Ultrasensitive Immune Complex Transfer Enzyme Immunoassay of HIV-1 p24 Antigen With Less Serum Interference Using 2,4-Dinitrophenyl-Anti-HIV-1 p24 IgG and Indirectly Immobilized (Anti-2,4-Dinitrophenyl Group) Fab'

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In the immune complex transfer enzyme immunoassay previously reported, the immune complex consisting of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-HIV-1 p24 Fab' conjugate, HIV-1 p24 antigen and monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate was trapped on polystyrene beads coated directly with affinity-purified (anti-2,4-dinitrophenyl group) IgG and was transferred to polystyrene beads coated with biotinyl-bovine serum albumin and streptavidin. The serum volume used was limited to 10 μ L due to serious serum interference, and the detection limit of HIV-1 p24 antigen was 240 fg/mL serum. In the present study, HIV-1 p24 antigen was incubated simultaneously with 2,4-dinitrophenyl-affinity-purified rabbit anti-

HIV-1 p24 IgG and monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate in the presence of excess nonspecific rabbit IgG. The immune complex of the three components formed was trapped on polystyrene beads coated successively with biotinyl-bovine serum albumin, streptavidin and biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab'. After washing, the immune complex was eluted from the polystyrene beads with excess ϵ N-2,4-dinitrophenyl-L-lysine and transferred to polystyrene beads coated with affinity-purified goat (antirabbit IgG) IgG. The serum volume used was increased to 90 μ L with only slight serum interference, and the detection limit of HIV-1 p24 antigen was lowered 9-fold to 26 fg/mL serum. *J. Clin. Lab. Anal.* 13:126–132, 1999. © 1999 Wiley-Liss, Inc.

Key words: human immunodeficiency virus type 1; p24 antigen; enzyme immunoassay; β -D-galactosidase; serum interference

INTRODUCTION

An ultrasensitive and specific enzyme immunoassay (immune complex transfer enzyme immunoassay) of HIV-1 p24 antigen has been described (1). The antigen was incubated simultaneously with 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-HIV-1 p24 Fab' conjugate and monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate, and the immune complex consisting of the three components formed was trapped on polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. After washing, the immune complex was eluted from the polystyrene beads with excess ϵ N-2,4-dinitrophenyl-L-lysine and was transferred to polystyrene beads coated with biotinyl-bovine serum albumin and streptavidin. The detection limit of the antigen was 2.4 fg (0.1 amol) per assay or

240 fg/mL serum. Recently, by the immune complex transfer enzyme immunoassay, HIV-1 p24 antigen and antibody IgGs to HIV-1 reverse transcriptase and p17 antigens in serum have been simultaneously detected to make earlier diagnosis of HIV-1 infection (2). The window period after HIV-1 infection, during which diagnosis of HIV-1 infection by detection of antibodies to HIV-1 is not possible due to the absence of detectable antibodies in the circulation, was shortened considerably, reducing the risk of HIV-1 infection by blood transfusion. However, the volume of serum samples used was

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limited to 10 μL in a total volume of 150 μL due to serious serum interference.

This article describes an ultrasensitive immune complex transfer enzyme immunoassay of HIV-1 p24 antigen with less serum interference using 2,4-dinitrophenyl-anti-HIV-1 p24 IgG and indirectly immobilized (anti-2,4-dinitrophenyl group) Fab'.

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 MgCl_2 , 1 g/L bovine serum albumin (fraction V, Intergen Company, Purchase, NY) and 1 g/L NaN_3 (buffer A).

Recombinant HIV-1 p24

Recombinant p24 antigen was produced in *Escherichia coli* transformed with expression plasmid carrying the corresponding cDNA and was purified as described previously (3). The recombinant proviral clone used was pNL4-3 (4), which contained DNA from HIV-1 isolates NY5 (GenBank accession number HIVNL43) and LAV (5), and the sequence for p24 antigen derived from NY5.

Antibodies

Monoclonal mouse anti-HIV-1 p24 IgG₁ (24C11) was obtained from Innogenetics N.V., Znijsnaarde, Belgium. Anti-HIV-1 p24 IgG was obtained by immunization of rabbit with recombinant HIV-1 p24 antigen (6). Anti-HIV-1 p24 F(ab')₂ was affinity-purified by elution from recombinant HIV-1 p24-nonspecific rabbit IgG-Sepharose 4B at pH 2.5 (1). (Anti-2,4-dinitrophenyl group) IgG was obtained by immunization of rabbit with 2,4-dinitrophenyl-bovine serum albumin and was affinity-purified by elution from 2,4-dinitrophenyl-bovine serum albumin-Sepharose 4B at pH 2.5 (7). Goat (antirabbit IgG) IgG (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan; 45 mg) in 1.0 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0, was affinity-purified by elution from a column (0.5 $\times$ 2.0 cm, 0.39 mL) of rabbit IgG-Sepharose 4B (20 mg IgG/g Sepharose 4B) with 0.2 mol/L glycine-HCl buffer, pH 2.5, at a flow rate of 15 mL/hr. The eluate (2.5 mL) containing 4.6 mg of the affinity-purified IgG was mixed with 0.5 mL of 1 mol/L Tris-HCl buffer, pH 8.0, and dialyzed against 0.1 mol/L sodium phosphate buffer, pH 7.0, containing 1 g/L NaN_3 . CNBr-activated Sepharose 4B for preparation of the protein-Sepharose 4B described above was obtained from Amersham Pharmacia Biotech, Buckinghamshire, England.

Monoclonal Mouse Anti-HIV-1 p24 Fab'- β -D-Galactosidase Conjugate

Thiol groups of monoclonal mouse anti-HIV-1 Fab' were allowed to react with maleimide groups introduced

into β -D-galactosidase molecules using *N,N*-*o*-phenylene-dimaleimide (1).

2,4-Dinitrophenyl-Biotinyl-Bovine Serum Albumin-Affinity-Purified Rabbit Anti-HIV-1 p24 Fab' Conjugate

Bovine serum albumin was treated with *N*-succinimidyl-*S*-acetylmercaptoacetate to introduce thiol groups and then allowed to react with α *N*-6-maleimidohexanoyl- ϵ *N*-2,4-dinitrophenyl-L-lysine and 6-maleimidohexanoyl-biotin (6,8). 2,4-Dinitrophenyl-biotinyl-bovine serum albumin was treated with *N*-succinimidyl-6-maleimidohexanoate to introduce melamine groups and then allowed to react with thiol groups of affinity-purified rabbit anti-HIV-1 p24 Fab' (1,6).

2,4-Dinitrophenyl-Affinity-Purified Rabbit Anti-HIV-1 p24 IgG

2,4-Dinitrophenyl-rabbit anti-HIV-1 p24 IgG

Rabbit anti-HIV-1 p24 IgG (2.4 mg, 16 nmol) in 0.57 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0, was incubated with 0.03 mL of 16 mmol/L *N*-succinimidyl-*S*-acetylmercaptoacetate (Pierce, Rockford, IL) in *N,N*-dimethylformamide at 30°C for 30 min. Then, the reaction mixture was incubated with 0.03 mL of 0.1 mol/L EDTA, pH 7.0, 0.03 mL of 1 mol/L Tris-HCl buffer, pH 7.0, and 0.02 mL of 4 mol/L hydroxylamine, pH 7.0, at 30°C for 5 min. Finally, the reaction mixture was incubated at 30°C for 30 min with 0.5 mL of α *N*-6-maleimidohexanoyl- ϵ *N*-2,4-dinitrophenyl-L-lysine solution, which had been prepared by incubation of 0.083 mL of 100 mmol/L ϵ *N*-2,4-dinitrophenyl-L-lysine (Sigma Chemical Co., Ltd., St. Louis, MO) in *N,N*-dimethylformamide with 0.056 mL of 100 mmol/L *N*-succinimidyl-6-maleimidohexanoate (Dojindo Laboratories, Kumamoto, Japan) in *N,N*-dimethylformamide and 0.42 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0, at 30°C for 30 min. The reaction mixture was subjected to gel filtration using a column (1.1 $\times$ 5.3 cm; 5.0 mL) of Sephadex G-50 fine (Amersham Pharmacia Biotech) in 0.1 mol/L sodium phosphate buffer, pH 7.0. The average number of 2,4-dinitrophenyl groups introduced per IgG molecule was 7.2, which was calculated from the absorbances at 280 nm and 360 nm (9).

2,4-Dinitrophenyl-affinity-purified rabbit anti-p24 IgG

2,4-Dinitrophenyl-rabbit anti-p24 IgG (1.7 mg, 11 nmol) in 1.2 mL of 0.1 mol/L sodium phosphate buffer, pH 7.0, was affinity-purified by elution from a column (0.33 $\times$ 1.2 cm, 0.1 mL) of recombinant HIV-1 p24-nonspecific rabbit IgG-Sepharose 4B (2 mg IgG/g Sepharose 4B) with 0.2 mol/L glycine-HCl buffer, pH 2.5, at a flow rate of 1.0 mL/hr (1). The eluate (0.5 mL) containing 0.05 mg of the affinity-purified IgG was mixed with 0.1 mL of 1 mol/L Tris-HCl buffer,

pH 8.0, and subjected to gel filtration on a column (1.0 × 45 cm) of Ultrogel AcA 34 (Biosepra, Villeneuve-la-Garenne, France) in 0.1 mol/L sodium phosphate buffer, pH 7.0.

Biotinyl-Affinity-Purified (Anti-2,4-Dinitrophenyl Group) Fab'

Biocytin (4 μmol) in 150 μL of 0.1 mol/L sodium phosphate buffer, pH 7.0, was incubated with *N*-succinimidyl-6-maleimidohexanoate (2 μmol) in 50 μL of *N,N*-dimethylformamide at 30°C for 1 hr. An aliquot (6 μL) of the 6-maleimidohexanoyl-biocytin solution was incubated with 150 μg (3.3 nmol) of affinity-purified (anti-2,4-dinitrophenyl group) Fab' in 560 μL of 0.1 mol/L sodium phosphate buffer, pH 6.0, containing 5 mmol/L EDTA at 30°C for 1 hr. The reaction mixture was subjected to gel filtration on a column (1.1 × 5.3 cm; 5 mL) of Sephadex G-50 fine (Amersham Pharmacia Biotech) in 0.1 mol/L sodium phosphate buffer, pH 7.0. The filtrate was stored at 4°C after addition of 1 g/L NaN₃ at a final concentration.

Polystyrene Beads

Polystyrene beads of 6.35 mm diameter were obtained from Immuno Chemical Inc., Okayama, Japan.

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

Polystyrene beads were allowed to stand in 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 10 μg/mL of affinity-purified (anti-2,4-dinitrophenyl group) IgG or affinity-purified (anti-rabbit IgG) IgG at 4°C overnight and were stored in buffer A until use.

Polystyrene Beads Coated With Biotinyl-Bovine Serum Albumin and Streptavidin

Polystyrene beads were allowed to stand first in 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 30 μg/mL of biotinyl-bovine serum albumin (6) at 4°C overnight and, then after washing with the same buffer, in the same buffer containing 30 μg/mL of streptavidin (Life Technologies, Rockville, MD) at 4°C overnight. The coated polystyrene beads were stored in buffer A until use.

Polystyrene Beads Coated Indirectly With Biotinyl-Affinity-Purified (Anti-2,4-Dinitrophenyl Group) Fab'

Polystyrene beads coated with biotinyl-bovine serum albumin and streptavidin described above were incubated in 150 μL of buffer A containing 3 pmol of biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab' with shaking at room temperature for 20 min.

Test of Serum Interference

Buffer A (90 μL) or serum samples (90 μL) were mixed successively with 10 μL of buffer A containing 100 amol of recombinant HIV-1 p24 antigen, 10 μL of buffer A containing 50 μg of inactive β-D-galactosidase (Mutein, Boehringer Mannheim GmbH, Mannheim, Germany), 20 μL of buffer A containing 10 fmol of monoclonal mouse anti-HIV-1 p24 Fab'-β-D-galactosidase conjugate, and 20 μL of buffer A containing 100 fmol of 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-HIV-1 p24 Fab' conjugate or 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG at 32°C for 4 hr. The mixtures (150 μL) were incubated with polystyrene beads (one bead/assay) coated directly with affinity-purified (anti-2,4-dinitrophenyl group) IgG or coated first with biotinyl-bovine serum albumin, then with streptavidin and finally indirectly with biotinyl-affinity-purified rabbit (anti-2,4-dinitrophenyl group) Fab' β-D-Galactosidase activity bound to the polystyrene beads was assayed for 1 hr by fluorometry using 4-methylumbelliferyl-β-D-galactoside as substrate (10). The fluorescence intensity of 1 × 10⁻⁸ mol/L 4-methylumbelliferone in 0.1 mol/L glycine-NaOH buffer, pH 10.3 was adjusted to 100.

Immune Complex Transfer Enzyme Immunoassay

Serum samples (90 μL) were mixed successively with 10 μL of buffer A containing recombinant HIV-1 p24 antigen, 10 μL of buffer A containing 50 μg of inactive β-D-galactosidase (Mutein, Boehringer Mannheim GmbH) and 100 μg of nonspecific rabbit IgG, 20 μL of buffer A containing 10 fmol of monoclonal mouse anti-HIV-1 p24 Fab'-β-D-galactosidase conjugate and 20 μL of buffer A containing 100 fmol of 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG. The mixtures (150 μL) were incubated at 32°C for 4 hr and then for 40 min with polystyrene beads (one bead/assay) coated first with biotinyl-bovine serum albumin, then with streptavidin and finally with biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab' (first solid phase). The mixtures contacted only a part of the polystyrene bead surface at any given time. However, the polystyrene beads were randomly rotated by shaking, so that the mixtures evenly contacted all parts of the polystyrene bead surface during the incubation to continuously mix the thin aqueous layer covering the polystyrene bead surface with the mixtures (11). The polystyrene beads were washed four times with 5 mL of buffer A and incubated with 150 μL of buffer A containing 2 mmol/L εN-2,4-dinitrophenyl-L-lysine for 15 min. The eluate was incubated with affinity-purified goat (antirabbit IgG) IgG-coated polystyrene beads (one bead per assay; second solid phase) as described above. β-D-Galactosidase activity bound to the second solid phase was assayed for 1 hr or 20 hr as described above. Incubations were performed at room temperature, unless otherwise specified.

Serum Samples

Serum samples were collected from 59 HIV-1 seronegative subjects (19 males aged 23 to 69 yr and 40 females aged 20 to 71 yr).

RESULTS AND DISCUSSION

Less Serum Interference With Indirectly Immobilized Biotinyl-(Anti-2,4-Dinitrophenyl Group) Fab'

Recombinant HIV-1 p24 antigen was incubated first simultaneously with 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-HIV-1 p24 Fab' conjugate and monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate in the absence or presence of human serum (90 μ L in a total volume of 150 μ L) and subsequently with a polystyrene bead coated directly with affinity-purified (anti-2,4-dinitrophenyl group) IgG by physical adsorption to trap the immune complex consisting of the three components formed (Fig. 1, left). β -D-Galactosidase activity bound to the polystyrene bead was much (69%) lower in the presence of

human serum than in its absence, indicating the presence of serious serum interference (Table 1).

For reduction of serum interference, polystyrene beads were coated first with biotinyl-bovine serum albumin by physical adsorption, then with streptavidin, and finally with biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab' and were substituted for those coated directly with affinity-purified (anti-2,4-dinitrophenyl group) IgG by physical adsorption described above (Fig. 1, right). As a result, little serum interference was observed (Table 1).

However, the immune complex of the three components was not efficiently eluted with excess *N*-2,4-dinitrophenyl-L-lysine for its transference to polystyrene beads coated with streptavidin. The immune complex of the three components must have bound not only to (anti-2,4-dinitrophenyl group) Fab' but also to streptavidin immobilized on the polystyrene beads. This difficulty was not satisfactorily overcome, even when the immune complex of the three components was trapped in the presence of excess biocytin on polystyrene beads, which had been coated successively with biotinyl-bovine serum albumin, streptavidin and biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab' as described above and then was treated with excess biocytin to block the binding sites of streptavidin.

Use of 2,4-Dinitrophenyl-Affinity-Purified Rabbit Anti-HIV-1 p24 IgG

Not only for reduction of serum interference but also for efficient elution and transference of the immune complex consisting of the three components, 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG was substituted for 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-HIV-1 p24 Fab' conjugate. Recombinant HIV-1 p24 antigen was incubated first with monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate and 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG in the absence or presence of human serum (90 μ L in a total volume of 150 μ L) and subsequently with a polystyrene bead coated directly with affinity-purified (anti-2,4-dinitrophenyl group) IgG by physical adsorption or a polystyrene bead coated first with biotinyl-bovine serum albumin, then with streptavidin and finally indirectly with biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab' (Fig. 1). β -D-Galactosidase activities bound to the indirectly coated polystyrene beads were similar in the absence and presence of human serum, while those bound to the directly coated polystyrene beads were considerably (4-fold) different (Table 1).

Thus, serum interference was markedly reduced using 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG and polystyrene beads coated indirectly with biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab'. Therefore, in the subsequent experiments, the immune complex transfer en-

Fig. 1. Immune complex transfer enzyme immunoassay of HIV-1 p24 antigen using 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-HIV-1 p24 Fab' conjugate (left) and 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG (right). DNP, 2,4-dinitrophenyl group; Stav, streptavidin; BSA, bovine serum albumin.

TABLE 1. Serum Interferences in Enzyme Immunoassays Using Polystyrene Beads Coated Directly With Affinity-Purified (Anti-2,4-Dinitrophenyl Group) IgG and Indirectly With Biotinyl-Affinity-Purified (Anti-2,4-Dinitrophenyl Group) Fab^a

Conjugate used	Polystyrene beads coated	Fluorescence intensity of bound β -D-galactosidase activity	
		Addition of 90 μ L serum	
		No	Yes
DNP-Biotinyl-BSA-anti-p24 Fab'	Directly with anti-DNP IgG	762 (100)	239 (31)
DNP-Biotinyl-BSA-anti-p24 Fab'	Indirectly with biotinyl-anti-DNP-Fab'	749 (100)	793 (106)
DNP-anti-p24 IgG	Directly with anti-DNP IgG	1,123 (100)	274 (24)
DNP-anti-p24 IgG	Indirectly with biotinyl-anti-DNP-Fab'	924 (100)	964 (104)

^aRecombinant HIV-1 p24 antigen (100 amol) was incubated with monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate and 2,4-dinitrophenyl-biotinyl-bovine serum albumin-affinity-purified rabbit anti-HIV-1 p24 Fab' conjugate (DNP-Biotinyl-BSA-anti-p24 Fab') or 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG (DNP-anti-p24 IgG) and then with polystyrene beads coated directly with affinity-purified (anti-2,4-dinitrophenyl group) IgG or coated first with biotinyl-bovine serum albumin, then with streptavidin and finally indirectly with biotinyl-affinity-purified rabbit (anti-2,4-dinitrophenyl group) Fab'. β -D-Galactosidase activity bound to the polystyrene beads was assayed by fluorometry for 1 hr. Values in parentheses are percentages. DNP: 2,4-dinitrophenyl group.

zyme immunoassay of HIV-1 p24 antigen was performed as follows (Fig. 1). Recombinant HIV-1 p24 antigen mixed with 90 μ L serum from HIV-1 seronegative subjects was incubated simultaneously with 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG and monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate and then with polystyrene beads coated first with biotinyl-bovine serum albumin, then with streptavidin and finally indirectly with biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab' (first solid phase) to trap the immune complex of the three components formed. The first solid phase after washing was incubated with excess ϵ N-2,4-dinitrophenyl-L-lysine to elute the immune complex of the three components. The eluate was incubated with polystyrene beads coated with affinity-purified goat (antirabbit IgG) IgG (second solid phase). β -D-Galactosidase activity was assayed by fluorometry.

Effect of Nonspecific Rabbit IgG on the Positive Signal

For further test of serum interference, 25 serum samples from HIV-1 seronegative subjects (serum Nos. 1 to 25) were mixed with recombinant HIV-1 p24 antigen and tested by the immune complex transfer enzyme immunoassay described above. The fluorescence intensity of β -D-galactosidase activity bound to the second solid phase (positive signal) was much lower with one serum sample (No. 25) than with other serum samples. And the low positive signal was markedly (6.3-fold) enhanced, when the serum sample mixed with recombinant HIV-1 p24 antigen was incubated with the two conjugates in the presence of excess nonspecific rabbit IgG (100 μ g) (Table 2). This suggested the presence of antibodies to rabbit IgG or rheumatoid factor in the human serum sample, which might have bound to 2,4-dinitrophenyl-affinity-purified anti-HIV-1 p24 IgG, preventing the binding of the immune complex to the first and second solid phases. However, the serum sample (No. 25) was not available for test of rheumatoid factor. Therefore, other serum samples (Nos. 26 and 27) containing rheumatoid

factor were tested. Fluorescence intensities for bound β -D-galactosidase activity bound to the second solid phase were not so low as with the serum sample (No. 25) and enhanced to only limited extents (1.2- to 1.5-fold) by addition of excess nonspecific rabbit IgG (Table 2). More experiments remain to be made for elucidation of the factor(s) interfering with the immune com-

TABLE 2. Effect of Nonspecific Rabbit IgG on the Positive Signals^a

Serum no.	Fluorescence intensity of β -D-galactosidase activity bound to the second solid phase in the presence of p24 antigen	
	Nonspecific rabbit IgG	
	—	100 μ g
1 to 24	333 $\pm$ 72 (SD) (range: 189–452)	—
18	427	444
19	452	440
20	391	395
21	342	352
22	245	251
23	290	260
24	189	270
25	63	397
26	283	341
27	178	261

^aRecombinant HIV-1 p24 antigen (100 amol) mixed with 90 μ L of serum samples was incubated with 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG and monoclonal mouse anti-HIV-1 p24 Fab'- β -D-galactosidase conjugate in the absence and presence of excess nonspecific rabbit IgG (100 μ g) and then with polystyrene beads coated first with biotinyl-bovine serum albumin by physical adsorption, then with streptavidin and finally indirectly with biotinyl-affinity-purified (anti-2,4-dinitrophenyl group) Fab' (first solid phase). After washing, the polystyrene beads were incubated with excess ϵ N-2,4-dinitrophenyl-L-lysine to elute the immune complex of the three components. The eluate was incubated with polystyrene beads coated with affinity-purified goat (anti-rabbit IgG) IgG (second solid phase). β -D-Galactosidase activity bound to the second solid phase was assayed to fluorometry for 1 hr.

plex transfer enzyme immunoassay using 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG.

Nonspecific Signal in the Absence and Presence of Nonspecific Rabbit IgG

On the basis of the above results, β -D-galactosidase activity bound to the second solid phase in the absence of recombinant HIV-1 p24 antigen, that is, the nonspecific signal in the immune complex transfer enzyme immunoassay was examined in the absence and presence of excess nonspecific rabbit IgG using 32 serum samples from HIV-1 seronegative subjects (serum Nos. 28 to 59) (Table 3). With 31 serum samples (Nos. 28 to 58) out of the 32, fluorescence intensities of β -D-galactosidase activity bound to the second solid phase (nonspecific signal) in the presence of excess nonspecific rabbit IgG were only slightly (28% on average) higher than those in its absence. However, bound β -D-galactosidase activity for one serum sample (No. 59) out of the 32 was 4.7-fold higher than the average value for other 31 serum samples in the absence of nonspecific rabbit IgG and increased 50-fold in the presence of excess nonspecific rabbit IgG. These results required a confirmation test to eliminate false positivity.

Confirmation Test

As a confirmation test, the immune complex transfer enzyme immunoassay was performed in the absence of 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG. The high fluorescence intensity of β -D-galactosidase activity bound to the second solid phase in the absence of HIV-1 p24 antigen with the serum sample (No. 59) was not changed by deletion of the 2,4-dinitrophenyl IgG, whereas those in the presence of recombinant HIV-1 p24 antigen with other serum samples from HIV-1 seronegative subjects were reduced to even lower levels than those in its absence (Table 4). Thus, test by deletion of 2,4-dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG could discriminate falsely positive samples from those showing positive signals.

TABLE 3. Effect of Nonspecific Rabbit IgG on the Negative Signals^a

Serum no.	Fluorescence intensity of β -D-galactosidase activity bound to the second solid phase in the presence of p24 antigen	
	Nonspecific rabbit IgG	
	—	100 μ g
28 to 58	3.6 $\pm$ 1.1 (SD) (range: 1.4 to 6.8)	4.6 $\pm$ 2.0 (SD) (range: 2.4 to 11)
59	17	848

^aSerum samples (90 μ L) from HIV-1 seronegative subjects were subjected to the immune complex transfer enzyme immunoassay under the indicated conditions. β -D-Galactosidase activity bound to the second solid phase was assayed by fluorometry for 20 hr.

TABLE 4. Confirmation Test for the Presence of HIV-1 p24 Antigen by Deletion of 2,4-Dinitrophenyl-Affinity-Purified Rabbit Anti-HIV-1 p24 IgG^a

Serum No.	Addition of recombinant HIV-1 p24 (100 amol)	Fluorescence intensity of β -D-galactosidase activity bound to the second solid phase	
		2,4-Dinitrophenyl-affinity-purified rabbit anti-HIV-1 p24 IgG	
		Added	Deleted
1	Yes	5,660	1.5
2	Yes	8,100	2.7
3	Yes	6,480	1.8
4	Yes	5,520	1.3
59	No	826	857

^aSerum samples (90 μ L) were tested by the immune complex transfer enzyme immunoassay under the indicated conditions. Excess nonspecific rabbit IgG (100 μ g) was added throughout. β -D-Galactosidase activities bound to the second solid phase were assayed by fluorometry for 20 hr.

Detection Limit of HIV-1 p24 Antigen

The detection limit of HIV-1 p24 antigen by the immune complex transfer enzyme immunoassay using 90 μ L serum samples mixed with excess nonspecific rabbit IgG was 0.1 amol (2.4 fg) per assay or 1.1 amol (26 fg) per mL serum. Thus, the immune complex transfer enzyme immunoassay of HIV-1 p24 antigen using 2,4-dinitrophenyl-affinity-purified anti-HIV-1 p24 IgG was 9-fold more sensitive than that using 10 μ L serum previously reported (1), although positive results had to be confirmed by deletion of 2,4-dinitrophenyl-affinity-purified anti-HIV-1 p24 IgG as described above.

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