Ultrasensitive and Rapid Enzyme Immunoassay (Thin Aqueous Layer Immune Complex Transfer Enzyme Immunoassay) for Antibody IgG to HIV-1 p17 Antigen

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The immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 p17 antigen was performed in two different ways (the present immunoassays I and II) within shorter periods of time than previously reported. In the present (simultaneous) immunoassay I, antibody IgG to HIV-1 p17 antigen in 10 μ L of serum samples was incubated simultaneously with 2,4-dinitrophenyl-maltose binding protein-recombinant p17(rp17) fusion protein and rp17- β -D-galactosidase conjugate in a total volume of 22 μ L for 10 min to form the immune complex comprising the three components. The reaction mixture was incubated with a polystyrene bead of 6.35 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 5 min in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) to trap the immune complex. After washing, the polystyrene bead was incubated with 30 μ L of ϵ N-2,4-dinitrophenyl-L-lysine solution in a polystyrene tube (12 $\times$ 75 mm) coated with affinity-purified (antihuman IgG γ -chain) IgG for 10 min to transfer the immune complex. In the present (sequential) immunoassay II, a polystyrene bead of 6.35 mm in diameter coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein was incubated in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) sequentially with antibody IgG to HIV-1 p17 antigen in 10 μ L of serum samples in a total volume of 16 μ L for 5 min and subsequently with rp17- β -D-galactosidase conjugate in a volume of 10 μ L for 5 and 10 min. The immune complex formed on the polystyrene bead was transferred to a polystyrene tube coated with affinity-purified (antihuman IgG γ -chain) IgG for 5 and 10 min in the same way as in the present immunoassay I. During the incubations,

the styrol test tubes containing the polystyrene beads and reaction mixtures were shaken, and the polystyrene test tubes were rotated with shaking, so that the polystyrene beads were rotated randomly, and small drops (16 to 30 μ L) of the reaction mixtures evenly contacted all parts of the solid phase surfaces during the incubations, though only small parts of the solid phase surfaces were contacted at one time. The intent was to continuously mix thin aqueous layers of the reaction mixtures covering the solid phase surfaces with the rest of the reaction mixtures. (Therefore, these immunoassays were called thin aqueous layer immunoassays.) β -D-Galactosidase activity bound to the polystyrene tubes was assayed by fluorometry for 30 and 60 min. The present immunoassays I and II, in which only 15 to 25 min were used for the immunoreactions, were as sensitive if not more so than the previous immune complex transfer enzyme immunoassay requiring 150 min for the immunoreactions. In these earlier immunoreactions, the immune complex comprising the three components formed by 30 min incubation was trapped onto two polystyrene beads (3.2 mm in diameter) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 60 min, and was then transferred to two polystyrene beads (3.2 mm in diameter) coated with affinity-purified (antihuman IgG γ -chain) IgG for 60 min in a total volume of 150 μ L. Furthermore, the present (sequential) immunoassay II (and probably I) could become approximately 10 times more sensitive by assaying bound β -D-galactosidase activity for a longer period of time (10 h), since β -D-galactosidase activity, bound nonspecifically in the presence of serum samples from HIV-1 seronegative subjects, was considerably low. *J. Clin. Lab. Anal.* 12:179–189, 1998. © 1998 Wiley-Liss, Inc.

Key words: antibody; human immunodeficiency virus type 1; p17; enzyme immunoassay

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INTRODUCTION

Ultrasensitive enzyme immunoassays (immune complex transfer enzyme immunoassays) for antibody IgGs to p17, p24, and reverse transcriptase (RT) of HIV-1 have been developed using recombinant p17, p24, and RT (rp17, rp24, and rRT) as antigens (1-7). Antibody IgGs to HIV-1 were reacted with 2,4-dinitrophenyl-recombinant protein conjugates and recombinant protein- β -D-galactosidase conjugates for 3 h, and the immune complexes formed, comprising the three components, were trapped onto polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG by overnight incubation. After washing, the immune complexes were eluted from the polystyrene beads with ϵ N-2,4-dinitrophenyl-L-lysine and were transferred to polystyrene beads coated with affinity-purified (antihuman IgG γ -chain) IgG by 3 h incubation. Bound β -D-galactosidase activity was assayed by fluorometry for 2.5 h. The volume of serum samples used was 10 μ L. These immunoassays have made the diagnosis of HIV-1 infection with urine (1-4) and whole saliva samples (5,6) reliable, and have been shown to be more useful as a corroborative test for HIV-1 infection with higher sensitivities and specificities than Western blotting (7). Notably, antibody IgG to p17 of HIV-1 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 p24 antigen of HIV-1 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 p24 antigen and antibody IgGs to p17 and RT (8). Recently, it has been made possible to perform more sensitive immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 p17 antigen within shorter periods of time (1 to 2.5 h for the immunoreactions described above) by incubation with shaking (11).

This report describes the immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 p17 antigen that can be performed within much shorter periods of time (15 to 25 min) and with a sensitivity as high as that of the previously reported ones.

MATERIALS AND METHODS

Buffers

The regularly used buffers were 10 mmol/L sodium phosphate buffer, pH 7.0, containing 1 mmol/L $MgCl_2$, 0.1% bovine serum albumin (fraction V, Intergen Company, Purchase, NY) and 0.1% NaN_3 (buffer A) and 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl, 1 mmol/L $MgCl_2$, 0.01% bovine serum albumin and 0.1% NaN_3 (buffer B).

2,4-Dinitrophenyl- β -D-Galactosidase

1. α N-6-Maleimidohehexanoyl- ϵ N-2,4-dinitrophenyl-L-lysine: An aliquot (0.45 ml) of 5 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine-HCl (Tokyo Kasei Kogyo, Tokyo, Japan) in 0.1 mol/L sodium phosphate buffer, pH 7.0, containing 5 mmol/L EDTA was incubated with 0.05 ml of 30 mmol/L *N*-succinimidyl-6-maleimidohehexanoate (Dojindo Laboratories, Kumamoto, Japan) in *N,N*-dimethylformamide at 30°C for 30 min.

2. 2,4-Dinitrophenyl- β -D-galactosidase: An aliquot (7.5 μ L, 22.5 nmol) of the α N-6-maleimidohehexanoyl- ϵ N-2,4-dinitrophenyl-L-lysine solution was incubated with β -D-galactosidase (Boehringer Mannheim GmbH, Mannheim, Germany) (2.7 mg, 5 nmol) in 0.3 ml of 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L EDTA at 30°C for 30 min. After the addition of 0.193 ml of 0.1 mol/L sodium phosphate buffer, pH 6.0, the reaction mixture was subjected to gel filtration using the centrifuged column procedure (12) with a column (1.1 $\times$ 5.3 cm) of Sephadex G-50 fine (Pharmacia Biotech AB, Uppsala, Sweden), equilibrated in 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 mol/L NaCl. The average number of dinitrophenyl groups introduced per β -D-galactosidase molecule was 3.9, which was calculated from the absorbance at 280 nm and 360 nm (13).

Recombinant p17 (rp17) Antigen of HIV-1

DNA fragment for the gene of HIV-1 p17 antigen was prepared by PCR using pNL4-3 (14) as a template and was inserted into pMAL-c2 (New England Biolabs, Beverly, MA) to produce rp17 as a fusion protein with maltose binding protein. The recombinant proviral clone used (pNL4-3) contained DNA from HIV-1 isolates NY5 (GenBank accession number HIVNL43) and LAV (15), and the sequence for p17 derived from NY5. Primers used for PCR were obtained from Bex Co., Ltd, Tokyo, Japan, and their sequences were 5'-CGGGATCCGGTATTGAAGGTCGTATGGGTGCG-AGAGCGTCGGTA and 5'-GGTCTAGATCAGTAAT-TTTGGCTGACCTGGCT for N- and C-termini, respectively, of p17 antigen. The plasmid and the PCR product were digested with Bam HI and Xba I (New England Biolabs) before the insertion.

Escherichia coli (competent, DH5 α , Life Technologies Oriental, Inc., Tokyo, Japan) was transformed with the plasmid containing the p17 DNA fragment. After induction with isopropyl-1-thio- β -D-galactopyranoside the transformed cells were sonicated in 15 mmol/L sodium phosphate buffer, pH 7.5, containing 5 mmol/L EDTA. The sonic supernatant was applied to a column of DEAE Sepharose (Pharmacia Biotech AB) equilibrated with the same buffer. The fusion protein was eluted from the column with the same buffer containing 75 mmol/L NaCl, and, following the instruction of New England Biolabs, was processed successively for affinity chromatography with amylose resin (New England Biolabs),

digestion with biotinyl factor Xa (New England Biolabs) and column chromatography with streptavidin gel (New England Biolabs). Subsequently, rp17 was separated from maltose binding protein by elution from a column of SP Sepharose Fast Flow (Pharmacia Biotech AB) with 15 mmol/L sodium phosphate buffer, pH 6.7, containing 0.5 mol/L NaCl after washing with the same buffer containing 0.1 mol/L NaCl. Homogeneity of the purified rp17 was confirmed by electrophoresis.

2,4-Dinitrophenyl-Maltose Binding Protein-Rp17 Fusion Protein

Maltose binding protein-rp17 fusion protein (1.2 mg) in 0.4 ml of 0.1 mol/L sodium phosphate buffer, pH 7.0, was incubated successively at 30°C for 30 min with 13.5 μ L of 50 mmol/L *N*-succinimidyl-*S*-acetylmercaptoacetate in *N,N*-dimethylformamide, for 5 min with 5 μ L of 1 mol/L glycylglycine, pH 7.0 and 20 μ L of 0.1 mol/L EDTA, pH 7.0, and for 10 min with 50 μ L of 1 mol/L NH_2OH , pH 7.0. The reaction mixture was incubated for 1 h with 150 μ L of α *N*-6-maleimidohexanoyl- ϵ *N*-2,4-dinitrophenyl-L-lysine solution, which had been prepared by incubating 30 μ L of 100 mmol/L ϵ *N*-2,4-dinitrophenyl-L-lysine in *N,N*-dimethylformamide, 15 μ L of 100 mmol/L *N*-succinimidyl-6-maleimidohexanoate in *N,N*-dimethylformamide and 105 μ L of 0.1 mol/L sodium phosphate buffer, pH 7.0 at 30°C for 1.5 h. The reaction mixture was subjected to gel filtration with Sephadex G-50 fine (Pharmacia Biotech AB) to separate 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein from low molecular weight-substances. The average number of 2,4-dinitrophenyl groups introduced per fusion protein molecule was 3.9, which was calculated from absorbances at 280 nm and 360 nm (13).

Rp17- β -D-Galactosidase Conjugate

Mercaptoacetylated rp17 antigen was conjugated to β -D-galactosidase (EC 3.2.1.23) from *E. coli* (Boehringer Mannheim, GmbH) using 2,4-phenylenedimaleimide (1).

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

Colored polystyrene beads with a diameter of 3.2 mm and white polystyrene beads with a diameter of 6.35 mm (Immuno Chemical, Okayama, Japan) were coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG (50 μ g/ml) by physical adsorption as described previously (16). White polystyrene beads with a diameter of 3.2 mm (Immuno Chemical) were coated with affinity-purified (antihuman IgG γ -chain) IgG (10 μ g/ml) by physical adsorption as described previously (16). The coated polystyrene beads were stored in buffer A containing 0.1 mol/L NaCl at 4°C until use.

Polystyrene Beads Coated With 2,4-Dinitrophenyl Maltose Binding Protein-Rp17 Fusion Protein

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 15 μ L/bead of buffer A containing 0.1 mol/L NaCl and various amounts of 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein in styrol test tubes (13.3 $\times$ 54 mm and 2.1 g, one bead/tube) for 5 min. The incubation was performed at room temperature with shaking as described below. The coated polystyrene beads were stored in buffer A containing 0.1 mol/L NaCl at 4°C until use.

Polystyrene Tubes Coated With Affinity-Purified (Anti-2,4-Dinitrophenyl Group) IgG and (Antihuman IgG γ -Chain) IgG

Polystyrene tubes (12 $\times$ 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% NaN_3 and 20 μ g of affinity-purified (anti-2,4-dinitrophenyl group) IgG or 10 μ g of affinity-purified (antihuman IgG γ -chain) IgG (16). The coated polystyrene tubes were stored with 0.5 ml of buffer A containing 0.1 mol/L NaCl at 4°C until use.

Binding of 2,4-Dinitrophenyl- β -D-Galactosidase to Affinity-Purified (Anti-2,4-Dinitrophenyl Group) IgG-Coated Solid Phases

Two different solid phases coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG were incubated with 2,4-dinitrophenyl- β -D-galactosidase in three different ways.

First, a polystyrene bead with a diameter of 6.35 mm coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG was incubated with 15, 50 and 150 μ L of buffer A containing 0.1 mol/L NaCl and 7.7 amol/ μ L of 2,4-dinitrophenyl- β -D-galactosidase in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) at room temperature for 1.25 and 2.5 min. The incubation was performed by shaking the test tube in a box (16 $\times$ 16 $\times$ 20 [depth] mm), so that the polystyrene bead was rotated randomly and the 2,4-dinitrophenyl- β -D-galactosidase solution contacted evenly all parts of the polystyrene bead surface during the incubation to continuously mix thin aqueous layer of the 2,4-dinitrophenyl- β -D-galactosidase solution with the rest. The shaking rate was 180 per min, and the shaking width was 25 mm. The polystyrene bead was washed three times with 2 ml of buffer B and was incubated with a mixture of 100 μ L of buffer B and 50 μ L of 3×10^{-4} mol/L 4-methylumbelliferyl- β -D-galactoside for 10 min. 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 for bound β -D-galactosidase activity was measured with a spectrofluoro-

photometer (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.

Second, a polystyrene bead with a diameter of 6.35 mm coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG was incubated with 150 μ L of buffer A containing 0.1 mol/L NaCl and 0.77 and 7.7 amol/ μ L of 2,4-dinitrophenyl- β -D-galactosidase in a small glass test tube (10 $\times$ 75 mm) at room temperature for 1.25 and 2.5 min. The small glass test tube was shaken in a box (15 $\times$ 15 $\times$ 60 [depth] mm). The shaking rate was 180 per min, and the shaking width was 25 mm. The whole surface of the polystyrene bead remained in the 2,4-dinitrophenyl- β -D-galactosidase solution during the incubation. β -D-galactosidase activity bound to the polystyrene bead was assayed as described above.

Third, a polystyrene test tube (12 $\times$ 75 mm) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG was incubated with 15, 50, and 150 μ L of buffer A containing 0.1 mol/L NaCl and 7.7 amol/ μ L of 2,4-dinitrophenyl- β -D-galactosidase at room temperature for 1.25 and 2.5 min. The polystyrene test tube was washed three times with 2 ml of buffer B, and β -D-galactosidase activity bound to the polystyrene test tube was assayed for 5 to 20 min by incubation with a mixture of 50 μ L of buffer B and 25 μ L 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 for bound β -D-galactosidase activity was measured as described above. All the incubations were performed by rotating the polystyrene test tubes in a box (15 $\times$ 32 $\times$ 20 [depth] mm), so that the 2,4-dinitrophenyl- β -D-galactosidase and 4-methylumbelliferyl- β -D-galactoside solutions contacted evenly all parts of the polystyrene test tube surface coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG during the incubation. The rotation rate was 40 per min.

Previous Immune Complex Transfer Enzyme Immunoassay Using Polystyrene Beads

The previous immune complex transfer enzyme immunoassay was performed as described previously (11). An aliquot (10 μ L) of serum samples was incubated for 0.5 h with 140 μ L of buffer A containing 0.4 mol/L NaCl, 50 μ g of inactive β -D-galactosidase (β -D-galactosidase-mutain, Boehringer Mannheim GmbH) and 100 fmol each of 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein and rp17- β -D-galactosidase conjugate and was incubated for 1 h with two colored polystyrene beads (3.2 mm in diameter) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG in a test tube. The colored polystyrene beads were washed three times with 2 ml of buffer B and were incubated for 1 h with two white polystyrene

beads (3.2 mm in diameter) coated with affinity-purified (antihuman IgG γ -chain) IgG in a test tube containing 150 μ L of buffer A containing 0.1 mol/L NaCl and 1 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine. β -D-galactosidase activity bound to the white polystyrene beads was assayed for up to 10 h as described above.

Present Immune Complex Transfer Enzyme Immunoassays (Thin Aqueous Layer Immune Complex Transfer Enzyme Immunoassays) I and II

Antibody IgG to HIV-1 p17 antigen was measured in two different ways (Fig. 1).

Present (simultaneous) immunoassay I

An aliquot (10 μ L) of serum samples was incubated in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) for up to 15 min with 12 μ L of buffer A containing 0.6 mol/L NaCl, 5 μ g of inactive β -D-galactosidase, 100 fmol of 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein, and 100 fmol of rp17- β -D-galactosidase conjugate, and subsequently incubated for up to 10 min with a polystyrene bead with a diameter of 6.35 mm coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. The polystyrene bead was washed three times with 2 ml of buffer B and was incubated for up to 20 min with 30 μ L of buffer A containing 0.1 mol/L NaCl and 1 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine in a polystyrene test tube (12 $\times$ 75 mm) coated with affinity-purified (antihuman IgG γ -chain) IgG. β -D-galactosidase activity bound to the polystyrene test tube was assayed for 60 min as described above.

Present (sequential) immunoassay II

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-maltose binding protein-rp17 fusion protein, was incubated in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) with a mixture of 10 μ L of serum samples and 6 μ L of buffer A containing 0.82 mol/L NaCl and 5 μ g of inactive β -D-galactosidase for up to 10 min and subsequently with 10 μ L of buffer A containing 0.4 mol/L NaCl and 200 fmol of rp17- β -D-galactosidase conjugate for up to 30 min. The polystyrene bead was washed three times with 2 ml of buffer B and was incubated with 30 μ L of buffer A containing 0.1 mol/L NaCl and 1 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine in a polystyrene test tube (12 $\times$ 75 mm) coated with affinity-purified (antihuman IgG γ -chain) IgG for up to 20 min. β -D-galactosidase activity bound to the polystyrene test tube was assayed for up to 10 h as described above.

All the incubations in the present immunoassays I and II were performed in the same way as described above for binding of 2,4-dinitrophenyl- β -D-galactosidase to affin-

Present (simultaneous) immunoassay I

Present (sequential) immunoassay II

Fig. 1. Present immune complex transfer enzyme immunoassays (thin aqueous layer immune complex transfer enzyme immunoassays) I (si-

multaneous) and II (sequential). **DNP**, 2,4-dinitrophenyl; **Ag**, antigen; **Ab**, antibody; **Enz**, enzyme.

ity-purified (anti-2,4-dinitrophenyl group) IgG-coated solid phases, except that polystyrene test tubes were rotated in a box (15 × 32 × 20 [depth] mm) with shaking in the presence of polystyrene beads so as to randomly rotate the polystyrene beads. In some experiments, β -D-galactosidase activity bound to the polystyrene beads was assayed for 10 min as 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 an HIV-1 seropositive subject and 8 HIV-1 seronegative subjects (2 males aged 24 to 39 yrs, and 6 females aged 32 to 57 yrs) 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

Rapid Binding to (Anti-2,4-Dinitrophenyl Group) IgG-Coated Solid Phases of 2,4-Dinitrophenyl- β -D-Galactosidase in Thin Aqueous Layers Covering the Solid Phase Surfaces

The time course of binding to affinity-purified (anti-2,4-dinitrophenyl group) IgG-coated solid phases of 2,4-dinitrophenyl- β -D-galactosidase in thin aqueous layers covering the solid phase surfaces was examined in three different ways.

First, a polystyrene bead of 6.35 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG was incubated with different volumes (15, 50, and 150 μ L) of 2,4-dinitrophenyl- β -D-galactosidase solution (7.7 amol/ μ L) in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) by shaking in a box (16 $\times$ 16 $\times$ 20 [depth] mm), so that the polystyrene bead was rotated randomly. The 2,4-dinitrophenyl- β -D-galactosidase solution evenly contacted all parts of the polystyrene bead surface during the incubation, although only a small part of the polystyrene bead surface was contacted at any given time. Random rotation served to continuously mix the thin aqueous layer covering the polystyrene bead surface with the rest of the 2,4-dinitrophenyl- β -D-galactosidase solution (Table 1). The shaking rate was 180 per min, and the shaking width was 25 mm.

Second, a polystyrene bead of 6.35 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG was incubated with 150 μ L of 2,4-dinitrophenyl- β -D-galactosidase solutions (0.77 and 7.7 amol/ μ L) in a smaller glass test tube (10 $\times$ 75 mm) with shaking (Table 1). In this experiment, the whole surface of the polystyrene bead always remained in the 2,4-dinitrophenyl- β -D-galactosidase solution during the incubation, although the incubation was performed with shaking as described above.

Third, a polystyrene tube (12 $\times$ 75 mm) coated with 0.4 ml

of affinity-purified (anti-2,4-dinitrophenyl group) IgG was incubated with different volumes (15, 50 and 150 μ L) of 2,4-dinitrophenyl- β -D-galactosidase solution (7.7 amol/ μ L) with rotation, so that the 2,4-dinitrophenyl- β -D-galactosidase solution evenly contacted all parts of the coated polystyrene tube surface during the incubation (Table 1). The rotation rate was 40 per min.

In the first and third experiments, the fraction of 2,4-dinitrophenyl- β -D-galactosidase bound to the solid phases was only 51% after 2.5 min incubation with 150 μ L of the 2,4-dinitrophenyl- β -D-galactosidase solution but increased up to 96-100% within 2.5 min, when the volume of the 2,4-dinitrophenyl- β -D-galactosidase solution was reduced to 15 μ L. By contrast, the fraction of 2,4-dinitrophenyl- β -D-galactosidase bound was only 16-19% in the second experiment (Table 1).

Two Different Thin Aqueous Layer Immune Complex Transfer Enzyme Immunoassays

On the basis of the above results, antibody IgG to HIV-1 p17 antigen was measured in two different ways (thin aqueous layer immune complex transfer enzyme immunoassays I and II abbreviated as the present immunoassays I and II) (Fig. 1).

In the present (simultaneous) immunoassay I, an aliquot (10 μ L) of serum samples containing antibody IgG to HIV-1 p17 antigen was incubated simultaneously with 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein and rp17- β -D-galactosidase conjugate in a volume of 12 μ L in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g). Subsequently, the reaction mixture (22 μ L) was incubated with a polystyrene bead of 6.35 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG as described above. The polystyrene bead was rotated randomly and a small drop of the reaction mixture (22 μ L) evenly contacted all parts of the

TABLE 1. Binding of 2,4-Dinitrophenyl- β -D-Galactosidase to Affinity-Purified (Anti-2,4-Dinitrophenyl Group) IgG-Coated Solid Phases

Solid phase coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG	2,4-Dinitrophenyl- β -D-galactosidase for incubation		Fraction of 2,4-dinitrophenyl- β -D-galactosidase bound (%)	
	Volume (μ L)	Concentration (amol/ μ L)	Incubation time (min)	
			1.25	2.5
Polystyrene bead with a diameter of 6.35 mm in a large styrol test tube (13.3 $\times$ 54 mm, 2.1 g)	15	7.7	78	100
	50	7.7	58	77
	150	7.7	30	51
Polystyrene bead with a diameter of 6.35 mm in a small glass test tube (10 $\times$ 75 mm)	150	0.77	12	19
	150	7.7	11	16
Polystyrene test tube (12 $\times$ 75 mm)	15	7.7	84	96
	50	7.7	57	77
	150	7.7	—	51

polystyrene bead surface during the incubation, although it contacted only a small part of the polystyrene bead surface at any given time. Random rotation served to continuously mix the thin aqueous layer covering the polystyrene bead surface with the rest of the reaction mixture. After washing, the polystyrene bead and 30 μ L of ϵ N-2,4-dinitrophenyl-L-lysine solution were added to a polystyrene tube (12 $\times$ 75 mm) coated with affinity-purified (antihuman IgG γ -chain) IgG, and the polystyrene tube was shaken with slow rotation in a box (15 $\times$ 35 $\times$ 20 [depth] mm) to elute and transfer the immune complex to the polystyrene tube surface.

In the present (sequential) immunoassay II, 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-maltose binding protein-rp17 fusion protein, was incubated with a mixture of 6 μ L of a buffer and 10 μ L of serum samples containing antibody IgG to HIV-1 p17 antigen and subsequently with 10 μ L of a buffer containing rp17- β -D-galactosidase conjugate in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) as described above. The immune complex formed on the polystyrene bead was transferred to a polystyrene tube coated with affinity-purified (antihuman IgG γ -chain) IgG as in the present immunoassay I described above.

Time Courses of the Immunoreactions in the Present (Simultaneous) Immunoassay I

Serum from an HIV-1 seropositive subject was diluted with serum from an HIV-1 seronegative subject, and 10 μ L of the diluted serum was incubated with 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein and rp17- β -D-galactosidase conjugate for up to 15 min to form the immune complex comprising the three components, and was subsequently incubated with a polystyrene bead coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 5 min to trap the immune complex. β -D-galactosidase activity bound to the polystyrene bead that was assayed for 10 min reached the maximum within 10 min, indicating that the formation of the immune complex was completed within 10 min.

The immune complex was formed for 10 min and was trapped for up to 10 min. Bound β -D-galactosidase activity reached the maximum within 3 min, indicating that trapping the immune complex was completed with 3 min.

The immune complex was formed for 10 min, trapped for 5 min, and was then transferred for up to 20 min. Transference of the immune complex was almost completed within 10 min.

Amount of 2,4-Dinitrophenyl-Maltose Binding Protein-Rp17 Fusion Protein for Coating Polystyrene Beads

Polystyrene beads of 6.35 mm in diameter were coated sequentially with affinity-purified (anti-2,4-dinitrophenyl

group) IgG and various amounts of 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein and were reacted with serum from an HIV-1 seropositive subject and rp17- β -D-galactosidase conjugate. β -D-galactosidase activity bound to the polystyrene beads increased with increasing amounts of 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein but was only approximately 20% higher when coated with 800 fmol than when coated with 400 fmol. In the following experiments, therefore, 800 fmol per bead were used for coating.

Time Courses of the Immunoreactions in the Present (Sequential) Immunoassay II

First, a polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein was incubated with the diluted serum for up to 10 min and subsequently with rp17- β -D-galactosidase conjugate for 5 min. Bound β -D-galactosidase activity reached the maximum within 5 min, indicating that the binding of antibody IgG to HIV-1 p17 antigen to the polystyrene bead was completed within 5 min (Fig. 2A).

Second, a polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein was incubated with the diluted serum for 5 min and subsequently with rp17- β -D-galactosidase conjugate for up to 30 min. Bound β -D-galactosidase activity continued to increase for 30 min, although only the results by up to 10 min incubations are shown in Fig. 2B.

Third, transference of the immune complex was almost completed within 10 min as described above (Fig. 2C).

Sensitivity of the Present Immunoassays I and II

On the basis of the above results, the present (simultaneous) immunoassay I was performed as follows. The immune complex was formed for 10 min, trapped for 5 min, and was then transferred for 10 min, followed by a 60 min assay of bound β -D-galactosidase activity. Serum from an HIV-1 seronegative subject and serum from an HIV-1 seropositive subject diluted with serum from an HIV-1 seronegative subject were tested. Bound β -D-galactosidase activities were similar to those by the previous immune complex transfer enzyme immunoassay for antibody IgG to HIV-1 p17 antigen, in which the immune complex was formed for 30 min, trapped for 60 min, and was transferred for 60 min using two polystyrene beads per tube with a diameter of 3.2 mm in a total volume of 150 μ L and bound β -D-galactosidase activity was assayed for 60 min (Table 2). Thus, the present immunoassay I using 25 min for the immunoreactions was as sensitive as the previous immune complex transfer enzyme immunoassay using 150 min for the immunoreactions and was approximately 10,000 times more sensitive than Western blotting for p17 band.

Fig. 2. Time courses of the immunoreaction in the present immune complex transfer enzyme immunoassay (thin aqueous layer immune complex transfer enzyme immunoassay) II. Serum from an HIV-1 seropositive subject was diluted 2×10^4 -fold with serum from an HIV-1 seronegative subject. **A**, A polystyrene bead with a diameter of 6.35 mm coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein was incubated with 10 μ L of the diluted serum in a total volume of 16 μ L for 2.5, 5 and 10 min and subsequently with 200 fmol of rp17- β -D-galactosidase conjugate in a volume of 10 μ L for 5 min; **B**, A polystyrene bead coated as described above

was incubated with 10 μ L of the diluted serum in a total volume of 16 μ L for 5 min and subsequently with 200 fmol of rp17- β -D-galactosidase conjugate in a volume of 10 μ L for 5 and 10 min; **C**, A polystyrene bead coated as described above was incubated with 10 μ L of the diluted serum in a total volume of 16 μ L for 5 min and with 200 fmol of rp17- β -D-galactosidase conjugate in a volume of 10 μ L for 5 and 10 min. After washing, the polystyrene bead was incubated with 30 μ L of ϵ N-2,4-dinitrophenyl-L-lysine in a polystyrene tube coated with affinity-purified (antihuman IgG γ -chain) IgG for 5 and 10 min. β -D-galactosidase activity bound to the polystyrene beads or polystyrene tubes was assayed for 10 min throughout.

The present (sequential) immunoassay II was also performed within a short period of time as follows. Polystyrene beads coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein were incubated with the serum samples described above for 5 min and subsequently incubated with rp17- β -D-galactosidase conjugate for 5 min. The

immune complex formed was transferred for 5 min. Bound β -D-galactosidase activities assayed for 60 min for the diluted serum samples from an HIV-1 seropositive subject were even slightly higher than those from the previous immune complex transfer enzyme immunoassay performed under the above condition, while bound β -D-galactosidase activity for serum from an HIV-1 seronegative subject was similarly low in both

TABLE 2. Signal (Fluorescence Intensity for Bound β -D-Galactosidase Activity) by the Present and Previous Immune Complex Transfer Enzyme Immunoassays Compared With Test Results by Western Blotting

Immune complex transfer enzyme immunoassay	Incubation time for							Signal					
	Total volume for immuno-reaction	formation of the immune complex	trapping of the immune complex	reaction with antibody IgG to p17	reaction with rp17-β-D-galactosidase	transferring of the immune complex	β-D-galactosidase assay	Serum from an HIV-1 seronegative subject	Dilution of serum from an HIV-1 seropositive subject with serum from an HIV-1 seronegative subject				
									2 × 10 ⁶	2 × 10 ⁵	2 × 10 ⁴	10 ³	3 × 10 ²
Previous	μL			min									
Present	150	30	60	–	–	60	60	0.0	4.1	35	365	–	–
I	22	10	5	–	–	10	60	0.0	3.9	32	373	–	–
II	26	–	–	5	5	5	60	0.0	4.7	47	439	–	–
II	26	–	–	5	10	10	30	0.0	3.5	38	328	–	–
Western blotting for p17 band		–	–	–	–	–	–	–	–	–	–	NG ^a	PS ^b

^aNG: negative.
^bPS: positive.

the present and previous immunoassays (Table 2). Thus, the present immunoassay II, in which the immunoreactions were performed within only 15 min in total, was even slightly more sensitive than the previous one using 150 min for the immunoreactions described above and was 10,000 times more sensitive than Western blotting for p17 band.

The sensitivity of the present immunoassay II did not significantly change even when the period of time for assay of bound β -D-galactosidase activity was reduced to 30 min, provided that the incubation with rp17- β -D-galactosidase conjugate and transference of the immune complex were performed both for a longer period of time (10 min) (Table 2). Thus, the present immunoassay II, performed within 55 min (25 min for the immunoreactions and 30 min for β -D-galactosidase assay), was as sensitive as the previous immune complex transfer enzyme immunoassay requiring 3.5 h (2.5 h for the immunoreactions and 1 h for β -D-galactosidase assay) and was 10,000 times more sensitive than Western blotting for p17 band.

Finally, in the present (sequential) immunoassay II, polystyrene beads coated with 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein were reacted with serum samples for 5 min and with rp17- β -D-galactosidase conjugate for 10 min, followed by a 10 min transfer of the immune complex, and β -D-galactosidase activity bound to polystyrene test tubes coated with affinity-purified (antihuman IgG γ -chain) IgG was assayed for 10 h. The fluorescence intensity in the presence of serum samples from HIV-1 seronegative subjects was 0.66 ± 0.27 (SD) (range, 0.4 to 1.2; $n=8$) (Table 3). This was only 0.14% of that for β -D-galactosidase activity bound to polystyrene beads coated with 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein and was as low as in the previous immune complex transfer enzyme immunoassay. By contrast, the fluorescence intensity in the presence of serum from an HIV-1 seropositive subject increased proportionally by increasing the period of time for β -D-galactosidase assay from 1 h to 10 h, and was approximately 35% of that for β -D-galactosidase activity bound to polystyrene beads coated with 2,4-dinitrophenyl-maltose binding protein-rp17 fusion pro-

tein. As a result, the sensitivity of the present (sequential) immunoassay II by 10 h assay of β -D-galactosidase activity was approximately 10 times higher than that by 1 h assay of β -D-galactosidase activity and was approximately 250 times higher than that when β -D-galactosidase activity bound to polystyrene beads coated with 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein was assayed without transference of the immune complex. This improved sensitivity was 100,000 times higher than that of Western blotting for p17 band.

DISCUSSION

The fraction of 2,4-dinitrophenyl- β -D-galactosidase bound to polystyrene beads and test tubes coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG increased when the polystyrene beads and test tubes were incubated with smaller volumes of 2,4-dinitrophenyl- β -D-galactosidase solution and the polystyrene beads were incubated in a larger test tube (Table 1). More specifically, the fraction of 2,4-dinitrophenyl- β -D-galactosidase bound increased with increasing ratios of the solid phase surface area to the solution volume and/or of the solid phase surface area out of the solution to that in the solution. This prompted us to shorten the period of time for the immune complex transfer enzyme immunoassay by using smaller volumes of reaction mixtures in combination with larger solid phase surfaces.

β -D-galactosidase activity bound to polystyrene tubes coated with affinity-purified (antihuman IgG γ -chain) IgG was higher in the present (sequential) immunoassay II than in the present (simultaneous) immunoassay I (Table 2). This was expected from theoretical consideration. When antibody IgG is reacted simultaneously with 2,4-dinitrophenyl-antigen and antigen-enzyme conjugate, the amount of the immune complex formed comprising the three components must be equal to the combined amount of the immune complex comprising one antibody IgG molecule and two 2,4-dinitrophenyl-antigen molecules and the immune complex comprising one antibody IgG molecule and two antigen-enzyme conjugate molecules. By contrast, the immune complex formed must

TABLE 3. Signal (Fluorescence Intensity for Bound β -D-Galactosidase Activity) by the Present and Previous Immune Complex Transfer Enzyme Immunoassays With a Prolonged Assay of Bound β -D-Galactosidase Activity

Immune complex transfer enzyme immunoassay	Total volume for immuno-reaction	Incubation time for reaction						Signal	
		formation of the immune complex	trapping of the immune complex	reaction with antibody IgG to p17	reaction with rp17- β -D-galactosidase	transferring of the immune complex	β -D-galactosidase assay	Serum samples from 8 HIV-1 seronegative subjects	Serum from an HIV-1 seropositive subject diluted 2×10^7 -fold with serum from an HIV-1 seronegative subject
	μ L			min				Mean $\pm$ SD (range)	
Previous	150	30	60	—	—	60	600	0.94 ± 0.24 (0.63–1.3)	4.9
Present II	26	—	—	5	10	10	600	0.66 ± 0.27 (0.4–1.2)	7.9

comprise the three components, when anti-2,4-dinitrophenyl group IgG-coated solid phase is reacted sequentially with 2,4-dinitrophenyl-antigen, antibody IgG, and antigen-enzyme conjugate.

In the present immunoassay II, polystyrene beads coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-maltose binding protein-rp17 fusion protein were reacted with serum samples and, without washing, with rp17- β -D-galactosidase conjugate as described above. Washing process after reaction with serum samples could be deleted. Alternatively, the polystyrene beads can be reacted with rp17- β -D-galactosidase conjugate after washing following reaction with serum samples, making it possible to use smaller amounts of rp17- β -D-galactosidase conjugate in smaller volumes.

Binding of rp17- β -D-galactosidase conjugate to antibodies against p17 antigen on the polystyrene beads depended not only on the incubation time but also on the concentration of the conjugate used. With higher concentrations of the conjugate and longer periods of time for binding of the conjugate, β -D-galactosidase activities bound increased, and the period of time for β -D-galactosidase assay could be shortened. Therefore, the concentration of the conjugate to be used remains to be optimized to obtain a higher sensitivity within a shorter period of time.

In the present immunoassays I and II, the immune complex was not efficiently transferred from affinity-purified (anti-2,4-dinitrophenyl group) IgG-coated polystyrene bead to affinity-purified (antihuman IgG γ -chain) IgG-coated polystyrene tube. The fractions of the immune complex transferred for 5 and 10 min were 26 and 35%, respectively. In preliminary experiments, an alternative transference of the immune complex was tested. The immune complex was trapped onto a polystyrene bead of 6.35 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG as in the present (sequential) immunoassay II. The polystyrene bead after washing was incubated with 35 μ L of 2 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g) for 15 min as described above to elute the immune complex, and the first eluate was transferred to a polystyrene test tube (12 $\times$ 75 mm) coated with affinity-purified (antihuman IgG γ -chain) IgG. The polystyrene bead was incubated with an additional 35 μ L of 2 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine for 1 min in the same way, and the second eluate was combined with the first eluate. The polystyrene test tube containing the combined eluate was rotated for 15 min as described above. The fraction of the immune complex transferred was 56.9%. The same result was obtained using polystyrene beads coated with 10 μ g/ml of affinity-purified (anti-2,4-dinitrophenyl group) IgG, while coated with 50 μ g/ml in the above experiments. The polystyrene test tube may be replaced by a polystyrene bead of 6.35 mm in diameter coated with affinity-purified (antihuman IgG γ -chain) IgG in a styrol test tube (13.3 $\times$ 54 mm and 2.1 g)

described above. However, no less than 30 min was used for transference of the immune complex. Solid-phase forms to shorten the period of time for transferring the immune complex remain to be developed.

In the present (sequential) immunoassay II, the nonspecific signal, that is, the fluorescence intensity for β -D-galactosidase activity bound to affinity-purified (antihuman IgG γ -chain) IgG-coated polystyrene tubes in the presence of serum samples from HIV-1 seronegative subjects, was 0.66 ± 0.27 (SD) (range, 0.4–1.2; $n=8$) (Table 3). This was 0.14% of that bound to affinity-purified (anti-2,4-dinitrophenyl group) IgG-coated polystyrene beads and could be measured only by 10 h assay since the highest sensitivity that could be achieved by the immune complex transfer enzyme immunoassay required 10 h (600 min) assay of the label enzyme. Therefore, the sensitivity of assay method for label enzyme remains to be improved 25- to 40-fold, since the immunoreactions can be performed within 15–25 min.

Finally, it should be noted that there are at least two factors to limit the reduction of the nonspecific signal by the immune complex transfer enzyme immunoassay. The first is the direct contact of affinity-purified (anti-2,4-dinitrophenyl group) IgG- and affinity-purified (antihuman IgG γ -chain) IgG-coated solid phases during transference of the immune complex. In the previous immune complex transfer enzyme immunoassay, a method to avoid direct contact of the two solid phases was tested. The immune complex was trapped onto colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. Subsequently, the immune complex was eluted from the colored polystyrene beads by incubation with ϵ N-2,4-dinitrophenyl-L-lysine, and the eluate was incubated with white polystyrene beads coated with affinity-purified (antihuman IgG γ -chain) IgG. β -D-galactosidase activity bound to the white polystyrene beads was approximately 50% lower than that observed when the colored and white polystyrene beads were incubated with ϵ N-2,4-dinitrophenyl-L-lysine in a test tube for transference of the immune complex. The second factor is nonspecific reaction(s) of the conjugates with serum samples from HIV-1 seronegative subjects, which remain to be analyzed.

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