samples of HIV-1-seropositive subjects by immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 using Ser-Ser-rp51 as antigen (Y) were well correlated to those obtained using rRT as antigen (X) (log Y = 0.99 log X + 0.23; r = 0.99). Thus, the use of rp51 as antigen was advantageous over that of rp66 and rRT in an immune complex transfer enzyme immunoassay of antibody IgG to HIV-1.

Recently, an ultrasensitive enzyme immunoassay (immune complex transfer enzyme immunoassay) for antibody immunoglobulin G (IgG) to HIV-1 using rRT as antigen and β-D-galactosidase from Escherichia coli as label has been developed (13). Antibody IgG to RT was allowed to react simultaneously with 2,4-dinitrophenyl-bovine serum albumin-rRT conjugate and rRT–β-D-galactosidase conjugate, and the immune complex of the three components formed was trapped on polystyrene beads coated with (anti-2,4-dinitrophenyl group) IgG. After washing, the immune complex was transferred to polystyrene beads coated with (anti-human IgG γ-chain) IgG in the presence of 2,4-dinitrophenyl-L-lysine. By this enzyme immunoassay, which was 300- to 1,000-fold more sensitive than Western blotting for p66 band, diagnosis and confirmation of HIV-1 infection using urine (7, 8, 13), whole saliva (19, 20), and serum (9) have become more reliable than by previous methods. Notably, diagnosis of HIV-1 infection was possible using even 1 µl of whole saliva (19). However, the following disadvantages were noted in the use of rRT as antigen. rRT had to be produced using a rather long (3,012-bp) DNA fragment of the whole HIV-1 pol gene (1, 26) and was not efficiently produced in widely used strains of E. coli. In addition, it was impossible to produce fusion proteins of RT for simple purification since rRT is a heterodimer of p66 and p51, as described above.

This report describes the preparation of recombinant HIV-1 p51 and its use as antigen in an immune complex transfer

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enzyme immunoassy of antibody IgG to HIV-1 in comparison with those of recombinant HIV-1 p66 and rRT.

MATERIALS AND METHODS

Enzymes and competent E. coli cells. Recombinant Taq DNA polymerase (Takara Taq) and ligase were obtained from Takara Shuzo Co., Kyoto, Japan. Restriction enzymes were obtained from New England Biolabs, Inc., Beverly, Mass. Competent cells of E. coli DH5α and BL21 were obtained from Life Technologies, Rockville, Md., and Novagen Inc., Madison, Wis., respectively.

Construction of plasmids. Plasmids for the production of recombinant HIV-1 protein (p66) and p51 (p51) were produced using HIV-1 p66 and p51 DNAs of pNL4-3 (25) by the emulsion-polymerase chain reaction (PCR) method as described from pNL4-3 by treatment with NcoI and SalI (29). The sequences of p66 and p51 derived from HIV-1 were used for the construction of pNL4-3 (29). The sequences of p66 and p51 derived from NY5. Oligodeoxynucleotides used were obtained from BEX, Tokyo, Japan.

(i) pMALBMBPSP2SP1Ser-Ser-p662121. The plasmid for producing a fusion protein of p66 having Ser-Ser at the N terminus with maltose binding protein (MBP) (MBP-Ser-Ser-p66-rp66) was constructed so as to link the two proteins with a spacer of 29 amino acids (Asn-Pro-Ser-Ser-Pro-Ser-Ser-Pro-Ser-Ser-Pro-Ser) derived from phi M13 (27). The factor Xa site was located in the common region of the p66 sequence and the p51 sequence in pMALBMBPSP2SP1Ser-Ser-p662121 and was inserted into pMAL-c2 and pSEp51 from pSE420.

(ii) pMALBMBPSP2SP1Ser-Ser-p667. The DNA fragment was treated with BglII-GATCCGTCTCATCGATCCTC and 5′-GGGTGAGAAGTTTCTGCTCCTATTA so as to have Ser-Ser-rp66 as described above for Ser-Ser-rp51 (Fig. 3). First, HIV-1 p66 DNA was prepared from pLITSer-Ser-p667 by treatment with HincII and was inserted into pMALBMBPSP2SP1Ser-Ser-p662121 after digestion with EcoRI and SpeI. The p66 derivative was produced from pLITSer-Ser-p667 by treatment with HincII and SpeI and inserted into pMALBMBPSP2SP1Ser-Ser-p662121 after digestion with EcoRI and SpeI.

(iii) pMALBMBPSP2SP1Ser-Ser-rp513121. The DNA fragment was treated with BglII-SalI and inserted into pMALBMBPSP2SP1Ser-Ser-p662121 after digestion with BglII and SalI.

(iv) pMALBMBPSP2SP1Ser-Ser-rp513121. The plasmid for producing a fusion protein of p51 with MBP (MBP-Ser-Ser-rp51) was constructed so as to link the two proteins with a spacer of 31 amino acids including two serine residues at the N terminus of p51 as described above for Ser-Ser-rp66 (Fig. 3). First, HIV-1 p51 DNA was produced by PCR using pNL5ab as template and two primers (5′-GACCGCGACCATCAGTTGC TATTGAGA and 5′-GGTCTGATCTAGATCTTTGAGTCATGC) so as to have SalI and XbaI sites at the 5′ and 3′ ends, respectively, and 1,680 bp (nucleotides 2,550 to 4,239) corresponding to p51 and was inserted into pMALBMBPSP2SP1 by treatment with SalI and XbaI (pMALBMBPSP2SP1p662001). Third, a DNA fragment (second spacer DNA, SP2) containing EcoRI and XbaI sites at its 5′ and 3′ ends, respectively, was produced by melting out two oligodeoxynucleotides (5′-AAATCGAAGAGATGACGATGAAGCTCAGCTGTCGACCCATTAGTCCTATGCCTGGATGCTCTGCTAAGCTGA and 5′-AATCGAAGAGATGACGATGAAGCTCAGCTGTCGACCCATTAGTCCTATGCCTGGATGCTCTGCTAAGCTGA) and inserting it into pMAL-c2 (New England Biolabs) after digestion with SalI and HindIII (pMALBMBPSP1102). Second, HIV-1 p66 DNA was produced by PCR using pNL5ab as template and two primers (5′-GACCGCGACCATCAGTTGC TATTGAGA and 5′-GGTCTGATCTAGATCTTTGAGTCATGC) so as to have SalI and XbaI sites at the 5′ and 3′ ends, respectively, and 1,680 bp (nucleotides 2,550 to 4,239) corresponding to p66 and was inserted into pMALBMBPSP1102 by treatment with SalI and XbaI (pMALBMBPSP1p662001).

Purification of recombinant proteins. Purification was carried out at 4°C throughout, except for the final chromatography with Ultrogel AcA44 (Biosepsis, Villeneuve la Garenne, France) at room temperature. Protein concentrations were determined with a bicinechonic acid protein assay reagent kit (Pierce, Rockford, Ill.).

(i) pSer-Ser-rp51. The cells from 18 liters of culture medium were suspended in 900 ml of buffer A, sonicated, and centrifuged at 25,000 × g for 20 min. The supernatant fluid (920 ml) was brought to 35% saturation of (NH4)2SO4 by addition of solid (NH4)2SO4 (192 g) and, after 20 min of stirring, centrifuged at 25,000 × g for 20 min. The supernatant fluid (1,000 ml) was brought to 60% saturation of (NH4)2SO4 by addition of solid (NH4)2SO4 (164 g) and, after 20 min of stirring, centrifuged at 12,000 × g for 20 min. The precipitate between the 35 and 60% saturations was dissolved in 150 ml of buffer B and dialyzed twice against 50 ml of buffer C. The dialysate (150 ml, 250 ml in volume of buffer B) was frozen at −20°C after the addition of solid 150 ml of buffer C (1.0 M NaCl, 10 mM EDTA, 10 mM sodium phosphate buffer, pH 7.0, containing 0.1% Triton X-100 (polyethylene glycol monor-n-octylphenyl ether) and was prepared for use.

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FIG. 1. Construction of plasmids for the production of MBP-Ser-Ser-rp66.
FIG. 2. Construction of pMALMBP2SP1SerSer662121 from pMALMBP2SP1p662101.

pLITp663

HIV-1 p66 →
Proile ***

5' *** AGATCT *** CGTC TCGACCATT *** A AGCTT ***
3' *** TCTAGA *** GCAGACCT GGGTAA *** TTCGA A ***

BgI II BsmB I Hind III

Fragment Ser-Ser

5' GATCT *** CGTCTCG ACCATT *** A AGCTT ***
3' *** TCTAG GCAGAGCTGGG TAA *** TTCGA A ***

BgI II BsmB I Hind III

yArgSerSe

BsmB I

yArgSerSe rProile ***

5' *** A GATCGCGTCTCATCGATCCTC ACCCATT *** A AGCTT ***
3' *** TCTAG GCAGAGCTAGCTAGGATGGG TAA *** TTCGA A ***

BgI II BsmB I Hind III

pLITSer-Ser-p667

Factor Xa Site HIV-1 p66 →

5' *** IleGluG1 yArgProile ***
3' *** ATCGAAGG TCGACCATT GGGTAA *** TTCGA A ***

Sal I Hind III

pMALMBPSP2SP1p662101
digested with Sal I and Hind III

BgI II/BsmB I

BsmB I-Hind III

fragment of
pLITSer-Ser-p667

pMALMBPSP2SP1-
Ser-Ser-p662121

Factor Xa Site HIV-1 p66 →

5' *** ATCGAAGG TCGACCCTC ACCCATT *** A AGCTT ***
3' *** TCTAGGCAGAGTAGCT AGGATGGG TAA *** TTCGA A ***

Sal I Hind III

pMALMBPSP2SP1-
Ser-Ser-p662121

yArgSerSe rProile ***

5' *** A GATCGCGTCTCATCGATCCTC ACCCATT *** A AGCTT ***
3' *** TCTAG GCAGAGCTAGCTAGGATGGG TAA *** TTCGA A ***

BgI II BsmB I Hind III
with buffer B, MBP-Ser-Ser-rp51 was eluted with 450 ml of buffer B containing 0.1 M NaCl. The eluate (125 ml) was applied to a column (5.0 by 6.0 cm) of Amylose resin in buffer B. After washing with buffer B containing 0.1 M NaCl, MBP-Ser-Ser-rp51 was eluted with buffer B containing 10 mM maltose. The eluate (41 ml) was adjusted to a concentration of 0.8 M (NH₄)₂SO₄ by addition of 440 µl of 10% Triton X-100 in water and 500 µl of a 1 mg/ml factor Xa solution at 23°C for 20 h. The reaction mixture was dialyzed against 2 liters of buffer H overnight, and the dialysate (50 ml, 129 mg of protein) was applied to a column (2.5 by 6.0 cm) of SP Sepharose FF in buffer H. After washing with buffer H, Ser-Ser-rp51 was eluted with 150 ml of 0.2 M sodium phosphate buffer, pH 6.8, containing 0.1% Triton X-100. The eluate fractions (18 and 23 ml, 17 mg of protein) were combined and concentrated with Centricon 30. The concentrated fractions (1.1 ml) were applied to a column (1.5 by 56 cm) of Ultrogel AcA44 in buffer G. Fractions 61 to 71 (11 ml) were concentrated to 1.7 ml containing 7.2 mg of protein with Centricon 30 and were used for labeling with 2,4-dinitrophenyl groups and β-D-galactosidase.

Antibodies. Rabbit (anti-2,4-dinitrophenyl–bovine serum albumin) serum and rabbit anti-MBP serum were obtained from Shibayagi, Gumma, Japan. Rabbit (anti-human IgG γ-chain) IgG was obtained from Medical and Biological Laboratories, Nagoya, Japan. IgG was prepared from serum by fractionation with Na₂SO₄ followed by passage through a column of diethylaminoethyl cellulose (17).

Coupling of proteins to Sepharose 4B. Rabbit (anti-MBP) IgG (10 mg), 2,4-dinitrophenyl–bovine serum albumin (10) (10 mg), and human IgG (10 mg) were coupled to 1.0 g of CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) in accordance with the manufacturer's instruction.

Affinity purification of antibodies. Affinity-purified (anti-2,4-dinitrophenyl–bovine serum albumin) IgG (22) and (anti-human IgG γ-chain) IgG (23) were prepared by elution at pH 2.5 from columns of 2,4-dinitrophenyl–bovine serum albumin–Sepharose 4B and human IgG-Sepharose 4B, respectively.

Coating of polystyrene beads with proteins. Colored and white polystyrene beads (3.2 mm in diameter; Immuno Chemical, Okayama, Japan) were coated by physical adsorption with affinity-purified (anti-2,4-dinitrophenyl–bovine serum albumin) IgG (0.1 g/liter) and affinity-purified (anti-human IgG γ-chain) IgG (0.1 g/liter), respectively (18).
Labeling of recombinant proteins with 2,4-dinitrophenyl groups. Mercaptoacetyl-Ser-Ser-rp51. Ser-Ser-rp51 (0.84 mg, 16 nmol) in 1.0 ml of buffer G was incubated with 30 μl of 7.2 mM N-succinimidyl-3-acylmercaptoacetate (Pierce) in N,N-dimethylformamide at 30°C for 30 min and was processed as described previously (14, 17). The average number of thiol groups introduced per Ser–Ser–rp51 molecule was 4.0 (17).

(i) 2,4-Dinitrophenyl-Ser–Ser–rp51 conjugate. Mercaptoacetyl-Ser-Ser-rp51 (0.48 mg, 9.5 nmol) in 1.2 ml of buffer G was incubated at 4°C for 20 h with N,N-6-maleimidohexanoyl-ε-N,2,4-dinitrophenyl-L-lysine solution, which had been prepared by incubation of ε-N,2,4-dinitrophenyl-L-lysine hydrochloride (Sigma) (0.18 mg, 0.46 μmol) in 91 μl of N,N-dimethylformamide with 0.25 μl of 0.1 M sodium hydroxide, pH 7.0, and N,N-succinimidyl-6-maleimidohexanoate (Dojindo Laboratories, Kumamoto, Japan) (0.12 mg, 0.38 μmol) in 38 μl of N,N-dimethylformamide at 30°C for 30 min. The reaction mixture was subjected to gel filtration on a Sephadex G-25 medium (Amersham Pharmacia Biotech) column (1.0 by 30 cm) in buffer G containing 0.1 M NaCl. The average number of 2,4-dinitrophenyl groups introduced per Ser-Ser-rp51 molecule was 3.1 (10). Fractions containing the conjugate were stored at 4°C after addition of bovine serum albumin (0.1 g/liter) and NaN₃ (1 g/liter) until use.

(ii) 2,4-Dinitrophenyl-Ser–Ser–rp56 conjugate. 2,4-Dinitrophenyl–Ser–Ser–rp56 conjugate was prepared using mercaptoacetyl-Ser-Ser-rp56 (0.24 mg, 3.7 nmol) and N,N-6-maleimidohexanoyl-ε-N,2,4-dinitrophenyl-L-lysine solution (71 μg, 109 nmol) as described above. HIV-1 rRT, prepared as described previously (26), was 2,4-dinitrophenylated using mercaptoacetyl-rRT (0.24 mg, 3.7 nmol) and N,N-6-maleimidohexanoyl-ε-N,2,4-dinitrophenyl-L-lysine solution (48 μg, 74 nmol) as described above.

Labeling of recombinant proteins with β-galactosidase. (i) Maleimide-β-D-galactosidase. β-D-Galactosidase from E. coli (Boehringer Mannheim, Mannheim, Germany) was treated with 4,4′-dithiodipyrined and N-succinimidyl-6-maleimidohexanoate as described previously (14). The average number of maleimide groups introduced per β-D-galactosidase molecule was 2.7 (17).

(ii) Mercaptoacetyl-Ser–Ser–rp51. Ser-Ser-rp51 (2.8 mg, 55 nmol) in 2.8 ml of buffer G of 0.1 M NaCl was incubated with 70 μl of 7.2 mM N-succinimidyl-3-acylmercaptoacetate in N,N-dimethylformamide at 30°C for 30 min and was processed as described previously (14, 17). The average number of thiol groups introduced per Ser-Ser–rp51 molecule was 1.8 (17).

(iii) Ser–Ser–rp51–β-D-galactosidase conjugate. Mercaptoacetyl-Ser-Ser-rp51 (0.77 mg, 15 nmol) in 2 ml of buffer G was incubated with maleimide-β-D-galactosidase (2.4 mg, 4.4 nmol) in 0.2 ml of buffer G at 4°C for 20 h. After incubation, the reaction mixture was incubated with 22 μl of 0.1 M NaCl and 0.01% Triton X-100. Fractions containing the conjugate were stored at 4°C until use. The average number of Ser–Ser–rp51 molecules conjugated per β-D-galactosidase molecule was 1.7 as calculated from the decrease in the number of maleimide groups (17). The amount of conjugate was calculated by β-galactosidase activity.

(iv) Ser–Ser–rp66–β-D-galactosidase and rRT–β-D-galactosidase conjugates. Ser–Ser–rp66–β-D-galactosidase and rRT–β-D-galactosidase conjugates were prepared using mercaptoacetyl-Ser-Ser-rp66 (0.15 mg, 23 nmol), maleimide-β-D-galactosidase (0.27 mg, 0.5 nmol), mercaptoacetyl-rRT (0.44 mg, 7.0 nmol), and N,N-succinimidyl-6-maleimidohexanoate (0.04 mg, 1.8 nmol) as described above for Ser–Ser–rp51–β-D-galactosidase conjugate.

Immune complex transfer enzyme immunoassay of antibody IgG to HIV-1. Immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 was performed essentially in the same way as described previously (11, 21). An aliquot (10 μl) of serum samples was incubated with 140 μl of buffer J containing 0.4 M NaCl and 0.01% Triton X-100. Fractions containing the conjugate were stored at 4°C until use. The average number of thiol groups introduced per Ser–Ser–rp51 molecule was 3.9 as described above.

RESULTS

Production and purification of recombinant HIV-1 proteins. In order to detect antibody IgG to HIV-1 RT (a heterodimer of p51 and p66), recombinant HIV-1 p51 and p66 with Ser-Ser at the N termini (Ser-Ser-rp51 and Ser-Ser-rp66) were produced as fusion proteins with MBP containing a factor Xa site between the two proteins in E. coli transformed with plasmids containing the corresponding DNAs and were purified from sonic extracts of the cells by successive processes of column chromatographies with DEAE Sepharose and Amylose resin, digestion with factor Xa, and column chromatographies with SF Sepharose, (anti-MBP) IgG-Sepharose, Affi-Gel heparin, hydroxyapatite, and Ultrogel AcA44.

Electrophoresis of sonic extracts indicated that MBP-Ser-Ser-rp51 was produced in much larger amounts per unit of culture medium volume than MBP-Ser-rp66. This was reflected in the fact that the amounts of MBP-Ser-Ser-rp51 and MBP-Ser-Ser-rp66 obtained from a culture medium volume of 18 liters which could be used for labeling with 2,4-dinitrophenyl groups and β-D-galactosidase were 7.2 and 1.0 mg (Peak II), respectively (Table 1). In addition, Ser-Ser-rp66 was converted to a mixture of almost equal amounts of Ser-Ser-rp66 and a smaller molecule, probably Ser-Ser-rp51, during purification and was polymerized to various extents in the eluate from a column of hydroxyapatite (Fig. 4). Polymerized Ser-Ser-rp66 tended to be precipitated on mercaptoacetylation for conjugation to β-D-galactosidase.

Comparison of Ser-Ser-rp51 with Ser-Ser-rp66 and rRT as antigens by immune complex transfer enzyme immunoassay. Ser-Ser-rp51 was compared with Ser-Ser-rp66 and rRT as antigens by immune complex transfer enzyme immunoassay of antibody IgG to HIV-1 using β-D-galactosidase from E. coli as label. rRT was produced in E. coli transformed with a plasmid containing the HIV-1 pol gene for protease, RT, and integrase and was purified as described previously (26). Serum samples (10 μl) were incubated with 2,4-dinitrophenyl–bovine serum albumin–antigen conjugate and antigen–β-D-galactosidase conjugate and subsequently with colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG to trap the immune complex of the three components formed. After washing, the colored polystyrene beads were incubated with 2,4-dinitrophenyl-L-lysine and white polystyrene beads coated with affinity-purified (anti-human IgG γ-chain) IgG to transfer the immune complex from the colored polystyrene beads to the white polystyrene beads was assayed by fluorometry.

(i) Dilution curves of two positive serum samples. When two serum samples from HIV-1-seropositive subjects serially diluted up to 10⁻³-fold from an HIV-1-seronegative subject were tested, the dilution curves obtained by using Ser-Ser-rp51 as antigen were almost completely parallel with those obtained by using rRT. However, Ser-Ser-rp66 showed much higher binding activity than rRT.
higher nonspecific and lower specific signals than Ser-Ser-rp51. Therefore, Ser-Ser-rp66 was not used in the following experiments.

(ii) Positive and negative signals for more serum samples.
The positive and negative signals were examined using serum samples from 79 HIV-1-seropositive subjects (50 asymptomatic carriers, 9 patients with ARC, and 20 patients with AIDS) and 200 HIV-1-seronegative subjects, respectively.

The positive signals obtained with Ser-Ser-rp51 as antigen \( (Y) \) were well correlated to those obtained with rRT as antigen \( (X) \) (log \( Y = 0.99 \log X + 0.23; r = 0.99 \)) (Fig. 5) and were up to 2.4-fold (1.6-fold, on average) higher than those obtained with rRT as antigen for 76 (96%) of the 79 seropositive subjects but equal or 1.2- to 1.7-fold lower for 3 seropositive subjects (4%) (Fig. 5 and 6). The negative signals obtained with Ser-Ser-rp51 (0.46 ± 0.34 [standard deviation]; range, 0 to 2.0) were not significantly different from those obtained with rRT (0.44 ± 0.34 [standard deviation]; range, 0 to 5.2). The lowest positive signals for the asymptomatic carriers and the patients with ARC and AIDS were 124,000-, 172,000-, and 13,000-fold, respectively, higher than the highest negative signal (Fig. 6). Namely, the sensitivity, specificity and positive and negative predictive values were all 100% (Table 2).

Comparison of immune complex transfer enzyme immunoassays using Ser-Ser-rp51 and rRT as antigens with Western blotting. Two serum samples from HIV-1-seropositive subjects serially diluted with serum from an HIV-1-seronegative subject were tested by immune complex transfer enzyme immunoassay using Ser-Ser-rp51 as antigen and Western blotting (Ortho HIV Western Blot Kit) for p51 and p66 bands. The former was 1,000- to 6,000-fold more sensitive than the latter.

### TABLE 1. Purification of Ser-Ser-rp66 and Ser-Ser-rp51

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol (ml)</th>
<th>Protein amt (mg)</th>
<th>Recovery (%)</th>
<th>Vol (ml)</th>
<th>Protein amt (mg)</th>
<th>Recovery (%)</th>
</tr>
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<tbody>
<tr>
<td>Culture medium</td>
<td>18,000</td>
<td>ND*</td>
<td></td>
<td>18,000</td>
<td>ND</td>
<td></td>
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<tr>
<td>Sonic extract</td>
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<td>ND</td>
<td></td>
<td>600</td>
<td>9,000</td>
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<tr>
<td>((\text{NH}_4\text{)}_2\text{SO}_4) fraction</td>
<td>183</td>
<td>ND</td>
<td></td>
<td>189</td>
<td>5,292</td>
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<tr>
<td>Eluate from DEAE Sepharose</td>
<td>295</td>
<td>ND</td>
<td></td>
<td>125</td>
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<td>Eluate from Amylose resin</td>
<td>19</td>
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<td>41</td>
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<tr>
<td>Eluate from butyl Sepharose</td>
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<td></td>
<td></td>
<td>44</td>
<td>131</td>
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<tr>
<td>Before factor Xa digestion</td>
<td>17</td>
<td>38</td>
<td>100</td>
<td>50</td>
<td>129</td>
<td>100</td>
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<tr>
<td>Eluate from SP Sepharose after factor Xa digestion</td>
<td>140</td>
<td>14</td>
<td>37</td>
<td>59</td>
<td>39</td>
<td>30</td>
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<tr>
<td>Flowthrough from (anti-MBP) IgG-Sepharose</td>
<td>151</td>
<td>13</td>
<td>34</td>
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<td>Eluate from Affi-gel heparin</td>
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<td>Eluate from hydroxyapatite</td>
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<td>6.3</td>
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<td>Eluate from Ultrogel AcA44</td>
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<td>1.0</td>
<td>2.6</td>
<td>4.1</td>
<td>2.6</td>
<td>1.1</td>
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</table>

* ND, not determined.

![FIG. 4. Elution profiles of Ser-Ser-rp66 (A) and Ser-Ser-rp51 (B) from a column of Ultrogel AcA44. The flow rate was 0.4 ml/min, and the fraction volume was 1.0 ml. See Materials and Methods for other conditions.](image1)

![FIG. 5. Correlation between signals by immune complex transfer enzyme immunoassays using Ser-Ser-rp51 and rRT as antigens. Circles, triangles, and squares indicate serum samples from HIV-1-asymptomatic carriers and patients with ARC and AIDS, respectively. The regression equation was log \( Y \) (signals with Ser-Ser-rp51) = 0.99 log \( X \) (signals with rRT) + 0.23, and the correlation coefficient was 0.99.](image2)
In addition, immune complex transfer enzyme immunoassays using Ser-Ser-rp51 and rRT as antigens were compared with Western blotting using the 79 serum samples from HIV-1-seropositive subjects and the 100 to 200 serum samples from HIV-1-seronegative subjects described above (Fig. 6 and Table 2). The detection rates of antibody IgG to gp160, gp41, p66, p51, p24, and p17 by Western blotting were 100, 99, 96, 90, 87, and 71%, respectively. The specificities and predictive values of Western blotting were less than 100% (82 to 99%), except that the specificity and positive predictive value for p66 band and the negative predictive values for gp160 and gp41 bands were 100%. In contrast, the detection rates of antibody IgG to HIV-1 by immune complex transfer enzyme immunoassays using Ser-Ser-rp51 and rRT as antigens and the specificities and predictive values of immune complex transfer enzyme immunoassays using Ser-Ser-rp51 and rRT were all 100%.

Comparison of immune complex transfer enzyme immunoassay using Ser-Ser-rp51 as antigen with the conventional ELISA using five recombinant proteins as antigen. Immune complex transfer enzyme immunoassay with Ser-Ser-rp51 as antigen was compared with the conventional ELISA using five recombinant proteins (gp120, gp41, p24, p17, and p15) as antigens using the 79 serum samples from HIV-1-seropositive subjects and the 131 to 200 serum samples from HIV-1-seronegative subjects described above (Table 2). The sensitivity, specificity, and predictive values of the immune complex transfer enzyme immunoassay were all 100%. However, the specificity and positive predictive value of the conventional ELISA were 99%. Furthermore, the lowest signals for the seropositive subjects by immune complex transfer enzyme immunoassay and the conventional ELISA were 13,000- and 124-fold, respectively, higher than the highest signals for the seronegative subjects.

**DISCUSSION**

Both Ser-Ser-rp51 and Ser-Ser-rp66 were readily produced as fusion proteins with MBP for easy purification using short

**TABLE 2. Sensitivity, specificity, and predictive values of various methods**

<table>
<thead>
<tr>
<th>Method and antigen(s) used</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no. positive/no. tested)</td>
<td>(no. negative/no. tested)</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>Immune complex transfer enzyme immunoassay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rRT</td>
<td>100 (79/79)</td>
<td>100 (200/200)</td>
<td>100</td>
</tr>
<tr>
<td>Ser-Ser-rp51</td>
<td>100 (79/79)</td>
<td>100 (200/200)</td>
<td>100</td>
</tr>
<tr>
<td>Western blotting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gp160</td>
<td>100 (79/79)</td>
<td>99 (99/100)</td>
<td>99</td>
</tr>
<tr>
<td>gp41</td>
<td>99 (78/79)</td>
<td>98 (98/100)</td>
<td>98</td>
</tr>
<tr>
<td>p66</td>
<td>96 (76/79)</td>
<td>100 (100/100)</td>
<td>100</td>
</tr>
<tr>
<td>p51</td>
<td>90 (71/79)</td>
<td>97 (97/100)</td>
<td>96</td>
</tr>
<tr>
<td>p24</td>
<td>87 (69/79)</td>
<td>85 (85/100)</td>
<td>82</td>
</tr>
<tr>
<td>p17</td>
<td>71 (56/79)</td>
<td>97 (97/100)</td>
<td>95</td>
</tr>
<tr>
<td>ELISA; gp120, gp41, p24, p17, p15</td>
<td>100 (79/79)</td>
<td>99 (130/131)</td>
<td>99</td>
</tr>
</tbody>
</table>
DNA fragments with 1,320 and 1,680 bp, respectively, while rRT had to be produced using a rather long DNA with 3,012 bp of the whole HIV-1 pol gene and could not be produced as a fusion protein with MBP due to the fact that RT is a heterodimer of p51 and p66 (1, 26). However, the use of Ser-Ser-rp51 as antigen in the enzyme immunoassay was advantageous over that of Ser-Ser-rp66 for the following reasons. Ser-Ser-rp51 was produced and purified in larger amounts (Table 1) and was much less polymerized (Fig. 4). In the immune complex transfer enzyme immunoassay, the conjugates of Ser-Ser-rp51 showed higher specific and lower nonspecific signals than those of Ser-Ser-rp66 and specific signals as high as those of rRT (Fig. 5 and 6).

The detection rate of antibody IgG to HIV-1 in 79 HIV-1-seropositive subjects by immune complex transfer enzyme immunoassay using Ser-Ser-rp51 as antigen was 100% (Fig. 6), while the detection rates of antibody IgGs to p51 and p66 by Western blotting in 79 HIV-1-seropositive subjects were 90 to 96% (both 96% in 50 asymptomatic carriers, both 100% in 9 patients with ARC, and 70% and 95%, respectively, in 20 patients with AIDS) (Table 2). This was consistent with a previous report that the detection rates of antibody IgG to HIV-1 by Western blotting for p51 and p66 bands were both 96% in asymptomatic carriers, 50 and 100%, respectively, in patients with ARC, and both 86% in patients with AIDS (13) and with the finding described above that the immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 using Ser-Ser-rp51 as antigen was 1,000- to 6,000-fold more sensitive than Western blotting for p66 and p51 bands when two serially diluted serum samples from HIV-1-seropositive subjects were tested.

In the immune complex transfer enzyme immunoassay using Ser-Ser-rp51 alone as antigen, the difference between the lowest signal for 79 HIV-1-seronegative subjects and the highest signal for 197 HIV-1-seropositive subjects was 13,000-fold (Fig. 6), while the difference was only 124-fold in the conventional ELISA using no less than five recombinant proteins (Fig. 6), while the difference was only 124-fold in the conventional ELISA using no less than five recombinant proteins (Fig. 5).

In summary, the use of Ser-Ser-rp51 as antigen was considered to be advantageous over the use of Ser-Ser-rp66 and rRT and the immune complex transfer enzyme immunoassay using Ser-Ser-rp51 as antigen seemed to be more competent for research and clinical purposes than Western blotting and the conventional ELISA.


