JOURNAL OF CLINICAL MICROBIOLOGY, June 1996, p. 1412–1419 Vol. 34, No. 6
0095-1137/96/$04.00+0

Flow Cytometric Immunofluorescence Assay for Detection of Antibodies to Human Immunodeficiency Virus Type 1 Using Insoluble Precursor Forms of Recombinant Polyproteins as Carriers and Antigens

YU-WEN HU,* PAUL BIRCH, EVAN BALASKAS, ABED ZEIBDAWI, VITO SCALIA, SUSAN ANNE THÉRIAULT-VALIN, PETER GILL, AND MAUNG T. AYE

Molecular Virology, National Testing Laboratory, The Canadian Red Cross Society, Ottawa, Ontario K1G 4J5, Canada

Received 19 June 1995/Returned for modification 30 August 1995/Accepted 28 February 1996

A new serological assay, the recombinant flow cytometric immunofluorescence assay (r-FIFA), was developed for the early detection of human immunodeficiency virus type 1 (HIV-1) antibodies by using recombinant insoluble forms of HIV-1 Gag-p45, Gag-gp41 chimeric protein, gp160, and Pol97 polyprotein as antigens and autologous carriers through flow cytometry. These recombinant proteins were expressed in insect cells by a baculovirus expression system. Eight anti-HIV-1 seroconversion panels, a low-titer anti-HIV-1 panel from Boston Biomedica Inc. (BBI), and three HIV-1 seroconversion specimens from the Provincial Health Laboratory of Ontario, Toronto, Ontario, Canada (PHL), were tested and analyzed by r-FIFA. In sensitivity comparisons between r-FIFA and tests licensed by the U.S. Food and Drug Administration, which were used to test all of the HIV-1 panels from BBI, detection of HIV-1 antibody by r-FIFA was on average greater than 20 days earlier than that by enzyme immunoassay. The sensitivity of r-FIFA has permitted the detection of HIV-1-specific immunoglobulin G (IgG), IgM, and IgA antibodies during seroconversion. A kinetic analysis of HIV-1 antibody production by r-FIFA has shown that either IgG or IgM, or both, can be detected, depending on the phase and type of the immune response in the HIV-1-infected individual. Both primary and secondary immune responses were observed during this period. The r-FIFA results suggest that implementation of r-FIFA may significantly reduce the "window" period from the time of infection to the time of seroconversion, with earlier detection of antibodies after initial infection. This would also make it possible for us to understand the immune response and the precise mechanisms of immunopathogenesis in the early period of HIV-1 infection.

Enzyme immunoassays (EIAs) and Western blot (immunoblot) assays (WB) have been routinely used to detect human immunodeficiency virus type 1 (HIV-1) infection since the tests were first licensed in 1985 (3). The implementation of these tests has significantly reduced the risk of transfusion-related HIV infection. However, some recent studies (14, 17) based on the detection of HIV DNA and RNA by PCR and virus isolation by viral culture techniques have demonstrated that some HIV-infected individuals do not have anti-HIV antibodies that are detectable by tests currently approved by the U.S. Food and Drug Administration (FDA). There is a "window" period estimated to span from a few weeks to several months between the time of initial HIV infection and the time of seroconversion. Reduction of the residual risk of HIV-1 infection from blood transfusion is still one of the most important issues in transfusion medicine. Another major concern is the reliability of the WB as a confirmatory test. Since the improved EIA was implemented in recent years, WB's levels of sensitivity and specificity for the detection of anti-HIV-1 antibodies during seroconversion have been called into question (22). A task force to identify acceptable alternatives to WB and immunofluorescence assays (IFAs) was recently recommended (13).

We have developed the recombinant flow cytometric indirect immunofluorescence assay (r-FIFA), which is based on the flow cytometric indirect immunofluorescence assay (FIFA) (24). The purified insoluble forms of the HIV-1 *gag* precursor, Gag-gp41 chimeric proteins, the *pol* precursor polyprotein Pol97, and cell-associated gp160 expressed in insect cells by baculovirus vectors instead of HIV-1-infected human cells (H9) were used directly as carriers and antigens for r-FIFA. The baculovirus expression system has several advantages over bacterial and mammalian systems, including superior yields of recombinant proteins, safety (baculovirus is not infectious to humans), and the fidelity of its products (9). HIV-1 *gag*, *pol*, and *env* are initially translated as precursor proteins (16). The antibodies to these precursors may be dominant and more specific than the antibodies to a linear sequence of amino acid residues of degraded viral gene products at the early stage of HIV-1 infection (1, 15). The HIV-1 *gag* precursor has potential for use as a carrier. Inserting defined relevant HIV-1 epitopes into the HIV-1 *gag* precursor could allow for the presentation of the selected epitopes by the particular antigen and carrier (12, 26). The HIV-1 Pol97 polyprotein, containing reverse transcriptase and integrase, is among the enzymatically active forms of the HIV-1 *pol* gene products (6). Pol97 is an excellent antigen for use in the detection of antibodies to HIV-1 and HIV-2 (20). Furthermore, the use of insoluble forms of HIV-1 recombinant protein for r-FIFA would solve the problems of antibody cross-reactivity to human cell antigens and the biohazard concerns of the original FIFA (24). In this report, we compare the sensitivity and specificity of r-FIFA with those of the licensed screening and confirmatory tests using HIV-1 seroconversion panels from Boston Biomedica Inc. (BBI) and

* Corresponding author. Mailing address: Molecular Virology, National Testing Laboratory, The Canadian Red Cross Society, 1800 Alta Vista Dr., Ottawa, Ontario K1G 4J5, Canada. Phone: (613) 739-2439. Fax: (613) 739-2426.

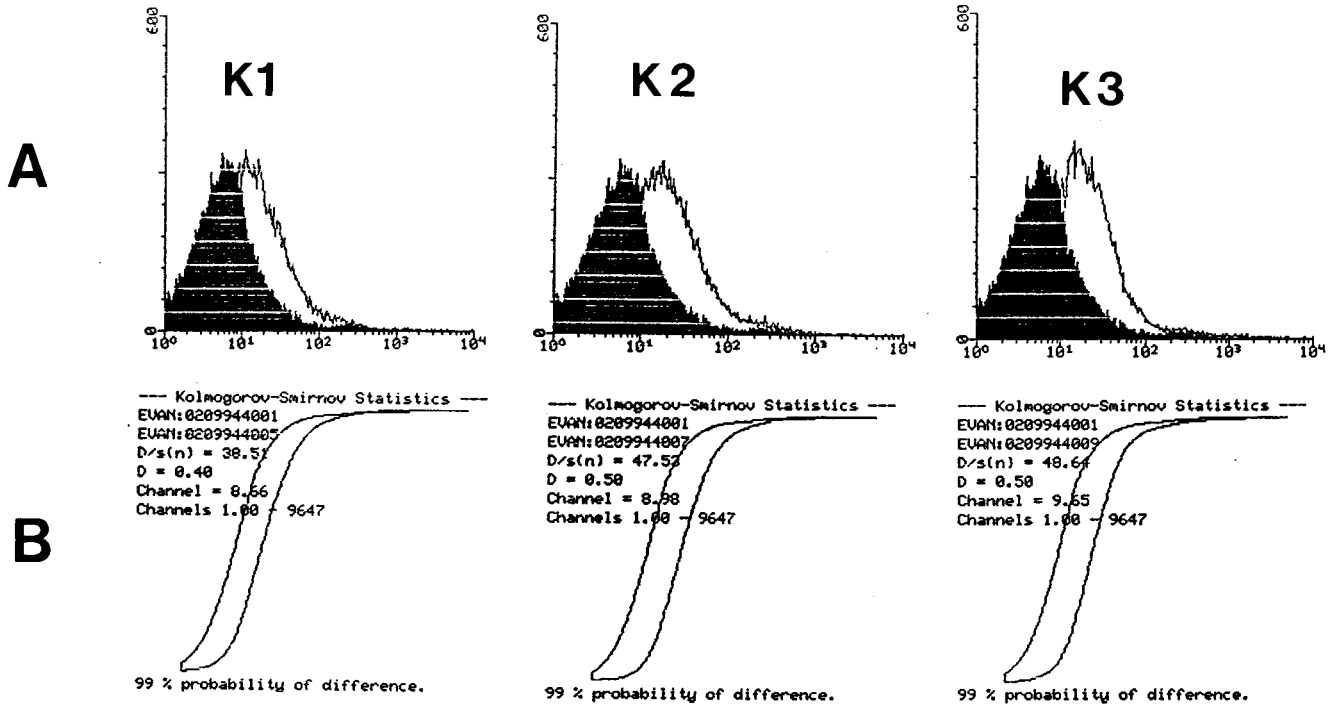

FIG. 1. Statistical analysis of histograms by the Kolmogorov-Smirnov test. (A) Histograms showing HIV-1 IgM antibody-positive signals (line peaks) of samples K1, K2, and K3 from BBI panel K and the negative background signal (solid black peaks) of the negative control are presented. (B) The Kolmogorov-Smirnov *D* statistics for comparison of the vertical displacement between distribution functions of the samples and control. The *D* statistics for samples K1, K2, and K3 are 0.40, 0.50, and 0.50, respectively, with a *P* value of ≤ 0.01 for all three samples, which were determined to be weakly positive (sample K1) and positive (samples K2 and K3) by r-FIFA.

the HIV-1 seroconversion specimens provided by the Provincial Public Health Laboratory of Ontario, Toronto, Ontario, Canada (PHL). We show that r-FIFA detects antibodies to HIV-1 significantly earlier than EIAs and confirmatory tests do. To our knowledge, this is the first time that the seroconversion profiles of anti-HIV-1 immunoglobulin G (IgG), IgM, and IgA antibody production during the window period have been determined by r-FIFA.

MATERIALS AND METHODS

Subjects. HIV-1 seroconversion panels obtained from BBI were used for the evaluation of r-FIFA. All of the panels were tested with several FDA-licensed EIAs (second-generation EIAs). BBI provided comprehensive data including EIA, WB, radioimmunoprecipitation assay, and antigen (p24) results for all BBI panels for comparative analysis. Each set of BBI HIV-1 seroconversion panels included 5 to 10 aliquots assembled from a repository of frozen plasma units (4% sodium citrate was used as the anticoagulant). The samples were from closely spaced serial bleedings from paid donors from clinics who were later determined to have seroconverted (BBI data). The following were analyzed by r-FIFA: a low-titer anti-HIV-1 panel (PRB104) in which the specimens are aliquots from serum or plasma units collected from asymptomatic blood donors; anti-HIV-1 seroconversion panels D (PRB904), E (PRB905), H (PRB908), J (PRB910), K (PRB911), P (PRB916), Q (PRB917), R (PRB918); and three HIV-1 seroconversion panels, as well as a series of negative specimens provided by PHL. To confirm the specificity of r-FIFA, 295 plasma or serum samples from random blood donors, 105 WB-positive samples, and 138 EIA-reactive, WB-indeterminate samples from National Testing Laboratory, The Canadian Red Cross Society, were also tested.

Construction of recombinant baculoviruses. A coding region of HIV-1 (including intact p17-, p24-, and part of p16-coding sequences) was isolated from plasmid pHxB-2D (19) and was inserted into the baculovirus genome under control of the polyhedrin gene promoter by the following procedure. A *Cla*I-*Bgl*II fragment was modified and subcloned into plasmid pUC19 by using synthetic oligonucleotide linkers. Linker 1 contained a *Bam*HI site and the missing sequence including the translation initiation codon (ATG) at the N-terminal part of the *gag* gene. Linker 2 created a translation termination codon (TAA) followed by a *Bam*HI site. The *Bam*HI fragment was isolated from pUC19-Gag-p45 and was inserted into the *Bam*HI site of the transfer vector pAcYM1 (25). The recombinant plasmid pAcYM1-Gag-p45 was used for cotransfection of *Spodoptera frugiperda* Sf9 cells with wild-type baculovirus AcNPV (*Autographa californica* nuclear polyhedrosis virus) DNA, and then the recombinant baculovirus Gag-p45 was isolated to express the recombinant protein Gag-p45. The recombinant baculovirus expressing the Gag-gp41 chimeric protein was constructed; this is referred to as Gag-gp41-C. The *gag* coding sequence used in the construction of Gag-gp41-C was the same as that of Gag-p45. The gp41-coding sequence (nucleotides 7737 to 8264; 176 amino acids) was inserted at the *Bgl*II site at the end of the Gag-p45 gene. The chimeric DNA was isolated and was inserted into the *Bam*HI cloning site of the transfer vector plasmid pVL 1393, and cotransfection was performed by the procedure provided with the BaculoGold system (PharMingen). The recombinant virus expressing Pol97 and gp160 was constructed as described previously (4, 6).

Cell culture and HIV-1 recombinant protein production. Recombinant baculoviruses were grown and assayed in Sf9 cells by using complete TNM-FH medium (Sigma) at 27°C as described previously (12). The cells were harvested at the optimum time of 72 h postinfection. The cell processing and the recombinant protein purification were carried out by using modifications of the procedure described previously (12). Briefly, the cell pellet was washed in 30 ml of phosphate-buffered saline (PBS) and was then centrifuged at 250 × *g* (GP centrifuge; Beckman) at room temperature (23 ± 5°C). The pellet was resuspended in 10 ml of PBS and was then sonicated on ice for 45 to 50 s (40% power setting) with a sonicating homogenizer (Cole-Parmer Ultrasonic Homogenizer 4710). The cell lysates were centrifuged in the GP centrifuge at 900 × *g* for 10 min. The pellet (fraction I) was discarded. The supernatant was centrifuged at 12,100 × *g* (Sorvall RC-5) and 4°C for 30 min. The pellet (fraction II) was resuspended in 1 to 2 ml of PBS and was stored at 4°C. The supernatant was centrifuged again at 120,000 × *g* (Beckman ultracentrifuge) and 20°C for 1.5 h. The resulting pellet (fraction III) was resuspended in 1 to 2 ml of PBS, and the supernatant was discarded. Fractions II and III were combined to form the intracellular protein stock. The protein stock was further purified by sucrose gradient (20 to 60%) ultracentrifugation at 120,000 × *g* for 3 h at 20°C.

Flow cytometry and r-FIFA assay for the detection of HIV-1 antibodies. (i) Flow cytometry. Flow cytometric analysis was performed on a Becton Dickinson FACSort instrument equipped with an argon ion laser tuned at 488 nm. Data acquisition was done with Lysis II software, version 1.1 (Becton Dickinson). Forward light scattering, orthogonal light scattering, and two fluorescence signals were determined on logarithmic settings for each of 20,000 events and were stored in data files. The detector settings had been optimized and were stored in data files for recall by the operator. Data analysis was also performed with the Lysis II software. A two-dimensional dot intensity plot of forward light scatter versus orthogonal light scatter was observed on ungated events. A region (R1)

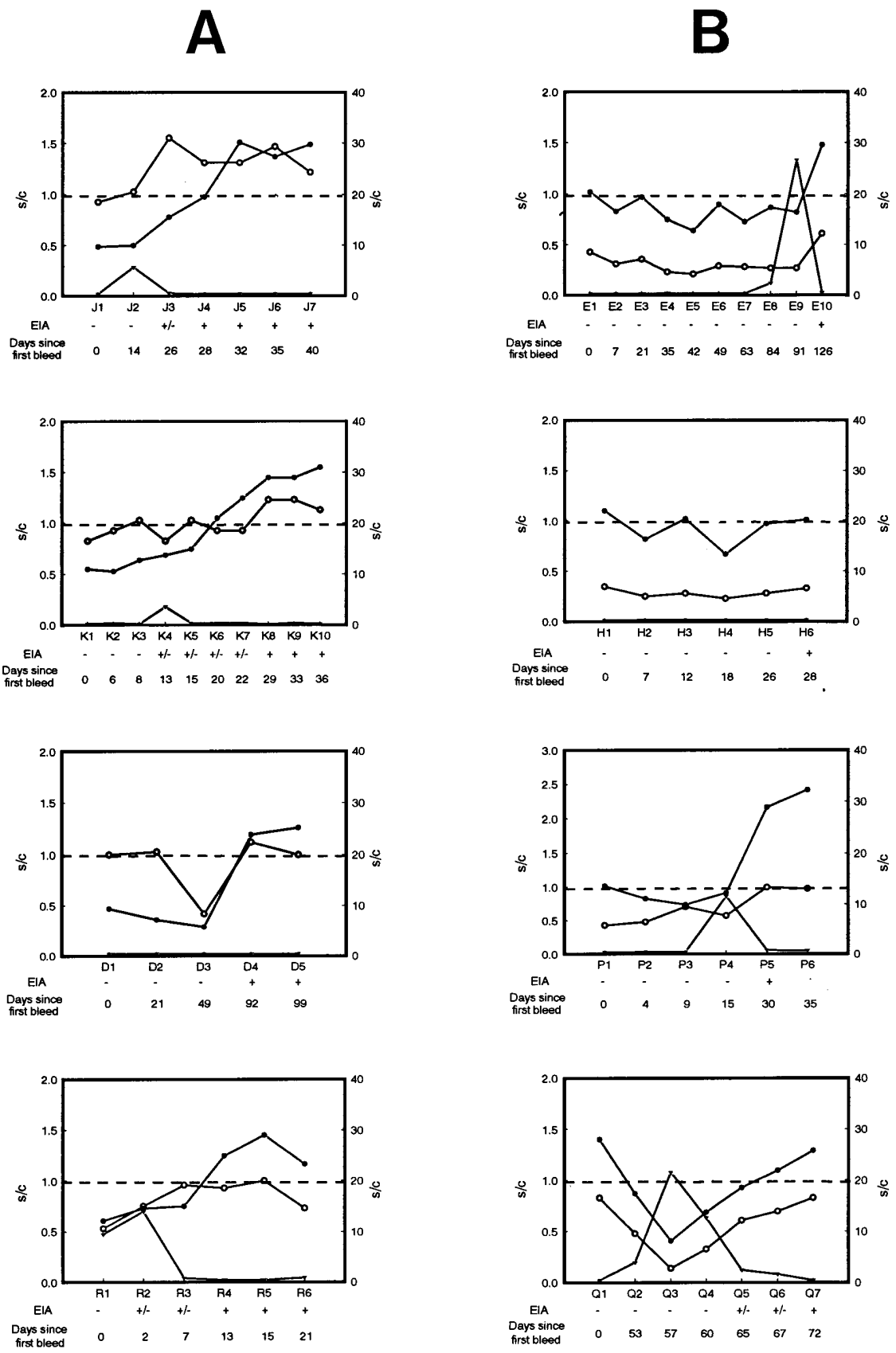

FIG. 2. Primary (A) and secondary (B) antibody response patterns during the window period of HIV-1 infection. The results from kinetic analysis of IgM and IgG antibody production are expressed as the median fluorescence intensity ratio of the *s/c* value (left *y* axis). The broken line represents an antibody *s/c* value of 1.0. An *s/c* value greater than or equal to 1.0 is considered positive. The right *y* axis represents the *s/c* value for the HIV-1 p24 antigen. EIA data, in parallel with results of r-FIFA, are a summary of data from 13 commercial tests: +, positive; +/−, positive by some tests and negative by others; −, negative. A mixture of Gag-p45 and Gag-gp41-C chimeric proteins was used as antigen. ○, IgM antibody to HIV-1; ●, IgG antibody to HIV-1; ▼, HIV-1 p24 antigen.

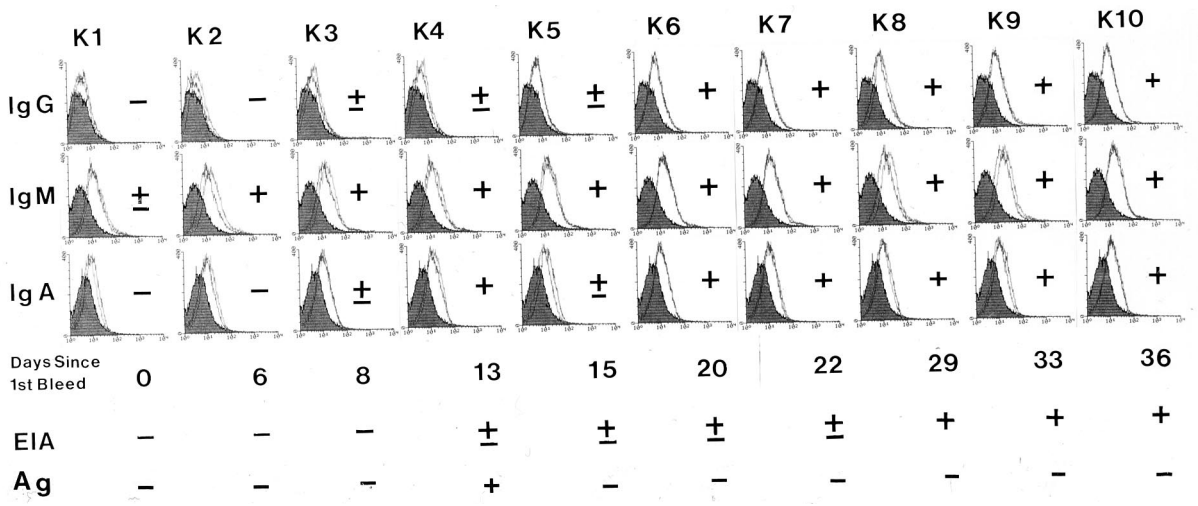

FIG. 3. Fluorescence histograms of samples in BBI panel K represent the profiles of IgG, IgM, and IgA antibody production at a very early stage of HIV-1 infection. Ag, antigen.

was set on the dot plot, and single-parameter histograms of FL1 (green emission for fluorescein isothiocyanate [FITC] is 530 nm) and FL2 (red emission for R-phycoerythrin [R-PE] E is 585 nm) were examined. The median value of fluorescence intensity (channel number) was used to determine whether the test samples were positive (10, 11). This analysis procedure has also been automated and has been stored as a command file.

(ii) r-FIFA procedure. In 1.5-ml microcentrifuge tubes (Sarstedt), 4 μl of control or sample (plasma or serum) and a predetermined amount of recombinant protein were combined with PBS (containing NaN_3) to a final volume of 100 μl. The tubes were gently vortexed and were then incubated at room temperature for 20 min on a rocker (model 1105; Adams Nutator). The mixture was washed three times in 1 ml of PBS per tube and was centrifuged at 12,700 × *g* (IEC Micro-MB centrifuge) for 5 min at room temperature. The supernatant was aspirated, and 10 μl of FITC or R-PE-conjugated goat antibody to human IgG (γ chain specific), IgM (μ chain specific), or IgA (α chain specific) (Southern Biotechnology) was added. The mixture was incubated for 20 min at room temperature in the dark and was then washed two times with 1 ml of PBS per tube, as before. After the second aspiration, 500 μl of PBS was added to each sample tube and the mixture was sonicated for 10 s with the sonicating homogenizer. The contents were then transferred to polystyrene tubes (12 by 75 mm; specific for the flow cytometer; Becton Dickinson), stored for 2 h in the dark at room temperature, and then read on the flow cytometer.

To determine the cutoff values of fluorescence intensity for r-FIFA for each of the antigens, 100 samples from healthy donors (50 serum and 50 plasma samples) were tested by using double staining with FITC-conjugated anti-human IgG and R-PE-conjugated anti-human IgM. Each sample was tested for median fluorescence intensity (*S*) in duplicate, and the result was reported in relation to the mean value of the median fluorescence intensity of a known seronegative control (*N*) (*S*/*N*). The seronegative control was used for all of the tests in the study. The cutoff value (*c*) was calculated by taking the mean *S*/*N* value of the population ($\bar{x}$)

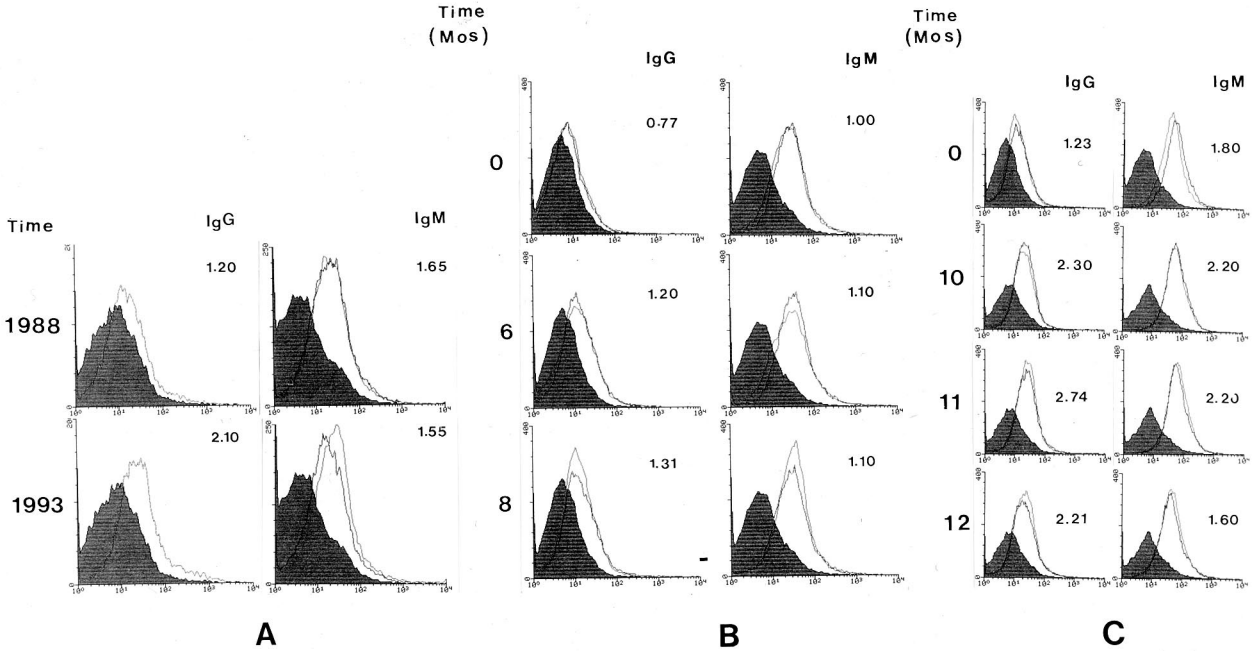

FIG. 4. Detection of early HIV-1 antibodies (IgG and IgM) by r-FIFA in the samples from three individuals (PHL-A, PHL-B, and PHL-C [panels A to C, respectively]) who were infected but seronegative by recently licensed screening tests with blood from the first bleeding (upper row). The numbers under "IgG" and "IgM" indicate the *s*/*c* values for the samples; a value of 1.0 or greater is considered positive. A mixture of Gag-p45 and Gag-gp41-C was used as the antigen for testing the samples.

and adding 3 standard deviations (SDs) as described previously (24). For example, the cutoff value of fluorescence intensity for Gag-p45 was calculated as follows: for IgG FITC, $\bar{x}$ *S/N* = 0.960, 1 SD = 0.266, and cutoff = $\bar{x}$ *S/N* + 3 SDs = 1.75; for IgM R-PE, $\bar{x}$ *S/N* = 1.666, 1 SD = 0.581, and cutoff = $\bar{x}$ *S/N* + 3 SDs = 3.41; and for IgA R-PE, $\bar{x}$ *S/N* = 1.270, 1 SD = 0.604, and cutoff = $\bar{x}$ *S/N* + 3 SDs = 3.08. Sample-to-cutoff ratios (*s/c*) of 1.0 or greater are considered positive.

RESULTS

Sensitivity of r-FIFA. In order to determine the optimal serum or plasma dilution required for the sensitivity test of r-FIFA, 20 samples found to be positive for HIV-1 by WB (10 serum and 10 plasma samples) were titrated with Gag-p45 as the antigen. Consistent results were obtained, indicating that a 1:25 sample dilution was optimal for r-FIFA. High levels of background noise occurred when dilutions lower than 1:25 were tested; thus, a dilution of 1:25 was used for further experiments.

Statistical analyses were carried out in a series of experiments with the flow cytometer and Lysis II software. Fifty-seven samples from eight BBI HIV-1 seroconversion panels were compared with a control which was used for all tests in the study. The IgG and IgM titers in each sample were measured. A total of 114 Kolmogorov-Smirnov *D* statistics were computed, and the histograms for each pair were plotted on a common graph. The results show that the histograms for the samples and the control tend to be clearly differentiated, with the samples shifted to the right of the control for all but eight pairs for IgG (BBI panel members Q3, P1, P2, P3, P4, K1, K2, and H2) and three pairs for IgM (BBI panel members Q3, H2, and H5), for which the samples were negative by all anti-HIV-1 assays used in the study, including r-FIFA. The sample sizes began at 12,907 for the control and 15,147 for the samples in the r-FIFA tests. By using the smallest sample sizes in the study, a *P* value of 0.05 would be achieved by a *D* value of 0.016 and a *P* value of 0.01 would be achieved by a *D* value of 0.019. The smallest *D* statistic in the study is 0.03, with a *P* value of 7×10^{-10}. Within the limits of machine accuracy, the *P* values in the present study are all effectively 0. The statistical analysis of the Kolmogorov-Smirnov test has resulted in strong proof of the significance of the histograms for the samples and the control (Fig. 1).

Analysis of serial samples from donors who were later determined to have seroconverted has proven to be very useful for the evaluation of serological assays and for documenting their enhanced sensitivities. The r-FIFA serological profiles of eight HIV-1-infected individuals during seroconversion are shown in Fig. 2. The results indicated that r-FIFA detected seroconversion on average more than 20 days earlier (range, 0 to 126 days) than the EIAs did. For example, the samples in panel Q were collected over 72 days, but 53 days elapsed between the first and second bleedings, making it appear probable that sample 1 was collected prior to infection; however, sample 2 was positive for the p24 antigen and a very faint p24 band appeared on WB of samples 1 through 5 (BBI data). The r-FIFA result shows that sample 1 was positive for HIV-1 IgG antibody and that sample 3, in which the p24 antigen concentration was increased, then became negative, suggesting that an antigen-antibody complex was formed at this time point. The result was confirmed by a radioimmunoprecipitation assay (RIPA) with ^{35}S-labelled HIV-1 recombinant gp160 (data not shown). Sample 1 in panel Q was positive and was therefore collected postinfection, not preinfection.

The high degree of sensitivity of r-FIFA and its multiple-detection feature have made it possible to study the profiles of the antibody response in the early stage of HIV-1 infection. As shown in Fig. 2, examples of the primary response were found

TABLE 1. Comparison of r-FIFA and FDA-licensed confirmatory tests on the basis of the sensitivity of detection of antibodies to HIV-1 proteins encoded in the three open reading frames of the HIV-1 genome in the low-titer anti-HIV-1 panel PRB104

PRB104 identification no.	Result of FDA-licensed test[a]																HIV-1 r-FIFA result			
	Bio-Rad WB				Ortho/Cambridge WB				Organon Teknika WB				HIV-1 RIPA RL15							
	gag	*pol*	*env*	Res.	*gag*	*pol*	*env*	Res.	*gag*	*pol*	*env*	Res.	*gag*	*pol*	*env*	Res.	*gag*	*env*[b]	*env*[b]	Res.
01	+	−	+	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+
02	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+
03	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+
04	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	+	+	+
05	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
06	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
07	+	−	−	IND	+	−	−	IND	+	−	−	IND	−	−	+	IND	+	+	+	+
08	−	−	−	−	+	−	−	IND	+	−	−	IND	−	−	+	IND	+	+	+	+
09	+	−	+	+	+	−	+	+	+	−	+	+	−	−	+	IND	+	+	+	+
10[c]	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−
11	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+	+
12	+	−	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+	−	+	+
13	+	−	+	+	+	−	−	IND	+	−	f160	+	−	−	+	IND	+	+	+	+
14	f24	−	−	IND	−	−	−	−	f24	−	−	IND	−	−	+	IND	+	−	−	IND
15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Total no. of positive results	12	1	10	10	12	2	9	9	13	3	10	10	8	4	13	8	13	8	12	12

[a] WB results were interpreted by using the criteria of the Centers for Disease Control and Prevention and the Association of State and Territorial Public Health Laboratory Directors. f, faint; +, positive; −, negative; IND, indeterminate; Res., result. By the Bio-Rad WB, Ortho/Cambridge WB, Organon Teknika WB, HIV-1 RIPA RL15, and HIV-1 r-FIFA assays, 71, 64, 71, 57, and 86% of the samples were found to be gag^+ env^+, respectively, and 7, 14, 21, 29, and 57% of the samples were found to be gag^+ pol^+ env^+, respectively.

[b] The chimeric Gag-gp41-C antigen was used to detect antibodies to *env*. Later, the results were confirmed by using insoluble gp160 polyprotein as the antigen in the r-FIFA.

[c] Sample 10 is the negative control.

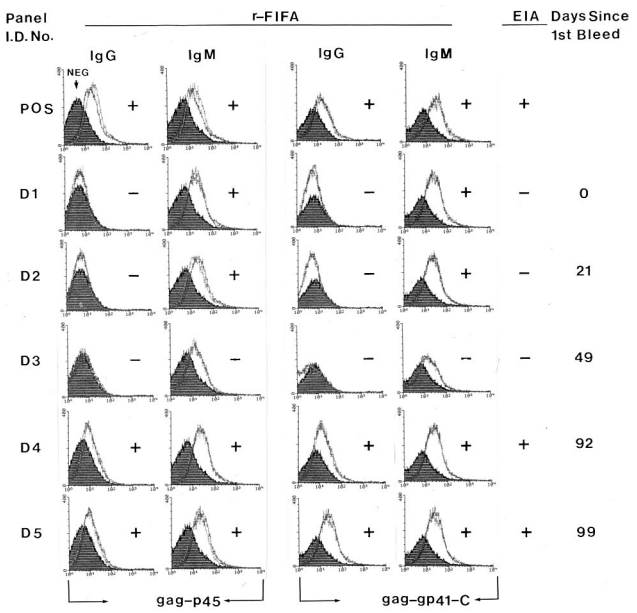

FIG. 5. Early HIV-1 antibody detection in the samples of BBI anti-HIV-1 seroconversion panel D by r-FIFA with two different antigens. The flow cytometric histograms show the anti-HIV-1 antibody signals of the samples (line peaks) and the fluorescence signal (background) of the negative sample as a control (solid grey peak).

for panels J and K. Panels D and R showed similar patterns of antibody production (Fig. 2A). However, for panels E, H, P, and Q (Fig. 2B), the HIV-1 IgG antibody level remained varied for a long period (longer than 1 to 3 months) prior to seroconversion, as detected by FDA-licensed tests. The IgM antibody became positive later (panel P). The pattern in panels E, H, P, and Q (Fig. 2B) is apparently different from the pattern in panels D, J, K, and R. Presumably, these individuals (panels E, H, P, and Q) were not recently infected with HIV-1. For some unknown reason, the virus antigen concentration rose, boosting the antibody response in which the anti-HIV-1 IgG antibodies increased to a higher level in a shorter time (as seen for panels E, P, and Q), indicating a possible secondary immune response. The fluorescence histograms of the samples for panel K (Fig. 3) show the kinetics of specific IgG, IgM, and IgA antibody production during the window period. The IgM antibodies were detected before the p24 antigen became detectable, and then IgA and IgG antibodies appeared as the p24 antigen level peaked. The IgM antibody response to HIV-1 overlapped with the HIV-1 antigen peak.

Three PHL HIV-1 seroconversion panels and a series of negative samples (data not shown) were evaluated in a blind test to confirm the sensitivity of r-FIFA. The first bleedings from all three panels were positive for IgG and/or IgM by r-FIFA but were negative by EIA (Fig. 4). The first bleedings of patients PHL-B and PHL-C were positive for HIV-1 p24 antigen (data not shown).

Furthermore, to compare the sensitivity of r-FIFA with those of FDA-licensed confirmatory tests, including WB and RIPA, the BBI low-titer anti-HIV-1 panel was tested by r-FIFA by using each of the antigens Gag-p45, Gag-gp41-C chimeric protein, and Pol97 (Table 1). Since the chimeric protein Gag-gp41-C has specific epitopes from HIV-1 *gag* and gp41 polyproteins, it is difficult to justify whether the samples tested have specific antibodies to *env*. To confirm that antibodies to *env* alone exist, all 15 of the samples were tested by r-FIFA with an insoluble form of gp160 (cell-associated gp160 expressed in insect cells) as the antigen. The results correlated 100% with the results from the assay with the Gag-gp41 chimeric protein as the antigen, as shown in Table 1. The antibodies to all three antigens, Gag-p45, Gag-gp41-C (or gp160), and Pol97, were detectable in 8 of the 14 samples (57%) by r-FIFA. However, the antibodies to *pol* gene products (p66, p51, and p31) were poorly detected in the FDA-licensed confirmatory tests. Only 1 to 4 of the 14 samples (7 to 29%) were found to have antibodies to all three proteins by the WB and RIPA. Statistical analysis as implemented by S-plus (Statistical Science) for comparison of antibodies to *gag*, *pol*, and *env* indicate that r-FIFA is more sensitive than the three licensed WB ($P = 0.0052$). However, the difference between r-FIFA and RIPA is not significant ($P = 0.1573$).

Reproducibility of r-FIFA. To evaluate the reproducibility of r-FIFA, two antigens, Gag-p45 and Gag-gp41-C, were used for the analysis of panel D samples (Fig. 5). Similar patterns of antibody production during the seroconversion were obtained by using the two antigens in separate tests. The duplicates of each assay were highly reproducible, as shown by the overlapping of two-line peaks, which represent the fluorescence inten-

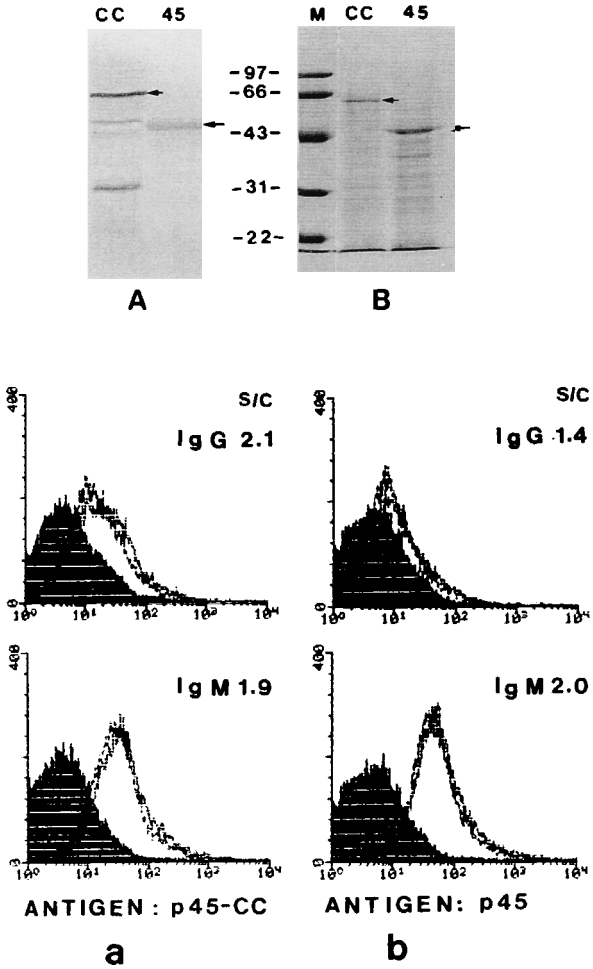

FIG. 6. Antibody detection by r-FIFA with antigen Gag-gp41-C (p45-CC; a) and Gag-p45 (p45; b) as antigens. The sample tested had weak antibodies to HIV-1 core antigen (A, lane 45) and strong antibodies to the Gag-gp41 antigen (A, lane CC) by WB analysis (anti-human IgG only). The Gag-p45 antigen (B, lane 45) and the Gag-gp41-C antigen (B, lane CC) were visualized by Coomassie blue staining of a 10% polyacrylamide gel.

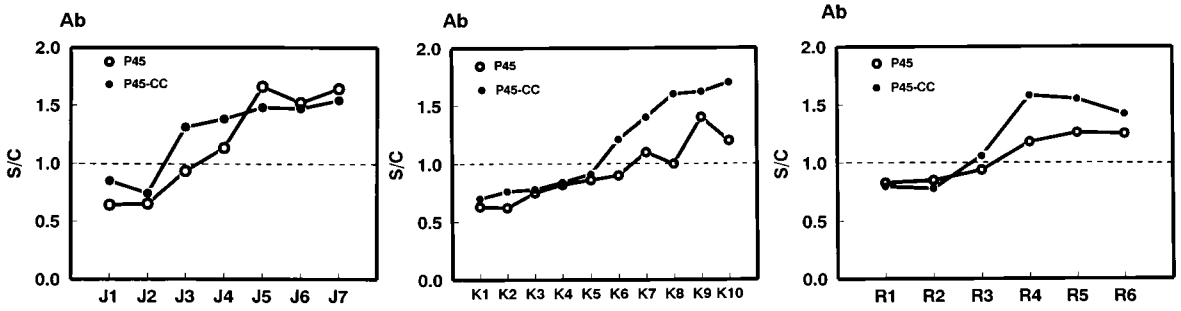

FIG. 7. Early HIV-1 antibody (Ab) (IgG) detection in the samples from panels J, K, and R by using the Gag-p45 (○; p45) and the Gag-gp41-C (●; p45-CC) antigens in r-FIFA.

sity of each duplicate of the sample. We found that the median fluorescence intensities of duplicates were similar (less than 10% difference) or identical for most of the samples tested.

Specificity of r-FIFA. A total of 295 random donor samples, 105 EIA- and WB-positive samples, and 128 samples reactive by repeat EIA and indeterminate by WB were tested by r-FIFA with the mixture of Gag-p45 and Gag-gp41-C as the antigen. Only 1 of the 295 random donor samples was weakly positive. The positive sample from the random donor was negative by EIA. All 105 EIA- and WB-positive samples were positive by r-FIFA. Only 30 of 128 samples (23%) repeatedly reactive by EIA and indeterminate by WB were still positive (most of those were expected to be false positive). These results suggest that r-FIFA's specificity may be higher than those of the EIAs and WB analysis; however, the very low percentage of false-positive results that do occur is probably due to antibody cross-reactivity with the antigens. The results for samples that test false positive will be confirmed further by r-FIFA with Gag-p45, p97, and gp160 as antigens in separate tests, as for Table 1.

Differences in antibody responses to different antigens, Gag-p45, Gag-gp41-C, and Pol97, in r-FIFA. Figure 6 shows that chimeric protein Gag-gp41-C binds more IgG antibodies than Gag-p45. Little difference in the detection of HIV-1 IgM antibodies by the antigens was found. We found that IgG antibody was detected earlier by Gag-gp41-C than by Gag-p45 (Fig. 7). In some of the seroconversion panels HIV-1 IgG antibodies to Pol97 were detected as early as those to Gag-gp41-C. The HIV-1 IgM antibodies to Pol97 were not detected as frequently as HIV-1 IgG antibodies during the window period (data not shown).

DISCUSSION

We have created a unique way to detect both IgG and IgM antibodies using insoluble forms of recombinant proteins as autologous carriers through flow cytometry. IgM antibody is one of the early markers of HIV-1 infection (5). This is probably one of the reasons why r-FIFA can detect HIV-1 antibody during the early stages of HIV-1 infection, as do the third-generation EIAs (28). However, in some HIV-1-infected individuals, only IgG antibodies were detectable by r-FIFA, such as in BBI panels H and E. These results confirm the previous findings of other investigators (18). These patients presumably had a secondary immune response at a stage of HIV-1 infection when the virus or the viral antigen was released at high concentrations in blood or body fluids. This suggests that the individual was not recently exposed to HIV-1 and had a longer window period. This could result from factors such as host genetics (7, 21), the route of viral transmission (2, 23, 27), the particular viral strains, and the initial viral load(s) involved in the course of HIV-1 infection (29). There is another possibility that the antibodies to *env* and *pol* were, strikingly, restricted to the IgG isotype (8).

The insoluble antigens used for r-FIFA are easily processed and purified without denaturation, keeping intact their natural molecular folding, which may optimize the presentation of antigen epitopes (15). This may be one of the explanations that can account for r-FIFA's higher sensitivity for the detection of early HIV-1 antibodies. Furthermore, an agglutination effect involving the reaction between IgM antibodies and the antigens in r-FIFA may enhance the sensitivity of the test for detecting specific antibodies because all the insoluble polyproteins used are particulate antigens.

r-FIFA is a simple test. By using the same principle described here, r-FIFA can be used to detect any antibody with an insoluble form of recombinant protein, such as hepatitis B virus core antigen or human T-cell leukemia type I or II *gag* chimeric proteins, as carriers (data not shown). We have constructed all three recombinant HIV-1 precursors encoded in the *gag*, *pol*, and *env* open reading frames of the HIV-1 genome. These are the only natural precursor forms produced in the viral life cycle and cover >90% of the regions of the viral structural proteins. Thus, r-FIFA may have the potential to be a better confirmatory test because of its high degrees of sensitivity and specificity and its ability to identify antibodies directed to individual HIV-1 polyproteins, and for these same reasons, it would also be a useful tool for basic research.

ACKNOWLEDGMENTS

This research was partially supported by a grant from the Miles (Bayer)/Canadian Red Cross Society Research and Development Fund.

We thank Catherine Dalzell, statistician, Fractionation Department, Canadian Red Cross, Ottawa, Ontario, Canada, for statistical evaluation of the data.

REFERENCES

1. **Benjouad, A., J.-C. Gluckman, L. Montagnier, and E. Bahraoui.** 1993. Specificity of antibodies produced against native or desialylated human immunodeficiency virus type 1 recombinant gp160. J. Virol. **67:**1693–1697.
2. **Borkowsky, W., K. Krasinski, H. Pollack, W. Hoover, A. Kaul, and T. Ilmet-Moore.** 1992. Early diagnosis of human immunodeficiency virus infection in children >6 months of age: comparison of polymerase chain reaction, culture, and plasma antigen capture techniques. J. Infect. Dis. **166:**616–619.
3. **Busch, M. P.** 1994. HIV and blood transfusions: focus on seroconversion. 23rd Congress of the International Society of Blood Transfusion. Vox Sang. **67**(Suppl. 3)**:**13–18.
4. **Farmer, J. L., R. G. Hampton, and E. Boots.** 1989. Flow cytometric assays for monitoring production of recombinant HIV-1 gp160 insect cells infected with a baculovirus expression vector. J. Virol. Methods **26:**279–290.
5. **Gallarda, J. L., D. R. Henrard, D. Liu, S. Harrington, S. L. Stramer, J. E.**

Valinsky, and P. Wu. 1992. Early detection of antibody to human immunodeficiency virus type 1 by using an antigen conjugate immunoassay correlates with the presence of immunoglobulin M antibody. J. Clin. Microbiol. **30:** 2379–2384.
6. **Hu, Y.-W., and C. Y. Kang.** 1991. Enzyme activities in four different forms of human immunodeficiency virus 1 *pol* gene products. Proc. Natl. Acad. Sci. USA **88:**4596–4600.
7. **Kashala, O., K. Kayembe, P. Kanki, P. Mukeba, M. Diese, M. Kalengayi, K.-W. Izzia, and M. Essex.** 1993. Humoral aspects of anti-HIV immune responses in Zairians with AIDS: lower antigenemia does not correlate with immune complex levels. AIDS Res. Hum. Retroviruses **9:**251–258.
8. **Khalife, J., B. Guy, M. Capron, M.-P. Kieny, J.-C. Ameisen, L. Montagnier, J.-P. Lecocq, and A. Capron.** 1988. Isotypic restriction of the antibody response to human immunodeficiency virus. AIDS Res. Hum. Retroviruses **4:**3–9.
9. **Kidd, I. M., and V. C. Emery.** 1993. The use of baculoviruses as expression vectors. Appl. Biochem. Biotechnol. **42:**137–159.
10. **Laerum, O. D., and R. Bjerknes.** 1992. Flow cytometry in hematology. Academic Press, London.
11. **Longobardi Givan, A.** 1992. Flow cytometry: first principles. Wiley-Liss Inc., New York.
12. **Luo, L., L. Yan, P. M. Cannon, K. Sunyoung, and C. Y. Kang.** 1992. Chimeric *gag*-V3 virus-like particles of human immunodeficiency virus induce virus-neutralizing antibodies. Proc. Natl. Acad. Sci. USA **89:**10527–10531.
13. **Martin, R., J. R. George, M. Hanson, J. Pearson, R. Turnicky, and J. Wethers.** 1993. Report of the Eighth Annual Conference on Human Retrovirus Testing. Association of State and Territorial Public Health Laboratory Directors.
14. **Mayo, D. J., A. M. Rose, S. E. Matchett, P. A. Hoppe, J. M. Solomon, and K. K. McCurdy.** 1991. Screening potential blood donors at risk for human immunodeficiency virus. Transfusion **31:**466–474.
15. **Nair, B. C., G. Ford, V. S. Kalyanaraman, M. Zafari, C. Fang, and M. G. Sarngadharan.** 1994. Enzyme immunoassay using native envelope glycoprotein (gp160) for detection of human immunodeficiency virus type 1 antibodies. J. Clin. Microbiol. **32:**1449–1456.
16. **Park, J., and C. D. Morrow.** 1992. The nonmyristylated $Pr160^{gag\text{-}pol}$ polyprotein of human immunodeficiency virus type 1 interacts with $Pr55^{gag}$ and is incorporated into viruslike particles. J. Virol. **66:**6304–6313.
17. **Petersen, L. R., G. A. Satten, R. Dodd, M. Busch, S. Kleinman, A. Grindon, B. Lenes, and the HIV Seroconversion Study Group.** 1994. Duration of time from onset of human immunodeficiency virus type 1 infectiousness to development of detectable antibody. Transfusion **34:**283–289.
18. **Race, E. M., K. M. Ramsey, H. L. Lucia, and M. W. Cloyd.** 1991. Human immunodeficiency virus infection elicits early antibody not detected by standard tests: implications for diagnostics and viral immunology. Virology **184:** 716–722.
19. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
20. **Reagan, K. J., C. C. Lile, Y. Devash, J. Turner, Y.-W. Hu, and C. Y. Kang.** 1990. Use of HIV-1 *pol* gene precursor to detect HIV-1 and HIV-2. Lancet **335:**236.
21. **Simmonds, P., D. Beatson, R. J. G. Cuthbert, H. Watson, B. Reynolds, J. F. Peutherer, J. V. Parry, C. A. Ludlam, and C. M. Steel.** 1991. Determinants of HIV disease progression: six-year longitudinal study in the Edinburgh haemophilia/HIV cohort. Lancet **338:**1159–1163.
22. **Simon, F., J. M. Pépin, and F. Brun-Vezinet.** 1992. Reliability of Western blotting for the confirmation of HIV-1 seroconversion. Lancet **340:**1541–1542.
23. **Sinicco, A., R. Fora, M. Sciandra, A. Lucchini, P. Caramello, and P. Gioannin.** 1993. Risk of developing AIDS after primary acute HIV-1 infection. J. Acquired Immune Defic. Syndr. **6:**575–581.
24. **Sligh, J. M., B. S. Stanford, T. Roodman, and C. C. Tsai.** 1989. Flow cytometric indirect immunofluorescence assay with high sensitivity and specificity for detection of antibodies to human immunodeficiency virus (HIV). Am. J. Clin. Pathol. **91:**210–214.
25. **Takehara, K., D. Ireland, and D. H. L. Bishop.** 1988. Co-expression of the hepatitis B surface and core antigens using baculovirus multiple expression vectors. J. Gen. Virol. **69:**2763–2777.
26. **Wagner, R., H. Fliessbach, G. Wanner, M. Motz, M. Niedrig, G. Deby, A. von Brunn, and H. Wolf.** 1992. Studies on processing particle formation and immunogenicity of the HIV-1 *gag* gene product: a possible component of a HIV vaccine. Arch. Virol. **127:**117–137.
27. **Willerford, D. M., J. J. Bwayo, M. Hensel, W. Emonyi, F. A. Plummer, E. N. Ngugi, N. Nagelkerke, W. M. Gallatin, and J. Kreiss.** 1993. Human immunodeficiency virus infection among high-risk seronegative prostitutes in Nairobi. J. Infect. Dis. **167:**1414–1417.
28. **Zaaijer, H. L., P. V. Exel-Oehlers, T. Kraaijeveld, E. Altena, and P. N. Lelie.** 1992. Early detection of antibodies to HIV-1 by third generation assays. Lancet **340:**770–772.
29. **Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho.** 1993. Genotypic and phenotypic characterization of HIV-1 in patients with primary infection. Science **261:**1179–1181.