Rapid Formation of the Immune Complexes on Solid Phase in the Immune Complex Transfer Enzyme Immunoassays for HIV-1 p24 Antigen and Antibody IgGs to HIV-1

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In order to perform the immune complex transfer enzyme immunoassays for HIV-1 p24 antigen and antibody IgGs to HIV-1 p17, reverse transcriptase and gp41 antigens as rapidly as possible, methods for rapid formation of the immune complexes on solid phase are described. HIV-1 p24 antigen was reacted with monoclonal anti-p24 Fab'- β -D-galactosidase conjugate at a high concentration and subsequently with polystyrene beads coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl bovine serum albumin-affinity-purified anti-p24 Fab' conjugate. Antibody IgGs to HIV-1 were reacted with polystyrene beads coated successively with affinity-purified

(anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-HIV-1 antigen conjugates and subsequently with HIV-1 antigen- β -D-galactosidase conjugates. The periods of time used for the formation of the immune complexes comprising the three components on the polystyrene beads (15–30 min) were much shorter than those used in the previous immune complex transfer enzyme immunoassays (90–300 min), and the sensitivities of the present and previous immune complex transfer enzyme immunoassays were similar. The detection limit of the HIV-1 p24 antigen by the present and previous methods were similar (3 to 10 zmol/assay). J. Clin. Lab. Anal. 12:227–237, 1998. © 1998 Wiley-Liss, Inc.

Key words: antibody; human immunodeficiency virus type 1; enzyme immunoassay; β -D-galactosidase

INTRODUCTION

Ultrasensitive enzyme immunoassays (immune complex transfer enzyme immunoassays) for antibody IgGs to HIV-1 p17, p24, and reverse transcriptase (RT) antigens have been developed using recombinant HIV-1 p17, p24, and RT (rp17, rp24, and rRT) as antigens (1–7). Antibody IgGs to HIV-1 were reacted simultaneously with 2,4-dinitrophenyl-recombinant HIV-1 antigen conjugates and recombinant HIV-1 antigen- β -D-galactosidase conjugates for 3 hr, and the immune complexes formed, comprising the three components, were trapped onto colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG by overnight incubation. After washing, the immune complexes were eluted from the colored polystyrene beads with ϵ N-2,4-dinitrophenyl-L-lysine and were transferred to white polystyrene beads coated with affinity-purified (anti-human IgG γ -chain) IgG by 3 hr incubation. Bound β -D-galactosidase activity was assayed by fluorometry for 2.5 hr. The volume of serum samples used was 10 μ L. These immunoassays have made reliable the diagnosis of HIV-1 infection with urine (1–4) and whole saliva samples (5,6) and have been shown to be more useful as

a confirmatory test for HIV-1 infection with higher sensitivities and specificities than Western blotting (7). Notably, antibody IgG to HIV-1 p17 antigen could be detected as early as or even earlier than antibodies to HIV-1 by conventional methods (7,8). In addition, an ultrasensitive enzyme immunoassay for HIV-1 p24 antigen has also been developed by applying the principle of immune complex transfer (9,10). On the basis of these developments, the window period after HIV-1 infection, during which diagnosis of the infection is not possible due to the absence of detectable antibodies to HIV-1, has been shortened by simultaneous detection of both HIV-1 p24 antigen and antibody IgGs to HIV-1 p17 and RT antigens (8). Recently, the periods of time for trapping and transferring of the immune complexes using the colored and white polystyrene beads have been shortened considerably by incubation with shaking (11). However, the periods of time for the formation of the immune

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complexes on polystyrene beads are still long (1.5–5 hr) in the immune complex transfer enzyme immunoassays (12,13).

This article describes methods for rapid formation of the immune complexes comprising the three components on polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG to perform the immune complex transfer enzyme immunoassays for HIV-1 p24 antigen and antibody IgGs to HIV-1 p17, reverse transcriptase and gp41 antigens as rapidly as possible.

MATERIALS AND METHODS

Buffers

The regularly used buffer was 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl, 1 mmol/L MgCl₂, 1 g/L NaN₃, and 1 g/L bovine serum albumin (fraction V, Interger Company, Purchase, NY) (buffer A). In other buffers, the concentration of NaCl was 0.4 mol/L (buffer B), and the concentration of bovine serum albumin was 0.1 g/L (buffer C).

2,4-Dinitrophenyl-Biotinyl-Bovine Serum Albumin-Affinity-Purified-Rabbit Anti-p24 Fab' Conjugate

Affinity-purified rabbit anti-p24 Fab' was reacted with 6-maleimidohehexanoyl-2,4-dinitrophenyl-biotinyl-bovine serum albumin (10).

Monoclonal Mouse Anti-p24 Fab'-β-D-Galactosidase Conjugate

Monoclonal mouse anti-p24 Fab' was conjugated to β-D-galactosidase from *Escherichia coli* using *o*-phenylenedimaleimide (10).

2,4-Dinitrophenyl-Maltose Binding Protein-rp17 Fusion Protein

Mercaptoacetylated maltose binding protein-rp17 fusion protein was reacted with αN-6-maleimidohehexanoyl-εN-2,4-dinitrophenyl-L-lysine (14).

2,4-Dinitrophenyl-Bovine Serum Albumin-rRT Conjugate

Mercaptoacetylated rRT was reacted with 6-maleimidohehexanoyl-2,4-dinitrophenyl-biotinyl-bovine serum albumin (1).

Synthetic HIV-1 gp41 Peptide

A synthetic HIV-1 gp41 peptide was obtained from Peptide Institute, Inc., Osaka, Japan. The amino acid sequence was H₂N-Cys-Arg-Leu⁵⁸⁸-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-Cys-Ser-Gly-Lys-Leu-Ile⁶¹⁰-COOH (Cys-Arg-env gp41(588–610)).

2,4-Dinitrophenyl-Bovine Serum Albumin-Synthetic HIV-1 gp41 Peptide Conjugate

Maleimide groups were introduced into 2,4-dinitrophenyl-bovine serum albumin molecules using *N*-succinimidyl-6-maleimidohehexanoate (2).

6-Maleimidohehexanoyl-2,4-dinitrophenyl-bovine serum albumin (0.65 mg, 9.9 nmol) in 0.45 mL of 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L EDTA was incubated with synthetic HIV-1 gp41 peptide (Cys-Arg-env gp41(588–610)) (145 μg, 50 nmol) in 0.05 mL of deionized water at 4°C for 20 hr. Subsequently, the reaction mixture was incubated with 10 μL of 0.1 mol/L 2-mercaptoethylamine in 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L EDTA at 30°C for 10 min and was subjected to gel filtration using a column (1.1 × 5.3 cm) of Sephadex G-50 fine (Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl. The average number of synthetic gp41 peptide molecules conjugated per albumin molecule was 4.0, which was calculated from the decrease in the number of maleimide groups. The amount of the conjugate was calculated from that of 2,4-dinitrophenyl-bovine serum albumin.

Recombinant HIV-1 Protein-β-D-Galactosidase Conjugates

Mercaptoacetylated rp17 and rRT antigens were reacted with maleimide groups introduced into β-D-galactosidase (EC 3.2.1.23) from *Escherichia coli* (Boehringer Mannheim GmbH, Mannheim, Germany) (1,15).

Synthetic HIV-1 gp41 Peptide-β-D-Galactosidase Conjugates

6-Maleimidohehexanoyl-β-D-galactosidase

β-D-Galactosidase from *Escherichia coli* (Boehringer Mannheim GmbH, Mannheim, Germany) (5.5 mg, 10.2 nmol) in 0.4 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0, was incubated with 21 μL of 40 mmol/L 4,4'-dithiodipyridine (Nacalai Tesque, Kyoto, Japan) in *N,N*-dimethylformamide at 30°C for 15 min. After incubation, the reaction mixture was subjected to gel filtration using a Sephadex G-50 fine column (1.1 × 5.3 cm) equilibrated with 0.1 mol/L sodium phosphate buffer, pH 7.0. The filtrate (500 μL) was incubated with 26.3 μL of 3.0 mmol/L *N*-succinimidyl-6-maleimidohehexanoate in *N,N*-dimethylformamide at 30°C for 15 min. After incubation, the reaction mixture was subjected to gel filtration using a Sephadex G-50 fine column (1.1 × 5.3 cm) equilibrated with 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5.0 mmol/L EDTA. The average number of maleimide groups introduced per β-D-galactosidase molecule was 3.4.

Synthetic HIV-1 gp41 peptide-β-D-galactosidase conjugate

6-Maleimidohehexanoyl-β-D-galactosidase (0.5 mg, 1.0 nmol) in 0.45 mL of 0.1 mol/L sodium phosphate buffer, pH

6.0 containing 5 mmol/L EDTA was incubated with synthetic gp41 peptide (Cys-Arg-*env* gp41(588–610)) (29 µg, 10 nmol) in 0.05 mL of deionized water at 4°C for 20 hr. Subsequently, the reaction mixture was incubated with 10 µL of 0.1 mol/L *N*-ethylmaleimide in 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L EDTA at 30°C for 10 min and was subjected to gel filtration using a column (1.1 × 5.3 cm) of Sephadex G-50 fine equilibrated with 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl. The average number of synthetic gp41 peptide molecules conjugated per β-D-galactosidase molecule was 3.4, which was calculated from the decrease in the number of maleimide groups. The amount of the conjugate was calculated from β-D-galactosidase activity.

Polystyrene Beads Coated With Affinity-Purified (Anti-2,4-Dinitrophenyl Group) IgG and Streptavidin

Colored polystyrene beads with a diameter of 3.2 mm and white polystyrene beads with a diameter of 6.35 mm (Immuno Chemical Inc., Okayama, Japan) were coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG (10 µg/mL) by physical adsorption (1). White polystyrene beads with a diameter of 3.2 mm (Immuno Chemical Inc.) were coated successively with biotinyl-bovine serum albumin (100 µg/mL) and streptavidin (100 µg/mL) by physical adsorption (9). The coated polystyrene beads were stored in buffer A at 4°C until use.

Polystyrene Beads Coated With 2,4-Dinitrophenyl-Biotinyl-Bovine Serum Albumin-Affinity-Purified Anti-p24 Fab' Conjugate

White polystyrene beads with a diameter of 6.35 mm coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG described above were incubated with 20 µL/bead of buffer A containing 200 and 400 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified anti-p24 Fab' conjugate in styrol test tubes (13.3 × 54 mm and 2.1 g, one bead/tube) for 5 min as described in the present immune complex transfer enzyme immunoassays. The coated polystyrene beads were stored in buffer A at 4°C until use.

Polystyrene Beads Coated With 2,4-Dinitrophenyl-HIV-1 Antigen Conjugates

White polystyrene beads with a diameter of 6.35 mm coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG as described above were incubated with buffer A containing 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein (400 and 800 fmol/15 µL/bead), 2,4-dinitrophenyl-bovine serum albumin-rRT conjugate (400 and 800 fmol/20 µL/bead) or 2,4-dinitrophenyl-bovine serum albumin-synthetic gp41 peptide conjugate (200 and 400 fmol/20 µL/bead) in styrol test tubes (13.3 × 54 mm and 2.1 g, one bead/tube) for 5 min as described in the present immune complex transfer enzyme

immunoassays. The coated polystyrene beads were stored in buffer A at 4°C until use.

Polystyrene Test Tubes Coated With Streptavidin

Polystyrene test tubes (12 × 75 mm, Maxisorp, A/S Nunc, DK-4000 Roskilde, Denmark) were coated by physical adsorption with 0.4 mL of 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 0.1% NaN₃ and 12 µg of biotinyl-bovine serum albumin and, after washing, with 0.4 mL of the same buffer containing 12 µg of streptavidin (9). The coated polystyrene tubes were stored with 0.5 mL of buffer A at 4°C until use.

Polystyrene Test Tubes Coated With Affinity-Purified (Anti-Human IgG γ-Chain) IgG

Polystyrene test tubes (12 × 75 mm, Maxisorp, A/S Nunc) were coated by physical adsorption with 0.4 mL of 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 0.1% NaN₃ and 4 µg of affinity-purified (anti-human IgG γ-chain) IgG (1). The coated polystyrene tubes were stored with 0.5 mL of buffer A at 4°C until use.

Previous (Simultaneous) Immune Complex Transfer Enzyme Immunoassay for HIV-1 p24 Antigen

The antigen, p24, in serum was measured as described previously (13). An aliquot (50 µL) of buffer B containing 50 µg of inactive β-D-galactosidase (β-D-galactosidase-mutain, Boehringer Mannheim GmbH) was mixed with 10 µL of non-specific rabbit serum and 50 µL of buffer B containing 5 fmol of monoclonal mouse anti-p24 Fab'-β-D-galactosidase conjugate and 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and was incubated with 40 µL of buffer B containing rp24 antigen for 3 hr. Subsequently, the reaction mixture was incubated with two colored polystyrene beads of 3.2 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 1 hr. After removing the reaction mixture, the colored polystyrene beads were washed and were incubated for 1 hr with two white polystyrene beads of 3.2 mm in diameter coated with streptavidin in 150 µL of buffer A containing 1 mmol/L *ε*N-2,4-dinitrophenyl-L-lysine. The incubations with polystyrene beads were performed at room temperature with 180 shakings per min throughout. The white polystyrene beads were washed, and β-D-galactosidase activity bound to the white polystyrene beads was assayed for 1 hr by incubation with a mixture of 100 µL of buffer C and 50 µL of 3 × 10⁻⁴ mol/L 4-methylumbelliferyl-β-D-galactoside. The enzyme reaction was stopped by addition of 2.5 mL of 0.1 mol/L glycine-NaOH buffer, pH 10.3, and the fluorescence intensity was measured with a spectrofluorophotometer (RF-510, Shimadzu Corporation, Kyoto, Japan) using 360 nm for excitation and 450 nm for emission analysis. The fluorescence

intensity of 1×10^{-8} mol/L 4-methylumbelliferone was adjusted to 100. The incubations were performed at room temperature without shaking throughout. β -D-Galactosidase activity bound to the colored polystyrene beads was also assayed for 1 hr, and that bound to the white polystyrene beads in the absence of p24 antigen was assayed for 20 hr.

Present (Sequential Thin Aqueous Layer) Immune Complex Transfer Enzyme Immunoassay for HIV-1 p24 Antigen

In a styrol test tube (13.3×54 mm, and 2.1 g), an aliquot (3.5 μ L) of buffer A containing 7.5 μ g of inactive β -D-galactosidase was mixed sequentially with 2.5 μ L of nonspecific rabbit serum, 1 μ L of buffer A containing 5 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate and was incubated for 10 min with 10 μ L of serum samples from HIV-1 seronegative subjects and 2 μ L of buffer A containing rp24. The reaction mixture (19 μ L) was incubated for 20 min with a polystyrene bead of 6.35 mm in diameter coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified anti-p24 Fab' conjugate. The incubation was performed by shaking the styrol test tube in a box ($16 \times 16 \times 20$ (depth) mm). The shaking rate was 180 per min, and the shaking width was 25 mm. As a result, the polystyrene bead was rotated randomly and a small drop (19 μ L) of the reaction mixture evenly contacted all parts of the polystyrene bead surface during the incubation, although it contacted only a small part of the polystyrene bead surface at a time, to continuously mix thin aqueous layer covering the polystyrene bead surface with the rest of the reaction mixture. The polystyrene bead was washed four times with 2 ml of buffer C and was incubated for 15 min with 35 μ L of buffer A containing 2 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine in a styrol test tube (13.3×54 mm and 2.1 g) as described above to elute the immune complex. The (first) eluate was transferred to a polystyrene test tube (12×75 mm) coated with streptavidin. The polystyrene bead in the styrol test tube was incubated for 1 min with an additional 35 μ L of buffer A containing 2 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine as described above, and the second eluate was combined with the first one. The polystyrene test tube containing the combined eluate was rotated in a box ($15 \times 32 \times 20$ (depth) mm) for 15 min, so that the combined eluate evenly contacted all parts of the coated polystyrene tube surface during the incubation. The polystyrene test tube was washed four times with 2 mL of buffer C, and β -D-galactosidase activity bound to the polystyrene test tube was assayed by incubation with a mixture of 50 μ L of buffer C and 25 μ L of 3×10^{-4} mol/L 4-methylumbelliferyl- β -D-galactoside as described above. The enzyme reaction was stopped by addition of 2.5 mL of 0.1 mol/L glycine-NaOH buffer, pH 10.3, and the fluorescence intensity was measured as described above. All the incubations were performed at

room temperature. β -D-Galactosidase activities bound to the polystyrene beads and the polystyrene test tubes in the presence of p24 antigen were assayed for 20 min, and those bound to the polystyrene test tubes in the absence of p24 antigen were assayed for 20 hr as described above in the previous immune complex transfer enzyme immunoassay for HIV-1 p24 antigen.

Previous (Simultaneous) Immune Complex Transfer Enzyme Immunoassays for Antibody IgGs to HIV-1 Antigens

The previous immune complex transfer enzyme immunoassays were performed as described previously (11,12). Serum samples from HIV-1 seropositive subjects were diluted with those from HIV-1 seronegative subjects 2×10^4 -fold for antibody IgG to p17 antigen, 2×10^3 -fold for antibody IgG to RT antigen and 2×10^2 -fold for antibody IgG to gp41 antigen. An aliquot (10 μ L) of the diluted serum samples was incubated with 140 μ L of buffer B containing 50 μ g of inactive β -D-galactosidase and 100 fmol each of 2,4-dinitrophenyl-HIV-1 antigen conjugate and HIV-1 antigen- β -D-galactosidase conjugate for antibody IgG to HIV-1 p17 antigen and 130 μ L of buffer B containing inactive β -D-galactosidase and the two conjugates and 10 μ L of nonspecific rabbit serum for antibody IgGs to HIV-1 RT and gp41 antigens. 2,4-Dinitrophenyl-HIV-1 antigen conjugates used were 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein, 2,4-dinitrophenyl-bovine serum albumin-rRT conjugate, and 2,4-dinitrophenyl-bovine serum albumin-synthetic gp41 peptide conjugate. The periods of time for incubation with antibody IgGs to p17, RT and gp41 antigens were 0.5, 4 and 0.5 hr, respectively. The reaction mixtures were incubated for 1 hr with two colored polystyrene beads (3.2 mm in diameter) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. The colored polystyrene beads were washed and incubated for 1 hr with two white polystyrene beads (3.2 mm in diameter) coated with affinity-purified (anti-human IgG γ -chain) IgG in 150 μ L of buffer A containing 1 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine. β -D-Galactosidase activity bound to the colored and white polystyrene beads was assayed as in the previous immune complex transfer enzyme immunoassay for HIV-1 p24 antigen described above.

Present (Sequential Thin Aqueous Layer) Immune Complex Transfer Enzyme Immunoassays for Antibody IgGs to HIV-1 Antigens

Serum samples from HIV-1 seropositive subjects were diluted as described above. A polystyrene bead with a diameter of 6.35 mm, which had been coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-HIV-1 antigen conjugate described above, was incubated for 5 min in a styrol test tube (13.3×54 mm and 2.1 g) with a mixture of 10 μ L of the diluted serum samples

described above, 5.25 μ L of buffer A containing 5 μ g of inactive β -D-galactosidase and 0.75 μ L of 5 mol/L NaCl for antibody IgG to HIV-1 p17 antigen and with a mixture of 10 μ L of the diluted serum samples described above, 2.5 μ L of non-specific rabbit serum, 7.75 μ L of buffer A containing 5 μ g of inactive β -D-galactosidase and 0.75 μ L of 5 mol/L NaCl for antibody IgGs to HIV-1 RT and gp41 antigens. Subsequently, 10 μ L of buffer A containing 200 fmol of HIV-1 rp17- β -D-galactosidase conjugate or 5 μ L of buffer A containing 100 fmol of rRT- β -D-galactosidase conjugate or 100 fmol of synthetic HIV-1 gp41 peptide- β -D-galactosidase conjugate was added, and the incubation was continued for 10 min as described above. Transferring of the immune complexes comprising the three components from the polystyrene bead to a polystyrene test tube coated with affinity-purified (anti-human IgG γ -chain) IgG and assay of bound β -D-galactosidase activity were performed as described in the present immune complex transfer enzyme immunoassays for HIV-1 p24 antigen described above.

Other Immunological Methods

The gelatin particle agglutination test for antibodies to HIV-1 was performed using a commercial kit with a lysate of HIV-1 as antigen (SERODIA-HIV, Fujirebio Inc., Tokyo, Japan). Western blotting for antibody IgGs to HIV-1 was performed using a commercial kit preblotted with nine proteins of HIV-1 (gp160, gp120, p66, p55, p51, gp41, p31, p24, and p17) (Ortho HIV Western Blot Kit, Ortho Diagnostic Systems Inc., Raritan, NJ).

Serum Samples From HIV-1 Seronegative and Seropositive Subjects

Serum samples were collected from two HIV-1 seropositive subjects and 8 HIV-1 seronegative subjects (2 males aged 24–39 yr, and 6 females aged 32–57 yr) and were stored at -20°C until use. Serum samples were tested by the gelatin particle agglutination test and the seropositivity was confirmed by Western blotting.

RESULTS AND DISCUSSION

Rapid Formation of the Immune Complex Comprising HIV-1 p24 Antigen and Labeled Anti-p24 Fab' Conjugates on Solid Phase

In the immune complex transfer enzyme immunoassay for HIV-1 p24 antigen, the immune complex comprising HIV-1 p24 antigen, 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity purified anti-p24 Fab' conjugate and monoclonal anti-p24 Fab'- β -D-galactosidase conjugate was formed on solid phase coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG and was transferred to another solid phase coated with streptavidin (Fig. 1). For rapid formation of the immune complex comprising the three components on the

Fig. 1. Previous (simultaneous) and present (sequential) immune complex transfer enzyme immunoassays for HIV-1 p24 antigen. DNP: 2,4-dinitrophenyl group. Gal: β -D-galactosidase.

solid phase, a polystyrene bead of 6.35 mm in diameter coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified anti-p24 Fab' conjugate (200 and 400 fmol/20 μ L/bead) was incubated for 20 min with a reaction mixture, in which HIV-1 p24 antigen had been incubated for 10 min with monoclonal anti-p24 Fab'- β -D-galactosidase conjugate at a high concentration (5 fmol/19 μ L). β -D-Galactosidase activity bound to the polystyrene bead with 400 fmol of the 2,4-dinitrophenylated conjugate was 20–33% higher than that with 200 fmol and was 2.5-fold higher than that by the previous method, in which two colored polystyrene beads of 3.2 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG was reacted for 1 hr with the immune complex formed by incubating HIV-1 p24 antigen for 3 hr simultaneously with 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified anti-p24 Fab' conjugate (100 fmol/150 μ L) and monoclonal anti-p24 Fab'- β -D-galactosidase conjugate (5 fmol/150 μ L) (Table 1). Thus, a larger amount of the immune complex comprising the three components was formed much more rapidly on the polystyrene bead than by the previous method.

Detection Limit of HIV-1 p24 Antigen

The immune complex was eluted from the polystyrene bead (6.35 mm in diameter) described above with ϵ N-2,4-dinitrophenyl-L-lysine for 15 min and was transferred to a

TABLE 1. Effect on Signals (Fluorescence Intensity for Bound β -D-Galactosidase Activity)^a in the Absence and Presence of HIV-1 p24 Antigen of the Immune Complex Transfer in the Present and Previous Immune Complex Transfer Enzyme Immunoassays^b

Immune complex transfer enzyme immuno-assay	Total volume for immuno-reaction	Incubation time for						Signal for anti-p24 Fab'-β-D-galactosidase conjugate used	Signal in the absence of p24 antigen		Signal in the presence of p24 antigen (100 amol)	
		Formation of the immune complex comprising the three components	Trapping of the immune complex comprising the three components	Formation of the immune complex comprising rp24 and anti-p24 Fab'-β-D-galactosidase conjugate	Trapping of the immune complex comprising rp24 and anti-p24 Fab'-β-D-galactosidase conjugate	Trans-ferring of the immune complex	Assay of β-D-galactosidase		Before transfer of the immune complex	After transfer of the immune complex	Before transfer of the immune complex	After transfer of the immune complex
Previous	μL 150	180	60	—	min —	60	1,200	1.28×10^6 (2.13×10^4) (5.57×10^6)	60 (1.0) (261)	0.23 — (1.0)	7,160 — (2.5)	2,880 — (1.0)
Present	19 to 70	—	—	10	20	30	1,200	1.28×10^6 (5.33×10^3) (3.20×10^6)	240 (1.0) (600)	0.40 — (1.0)	17,760 — (1.9)	9,288 — (1.0)

^aThe amounts of 2,4-dinitrophenyl-biotinyl-bovine serum albumin affinity-purified rabbit and anti-p24 Fab' conjugate monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate were 100 and 5 fmol, respectively, in the previous immunoassay and 400 and 5 fmol, respectively, in the present immunoassay. The volumes of nonspecific rabbit serum used in the previous and present immunoassays were 10 and 2.5 μ L, respectively. β -D-Galactosidase activity was assayed for 20 to 60 min and was expressed as that assayed for 20 hr, except that β -D-galactosidase activity after transfer of the immune complex in the absence of p24 antigen was assayed for 20 hr.

^bValues in parentheses are the ratios of signals to those for β -D-galactosidase activity bound to the polystyrene beads and test tubes before and after transfer of the immune complex.

polystyrene test tube coated with streptavidin for 15 min. β -D-Galactosidase activity bound to the polystyrene test tube was 3.2-fold higher than that by the previous method, in which the two colored polystyrene beads (3.2 mm in diameter) described above were incubated for 1 hr with two white polystyrene beads of 3.2 mm in diameter coated with streptavidin in the presence of ϵ N-2,4-dinitrophenyl-L-lysine (Table 1). In the absence of HIV-1 p24 antigen, β -D-galactosidase activity bound to the polystyrene test tube was 1.7-fold higher than that bound to the white polystyrene beads in the previous method (Table 1). As a result, the detection limits of HIV-1 p24 antigen by the present and previous methods were similar (3–10 zmol/assay).

Rapid Formation of the Immune Complexes Comprising Antibody IgGs to HIV-1 and Labeled HIV-1 Antigen Conjugates on Solid Phase

In the immune complex transfer enzyme immunoassays for antibody IgGs to HIV-1 antigens, the immune complexes comprising antibody IgGs to HIV-1 antigens, 2,4-dinitrophenyl-HIV-1 antigen conjugates and HIV-1 antigen- β -D-galactosidase conjugates were formed on solid phase coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG and were transferred to another solid phase coated with affinity-purified

(anti-human IgG γ -chain) IgG (Fig. 2). For rapid formation of the immune complexes comprising the three components on solid phase, a polystyrene bead of 6.35 mm in diameter coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-HIV-1 antigen conjugates (200 to 800 fmol/15 to 20 μ L/bead) was incubated for 5 min with serum containing antibody IgGs to HIV-1 antigens and subsequently for 10 min with HIV-1 antigen- β -D-galactosidase conjugates at high concentrations (100–200 fmol/26 μ L).

For antibody IgG to HIV-1 p17 antigen, 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein (400 and 800 fmol/15 μ L/bead) and rp17- β -D-galactosidase conjugate (200 fmol/26 μ L) were used. β -D-Galactosidase activity bound to the polystyrene bead with 800 fmol of the 2,4-dinitrophenylated fusion protein was 19–23% higher than that with 400 fmol and was 2.7-fold higher than that by the previous method, in which two colored polystyrene beads of 3.2 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG were reacted for 1 hr with the immune complex formed by incubating serum containing antibody IgG to HIV-1 p17 antigen simultaneously with 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein (100 fmol/150 μ L) and rp17- β -D-galactosidase conjugate (100 fmol/150 μ L) for 30 min (Table 2). Thus, a larger amount of the immune complex comprising the three components was formed on the polystyrene bead (6.35 mm in diameter) much more rapidly by sequential reaction of the three components than that trapped on the two colored polystyrene beads from a reaction mixture in which the three components had been simultaneously incubated.

For antibody IgG to HIV-1 RT antigen, 2,4-dinitrophenyl-bovine serum albumin-rRT conjugate (400 and 800 fmol/20 μ L/bead) and rRT- β -D-galactosidase conjugate (100 fmol/26 μ L) were used. β -D-Galactosidase activity bound to the polystyrene bead (6.35 mm in diameter) with 400 fmol of the 2,4-dinitrophenyl conjugate was similar to that with 800 fmol and was 1.8-fold lower than that by the previous method, in which affinity-purified (anti-2,4-dinitrophenyl group) IgG-coated colored polystyrene beads (2 beads/assay) of 3.2 mm in diameter were reacted for 1 hr with the immune complex formed by incubating serum containing antibody IgG to HIV-1 RT antigen simultaneously with the two conjugates (100 fmol each/150 μ L) for 4 hr (Table 3). Thus, the immune complex comprising the three components was formed on the polystyrene bead (6.35 mm in diameter) much more rapidly by sequential reaction of the three components than by the previous method, although the amount of the immune complex formed was slightly smaller than that trapped onto the two colored polystyrene beads (3.2 mm in diameter) from a reaction mixture in which the three components had been simultaneously incubated.

For antibody IgG to HIV-1 gp41 antigen, 2,4-dinitrophenyl-bovine serum albumin-synthetic HIV-1 gp41 peptide conjugate (200 and 400 fmol/20 μ L/bead) and synthetic HIV-1 gp41

Fig. 2. Previous (simultaneous) and present (sequential) immune complex transfer enzyme immunoassays for antibody IgG. DNP: 2,4-dinitrophenyl group. Ag: antigen. Ab: antibody. Gal: β -D-galactosidase.

TABLE 2. Effect on Signals (Fluorescence Intensity for Bound β -D-Galactosidase Activity) in the Absence and Presence of Antibodies to HIV-1 p17 Antigen of the Immune Complex Transfer^a in the Present and Previous Immune Complex Transfer Enzyme Immunoassays^b

Immune complex transfer enzyme immuno-assay	Total volume for immuno-reaction	Incubation time for						Signal for rp17-β-D-galacto-sidase conjugate used	Signal in the absence of antibodies to p17 antigen		Signal in the presence of antibodies to p17 antigen	
		Formation of the immune complex	Trapping of the immune complex	Reaction with antibody IgG to p17	Reaction with rp17-β-D-galacto-sidase conjugate	Trans-ferring of the immune complex	β-D-galacto-sidase assay		Before transfer of the immune complex	After transfer of the immune complex	Before transfer of the immune complex	After transfer of the immune complex
Previous	μL 150	30	60	—	min —	60	1,200	1.98×10^7 (9.52×10^4) (2.08×10^7)	208 (1.0) (219)	0.95 — (1.0)	10,387 — (2.0)	5,217 — (1.0)
Present	16 to 70	—	—	5	10	30	1,200	3.97×10^7 (4.31×10^4) (3.61×10^7)	918 (1.0) (835)	1.1 — (1.0)	28,440 — (1.9)	15,260 — (1.0)

^aSerum from an HIV-1 seropositive subject was diluted 2×10^4 -fold with serum from an HIV-1 seronegative subject, and 10 μ L of the diluted serum was used. The amounts of 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein and rp17- β -D-galactosidase conjugate were both 100 fmol/assay in the previous immunoassay and 800 and 200 fmol, respectively, in the present immunoassay. β -D-Galactosidase activity was assayed for 20 to 60 min and was expressed as that assayed for 20 hr, except that β -D-galactosidase activity after transfer of the immune complex in the absence of antibody IgG to HIV-1 p17 antigen was assayed for 20 hr.

^bValues in parentheses are the ratios of signals to those for β -D-galactosidase activity bound to the polystyrene beads and test tubes before and after transfer of the immune complex.

TABLE 3. Effect on Signals in the Absence and Presence of Antibodies to HIV-1 RT Antigen of the Immune Complex Transfer^a in the Present and Previous Immune Complex Transfer Enzyme Immunoassays^b

Immune complex transfer enzyme immuno-assay	Total volume for immuno-reaction	Incubation time for						Signal for rRT β -D-galacto-sidase conjugate used	Signal in the absence of antibodies to RT		Signal in the presence of antibodies to RT	
		Formation of the immune complex	Trapping of the immune complex	Reaction with antibody IgG to RT	Reaction with rRT β -D-galacto-sidase conjugate	Trans-ferring of the immune complex	β -D-galacto-sidase assay		Before transfer of the immune complex	After transfer of the immune complex	Before transfer of the immune complex	After transfer of the immune complex
	μ L				min							
Previous	150	240	60	—	—	60	1,200	1.87×10^7 (3.05×10^4) (2.92×10^6)	612 (1.0) (96)	3.3 — (1.0)	28,080 — (1.7)	14,946 — (1.0)
Present	21 to 70	—	—	5	10	30	1,200	1.87×10^7 (1.41×10^4) (1.56×10^7)	1,326 (1.0) (1,105)	1.2 — (1.0)	15,330 — (1.6)	9,408 — (1.0)

^aSerum from an HIV-1 seropositive subject was diluted 2×10^3 -fold with serum from an HIV-1 seronegative subject, and 10 μ L of the diluted serum was used. The amounts of 2,4-dinitrophenyl-bovine serum albumin-rRT conjugate and rRT- β -D-galactosidase conjugate used were both 100 fmol/assay in the previous immunoassay and 400 and 100 fmol, respectively, in the present immunoassay. The volumes of nonspecific rabbit serum used in the previous and present immunoassays were 10 and 2.5 μ L, respectively. β -D-Galactosidase activity was assayed for 20 to 60 min and was expressed as that assayed for 20 hr, except that β -D-galactosidase activity after transfer of the immune complex in the absence of antibody IgG to HIV-1 RT antigen was assayed for 20 hr.

^bValues in parentheses are the ratios of signals to those for β -D-galactosidase activity bound to the polystyrene beads and test tubes before and after transfer of the immune complex.

TABLE 4. Effect on Signals in the Absence and Presence of Antibodies to HIV-1 gp41 Antigen of the Immune Complex Transfer^a in the Present and Previous Immune Complex Transfer Enzyme Immunoassays^b

Immune complex transfer enzyme immuno-assay	Total volume for immuno-reaction	Incubation time for						Signal for gp41- β -D-galactosidase conjugate used	Signal in the absence of antibodies to gp41		Signal in the presence of antibodies to gp41	
		Formation of the immune complex	Trapping of the immune complex	Reaction with antibody IgG to gp41	Reaction with gp41- β -D-galactosidase conjugate	Transferring of the immune complex	β -D-galactosidase assay		Before transfer of the immune complex	After transfer of the immune complex	Before transfer of the immune complex	After transfer of the immune complex
	μ L				min							
Previous	150	30	60	—	—	60	1,200	1.91×10^7 (4.03×10^4) (8.30×10^6)	474 (1.0) (193)	2.3 — (1.0)	15,540 — (2.6)	6,000 — (1.0)
Present	21 to 70	—	—	5	10	30	1,200	1.91×10^7 (6.37×10^3) (4.06×10^6)	3,000 (1.0) (638)	4.7 — (1.0)	61,260 — (5.8)	10,596 — (1.0)

^aSerum from an HIV-1 seropositive subject was diluted 2×10^2 -fold with serum from an HIV-1 seronegative subject, and 10 μ L of the diluted serum was used. The amounts of 2,4-dinitrophenyl-bovine serum albumin-synthetic gp41 peptide conjugate and synthetic gp41 peptide- β -D-galactosidase conjugate used were both 100 fmol/assay in the previous immunoassay and 200 and 100 fmol, respectively, in the present immunoassay. The volumes of nonspecific rabbit serum used in the previous and present immunoassays were 10 and 2.5 μ L, respectively. β -D-Galactosidase activity was assayed for 20 to 60 min and was expressed as that assayed for 20 hr, except that β -D-galactosidase activity after transfer of the immune complex in the absence of antibody IgG to HIV-1 gp41 antigen was assayed for 20 hr.

^bValues in parentheses are the ratios of signals to those for β -D-galactosidase activity bound to the polystyrene beads and test tubes before and after transfer of the immune complex.

peptide- β -D-galactosidase conjugate (100 fmol/26 μ L) were used. β -D-Galactosidase activity bound to the polystyrene bead with 200 fmol of the 2,4-dinitrophenyl conjugate was 17% higher than that with 400 fmol and was 3.9-fold higher than that by the previous method, in which affinity-purified (anti-2,4-dinitrophenyl group) IgG-coated colored polystyrene beads (2 beads/assay) of 3.2 mm in diameter were reacted for 1 hr with the immune complex formed by incubating serum containing antibody IgG to HIV-1 gp41 antigen with the two conjugates (100 fmol each/150 μ L) for 30 min (Table 4). Thus, a much larger amount of the immune complex comprising the three components was formed on the polystyrene bead (6.35 mm in diameter) much more rapidly by sequential reaction of the three components than by trapping the immune complex on the two colored polystyrene beads (3.2 mm in diameter) from a reaction mixture in which the three components had been simultaneously incubated.

Detection Limits of Antibody IgGs to HIV-1

The immune complexes comprising antibody IgGs to HIV-1 and the two conjugates were eluted from the polystyrene beads of 6.35 mm in diameter described above with 35 μ L of ϵ N-2,4-dinitrophenyl-L-lysine for 15 min (the first eluate) and, after removing the first eluate, with another 35 μ L for 1 min (the second eluate). The first and second eluates were incubated with a polystyrene test tube coated with affinity-purified (anti-human IgG γ -chain) IgG for 15 min. β -D-Galactosidase activity bound to the polystyrene test tube in the presence of antibody IgG to HIV-1 gp41 antigen after the transfer was 5.8-fold lower than that bound to the corresponding polystyrene bead before the transfer, although those in the presence of antibody IgGs to HIV-1 p17 and RT antigens were only 1.9 and 1.6-fold, respectively, lower (Tables 2–4). The immune complex with the synthetic HIV-1 gp41 peptide conjugates might have been dissociated to a greater extent than those with recombinant p17 and RT conjugates. This was supported by the fact that β -D-galactosidase activity bound to the polystyrene bead in the presence of antibody IgG to HIV-1 gp41 antigen decreased more rapidly (48% and 67% within 30 and 60 min, respectively) when it was allowed to stand in a neutral buffer than those in the presence of antibody IgGs to HIV-1 p17 and RT antigens (4 to 15% and 6 to 24% within 30 and 60 min, respectively) (Table 5). Notably, β -D-galactosidase activity bound to the polystyrene bead of 6.35 mm in diameter in the presence of antibody IgG to HIV-1 gp41 antigen decreased to a greater extent (67% within 60 min) than that bound to the polystyrene beads of 3.2 mm in diameter (35% within 60 min), suggesting that the immune complex formed with the β -D-galactosidase conjugate at a high concentration of 100 fmol/26 μ L was dissociated to a greater extent than that formed with the same conjugate at a low concentration of 100 fmol/150 μ L (Table 5).

β -D-Galactosidase activities bound to the polystyrene test

TABLE 5. Decrease of β -D-Galactosidase Activity Bound to Affinity-Purified (Anti-2,4-Dinitrophenyl Group) IgG-Coated Polystyrene Beads Allowed to Stand in Buffer A

Time in buffer A	Fluorescence intensity for β -D-galactosidase activity bound to affinity-purified (anti-2,4-dinitrophenyl group) IgG-coated polystyrene beads in the presence of			
	Anti-RT (A)	Anti-p17 (A)	Anti-gp41	
			(A)	(B)
min			%	
0	100	100	100	100
30	96	85	52	—
60	94	76	33	65

(A) Polystyrene beads of 6.35 mm in diameter coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-HIV-1 antigen conjugates were incubated with serum samples containing antibody IgGs to HIV-1 for 5 min and subsequently with HIV-1 antigen- β -D-galactosidase conjugates for 10 min as in the present immune complex transfer enzyme immunoassays and, after washing, were allowed to stand in buffer A at room temperature for indicated periods of time.

(B) Serum sample containing antibody IgG to HIV-1 gp41 antigen was incubated simultaneously with the two conjugates for 30 min and subsequently with two colored polystyrene beads of 3.2 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 1 hr as in the previous immune complex transfer enzyme immunoassay and, the two colored polystyrene beads after washing were allowed to stand in buffer A at room temperature for the indicated period of time.

tubes for antibody IgGs to HIV-1 p17, RT and gp41 antigens were 2.9, 0.63, and 1.8 times, respectively, as high as those obtained by the previous methods, in which the immune complexes comprising antibody IgGs to HIV-1 were transferred from the two colored polystyrene beads (3.2 mm in diameter) described above to two white polystyrene beads (3.2 mm in diameter) coated with affinity-purified (anti-human IgG γ -chain) IgG by incubating the four polystyrene beads in 150 μ L of ϵ N-2,4-dinitrophenyl-L-lysine for 1 hr. β -D-Galactosidase activities bound to the polystyrene test tubes in the absence of antibody IgGs to HIV-1 were only slightly higher or lower than those bound to the two white polystyrene beads by the previous methods. As a result, the detection limits of antibody IgGs to HIV-1 by the present methods, in which the immune complexes were more rapidly formed on the polystyrene beads and were more rapidly transferred to the polystyrene test tubes, were similar to those by the previous methods, in which longer periods of time were used for the formation of the immune complexes on the polystyrene beads.

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