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Measurement of Human Immunodeficiency Virus Type 1 p24 in Serum by an Ultrasensitive Enzyme Immunoassay, the Two-Site Immune Complex Transfer Enzyme Immunoassay

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Human immunodeficiency virus type 1 (HIV-1) p24 antigen was measured by an ultrasensitive enzyme immunoassay (two-site immune complex transfer enzyme immunoassay). The antigen was reacted simultaneously with 2,4-dinitrophenyl–biotinyl–bovine serum albumin–anti-recombinant p24 (rp24) Fab′ conjugate and anti-rp24 Fab′–β-D-galactosidase conjugate. The complex that was formed, comprising the three components, was transferred from polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) immunoglobulin G (IgG) to polystyrene beads coated with streptavidin. The detection limit of rp24 was 2.4 fg (0.1 amol) per assay or 0.24 pg/ml with as little as 10 μl of serum. When sera were treated at low pH, p24 was detected in 34 (68%) of 50 serum samples from asymptomatic carriers, in 25 (86%) of 29 serum samples from patients with advanced HIV-1 infection, and in none of 117 serum samples from HIV-1-seronegative individuals. Levels of p24 in serum were inversely correlated to those of anti-HIV-1 p24 IgG, and the recovery of rp24 added to serum decreased to zero with increasing levels of anti-HIV-1 p24 IgG in serum. This sensitive method may be used as a powerful tool for investigating the disease.

A core protein, p24, of human immunodeficiency virus type 1 (HIV-1) can be detected at various stages of HIV-1 infection and has been implicated as a surrogate marker for different purposes such as the diagnosis of HIV-1 infection, prediction of disease progression, and monitoring the effects of therapeutic drugs (2–4, 13, 15, 16, 19, 20, 26). The core protein appears in serum and plasma prior to antibody seroconversion, which is currently used for the diagnosis of HIV-1 infection (4, 19, 26). By long-term follow-up, persistent p24 antigenemia has been shown to be associated with the earlier development of AIDS (15, 16). The effect of the antiviral therapy with 3′-azido-3′-deoxythymidine and dideoxyinosine has been assessed by measuring declines in p24 levels in serum and plasma (2, 3, 13). However, the rate of detection of p24 in serum and plasma is rather low. By the conventional two-site enzyme immunoassays with as much as 100 to 200 μl of serum or plasma, p24 has been detected in 8 to 10 and 38 to 48% of asymptomatic carriers before and after acid treatment of serum or plasma, respectively (4, 15, 17, 21). This is at least partly due to the low levels of sensitivity of the methods used (10 to 50 pg/ml) (2, 4, 13, 15–17, 19, 21, 25, 26). Recently, HIV-1 RNA in serum and plasma detected by the branched DNA signal amplification assay (18) and reverse transcription PCR assay (9) has been shown to correlate with p24 positivity in serum and has been suggested as one of the potential surrogate markers for measuring disease progression and monitoring antiviral therapy. However, its clinical application appears to be hampered by several problems such as cost-benefit balance, quantification, and contamination, which reduces the reliability of assay results. Therefore, the development of a sensitive method to provide a reliable surrogate marker at a reasonable cost is desirable.

We describe here the measurement of p24 by an ultrasensitive enzyme immunoassay with a detection limit of 0.24 pg/ml with as little as 10 μl of serum.

MATERIALS AND METHODS

Buffers. The regularly used buffers were 0.1 mol of sodium phosphate buffer (pH 7.0) per liter (buffer A), 0.1 mol of sodium phosphate buffer (pH 6.0) per liter containing 5 mmol of EDTA per liter (buffer B), and 10 mmol of sodium phosphate buffer (pH 7.0) per liter containing 0.1 g of bovine serum albumin (fraction V; Intergen Company, Purchase, N.Y.) per liter and 1.0 mmol of $MgCl_2$ per liter and 1.0 g of NaN_3 per liter (buffer C).

rp24 of HIV-1. Recombinant p24 (rp24) was produced in *Escherichia coli* transformed with an expression plasmid carrying the corresponding cDNA and was purified as described previously (24). The recombinant proviral clone used was pNL4-3 (1), which contained DNA from HIV-1 isolates NY5 (GenBank accession number HIVNL43) and LAV (27), and the sequence for p24 was derived from NY5.

Anti-rp24 antibodies. Recombinant p24 of HIV-1 in saline (0.2 mg/0.5 ml) was emulsified with 0.5 ml of Freund's complete adjuvant. Female New Zealand albino rabbits (weight, 1.5 to 2.0 kg) were injected with 1 ml of the emulsion at multiple intradermal sites. For booster injections, rp24 in saline (0.2 mg/0.5 ml) was emulsified with 0.5 ml of Freund's incomplete adjuvant, and 1 ml of the emulsion was injected three times at 3-week intervals as described above for the initial immunization. Blood was collected 2 weeks after the last booster injection. Immunoglobulin G (IgG) and Fab′ were prepared as described previously (11).

Biotinyl-bovine serum albumin. Maleimide groups were introduced into bovine serum albumin molecules by using *N*-succinimidyl-6-maleimidohexanoate (7), and the molecules were reacted with *N*-biotinyl-2-mercaptoethylamine (14). The average number of biotin residues introduced per albumin molecule was 13, which was calculated from the decrease in the number of maleimide groups (11).

Protein-coated polystyrene beads. Polystyrene beads (3.2 mm in diameter; Immuno Chemical Inc., Okayama, Japan) were coated with proteins by physical adsorption (12). Colored polystyrene beads were coated with affinity-purified

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(anti-2,4-dinitrophenyl–bovine serum albumin) IgG (0.1 g/liter) (7). White polystyrene beads were coated with biotinyl-bovine serum albumin (0.1 g/liter) and anti-rp24 IgG (0.1 g/liter). Biotinyl-bovine serum albumin-coated polystyrene beads were coated with streptavidin (0.1 g/liter) (8).

2,4-Dinitrophenyl–biotinyl–bovine serum albumin–anti-rp24 Fab′ conjugate. (i) Mercaptoacetyl-2,4-dinitrophenyl–bovine serum albumin. 2,4-Dinitrophenyl-bovine serum albumin was reacted with *N*-succinimidyl-*S*-acetylmercaptoacetate (7). The average number of thiol groups introduced per albumin molecule was 5.2.

(ii) 6-Maleimidohexanoyl-biocytin. An aliquot (0.45 ml) of 17 mmol of biocytin (Sigma Chemical Co., St. Louis, Mo.) per liter in buffer A was incubated with 50 μl of 100 mmol of *N*-succinimidyl-6-maleimidohexanoate per liter in *N*,*N*-dimethylformamide at 30°C for 30 min.

(iii) 2,4-Dinitrophenyl–biotinyl–bovine serum albumin. Mercaptoacetyl-2,4-dinitrophenyl–bovine serum albumin (6.6 mg, 0.1 μmol) in 0.1 ml of buffer B was incubated with 6-maleimidohexanoyl–biocytin solution (0.2 ml, 2 μmol) at 30°C for 30 min and subsequently with 50 μl of 0.1 mol of *N*-ethylmaleimide per liter in buffer B at 30°C for 15 min. The reaction mixture was subjected to gel filtration on a column (1.1 by 5.3 cm) of Sephadex G-50 fine (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with buffer A. The average number of biocytin molecules conjugated per albumin molecule was 5.2, which was calculated from the decrease in the number of thiol groups (11).

(iv) 6-Maleimidohexanoyl-2,4-dinitrophenyl–biotinyl–bovine serum albumin. 2,4-Dinitrophenyl–biotinyl–bovine serum albumin was reacted with *N*-succinimidyl-6-maleimidohexanoate (7). The average number of maleimide groups introduced per albumin molecule was 5.1.

(v) 2,4-Dinitrophenyl–biotinyl–bovine serum albumin–anti-rp24 Fab′ conjugate. Anti-rp24 Fab′ (0.61 mg, 13 nmol) in 0.6 ml of buffer B was incubated with 6-maleimidohexanoyl-2,4-dinitrophenyl–biotinyl–bovine serum albumin (0.17 mg, 2.6 nmol) in 0.1 ml of buffer B at 4°C for 20 h and subsequently with 10 μl of 0.1 mol of 2-mercaptoethylamine per liter in buffer B at 30°C for 15 min and with 20 μl of 0.1 mol of *N*-ethylmaleimide per liter in buffer B at 30°C for 15 min. The reaction mixture was subjected to gel filtration on a column (1.5 by 45 cm) of Ultrogel AcA 34 (IBF Biotechnics, Villeneuve-la-Garenne, France) by using 10 mmol of sodium phosphate buffer (pH 7.0) per liter containing 0.1 mol of NaCl per liter. The average number of anti-rp24 Fab′ molecules conjugated per albumin molecule was 2.8, which was calculated from the decrease in the number of maleimide groups (11). The amount of the conjugate was calculated as described above for 2,4-dinitrophenyl–bovine serum albumin (7).

Anti-rp24 Fab′–β-D-galactosidase conjugate. Anti-rp24 Fab′ was conjugated to β-D-galactosidase (EC 3.2.1.23) from *E. coli* with *N*,*N*′-*o*-phenylenedimaleimide (11). The average number of Fab′ molecules conjugated per β-D-galactosidase molecule was 3.2, and the amount of the conjugate was calculated from β-D-galactosidase activity (11).

Acid treatment of serum. An aliquot (10 μl) of serum acidified with 25 μl of 0.1 mol of HCl per liter was allowed to stand at room temperature overnight, was neutralized with 25 μl of 0.1 mol of NaOH per liter–10 μl of 1 mol of sodium phosphate buffer (pH 7.0) per liter, and was mixed with 30 μl of buffer C containing 1 mol of NaCl per liter.

Two-site immune complex transfer enzyme immunoassay for p24. The antigen, p24 or rp24, in 10 μl of serum mixed with 90 μl of buffer C containing 0.4 mol of NaCl per liter or 100 μl of acid-treated serum was incubated for 4 h with 50 μl of buffer C containing 0.4 mol of NaCl per liter, 2,4-dinitrophenyl–biotinyl–bovine serum albumin–anti-rp24 Fab′ conjugate (100 fmol), anti-rp24 Fab′–β-D-galactosidase conjugate (100 fmol), and nonspecific rabbit $F(ab')_2$ (0.1 mg). Two colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG were added to the reaction mixture, and the incubation was continued overnight. The colored polystyrene beads were washed twice by the addition and aspiration of 2 ml of buffer C containing 0.1 mol of NaCl per liter and were incubated for 3 h with 150 μl of buffer C containing 0.1 mol of NaCl per liter and 1.0 mmol of ε*N*-2,4-dinitrophenyl–L-lysine per liter and two white polystyrene beads coated with streptavidin. All of the processes were performed at room temperature. After removing the colored polystyrene beads, the white polystyrene beads were washed as described above, and bound β-D-galactosidase activity was assayed at 30°C for 20 h by fluorometry by using 4-methylumbelliferyl-β-D-galactoside as the substrate (10). The fluorescence intensity was measured relative to that of 10^{-8} mol of 4-methylumbelliferone per liter.

Conventional two-site enzyme immunoassay for p24. The antigen, p24 or rp24, in 10 μl of serum mixed with 140 μl of buffer C containing 0.4 mol of NaCl per liter was incubated with a polystyrene bead coated with anti-rp24 IgG at room temperature overnight. The polystyrene bead was washed as described above and was incubated with 150 μl of buffer C containing 0.1 mol of NaCl per liter, anti-p24 Fab′–β-D-galactosidase conjugate (100 fmol), and nonspecific rabbit $F(ab')_2$ (0.1 mg) at room temperature for 4 h. The polystyrene bead was washed as described above, and bound β-D-galactosidase activity was assayed at 30°C for 1 h as described above.

Enzyme immunoassay for anti-HIV-1 p24 IgG. Anti-HIV-1 p24 IgG was measured as described previously (6). Ten microliters of serum mixed with 90 μl of buffer C containing 0.4 mol of NaCl per liter or 100 μl of acid-treated serum was incubated with 2,4-dinitrophenyl–bovine serum albumin–rp24 conjugate and rp24–β-D-galactosidase conjugate. The immune complex that was formed, comprising the three components, was trapped onto colored polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG and was transferred to white polystyrene beads coated with affinity-purified (anti-human IgG gamma chain) IgG. Bound β-D-galactosidase activity was assayed at 30°C for 2.5 h as described above.

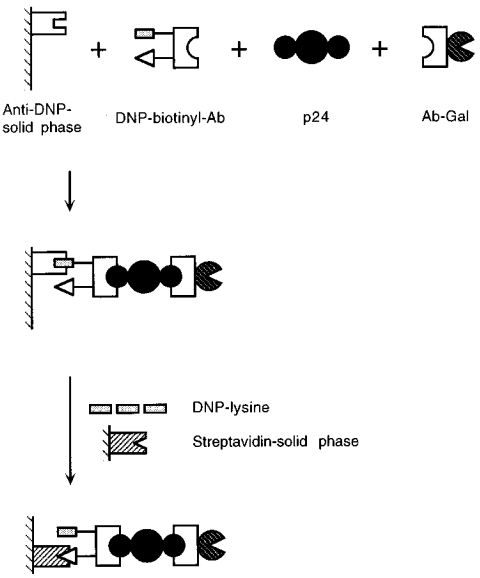

FIG. 1. Schematic representation of the two-site immune complex transfer enzyme immunoassay for HIV-1 p24. DNP, 2,4-dinitrophenyl group; Ab, antibody; Gal, β-D-galactosidase.

Serum samples. Serum samples were collected from HIV-1-seropositive and HIV-1-seronegative individuals whose serologic status was discriminated by the gelatin particle agglutination test by using a commercial kit (SERODIA-HIV; Fujirebio Inc., Tokyo, Japan) (6). Seropositivity was confirmed by Western blotting (immunoblotting) with a commercial kit (Ortho HIV Western Blot Kit; Ortho Diagnostic Systems Inc., Raritan, N.J.) (6).

The number of $CD4^+$-positive T lymphocytes in peripheral blood was determined by an indirect immunofluorescence technique with monoclonal antibody (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) and a flow cytometry system (Coulter, Hialeah, Fla.).

RESULTS

HIV-1 p24 antigen in serum was measured by an ultrasensitive enzyme immunoassay (two-site immune complex transfer enzyme immunoassay) as shown schematically in Fig. 1. The antigen was reacted simultaneously with 2,4-dinitrophenyl–biotinyl–bovine serum albumin–anti-rp24 Fab′ conjugate and anti-rp24 Fab′–β-D-galactosidase conjugate. The complex that was formed, comprising the three components, was trapped onto polystyrene beads coated with affinity-purified (anti-2,4-dinitrophenyl group) IgG. The polystyrene beads were washed to eliminate excess anti-rp24 Fab′–β-D-galactosidase conjugate. The complex was eluted from the polystyrene beads with excess ε*N*-2,4-dinitrophenyl–L-lysine and was transferred onto polystyrene beads coated with streptavidin to eliminate nonspecifically bound anti-rp24 Fab′–β-D-galactosidase conjugate more completely. The β-D-galactosidase activity bound to the latter beads was assayed by fluorometry.

Serum interference. The recovery of rp24 in the presence of serum was tested at three different levels of rp24. Three different serum samples (1 ml) from HIV-1-seronegative individuals were mixed with 0.7, 9.0, and 125 pg of rp24, respectively, and were subjected to the present enzyme immunoassay before and after mixing with additional rp24 (2.0, 10, and 200 pg). The recoveries of rp24 were 94 to 119% with 5 and 10 μl of serum per tube and 80 to 96% with 20 μl of serum per tube. In addition, two different serum samples from seronegative indi-

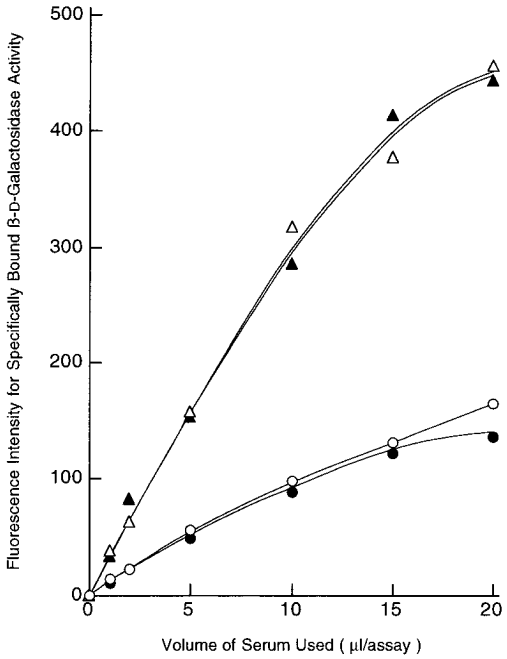

FIG. 2. Effect of serum volumes in the two-site immune complex transfer enzyme immunoassay for rp24. Two different serum samples (1 ml; represented by triangles and circles, respectively) from HIV-1-seronegative individuals were mixed with 30 and 100 pg of rp24 in 10 μl of buffer C containing 0.4 mol of NaCl per liter, and various volumes of the sera were tested. Open and closed symbols indicate signals before and after acid treatment, respectively.

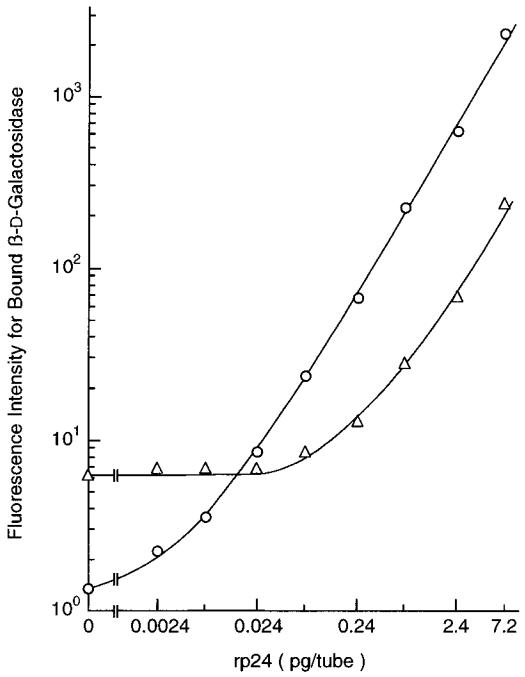

FIG. 3. Dose-response curves of rp24 by the two-site immune complex transfer enzyme immunoassay (circles) and the conventional two-site enzyme immunoassay (triangles).

viduals were mixed with rp24 and were subjected to the present enzyme immunoassay by using various volumes of serum. As the serum volume was increased, the signal was enhanced almost linearly with up to 10 μl of serum but to a lesser extent with more than 10 μl of serum (Fig. 2). Therefore, 10 μl of serum was used in all the experiments described below.

Detection limit and assay range of p24. The detection limit of rp24 per assay by the present enzyme immunoassay was 0.1 amol (2.4 fg) and was 30-fold less than that by the conventional two-site enzyme immunoassay with polystyrene beads coated with anti-rp24 IgG and anti-rp24 Fab′–β-D-galactosidase conjugate (Fig. 3). The assay range of p24 in serum by the present enzyme immunoassay was 0.24 to 720 pg/ml, since the signal increased linearly with up to 7.2 pg per tube and 10 μl of serum could be used without serum interference as described above.

Assay variation. The reproducibility of the present enzyme immunoassay was examined at three different levels over the range of 0.8 to 66 pg/ml of rp24 for within-assay variation and at three different levels over the range of 0.9 to 66 pg/ml of rp24 for between-assay variation. The coefficients of within-assay and between-assay variations were 5.1 to 7.3% (n = 20) and 6.2 to 8.8% (n = 20), respectively.

Measurement of p24 in sera from seropositive and seronegative individuals. Seventy-nine serum samples were collected from HIV-1-seropositive individuals aged 10 to 61 years (50 asymptomatic carriers, 9 patients with AIDS-related complex [ARC], and 20 patients with AIDS), and 117 serum samples were collected from HIV-1-seronegative individuals aged 24 to 68 years.

Without acid treatment of sera, p24 was detected in 24 (48%) of 50 serum samples from the asymptomatic carriers, 7 (78%) of 9 serum samples from the patients with ARC, 18 (90%) of 20 serum samples from the patients with AIDS, and 6 (5%) of 117 serum samples from the seronegative individuals.

When sera were treated at low pH to inactivate anti-HIV-1 p24 IgG with less impairment of p24 immunoreactivity (17, 25), p24 levels in serum were enhanced 1.4- to 17.4-fold in 19 (1.4- to 2.0-fold in 5 samples and more than 2.0-fold in 14 samples) of 50 serum samples from the asymptomatic carriers, 1.3- to 24.0-fold in 6 (1.3-fold in 1 sample and more than 2.0-fold in 5 samples) of 9 serum samples from the patients with ARC, and 1.4- to 30.7-fold in 11 (1.4- to 2.0-fold in 3 samples and more than 2.0-fold in 8 samples) of 20 serum samples from the patients with AIDS. As a result, p24 was detected in 34 (68%) of 50 serum samples from the asymptomatic carriers, 7 (78%) of 9 serum samples from the patients with ARC, 18 (90%) of 20 serum samples from the patients with AIDS, and none of 117 serum samples from the seronegative individuals (Fig. 4).

Thus, the specificity of the present enzyme immunoassay was 95% without acid treatment of serum but improved to 100% with acid treatment. The sensitivity for the asymptomatic carriers was 48% without acid treatment and improved to 68% with acid treatment. The sensitivities for the patients with ARC and AIDS were 78 and 90%, respectively, regardless of acid treatment, although p24 levels in serum in 55 to 67% of the samples were significantly enhanced by acid treatment, as described above.

Correlation of p24 levels with anti-HIV-1 p24 IgG levels and $CD4^+$ T-lymphocyte counts. Anti-HIV-1 p24 IgG in sera from the HIV-1-seropositive individuals was measured by the immune complex transfer enzyme immunoassay described previously (6) and was compared with the p24 levels in serum described above (Fig. 4). In the asymptomatic carriers and the patients with ARC, the levels of p24 in serum before and after acid treatment tended to be inversely correlated with those of anti-HIV-1 p24 IgG. The regression equations and correlation

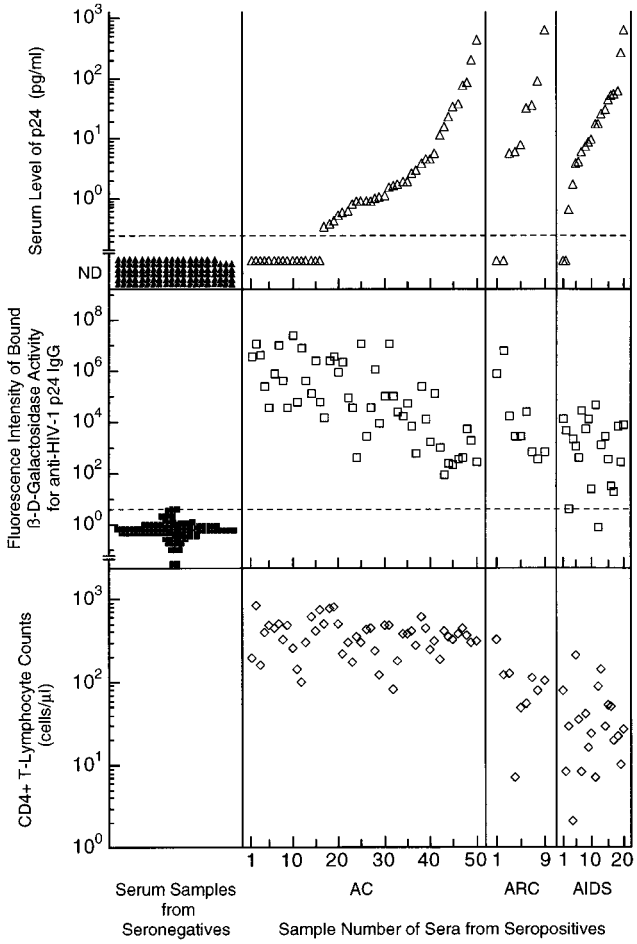

FIG. 4. Measurement of p24 in serum by the two-site immune complex transfer enzyme immunoassay. Serum samples from 79 HIV-1-seropositive individuals and 117 HIV-1-seronegative individuals were tested. Open and closed symbols indicate the values for seropositive and seronegative individuals, respectively. Triangles indicate p24 levels in serum after acid treatment. Squares indicate anti-HIV-1 p24 IgG levels in serum. The broken lines indicate tentative cutoff values. $CD4^+$ T-lymphocyte counts are shown at the bottom. AC, asymptomatic carriers; ARC, patients with AIDS-related complex; AIDS, patients with AIDS; ND, not detectable.

coefficients for the correlation between the levels of p24 in serum after acid treatment (y) and anti-HIV-1 p24 IgG (x) were $\log y = -0.37 \log x + 2.1$ ($r = 0.66$) for 34 of the 50 asymptomatic carriers, in whom p24 was detectable, and $\log y = -0.59 \log x + 3.5$ ($r = 0.56$) for 7 of the 9 patients with ARC, in whom p24 was detectable. However, this was the case only for some of the patients with AIDS, and for other patients with AIDS, both the levels of p24 in serum and anti-HIV-1 p24 IgG levels were low ($\log y = 0.063 \log x + 1.1$; r = 0.12). Little inverse correlation was observed between p24 levels in serum and $CD4^+$ T-lymphocyte counts ($r < 0.05$) (Fig. 4).

p24 levels in sera of seropositive individuals corrected by the recovery of added rp24. Anti-HIV-1 p24 IgG was not completely inactivated by acid treatment, and 3.6 to 8.4% of its activity was detected after acid treatment. Consequently, with increasing levels of anti-HIV-1 p24 IgG in serum, the levels of the remaining anti-HIV-1 p24 IgG in serum after acid treatment were enhanced, and the recoveries of rp24 added to sera containing anti-HIV-1 p24 IgG decreased even after acid treatment (Table 1). From the recoveries of rp24 added to sera, p24 levels in serum after acid treatment could be corrected to higher levels (Table 1). When sera contained extremely high levels of anti-HIV-1 p24 IgG, p24 levels in serum were undetectable or the recoveries of rp24 added to sera were almost zero, preventing the calculation of corrected values (Table 1).

DISCUSSION

The present enzyme immunoassay was much more sensitive than the conventional two-site enzyme immunoassay. The detection limit of rp24 per assay by the present enzyme immunoassay (0.1 amol or 2.4 fg) was 630- to 4,200-fold less than those by previously reported conventional two-site enzyme immunoassays (2, 4, 13, 15–17, 19, 21, 25, 26). The limit of detection of rp24 in serum by the present enzyme immunoassay (0.24 pg/ml with as little as 10 μl of serum) was 42- to 210-fold less than those in previous reports (10 to 50 pg/ml with as much as 100 to 200 μl of serum or plasma) (2, 4, 13, 15–17, 19, 21, 25, 26).

The detection rates of p24 in the sera of HIV-1-seropositive individuals by the conventional two-site enzyme immunoassays were lower than those by the present enzyme immunoassay. The detection rates by the conventional two-site enzyme immunoassays before and after acid treatment of serum were 8 to 10% (4, 17, 21) and 38 to 48% (17, 21), respectively, for asymptomatic carriers, 12% (21) and 59% (21), respectively, for patients with ARC, and 37 to 86% (4, 15, 21) and 86% (21), respectively, for patients with AIDS. By the present enzyme immunoassay, the detection rate for the asymptomatic carriers was 48% before acid treatment and improved to 68% with acid treatment, and the detection rates for the patients with ARC and AIDS were 78 and 90%, respectively, with no improvement with acid treatment. In the nine patients with ARC, the levels of p24 in serum were in a range similar to that in the asymptomatic carriers and tended to be inversely correlated with those of anti-HIV-1 p24 IgG both before and after acid treatment, as was the case in the asymptomatic carriers (Fig. 4). Therefore, improvement of the detection rate by acid treatment may be observed by testing a larger number of patients with ARC, as was the case with the 50 asymptomatic carriers. In 90% of the 20 patients with AIDS, p24 was detected without acid treatment, and in 2 (10%) of them p24 was not detectable even after acid treatment with low levels of anti-HIV-1 p24 IgG, which may imply no improvement by acid treatment because of the absence of p24 caused by the severe depression of p24 production (Fig. 4).

Without acid treatment of sera, false positivity was observed for 5% of the sera from HIV-1-seronegative individuals as described above. On the other hand, in some of the HIV-1-seropositive individuals p24 levels in serum were slightly lower after acid treatment than before acid treatment. These two results might have been due to the presence of anti-β-D-galactosidase antibodies and another nonspecific reaction(s), since the rate of false positivity in 5% of the seronegative individuals was reduced partly by preincubation of sera with inactive β-D-galactosidase (β-D-galactosidase-Mutein; Boehringer Mannheim GmbH, Mannheim, Germany) (data not shown) and was totally eliminated by acid treatment.

In contrast to p24 levels in serum, the level of antibodies to p24 in serum has been reported to decline with progression of the disease (16) and, therefore, to correlate inversely to the level of p24 in serum (15–17, 20, 21, 25). Similar results were obtained in the present study except for those for patients with AIDS (Fig. 4). In some of the patients with AIDS, the levels of both p24 and anti-HIV-1 p24 IgG in serum were low. This might have been because the production of both p24 and

TABLE 1. Recovery of rp24 added to sera containing different levels of anti-HIV-1 p24 IgG

Serum sample no.	Level of anti-HIV-1 p24 IgG in serum[a]		Level of p24 in serum (pg/ml)		Recovery of rp24 added to serum (%)[b]		Level of p24 in serum corrected by recovery (pg/ml)[c]	
	Before acid treatment	After acid treatment	Before acid treatment	After acid treatment	Before acid treatment	After acid treatment	Before acid treatment	After acid treatment
1	2.4×10^2	1.5×10^1 (6.2)[d]	2.4	17.0	61.0	93.7	3.9	18.1
2	1.8×10^3	6.5×10^1 (3.6)	11.5	31.8	26.0	80.3	44.2	39.6
3	1.4×10^4	1.1×10^3 (7.9)	0.6	6.3	22.3	40.3	2.7	15.6
4	3.3×10^4	2.7×10^3 (8.2)	2.4	4.0	2.6	19.5	92.3	20.5
5	1.5×10^6	7.5×10^4 (5.0)	ND[e]	ND	0.0	0.0		
6	3.8×10^6	2.6×10^5 (6.8)	ND	ND	0.0	0.0		

[a] Levels of anti-HIV-1-p24 IgG were expressed as fluorescence intensities for bound β-D-galactosidase activity.
[b] An aliquot (10 μl) of serum from HIV-1-seropositive individuals mixed with 0.1 pg of rp24 in 10 μl of buffer C containing 0.4 mol of NaCl per liter was allowed to stand at room temperature for 1 h and was subjected to the present enzyme immunoassay with or without acid treatment.
[c] The levels of p24 in serum before the addition of rp24 were corrected by the recovery of rp24 added to the sera.
[d] Values in parentheses are in percent.
[e] ND, not detectable.

anti-HIV-1 p24 IgG was depressed with progression of the disease. This was supported by decreased counts of $CD4^+$ T lymphocytes in those patients (Fig. 4).

The recovery of rp24 added to serum was examined, and p24 levels in serum were corrected by the recoveries. The corrected values appeared to be within a narrow range (15.6 to 39.6 pg/ml) (Table 1) and might be closer to true values. When sera contained extremely high concentrations of anti-HIV-1 p24 IgG, the anti-HIV-1 p24 IgG remaining after acid treatment (the addition of one-fourth volume of 0.1 mol of HCl per liter to pH 1.8) reduced the recovery of rp24 added to serum to zero, and no corrected values could be calculated (Table 1). This was because anti-HIV-1 p24 IgG was not completely (91.8 to 96.4%) inactivated by acid treatment. These results do not indicate the absence of p24 in those sera but do suggest the possibility that p24 is present in all sera of HIV-1-seropositive individuals, including asymptomatic carriers.

The amino acid sequence of rp24 used is identical to that of HIV-1 NY5 p24 as described in the Materials and Methods section. The homology of the amino acid sequence of rp24 used to that of p24 of other HIV-1 strains (LAV, RF, and MN; GenBank accession numbers HIVBRUCG, HIVRF, and HIVMNCG, respectively) is 97 to 98%. The homologies of the amino acid sequence of rp24 used to that of HIV-2 p26 and human T-cell leukemia virus types I and II p24 are 69, 20, and 20%, respectively (5, 22, 23). Therefore, the present enzyme immunoassay may measure p24 of other HIV-1 strains with the same sensitivity that it measures the p24 protein of NY5, the p26 protein of HIV-2 with a lower sensitivity, and the p24 proteins of human T-cell leukemia types I and II with an extremely lower sensitivity, although it remains to be tested.

Thus, the present sensitive method for the detection of p24 may become a powerful tool for investigating HIV-1 disease, although further longitudinal clinical trials to assess the usefulness of the method remain to be performed.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Chaisson, R. E., M. D. Leuther, J.-P. Allain, S. Nusinoff-Lehrman, G. S. Boone, D. Feigal, and P. Volberding.** 1988. Effect of zidovudine on serum human immunodeficiency virus core antigen levels. Arch. Intern. Med. **148:** 2151–2153.
3. **Drusano, G. L., G. J. Yuen, J. S. Lambert, M. Seidlin, R. Dolin, and F. T. Valentine.** 1992. Relationship between dideoxyinosine exposure, CD4 counts, and p24 antigen levels in human immunodeficiency virus infection. Ann. Intern. Med. **116:**562–566.
4. **Goudsmit, J., F. DeWolf, D. A. Paul, L. G. Epstein, J. M. A. Lange, W. J. A. Krone, H. Speelman, E. C. Wolters, J. Van Der Noordaa, J. M. Oleske, H. J. Van Der Helm, and R. A. Coutinho.** 1986. Expression of human immunodeficiency virus antigen (HIV-Ag) in serum and cerebrospinal fluid during acute and chronic infection. Lancet **ii:**177–180.
5. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. Nature (London) **326:**662–669.
6. **Hashida, S., K. Hashinaka, K. Hirota, A. Saitoh, A. Nakata, H. Shinagawa, S. Oka, K. Shimada, J. Mimaya, S. Matsushita, and E. Ishikawa.** 1994. Detection of antibody IgG to HIV-1 in urine by ultrasensitive enzyme immunoassay (immune complex transfer enzyme immunoassay) using recombinant p24 as antigen for diagnosis of HIV-1 infection. J. Clin. Lab. Anal. **8:**86–95.
7. **Hashida, S., K. Tanaka, N. Yamamoto, T. Uno, K. Yamaguchi, and E. Ishikawa.** 1991. Detection of one attomole of [Arg^8]-vasopressin by novel noncompetitive enzyme immunoassay (hetero-two-site complex transfer enzyme immunoassay). J. Biochem. **110:**486–492.
8. **Hashida, S., K. Tanaka, N. Yamamoto, T. Uno, K. Yamaguchi, and E. Ishikawa.** 1991. Novel and sensitive noncompetitive enzyme immunoassay (hetero-two-site enzyme immunoassay) for arginine vasopressin in plasma. Anal. Lett. **24:**1109–1123.
9. **Holodniy, M., D. A. Katzenstein, S. Sengupta, A. M. Wang, C. Casipit, D. H. Schwartz, M. Konrad, E. Groves, and T. C. Merigan.** 1991. Detection and quantification of human immunodeficiency virus RNA in patient serum by use of the polymerase chain reaction. J. Infect. Dis. **163:**862–866.
10. **Imagawa, M., S. Hashida, Y. Ohta, and E. Ishikawa.** 1984. Evaluation of β-D-galactosidase from *Escherichia coli* and horseradish peroxidase as labels by sandwich enzyme immunoassay technique. Ann. Clin. Biochem. **21:**310–317.
11. **Ishikawa, E., M. Imagawa, S. Hashida, S. Yoshitake, Y. Hamaguchi, and T. Ueno.** 1983. Enzyme-labeling of antibodies and their fragments for enzyme immunoassay and immunohistochemical staining. J. Immunoassay **4:**209–327.
12. **Ishikawa, E., and K. Kato.** 1978. Ultrasensitive enzyme immunoassay. Scand. J. Immunol. **8**(Suppl. 7)**:**43–55.
13. **Jackson, G. G., D. A. Paul, L. A. Falk, M. Rubenis, J. C. Despotes, D. Mack, M. Knigge, and E. E. Emeson.** 1988. Human immunodeficiency virus (HIV) antigenemia (p24) in the acquired immunodeficiency syndrome (AIDS) and the effect of treatment with zidovudine (AZT). Ann. Intern. Med. **108:**175–180.
14. **Kohno, T., E. Ishikawa, S. Sugiyama, and S. Nakamura.** 1988. Novel enzyme immunoassay of anti-insulin IgG in human serum. J. Clin. Lab. Anal. **2:**19–24.
15. **MacDonell, K. B., J. S. Chmiel, L. Poggensee, S. Wu, and J. P. Phair.** 1990. Predicting progression to AIDS: combined usefulness of CD4 lymphocyte counts and p24 antigenemia. Am. J. Med. **89:**706–712.
16. **Moss, A. R., P. Bacchetti, D. Osmond, W. Krampf, R. E. Chaisson, D. Stites, J. Wilber, J.-P. Allain, and J. Carlson.** 1988. Seropositivity for HIV and the development of AIDS or AIDS related condition: three year follow up of the San Francisco General Hospital cohort. Br. Med. J. **296:**745–750.
17. **Nishanian, P., K. R. Huskins, S. Stehn, R. Detels, and J. L. Fahey.** 1990. A simple method for improved assay demonstrates that HIV p24 antigen is present as immune complexes in most sera from HIV-infected individuals. J. Infect. Dis. **162:**21–28.
18. **Pachl, C., M. Saxer, T. Elbeik, M. Stempien, S-J. Fong, D. Kern, P. Sheridan, B. Hoo, D. Besemer, R. Kokka, P. Neuwald, M. Urdea, M. Fineberg, and J. Todd.** 1993. Quantitation of HIV-1 RNA in plasma using a branched DNA

(bDNA) signal amplification assay: evaluation of specimen collection and stability, abstr. 312, p. 110. *In* Abstracts of the 1st National Conference on Human Retroviruses and Related Infections 1993. National Foundation of Infectious Diseases and American Society for Microbiology, Washington, D.C.

19. **Paul, D. A., L. A. Falk, H. A. Kessler, R. M. Chase, B. Blaauw, D. S. Chudwin, and A. L. Landay.** 1987. Correlation of serum HIV antigen and antibody with clinical status in HIV-infected patients. J. Med. Virol. **22:**357–363.
20. **Rathbun, R. C.** 1993. Surrogate markers for assessing treatment response in HIV disease. Ann. Pharmacother. **27:**450–455.
21. **Rodríguez-Iglesias, M. A., J. R. Alvarez, A. Vergara, M. S. Garcia-Valdivia, I. Jesús, and J. Mira.** 1992. Improved detection of HIV p24 antigen in serum after acid pretreatment. Eur. J. Clin. Microbiol. Infect. Dis. **11:**849–850.
22. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. USA **80:**3618–3622.
23. **Shimotohno, K., Y. Takahashi, N. Shimizu, T. Gojobori, D. W. Golde, I. S. Y. Chen, M. Miwa, and T. Sugimura.** 1985. Complete nucleotide sequence of an infectious clone of human T-cell leukemia virus type II: an open reading frame for the protease gene. Proc. Natl. Acad. Sci. USA **82:**3101–3105.
24. **Tanaka, N., A. Saitoh, A. Nakata, and H. Shinagawa.** 1992. A simple method for overproduction and purification of p24 gag protein of human immunodeficiency virus type 1. Microbiol. Immunol. **36:**823–831.
25. **Vasudevachari, M. B., N. P. Salzman, D. R. Woll, C. Mast, K. W. Uffelman, G. Toedter, D. Hoefheinz, J. A. Metcalf, and H. C. Lane.** 1993. Clinical utility of an enhanced human immunodeficiency virus type 1 p24 antigen capture assay. J. Clin. Immunol. **13:**185–192.
26. **Von Sydow, M., H. Gaines, A. Sönnerborg, M. Forsgren, P. O. Pehrson, and Ö. Strannegård.** 1988. Antigen detection in primary HIV infection. Br. Med. J. **296:**238–240.
27. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40:**9–17.