Optimal Conditions of Immune Complex Transfer Enzyme Immunoassay for p24 Antigen of HIV-1

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In the immune complex transfer enzyme immunoassay for HIV-1 p24 antigen, different preparations of anti-p24 Fab'- β -D-galactosidase conjugate, various periods of time for immunoreactions involved, and shaking for incubations with polystyrene beads were tested. On the basis of the results of these experiments, p24 antigen was measured as follows. The antigen was reacted simultaneously with 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and highly polymerized monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate at 37°C for 2 hr. The immune complex formed comprising the three components was trapped onto colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 1.5 hr and was transferred to white polystyrene beads coated with streptavidin in the presence of ϵ N-2,4-dinitrophenyl-L-lysine for 1.5

hr. The incubations with polystyrene beads were performed at room temperature with shaking. β -D-Galactosidase activity bound to the white polystyrene beads was assayed by fluorometry at 30°C for 2 hr. The detection limit of p24 antigen (0.1 amol/tube and 10 amol (0.24 pg)/ml of serum) was equal to that obtained when the formation, trapping, and transferring of the immune complex were performed for 4, 16, and 3 hr, respectively, by incubation without shaking. Namely, the period of time required for the immune complex transfer enzyme immunoassay of p24 antigen was markedly shortened (25.5–7 hr) without loss of the sensitivity. By the improved immune complex transfer enzyme immunoassay, p24 antigen was detected 12–20 days earlier than the detection of antibodies to HIV-1, i.e., seroconversion by the conventional ELISA. *J. Clin. Lab. Anal.* 12:115–120, 1998. © 1998 Wiley-Liss, Inc.

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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 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, the immune complex transfer enzyme immunoassay for p24 antigen of HIV-1 also has been developed (9,10). On the basis of these results, the window period after 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 shortened by simultaneous detection of both p24 antigen and antibody IgGs to p17 and reverse transcriptase of HIV-1 (8). In these immunoassays, however, the period of time used for the immunoreactions involved was considerably long. In the immune complex transfer enzyme immunoassay for p24 antigen of HIV-1, the immune complex, comprising 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 were trapped onto polystyrene beads coated with affinity-purified (anti-2,4-

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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 within shorter periods of time (15–60 min each for the formation, trapping, and transferring of the immune complex) by incubations for the immunoreactions with shaking (11).

This report describes optimal conditions of the immunoreactions involved to perform the immune complex transfer enzyme immunoassay for p24 antigen of HIV-1 within shorter periods of time without loss of the sensitivity.

MATERIALS AND METHODS

Recombinant Proteins of HIV-1

Recombinant p24 (rp24) was produced in *Escherichia coli* transformed with expression plasmid carrying the corresponding cDNA and was purified as described previously (12). The recombinant proviral clone used was pNL4-3 (13), which contained DNA from HIV-1 isolates NY5 (GenBank accession number HIVNL43) and LAV (14), and the sequences for rp24 derived from NY5.

Anti-p24 Antibodies

Rabbit anti-p24 serum was prepared by immunization with rp24 (9). Monoclonal mouse anti-p24 IgG₁ (24C11) was obtained from Innogenetics N.V. (Zwijnaarde, Belgium).

Buffer A

The regularly used buffer was 10 mmol/L sodium phosphate buffer, pH 7.0, containing 1.0 g/L bovine serum albumin (fraction V, Interger, Purchase, NY), 1.0 mmol/L MgCl₂, and 1.0 g/L NaN₃ (buffer A).

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).

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

Monoclonal mouse anti-p24 Fab' was conjugated with β -D-galactosidase from *E. 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 μ l) of p24 antigen di-

luted with buffer A containing 0.4 mol/L NaCl was incubated for 4 hr with 40 μ l 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, 5 or 100 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate and 50 μ g of inactive β -D-galactosidase (β -D-galactosidase-mutein, Boehringer Mannheim GmbH, Mannheim, Germany) and 10 μ l of nonspecific rabbit serum. To the reaction mixture, 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 overnight. 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 μ l of buffer A containing 0.1 mol/L NaCl and 1 mmol/L ϵ N-2,4-dinitrophenyl-L-lysine. The incubations were performed at room temperature without shaking throughout. After removing the colored polystyrene beads, the white polystyrene beads were washed, and bound β -D-galactosidase activity was assayed by fluorometry using 4-methylumbelliferyl- β -D-galactoside as substrate at 30°C for 2.5 hr. The fluorescence intensity was measured relative to 1×10^{-8} mol/L 4-methylumbelliferone (15).

Present Immune Complex Transfer Enzyme Immunoassay

The present immune complex transfer enzyme immunoassay was performed in the same way as the previous one described above except for the following modifications. An aliquot (100 μ l) of p24 antigen diluted with buffer A containing 0.4 mol/L NaCl was incubated with the two conjugates, inactive β -D-galactosidase and nonspecific rabbit serum for up to 16 hr and subsequently with two colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for up to 4 hr. The colored polystyrene beads after washing were incubated for up to 2 hr with streptavidin-coated polystyrene beads in the presence of ϵ N-2,4-dinitrophenyl-L-lysine. The incubations with polystyrene beads were performed at room temperature with 180 shakings per min throughout. Bound β -D-galactosidase activity was assayed at 30°C for up to 2 hr.

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, Tokyo, Japan). The conventional ELISA for antibodies to HIV-1 was performed using a commercial kit with two recombinant proteins of HIV-1 (gp41 and p24) as antigens (Abbott Recombinant HIV-1/HIV-2 3rd Generation EIA, Abbott Laboratories, North Chicago, IL).

Seroconversion Serum Panels

Three seroconversion serum panels, J, P, and Z, were obtained from Boston Biomedica (West Bridgewater, MA).

Serum Samples From HIV-1 Seronegative Subjects

Serum samples were collected from 50 HIV-1 seronegative subjects (39 males ages 27–79 yr and 11 females ages 35–63 yr) and were stored at -20°C until use. The seronegativity was tested by the gelatin particle agglutination test.

RESULTS AND DISCUSSION

The present immune complex transfer enzyme immunoassay for p24 antigen of HIV-1 was performed as follows. The antigen was reacted simultaneously with 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified

rabbit anti-p24 Fab' conjugate and 5 or 100 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate. Two different preparations of monoclonal anti-p24 Fab'- β -D-galactosidase conjugate, that is, highly and less polymerized conjugates were used (Fig. 1). The immune complex formed comprising the three components was trapped onto colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. The immune complex was eluted from the colored polystyrene beads with excess of ϵ N-2,4-dinitrophenyl-L-lysine and was transferred to white polystyrene beads coated with streptavidin (Fig. 2). The immunoreactions using polystyrene beads were performed at room temperature with shaking. β -D-Galactosidase activity bound to the white polystyrene beads was assayed by fluorometry. In order to perform the immune complex transfer enzyme immunoassay within as short a period of time as possible, time courses of the immunoreactions were examined (Fig. 3).

Fig. 1. Elution profile of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate from a column of Ultrogel AcA22. Maleimide- β -D-galactosidase (1.7 mg, 3.2 nmol) was reacted with monoclonal mouse anti-p24 Fab' (0.65 mg, 13.0 nmol) in 1.6 ml of 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 1 mmol/L EDTA at 4°C for 16 hr. The reaction mixture was applied to a column of Ultrogel AcA22 (1.5 x 60 cm) using 10 mmol/L sodium phosphate buffer, pH 7.0, containing 0.1 g/L bovine serum albumin, 0.1 mol/L NaCl, 1 mmol/L MgCl_2 , and 1 g/L NaN_3 . The flow rate was 25 ml/hr, and the fraction volume was 1.0 ml. Closed circles indicate the fluorescence intensity for β -D-galactosidase activity in an aliquot (0.01 μl) of each fraction, assayed at 30°C for 10 min by fluorometry using 4-methylumbelliferyl- β -D-galactoside as substrate. The fluorescence intensity was measured relative to 1×10^{-7} mol/L 4-methylumbelliferone in 0.1 mol/L glycine-NaOH buffer, pH 10.3. Open circles indicate the absorbance at 280 nm of each fraction.

Fig. 2. Immune complex transfer enzyme immunoassay for HIV-1 p24 antigen. DNP: 2,4-dinitrophenyl group. Gal: β -D-galactosidase.

Fig. 3. Time courses for the formation, trapping and transferring of the immune complex comprising 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate, HIV-1 p24 antigen and monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate. **A.** p24 antigen (100 amol) was incubated with 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and 5 or 100 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate at 25°C (broken lines) and 37°C (solid lines) for up to 16 hr and subsequently with two colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG at room temperature for 20 min with shaking. **B.** p24 antigen (100 amol) was incubated with the two conjugates at 37°C for 2 hr as described above and subsequently with the two colored polystyrene beads

at room temperature for up to 4 hr with shaking. **C.** p24 antigen (100 amol) was incubated with the two conjugates at 37°C for 2 hr as described above and subsequently with the two colored polystyrene beads at room temperature for 2 hr with shaking. The colored polystyrene beads after washing were incubated with white polystyrene beads coated with streptavidin in the presence of ϵ N-2,4-dinitrophenyl-L-lysine at room temperature for up to 2 hr with shaking. β -D-Galactosidase activity bound to the polystyrene beads was assayed at 30°C for 1 hr. Circles and squares indicate results obtained using 5 fmol of highly polymerized monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate and 100 fmol of less polymerized one (Fig. 1), respectively. Open and closed symbols indicate results obtained in the presence and absence, respectively, of p24 antigen (100 amol).

Time Course of the Formation of the Immune Complex

The antigen p24 was reacted with the two conjugates at 25 and 37°C for up to 16 hr, and the immune complex formed was trapped for 20 min. β -D-Galactosidase activity bound to the colored polystyrene beads in the presence of p24 antigen was 2-fold higher with 5 fmol of highly polymerized anti-p24 Fab'- β -D-galactosidase conjugate than with 100 fmol of less polymerized one and continued to increase at 25°C for 4 hr. However, by reacting p24 antigen with the two conjugates at 37°C, bound β -D-galactosidase activity in the presence of p24 antigen reached values close to the maximum within 2 hr.

Time Course of Trapping of the Immune Complex

The antigen p24 was reacted with the two conjugates at 37°C for 2 hr as described above, and the immune complex formed was trapped for up to 4 hr. β -D-Galactosidase activity bound to the colored polystyrene beads in the presence of p24 antigen was 2.3-fold higher with 5 fmol of highly polymerized anti-p24 Fab'- β -D-galactosidase conjugate than with 100 fmol of less polymerized one and reached values close to the maximum within 2 hr with less polymerized anti-p24 Fab'-

β -D-galactosidase conjugate but continued for 4 hr with highly polymerized one.

Time Course of Transferring of the Immune Complex

The antigen p24 was reacted with the two conjugates at 37°C for 2 hr as described above. Subsequently, the immune complex formed was trapped at room temperature for 2 hr and was transferred at room temperature for up to 2 hr. β -D-Galactosidase activity bound to the white polystyrene beads in the presence of p24 antigen was 1.5-fold higher with 5 fmol of highly polymerized anti-p24 Fab'- β -D-galactosidase conjugate than with 100 fmol of less polymerized one and reached values close to the maximum within 1.5 to 2 hr, whether highly or less polymerized anti-p24 Fab'- β -D-galactosidase conjugate was used.

Detection Limit of p24 Antigen by the Present Immune Complex Transfer Enzyme Immunoassay

On the basis of the above results, p24 antigen was reacted with the two conjugates at 37°C for 2 hr as described above, and the immune complex formed was trapped and transferred

Fig. 4. Dose-response curves of recombinant p24 antigen of HIV-1 by the immune complex transfer enzyme immunoassays. Open symbols indicate dose-response curves and values for serum samples from HIV-1 seronegative subjects by the present immune complex transfer enzyme immunoassay for p24 antigen. The p24 antigen and serum samples (10 μ l) from HIV-1 seronegative subjects were incubated with 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and 5 or 100 fmol of monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate at 37°C for 2 hr and subsequently with two colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 1.5 hr. The colored polystyrene beads after washing were incubated with two white polystyrene beads coated with streptavidin in the presence of ϵ N-2,4-dinitrophenyl-L-lysine for 1.5 hr. The incubations with polystyrene beads were performed at room temperature with shaking throughout. After washing, β -D-galactosidase activity bound to the white polystyrene beads was assayed at 30°C for 2 hr. Triangles indicate values obtained with serum samples from HIV-1 seronegative subjects using 5 fmol of highly polymerized monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate. Closed symbols indicate dose-response curves by the previous immune complex transfer enzyme immunoassay for p24 antigen. The antigen was incubated with the two conjugates for 4 hr as described above and subsequently with the two colored polystyrene beads for 16 hr. The colored polystyrene beads after washing were incubated with the two white polystyrene beads in the presence of ϵ N-2,4-dinitrophenyl-L-lysine for 3 hr. The incubations were performed at room temperature without shaking throughout. Bound of β -D-galactosidase activity was assayed at 30°C for 2.5 hr. Circles indicate dose-response curves obtained using 5 fmol of highly polymerized monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate, and squares indicate dose-response curves obtained using 100 fmol of less polymerized one (see Fig. 1).

Fig. 5. Test results of HIV-1 seroconversion serum panels by the immune complex transfer enzyme immunoassay for p24 antigen of HIV-1 and a conventional ELISA. Circles indicate test results of three HIV-1 seroconversion serum panels by the present immune complex transfer enzyme immunoassay for p24 antigen. Serum samples (10 μ l) were incubated with 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-p24 Fab' conjugate and 5 fmol of highly polymerized monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate (see Fig. 1) at 37°C for 2 hr and subsequently with two colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG for 1.5 hr. The colored polystyrene

beads after washing were incubated with two white polystyrene beads coated with streptavidin in the presence of ϵ N-2,4-dinitrophenyl-L-lysine for 1.5 hr. The incubations with polystyrene beads were performed at room temperature with shaking throughout. After washing, β -D-galactosidase activity bound to the white polystyrene beads was assayed at 30°C for 2 hr. Squares indicate test results of three HIV-1 seroconversion serum panels by a conventional ELISA (Abbott 3rd generation). The broken line indicates p24 antigen level corresponding to the highest signal among 50 serum samples from HIV-1 seronegative subjects. **A.** Panel J. **B.** Panel P. **C.** Panel Z.

by incubation at room temperature for 1.5 hr with shaking. Bound β -D-galactosidase activity was assayed at 30°C for 2 hr. The detection limits of rp24 were 0.1 amol/tube and 10 amol (0.24 pg)/ml of serum using 10 μ l of serum samples. These were equal to those of the previous immune complex transfer enzyme immunoassay, in which p24 antigen was reacted with the two conjugates at room temperature for 4 hr and trapping and transferring of the immune complex were performed at room temperature for 16 hr and 3 hr, respectively, without shaking, followed by 2.5 hr assay of bound β -D-galactosidase activity (Fig. 4). Namely, the period of time required for the immune complex transfer enzyme immunoassay of p24 antigen was markedly shortened (25.5–7 hr) without loss of the sensitivity.

Detection of p24 Antigen in Seroconversion Serum Panels by the Present Immune Complex Transfer Enzyme Immunoassay

Three HIV-1 seroconversion serum panels were tested by the present immune complex transfer enzyme immunoassay, in which serum samples were reacted with the two conjugates at 37°C for 2 hr as described above, and the immune complex formed was trapped and transferred by incubation at room temperature for 1.5 hr with shaking. Highly polymerized monoclonal mouse anti-p24 Fab'- β -D-galactosidase conjugate (5 fmol/tube) was used. Bound β -D-galactosidase activity was assayed at 30°C for 2 hr. HIV-1 p24 antigen was detected 12–20 days earlier than the detection of antibodies to HIV-1, that is, seroconversion by a conventional ELISA (Abbott 3rd generation) (Fig. 5).

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