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Enzyme Immunoassay System for Detecting Antibodies in Complexes with HIV-1 Antigens

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An enzyme immunoassay is proposed for detection of antibodies in complexes with antigens. Specific anti-HIV antibodies were detected in sera which were considered HIV-negative.

Key Words: *HIV infection; enzyme immunoassay; immune complexes; antibodies*

Detection of anti-HIV antibodies by enzyme immunoassay is based on specific binding of antibodies to antigens (peptides, recombinant proteins, and viral lysates) adsorbed on a solid phase [6]. Detection of antibodies in these systems implies that active centers of antibodies are free (not bound to antigen), while adsorbed antigenic structures should reproduce at least part of the immunogen epitopes. These conditions are neglected during so-called "seronegative window" a period when there are no free antibodies [8], and in case of extra production of antibodies specific to HIV-1 protein epitopes which are not reproduced *in vitro*. The first of these conditions is hazardous for banks of blood preparations and the second is interesting for studies of the pathogenesis of HIV infection.

We propose an enzyme immunoassay system detecting antibodies in specific complexes with HIV-1 proteins, circulating in blood plasma (Fig. 1). Antibody Fc fragments specifically reacting with *St. aureus* protein A are recognized. This approach is based on the possibility of immunoaffine separation of such complexes from all other ones if at least some antigenic determinants are free. Immunoadsorbent is represented by $F(ab')_2$ fragments of HIV-specific human antibodies. A new feature of this method is that antibodies can be detected long before they are produced in excess. Moreover, antibodies are recognized in the organism with the conformation-dependent antigenic determinants of viral proteins present in their native form.

The use of $F(ab')_2$ fragments cancels the limitations for testing the sera containing the rheumatoid factor, because this factor specificity is confined to Fc fragments of IgG [4].

MATERIALS AND METHODS

Pooled plasma from HIV-1 infected subjects was the source of anti-HIV antibodies for preparing the immunoadsorbent. The specific anti-HIV titer of pooled plasma was 4.3/mg protein. Immunoglobulin preparation (1.2 mg/ml) precipitated with 1 M ammonium sulfate had a specific titer of 106.6/μg protein. The efficacy of isolated immunoglobulins as the "capture" antibodies is shown in Fig. 2. After dialysis against 0.1 M sodium acetate buffer (pH 4.5), immunoglobulins were fragmented with pepsin (Sigma) into $F(ab')_2$ and Fc fragments (the latter were hydrolyzed into multiple subfragments under the same conditions [10]): one pepsin fraction (15

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mAnson/mg) against 49 fractions of the preparation protein (20 h at 37°C).

The resultant $F(ab')_2$ fragments were adsorbed 1:500 on Greiner plates for 16 h at room temperature in 20 mM carbonate-bicarbonate buffer, pH 9.6. After washing in phosphate-buffered saline (PBS) with 0.1% Tween-20 and 1-h blocking with 1% bovine serum albumin in PBS, 100 µl test sera was added to wells (1:100 in buffer with albumin). HIV-specific antibodies in bound immune complexes were detected by protein A (*St. aureus*) conjugated with horseradish peroxidase (Bio-Rad). Orthophenylene diamine was the peroxidase substrate. Cut-off was calculated as the mean value of light absorbance for 6 HIV-negative sera (492 nm) and 2.5 standard deviations.

The specificity of the reaction between the immunoadsorbent and antigen was confirmed by the competitive enzyme immunoassay (EIA). Serum (1:100) was preincubated with HIV-1-specific $F(ab')_2$ fragments (1:100) for 30 min at 37°C. The reaction was considered specific if the signal in competitive EIA decreased at least 2-fold in comparison with the noncompetitive variant.

RESULTS

Six sera from HIV-negative donors (D1-D6) were negative controls and 6 sera from patients infected with HIV-1 at different stages of infection (Table 1). Two sera (PS1 and PS2) were from a boy whose mother was infected with HIV-1 (PM). At the age of 18 months, despite the negative serological tests, HIV infection was diagnosed by the nest DNA polymerase chain reaction [2]. PS1 serum was collected at the age of 18 months and PS2 and PM, at the age of 47 months. PS1 and PS2 sera contain HIV-1 structural elements: capsid protein p24 and virion RNA. This indicates virus reproduction and hematogenous dissemination in the organism. PS1 and PS2 in high dilutions neutralize HIV-1 *in vitro*.

Figure 3 shows that the proposed EIA variant detects antibodies in PS1 and PS2 despite negative results of common tests for antibodies. If the sera are clearly positive, no antibodies are detected in the immune complexes. This may be due to the absence of complexes or complete shielding of antigenic determinants of viral proteins with antibodies.

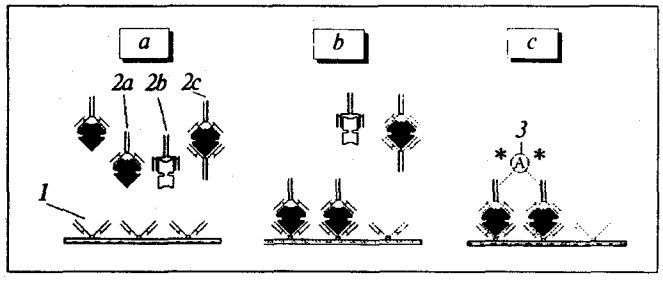

Fig. 1. Enzyme immunoassay for direct detection of antibodies in immune complexes. *a*) incubation of specimen with $F(ab')_2$ fragments adsorbed on solid phase; *b*) washing from unbound immune complexes; *c*) incubation with protein A conjugated with enzyme. *1*) specific $f(ab')_2$ fragments; *2a*) immune complex (IC) with HIV antigens; *2b*) IC with any other antigens; *2c*) IC with HIV antigens representing all epitopes; *3*) protein A conjugate with enzyme.

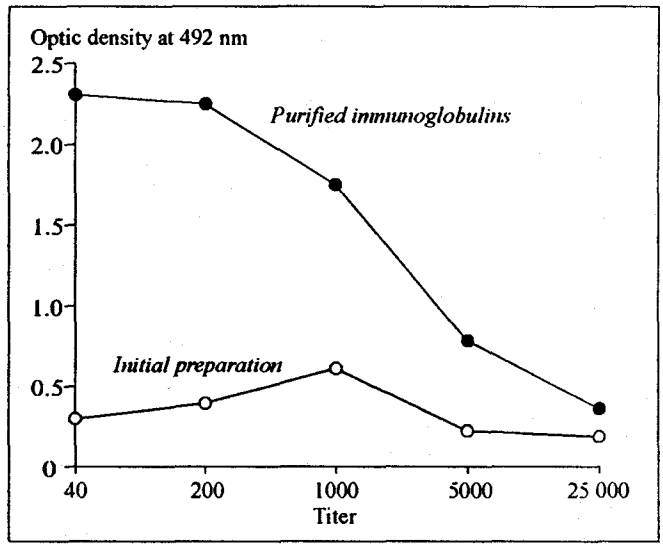

Fig. 2. Curves representing titration of the initial preparation and purified immunoglobulins as immunoadsorbents. Antigen, recombinant HIV-1 p24 (1 ng/ml, Vektor, Novosibirsk), was detected by anti-HIV-1 conjugated with horseradish peroxidase [3].

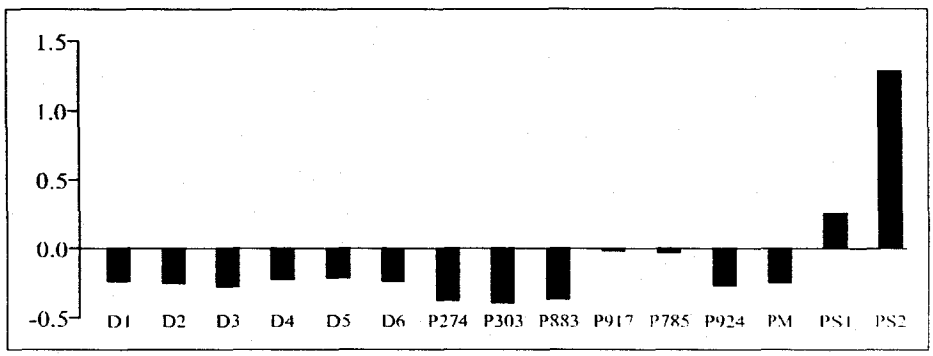

Fig. 3. Content of antibodies in complexes with HIV-1 antigens. Cut-off=0.407.

TABLE 1. The Sera

Serum code	Category	Titer in Screen-HIV, $\times 10^3$	Titer in Eco-Lab	Titer in Peptoscreen-2, $\times 10^2$	Antibody spectrum in Blot-HIV[1]	[p24] after destruction of immune complex, ng/ml serum	HIV RNA in serum[2]	Titer of HIV-1_{RF} complete neutralization[3]
D1-D6	HIV$^-$	—	—	—	—	0	—	—
P274	HIV$^+$	32	5 000	50	GAG, ENV_1, POL	0	+	20
P303	HIV$^+$	16	5 000	5	GAG, ENV_1, POL	0.12	+	40
P785	HIV$^+$	50	2 500	250	GAG, ENV_1, POL	0.09	+	—
P883	HIV$^+$	50	1 250	25	GAG, ENV_1, POL	0.23	+	—
P917	HIV$^+$	50	2 500	50	GAG, ENV_1, POL	0	+	40
P924	HIV$^+$	50	1 250	25	GAG, ENV_1, POL	0	+	80
PM	HIV$^+$	50	6 400	100	GAG, ENV_1, POL	0.14	+	320
PS1	HIV$^+$?	—	—	—	—	0.10	+	320
PS2	HIV$^+$?	—	—	—	—	0.11	+	320

Note. —: negative result. [1]This test system (dot-EIA) detects antibodies specific to recombinant HIV-1 GAG (p24, p17), ENV_1 (gp120, gp41), and POL (p51) and HIV-2 ENV_2 (gp110, gp38). [2]Nest RNA-polymerase chain reaction to HIV-1 gag gene sequence: SK39/SK146 external primers and SK104/SK436 internal primers [1]. [3]HIV-1_{RF} (40 $TCID_{50}$) in H9 lymphoblastoid cells was used. Negative result: the serum did not completely neutralize HIV-1_{RF} even in the minimum dilution 1:10.

Anti-HIV antibodies in PS1 and PS2 are detected only in immune complexes which probably include whole viral particles. Part of antigenic determinants in these complexes is free, and their blocking in competitive EIA decreases the signal intensity by 2.5 times.

These results reflect the period of "seronegative window" which may last for more than 6 months in HIV infection [11]. The period between the manifestation of acute symptoms and seroconversion in viral infections is usually 10-14 days. A much longer duration of this period in HIV infection requires detailed studies of the initial stage of humoral immune response in this disease.

Another explanation is possible. Neutralizing activity of PS1 and PS2 sera may indicate the production of neutralizing anti-HIV antibodies. These antibodies are usually specific to the epitopes of HIV-1 surface proteins gp120-gp41 [9]. It is obvious that such antigenic structures cannot be the components of diagnostic agents for enzyme immunoassay and, as a result, antibodies of such specificity are not detected by the available test systems. Intrauterine infection with development of tolerance of stable and structurally simple antigenic determinants of the virus might have led to excessive production of antibodies to the oligomer. The lack of reaction of PS1 and PS2 to the peptides simulating the linear epitope variants of the third variable domain of gp120 confirms the probability of such specificity [5,7].

The proposed method can be used in the studies of long "seronegative window" in HIV infection. Studies of seronegative HIV infection will help elucidate the pathogenesis of this infection and AIDS.

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