Rapid and Ultrasensitive Enzyme Immunoassay (Thin Aqueous Layer Immune Complex Transfer Enzyme Immunoassay) for HIV-1 p24 Antigen

Setsuko Ishikawa, Seiichi Hashida, Kazuya Hashinaka, and Eiji Ishikawa*

Department of Biochemistry, Miyazaki Medical College, Kiyotake, Miyazaki, Japan

The immune complex transfer enzyme immunoassay for HIV-1 p24 antigen was performed in three different ways (in the present immunoassays I, II, and III) within much shorter periods of time than previously reported. In the present (simultaneous) immunoassay I, p24 antigen was incubated simultaneously with 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate in a total volume of 19 μ L for 15 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 to trap the immune complex. After washing, the polystyrene bead was incubated with 35 μ L of ϵ N-2,4-dinitrophenyl-L-lysine for 15 min to elute the immune complex (the first eluate) and, after removing the first eluate, with an additional 35 μ L of ϵ N-2,4-dinitrophenyl-L-lysine for 1 min (the second eluate). The first and second eluates were incubated with a polystyrene test tube (12 $\times$ 75 mm) coated with streptavidin for 15 min. In the present (sequential) immunoassay II, a polystyrene bead of 6.35 mm in diameter successively coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate was incubated with p24 antigen in a total volume of 20 μ L for 5 min and subsequently with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate in a volume of 5 μ L for 20 min. The immune complex formed on the polystyrene bead was transferred to a polystyrene test tube coated with streptavidin as described above. In the present (sequential) immunoassay III, p24 antigen was incubated with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate in a total volume of 19 μ L for 10 min and 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 rabbit anti-p24 Fab' conjugate for 20 min. The immune complex formed on the polystyrene bead was transferred as described above. The incubations were performed at room temperature either by shaking the polystyrene beads (one/assay) and the reaction mixtures in styrol test tubes (13.3 $\times$ 54 mm and 2.1 g) so as to randomly rotate the polystyrene beads or by rotating the polystyrene test tubes (12 $\times$ 75 mm) containing the reaction mixtures, so that small drops (19 to 70 μ L) of the reaction mixtures evenly contacted all parts of the solid phase surfaces during the incubations (although they contacted only small parts of the solid phase surfaces at a time) to continuously mix thin aqueous layers covering the solid phase surfaces with the rest of the reaction mixtures. (Therefore, these immunoassays are called thin aqueous layer immunoassays.) The detection limits of p24 antigen by 1 hr assay of bound β -D-galactosidase activity in the present immunoassays I, II, and III were 0.1, 0.2 and 0.1 amol/assay, respectively, and were slightly higher than or equal to that by the previously reported immune complex transfer enzyme immunoassay, in which the immune complex was formed for 4 hr, was trapped for 16 hr, and was transferred for 3 hr followed by 1-hr assay of bound β -D-galactosidase activity. By 20-hr assay of bound β -D-galactosidase activity, the detection limit of p24 antigen was further lowered to 10 zmol/assay in the present (simultaneous) immunoassay I and to 3 zmol/assay in the present (sequential) immunoassay III. However, the nonspecific reaction(s) with serum samples from HIV-1 seronegative subjects hampered the improvement of the detection limit by 20-hr assay of bound β -D-galactosidase activity. *J. Clin. Lab. Anal.* 12:205–212, 1998. © 1998 Wiley-Liss, Inc.

*Correspondence to: Eiji Ishikawa, M.D., Ph.D., Professor of Biochemistry, Department of Biochemistry, Miyazaki Medical College, Kiyotake, Miyazaki 889-1692, Japan.

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INTRODUCTION

Ultrasensitive enzyme immunoassays (immune complex transfer enzyme immunoassays) for antibody IgGs to HIV-1 have been developed using recombinant proteins of HIV-1 as antigens (1–7). 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 with higher sensitivities and specificities than Western blotting (7). Notably, antibody IgG to HIV-1 p17 antigen was detected as early as or even earlier than antibodies to HIV-1 by conventional methods (7,8). In addition, the immune complex transfer enzyme immunoassay for HIV-1 p24 antigen has also been developed (9,10). On the basis of these results, the period of time immediately following HIV-1 infection, during which the diagnosis of HIV-1 infection is not possible due to the absence of detectable antibodies to HIV-1, has been considerably shortened by simultaneous detection of both p24 antigen and antibody IgGs to p17 antigen and reverse transcriptase of HIV-1 (8). In these immunoassays, however, the periods of time used for the immunoreactions involved were considerably long. In the immune complex transfer enzyme immunoassay for HIV-1 p24 antigen, the immune complex (comprised of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-anti-p24 Fab' conjugate, the antigen and anti-p24 Fab'- β -D-galactosidase conjugate) was formed by 4-hr incubation and was trapped onto polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG by overnight incubation. After washing, the immune complex was eluted from the polystyrene beads with ϵ N-2,4-dinitrophenyl-L-lysine and was transferred to polystyrene beads coated with streptavidin by 3-hr incubation. Bound β -D-galactosidase activity was assayed by fluorometry for 2.5 hr. Recently, it has been made possible to perform more sensitive immune complex transfer enzyme immunoassays for antibody IgG to p17 antigen within shorter periods of time (15 to 60 min each for the formation, trapping, and transferring of the immune complex) by incubations with shaking for the immunoreactions (11).

This report describes ultrasensitive and rapid enzyme immunoassays (thin aqueous layer immune complex transfer enzyme immunoassays) for HIV-1 p24 antigen that can be performed within much shorter periods of time with as high sensitivity as that of the previously reported immune complex transfer enzyme immunoassay.

MATERIALS AND METHODS

Buffer

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 bovine serum albumin (fraction V,

Intergen Company, Purchase, NY). The concentrations of bovine serum albumin were 1 g/L (buffer A) and 0.1 g/L (buffer B).

Anti-p24 Antibodies

Rabbit anti-p24 serum was prepared as described previously (9). Monoclonal mouse anti-p24 IgG₁ (24C11) was obtained from Innogenetics N.V., Zwijnaarde, Belgium.

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, Okayama, Japan) were coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG (50 μ g/ml) by physical adsorption as described previously (9). White polystyrene beads with a diameter of 3.2 mm (Immuno Chemical) were coated successively with biotinyl-bovine serum albumin (100 μ g/ml) and streptavidin (100 μ g/ml) by physical adsorption as described previously (9). The coated polystyrene beads were stored in buffer A at 4°C until use.

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

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

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 (50 μ g/ml) as described above were incubated with 20 μ L/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 $\times$ 54 mm, one bead/assay) for 5 min as described in the present immune complex transfer enzyme immunoassay I. The coated polystyrene beads were stored in buffer A at 4°C until use.

Polystyrene Test Tubes Coated With Streptavidin

Polystyrene test tubes (12 $\times$ 75 mm, Maxisorp, A/S Nunc, DK-4000 Roskilde, Denmark) were coated by physical adsorption with 0.4 μ L of 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 0.1% NaN₃ and 12 μ g 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.

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

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

Previous Immune Complex Transfer Enzyme Immunoassay

The antigen, p24, in serum was measured as described previously (10). An aliquot (100 μL) of buffer A containing 0.4 mol/L NaCl, 50 μg of inactive β -D-galactosidase (Mutein, Boehringer Mannheim GmbH, Mannheim, Germany) and recombinant p24 (rp24) antigen was incubated for 4 hr with 10 μL of nonspecific rabbit serum and 40 μL of buffer A containing 0.4 mol/L NaCl, 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and 5 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate. To the reaction mixture (150 μL), two colored polystyrene beads (3.2 mm in diameter) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG were added, and the incubation was continued for 16 hr. After removing the reaction mixture, the colored polystyrene beads were washed and were incubated for 3 hr with two white polystyrene beads (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 white polystyrene beads were washed three times with 2 ml of buffer B and were incubated for 1 hr with a mixture of 100 μL of buffer B 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 for bound β -D-galactosidase activity 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.

Present Immune Complex Transfer Enzyme Immunoassay I

In a styrol test tube (13.3 $\times$ 54 mm and 2.1 g), an aliquot (2.5 μL) of buffer A containing 7.5 μg of inactive β -D-galactosidase was mixed sequentially with 2.5 μL of nonspecific rabbit serum, 1 μL of buffer A containing 5 fmol of monoclonal mouse anti-p24 Fab' β -D-galactosidase conjugate, 1 μL of buffer A containing 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-

p24 Fab' conjugate, 10 μL of serum samples from HIV-1 seronegative subjects, and 2 μL of buffer A containing rp24. The reaction mixture (19 μL) was allowed to stand for up to 30 min to form the immune complex comprising the three components and was incubated for up to 5 min with a polystyrene bead (6.35 mm in diameter) coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. The incubation was performed by shaking the styrol test tube in a box (16 $\times$ 16 $\times$ 20 [depth] mm), so that the polystyrene bead was rotated randomly and a small drop (19 μL) 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 shaking rate was 180 per min, and the shaking width was 25 mm. The polystyrene bead was washed four times with 2 ml of buffer B and was incubated for up to 15 min with 35 μL of buffer A containing 2 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine in a styrol test tube (13.3 $\times$ 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 $\times$ 75 mm) coated with streptavidin. The polystyrene bead in the styrol test tube was incubated for 1 min with an additional 35 μL 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 (40 rotations per min) for up to 20 min, so that the ϵ N-2,4-dinitrophenyl-L-lysine solution evenly contacted the polystyrene test tube surface coated with streptavidin during the incubation. The polystyrene test tube was washed four times with 2 ml of buffer B, and β -D-galactosidase activity bound to the polystyrene test tube was assayed for 1 or 20 hr by incubation with a mixture of 50 μL of buffer B and 25 μL 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 for bound β -D-galactosidase activity was measured as described above. All the incubations were performed at room temperature.

Present Immune Complex Transfer Enzyme Immunoassay II

In a styrol test tube (13.3 $\times$ 54 mm and 2.1 g), 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 rabbit anti-p24 Fab' conjugate was incubated with a mixture of 2.5 μL of nonspecific rabbit serum, 10 μL of serum samples from HIV-1 seronegative subjects and 7.5 μL of buffer A containing 7.5 μg of inactive β -D-galactosidase and rp24 for 5 min, and with 5 μL of buffer A containing 5 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate for

up to 30 min. After washing the polystyrene bead, the immune complex was transferred from the polystyrene bead to a polystyrene test tube coated with streptavidin as described above.

Present Immune Complex Transfer Enzyme Immunoassay III

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, and 1 μ L of buffer A containing 5 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate and was incubated for up to 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 up to 30 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 as described above. After washing the polystyrene bead, the immune complex was transferred from the polystyrene bead to a polystyrene test tube coated with streptavidin as described above.

Serum Samples

Serum samples were collected from 6 healthy subjects (3 males aged 24–63 yr and 3 females aged 32–61 yr) with very low risk for HIV infection and were stored at -20°C until use.

RESULTS AND DISCUSSION

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

The immune complex transfer enzyme immunoassay for HIV-1 p24 antigen was performed at room temperature in three different ways (Fig. 1).

In the present (simultaneous) immunoassay I, an aliquot (2.5 μ L) of nonspecific rabbit serum was mixed successively with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate to a total volume of 7 μ L and was incubated with 10 μ L of serum from an HIV-1 seronegative subject and rp24 antigen in a volume of 2 μ L in a styrol test tube (13.3×54 mm and 2.1 g) to form the immune complex comprising the three components. Subsequently, the reaction mixture (19 μ L) was incubated with a polystyrene bead of 6.35 mm in diameter coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG to trap the immune complex. The incubation with the polystyrene bead was performed by shaking the styrol test tube in a box ($16 \times$

Fig. 1. Present immune complex transfer enzyme immunoassays (thin aqueous layer immune complex transfer enzyme immunoassays) I (simultaneous) and III (sequential) for HIV-1 p24 antigen. DNP: 2,4-dinitrophenyl group. Gal: β -D-galactosidase

16×20 (depth) mm). The shaking rate and width were 180/min and 25 mm, respectively. As a result, the polystyrene bead was rotated randomly in the styrol test tube, and a small drop (19 μ L) of the reaction mixture evenly contacted all parts of the polystyrene bead surface during the incubation, although contacted only its small part at a time, to continuously mix thin aqueous layer covering the polystyrene bead surface with the rest of the reaction mixture. After washing, the polystyrene bead was incubated with 35 μ L of 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 added to a polystyrene test tube (12×75 mm) coated with streptavidin. The polystyrene bead in the styrol test tube was incubated with an additional 35 μ L of ϵ N-2,4-dinitrophenyl-L-lysine with shaking. The second eluate was also added to the polystyrene test tube. The polystyrene test tube containing the combined eluate was rotated as described in Materials and Methods.

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-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate was incubated sequentially with rp24 antigen in the presence of 2.5 μ L of nonspecific rabbit serum and 10 μ L of serum from an HIV-1 seronegative subject in a total volume of 20 μ L and with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate in a volume of

5 μ L with shaking as described above. The immune complex was transferred as described above.

In the present (sequential) immunoassay III, rp24 antigen was incubated with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate in the presence of 2.5 μ L of nonspecific rabbit serum and 10 μ L of human serum from an HIV-1 seronegative subject in a total volume of 19 μ L and subsequently 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 rabbit anti-p24 Fab' conjugate with shaking as described above. The immune complex was transferred as described above.

Time Course of the Immune Complex Formation on Polystyrene Beads in the Present (Simultaneous) Immunoassay I

Time course of the formation of the immune complex

The antigen, rp24, was incubated with the two conjugates for 15 and 30 min to form the immune complex and subsequently with the polystyrene bead for 5 min to trap the immune complex. β -D-Galactosidase activity bound to the polystyrene bead reached the maximum by the 15 min incubation. Namely, the formation of the immune complex was completed within 15 min.

Time course of trapping the immune complex

The antigen, rp24, was incubated with the two conjugates for 15 min to form the immune complex and subsequently with the polystyrene bead for 3 and 5 min to trap the immune complex. β -D-Galactosidase activity bound reached the maximum by the 3 min incubation. Namely, trapping of the immune complex was completed within 3 min.

Amount of 2,4-Dinitrophenyl-Biotinyl-Bovine Serum Albumin-Affinity-Purified Rabbit Anti-p24 Fab' Conjugate for Coating Polystyrene Beads

In the present immunoassay II, a polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 200 or 400 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate was incubated sequentially with rp24 antigen for 5 min and with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate for 20 min. β -D-Galactosidase activity bound to the polystyrene bead was 16–31% higher when coated with 200 fmol than when coated with 400 fmol. In the present immunoassay III, rp24 antigen was incubated with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate for 10 min and subsequently with a polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 200 or 400 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-pu-

rified rabbit anti-p24 Fab' conjugate for 20 min. β -D-Galactosidase activity bound to the polystyrene bead was 20–33% higher when coated with 400 fmol than when coated with 200 fmol. In the present immunoassays II and III, therefore, the amounts of the conjugate used for coating were 200 and 400 fmol/bead, respectively.

Time Course of the Immune Complex Formation on Polystyrene Beads in the Present (Sequential) Immunoassay II

A polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate was incubated with rp24 antigen for 5 min and subsequently with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate for 15 and 30 min. β -D-galactosidase activity bound to the polystyrene bead by the 30 min incubation was 21% higher than that by the 15 min incubation.

Time Course of the Immune Complex Formation on Polystyrene Beads in the Present (Sequential) Immunoassay III

The antigen rp24 was incubated with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate for 5 and 10 min and with a polystyrene bead coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate for 20 min. β -D-galactosidase activity bound to the polystyrene bead by the 10 min incubation was 25% higher than that by the 5 min incubation. When rp24 antigen was incubated with the β -D-galactosidase conjugate for 10 min and with the polystyrene bead for 20 and 30 min, β -D-galactosidase activity bound by the 30 min incubation was 29% higher than that by the 20 min incubation.

Time Course of Transferring the Immune Complex

The antigen rp24 was incubated with monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate for 10 min and subsequently with a polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate for 20 min. After washing, the polystyrene bead was incubated with ϵ N-2,4-dinitrophenyl-L-lysine for 10 and 15 min. β -D-galactosidase activity bound to the polystyrene bead decreased by 70 and 76%, respectively. Namely, the immune complex was eluted 70–76% within 10 to 15 min.

A polystyrene bead coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG, on which the immune complex had been formed as described above, was incubated with 35 μ L of ϵ N-2,4-dinitrophenyl-L-lysine for 15 min (the first eluate)

TABLE 1. Signal (Fluorescence Intensity for β -D-Galactosidase Activity) by the Present and Previous Immune Complex Transfer Enzyme Immunoassays for p24 Antigen

		Incubation time for										
Immune complex transfer enzyme immunoassay	Total volume for immuno-reaction	Formation of the immune complex comprising the three components	Trapping of the immune complex comprising the three components	Reaction with rp24	Reaction with anti-p24 Fab'-β-D-galactosidase conjugate	Formation of the immune complex comprising rp24 and anti-p24Fab'-β-D-galactosidase conjugate	Trapping of the immune complex comprising rp24 and anti-p24 Fab'-β-D-galactosidase conjugate	Transferring of the immune complex	Assay of β-D-galactosidase	Signal		Serum samples from HIV-1 seronegative subjects
										rp24 (amol)		
										0	100	
Present 0.0 to 0.59	μL	min										
	I	19 to 70	15	5	—	—	—	—	30	60	0.0	353
— 0.0 to 0.27	II	20 to 70	—	—	5	20	—	—	30	60	0.0	126
	III	19 to 70	—	—	—	—	10	20	30	60	0.0	424
Previous —		150	240	960	—	—	—	—	180	60	0.0	94

The amounts of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified anti-p24 Fab' conjugate used in the present immune complex transfer enzyme immunoassays I, II and III were 100, 200, and 400 fmol/assay, respectively, and the amount of monoclonal anti-p24 Fab'- β -D-galactosidase conjugate used was 5 fmol/assay in all the immunoassays. In the present immunoassays, the immune complex was eluted for 15 min and was transferred for 15 min.

Fig. 2. Dose-response curves of HIV-1 rp24 antigen by the present immune complex transfer enzyme immunoassay I. The antigen was incubated simultaneously with 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and 5 fmol of monoclonal mouse anti-p24 Fab'-β-D-galactosidase conjugate in the presence of 2.5 μL of nonspecific rabbit serum and 10 μL of human serum from an HIV-1 seronegative subject in a total volume of 19 μL for 15 min and 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. The immune complex was transferred as described in Table 1. Bound β-D-galactosidase activity was assayed for 1 hr and 20 hr (small figure).

and, after removing the first eluate, with another 35 μL for 1 min (the second eluate). The first and second eluates were incubated with a polystyrene test tube coated with streptavidin for 10, 15 and 20 min. β-D-galactosidase activity bound to the polystyrene test tube by the 15 min incubation was 11%

higher than that by the 10 min incubation and was equal to that by the 20 min incubation. Namely, trapping of the immune complex was completed within 15 min.

Sensitivity of the Present Immunoassays

On the basis of the above results, the present immunoassays I, II, and III were performed as follows (Table 1). In the present (simultaneous) immunoassay I, rp24 antigen was incubated with the two conjugates for 15 min and subsequently with a polystyrene bead coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 5 min, and the immune complex was eluted for 15 min and was transferred for 15 min. In the present (sequential) immunoassay II, a polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate (200 fmol/bead) was incubated with rp24 antigen for 5 min and subsequently with monoclonal mouse anti-p24 Fab'-β-D-galactosidase conjugate for 20 min, and the immune complex was transferred as described above. In the present (sequential) immunoassay III, rp24 antigen was incubated with monoclonal mouse anti-p24 Fab'-β-D-galactosidase conjugate for 10 min and subsequently with a polystyrene bead coated successively with affinity-purified (anti-2,4-dinitrophenyl group) IgG and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate (400 fmol/bead) for 20 min, and the immune complex was transferred as described above.

In the present (simultaneous) immunoassay I, the detection limit of rp24 antigen was 0.1 amol/assay by 1 hr assay of bound β-D-galactosidase activity and was lowered to 10 zmol by 20 hr assay of bound β-D-galactosidase activity (Fig. 2).

In the present (sequential) immunoassay II, the detection limit of rp24 antigen was 0.2 amol/assay by 1 hr assay of bound β-D-galactosidase activity.

In the present (sequential) immunoassay III, β-D-galactosidase activity bound to polystyrene beads coated with affini-

TABLE 2. Effect of the Immune Complex Transfer on Signals for β-D-Galactosidase Activity Bound to Polystyrene Test Tubes Coated with Streptavidin in the Presence and Absence of p24 Antigen in the Present Immunoassay III^a

Ratio of signal ^b	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)	
		Before transfer of the immune complex	After transfer of the immune complex	Before transfer of the immune complex	After transfer of the immune complex
—	1.28×10^6	240	0.4	17,760	9,288
Ratio of signal for the conjugate used to that before transfer	5.33×10^3	1.0	—	—	—
Ratio of signals for the conjugate used and before transfer to that after transfer	3.20×10^6	600	1.0	1.9	1.0

^aThe present immunoassay III was performed as described in Table 1.

^bThe signals are expressed as the fluorescence intensities for β-D-galactosidase activity assayed for 20 hr.

Fig. 3. Dose-response curves of HIV-1 rp24 antigen by the present immune complex transfer enzyme immunoassay III. The antigen was incubated with 5 fmol of monoclonal mouse anti-p24 Fab'-β-D-galactosidase conjugate in the presence of 2.5 μL of nonspecific rabbit serum and 10 μL of human serum from an HIV-1 seronegative subject in a total volume of 19 μL for 10 min, and was incubated 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 rabbit anti-p24 Fab' conjugate (400 fmol/bead) for 20 min. The immune complex was transferred as described in Table 1. Bound β-D-galactosidase activity was assayed for 1 hr and 20 hr (small figure).

ity-purified (anti-2,4-dinitrophenyl group) IgG in the absence of p24 antigen was 5,330-fold lower than that of monoclonal mouse anti-p24 Fab'-β-D-galactosidase conjugate used, and β-D-galactosidase activity bound to polystyrene test tubes coated with streptavidin in the absence of p24 antigen was 600-fold lower than that bound to the polystyrene beads. By contrast, β-D-galactosidase activity bound to the polystyrene test tubes in the presence of p24 antigen was only 1.9-fold lower than that bound to the polystyrene beads (Table 2). As a result, the detection limit of p24 antigen was lowered 300-fold by transferring of the immune complex and was 0.1 amol by 1 hr assay of bound β-D-galactosidase activity. This was further lowered to 3 zmol by 20 hr assay of bound β-D-galactosidase activity (Fig. 3).

However, the highest bound β-D-galactosidase activity in the presence of serum samples from HIV-1 seronegative sub-

jects was only slightly lower than that corresponding to 0.2 amol of rp24 antigen in the present immunoassay I and 0.1 amol of rp24 antigen in the present immunoassay III. Namely, the sensitivity of the present immunoassays were limited by unknown reaction(s) of the conjugate(s) with serum samples from HIV-1 seronegative subjects.

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