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Comparative Humoral Responses to Human Immunodeficiency Virus Type 1-p24^{gag} Linear B-Cell Epitopes among Individuals Showing Atypical Western Immunoblotting Reactions and Implications for Diagnosis

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Serum specimens from 25 individuals with an isolated human immunodeficiency virus type 1 (HIV-1) core antigen reactivity in a Western immunoblot test were examined for their reactivities with HIV-1 virions, control cellular antigens, HIV-1-Bru p24^{gag} recombinant protein (p24^{gag}), and a panel of 22 p24^{gag}-derived peptides. The results were as follows: (i) serum specimens from eight HIV-1-uninfected subjects did bind to virions but failed to bind to p24^{gag}; (ii) sera from 13 HIV-1-uninfected subjects and from one HIV-2-infected patient reacted with HIV-1 virions and p24^{gag} but failed to bind to any of the peptides expressing major p24^{gag} epitopes; and (iii) 3 serum specimens obtained from one neonate carrying anti-HIV-1 maternal antibody and from two HIV-1-infected subjects who had seroconverted during the study reacted with HIV-1 virions, p24^{gag}, and one or more peptides containing the major p24^{gag} epitopes. Our data suggest that the combination of p24^{gag} and appropriate peptides could be useful for resolution when atypical Western immunoblot results are encountered.

The strategy routinely used for the diagnosis of human immunodeficiency virus type 1 (HIV-1) infection consists of the determination of seropositivity to HIV-1 antigens by an enzyme-linked immunosorbent assay (ELISA), the result of which is subsequently confirmed by a Western immunoblot (WB) to viral proteins. The sensitivity and specificity of this procedure have been reported to be over 97% (4). However, sera from some subjects present incomplete WB patterns most frequently limited to the 17- or 24-kDa band. These sera are therefore regarded as indeterminate. Indeterminate patterns could be due to (i) early seroconversion with an immunological response that is restricted to the major HIV-1 immunogens (7), (ii) infection with a retrovirus presenting restricted antigenic conservation with HIV-1 and/or HIV-2 (6), (iii) infection with a defective HIV (9), (iv) reactivity with a protein encoded by one of the endogenous retrovirus-related sequences that exists within the normal genomic DNA of eukaryotes (17), (v) recognition of a cross-reactive epitope shared between an unidentified infectious agent and an HIV-encoded antigen (3), (vi) reactivity against an HIV-unrelated host cell-derived molecule of the viral lysate comigrating with an HIV-1 antigen in the WB (18), or (vii) reactivity with a cellular antigen that is overexpressed under HIV-1 transactivation (1) and that acts as a foreign antigen.

In routine use, the percentage of sera with an indeterminate WB test result varies according to the specimen series studied and the tests used (14, 19, 24). Most commonly, subjects with indeterminate HIV-1 serological status show a repeated negative assay for p24 antigenemia and HIV-1 isolation (7, 10, 13) and are considered to be uninfected. Nevertheless, these subjects require long-term serological follow-up or additional testing to ensure that they are not infected. Therefore, it is of highly practical importance to understand the reasons for such frequent indeterminate WB reactions and to distinguish, as early as possible, HIV-1-infected patients from uninfected individuals. To achieve this goal, we tested serum specimens from individuals with indeterminate WB results for their fine reactivities with viral antigens.

Twenty-five serum specimens (serum specimens 1 to 25 from subjects 1 to 25, respectively) were selected for their HIV-1 positivity in an ELISA (ELAVIA-Ac-Ab-AkI; Pasteur Diagnostic, Marnes-la-Coquette, France) and for their reactivities limited to HIV-1 core proteins in a WB test (Biotech-Du Pont, Wilmington, Del.). Among these, 15 serum specimens (serum specimens 2 to 4, 6, 7, 11, 12, 15, 17 to 20, 21, 23, and 24) reacted with an isolated 24-kDa band, and the 10 other specimens (serum specimens 1, 5, 8 to 10, 13, 14, 16, 22, and 25) reacted with both the 24- and 55-kDa bands. This series of serum specimens was tested by a blinded investigator for their reactivities with HIV-1-Bru viral antigens, control cellular antigens, HIV-1-Bru p24^{gag} recombinant protein, and a panel of 22 overlapping peptides wholly covering the HIV-1-Bru p24^{gag} amino acid sequence. Clinical information relevant to these samples was made available to the blinded investigator only at the end of the work; it included a 6-month serological follow-up for all subjects. The WB test results for HIV-1 showed that subjects 14 and 21 had seroconverted to HIV-1 positivity and

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TABLE 1. Summary of the results of serological tests

Serum specimen type and no.	Binding to[a]:				No. of serum specimens with this pattern/total no. of serum specimens (%)
	HIV-1	CEM[Ag]	p24[gag b]	PX[c]	
Indeterminate					
8, 13, 17, 18, 20, and 24	+	−	−	NT[d]	6/25 (24)
15 and 23	+	+	−	NT	2/25 (8)
1, 5, 6, 7, 16, 22, and 25	+	−	+	−	7/25 (28)
2, 3, 4, 9, 10, 11, 14, 19, and 21	+	−	+	+	9/25 (36)
12	+	+	+	+	1/25 (4)
Negative (26 to 33)	−	−	−	−	8/8 (100)
Positive (34 to 45)	+	−	+	+	12/12 (100)

[a] Serum specimen binding to HIV-1-Bru antigens (HIV-1), CEM cell antigens (CEM[Ag]), HIV-1-Bru p24[gag] recombinant molecules (p24[gag]), and HIV-1-Bru p24[gag]-derived peptides (PX) was tested by ELISA. +, a positive ELISA reaction (A_{492} over three times the background value); −, a negative reaction. Background, $0.1 < A_{492} < 0.15$.

[b] Comparison of immunoreactivity of p24[gag] versus that of viral p24 has been reported previously (21, 22).

[c] Binding to one or more peptides (P14 to P35). See Table 2 for details.

[d] NT, not tested.

that subject 9 (a child born to an HIV-1-infected mother) had seroreverted at 12 months of age. The WB test results for HIV-2 revealed the seropositivity of subject 1. Additional biological information such as diagnosis of an infection with a pathogenic agent other then HIV was indicated when available. Finally, the follow-up information indicated that serum specimens 3 and 4 were obtained from a healthy mother and her neonate, respectively. Controls included eight serum specimens obtained from HIV-1 antibody-negative healthy subjects (serum specimens and subjects 26 to 33) and 12 serum specimens obtained from HIV-1-infected patients who were either asymptomatic or symptomatic (serum specimens and subjects 34 to 45). The specificities of serum antibodies from individuals who had an indeterminate HIV-1 WB test result were analyzed by direct ELISA as described previously (20). Briefly, plates were coated overnight at 4°C with 10 μg (per ml) of either HIV-1-Bru virus (2) propagated on CEM cells and concentrated from the cell-free supernatant by ultracentrifugation as described previously (5), concentrated cell-free supernatant from uninfected CEM cells, or HIV-1 p24[gag] recombinant protein in 100 mM sodium carbonate buffer (pH 9.6). After washing and saturation with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), 100 μl of serum (diluted 75-fold in PBS–0.2% BSA containing 50 μM EDTA, 10 μM aprotinin, and 10 μM trypsin inhibitor) was added, and the mixture was incubated overnight at 4°C. The plates were washed five times with PBS–0.05% Tween 20, and bound immunoglobulins (Ig's) were detected by incubation with 100 μl of goat anti-human Ig (heavy and light chains) peroxidase conjugate (diluted 3,500-fold; Amersham, Buckinghamshire, England) for 1 h and by subsequent incubation with *o*-phenylenediamine (Sigma, St. Louis, Mo.) as the substrate. The A_{492} was then measured. As summarized in Table 1, 17 of 25 serum specimens (68%) reacted with the p24[gag] recombinant protein, whereas 8 (32%) were unreactive. Finally, two serum specimens (8%) that did not react with the HIV-1-Bru p24[gag] recombinant protein reacted with control cellular antigens. Control serum specimens from HIV-1-seropositive patients and seronegative healthy subjects were respectively positive and negative for HIV-1-Bru p24[gag] recombinant protein binding.

The serum specimens that reacted with the HIV-1-Bru p24[gag] recombinant protein were tested by direct ELISA for their reactivities with the p24[gag]-derived oligopeptides. The 22 p24[gag]-derived linear synthetic peptides (purity, over 85%) used in the present study (called P14 to P35) have been described previously (22). Their sequences are as follows: P14, PIVQNIQGQMVHQAIS; P15, MVHQAISPRTLNAWV; P16, SPRTLNAWVKVVEEK; P17, AWVKVVEEKAFSPEVIPMF; P18, IPMFSALSEGATPQDL; P19, SEGATPQDLNTMLNTVG; P20, GHQAAMQMLKETINEE; P21, LKETINEEAAEWDRVHPV; P22, HAGPIAPGQMREPRG; P23, GSDIAGTTSTLQEQIGWMTNN; P24, EQIGWMTNNPPIPVGEI; P25, IPVGEIYKRWIILGL; P26, KRWIIVGLNKIVRMY; P27, LNKIVRMYSPTSILDIRQ; P28, IRQGPKEPFRDYVDRFYKTL; P29, FRDYVDRFYKTLRAEQAS; P30, KTLRAEQASQEVKNWMTET; P31, ETLLVQNANPDCKTILKAK; P32, DCTILKALGPAATLE; P33, KALGPAATLEEMMTACQ; P34, MMTACQGVGGPGHKA; P35, GHKARVLAEAMSQVTN. The direct ELISA on a peptide-coated solid phase was performed as described above, and the A_{492} was measured to evaluate the amount of bound Ig. The results were normalized by calculating an index of reactivity by the Student-Fisher statistical test (23), which allows comparison of the patterns of reactivity between sera without the need to take into account their respective gamma globulin levels. Reactivity with a peptide was interpreted as positive for index values over 0.3, and an index value of 0 corresponded to the mean absorbance with the different peptides. Seven of the 17 serum specimens that reacted with the HIV-1-Bru p24[gag] recombinant protein and that had an indeterminate WB test result did not bind to any p24[gag]-derived peptide (Table 2). This could indicate that their respective target epitopes have conformational requirements. The last 10 serum specimens reacted with one or more peptides. Only a few peptides were recognized by more than one serum specimen. For instance, the P31, P21, and P23 peptides were recognized by serum specimens 3 and 4, 9 and 21, and 10 and 11, respectively. Peptide reactivity was also tested by peptide inhibition in the antibody-binding assay. To this end, each serum specimen (100 μl of serum diluted 75-fold) was mixed with peptide (concentration range, 0.1 nM to 10 μM) in PBS–0.2% BSA solution containing 50 μM EDTA, 10 μM aprotinin, and 10 μM trypsin inhibitor before being added to the peptide-coated wells. After 1 h of incubation at room temperature, plates were washed five times and bound Ig was detected. Only a few serum specimens (specimens 2, 3, 4, and 9) reacted by ELISA to a peptide (P35, P31, or P20) were inhibited by the homologous peptide (Table 2). This observation may indicate that the majority of the antigen-antibody reactions that we analyzed were of low affinity.

Since the specificities of the reactions of serum specimens 3 and 4 with P31 and serum specimen 2 with P35 were demonstrated by competitive inhibition of binding by using the homologous peptides (see above) and the HIV-1-Bru p24[gag] recombinant protein (data not shown), we searched for sequence homologies between these regions of HIV-1-Bru p24[gag] and non-HIV-encoded antigens using the compilation of sequences of the National Biomedical Research Foundation data bank. A comparison based on five consec-

TABLE 2. Reactivity of sera indeterminate by WB with HIV-1-Bru $p24^{gag}$-derived peptides

Serum specimen no.	Ig concn (g/liter)		PX[a]	IR[b]	% Binding inhibition to PX by 10 mM:	
	IgG	IgM			PX[a]	P15[c]
12	NT[d]	NT	P18	0.31	<10[e]	<10
9	NT	NT	P20	0.83	29	<10
9	NT	NT	P21	0.52	<10	<10
21	10.6	3.9	P21	1.55	<10	<10
10	14.8	3.6	P23	0.36	<10	<10
11	15.9	1.7	P23	0.58	<10	<10
19	NT	NT	P24	0.44	NA[f]	NA
14	NT	NT	P29	0.40	<10	<10
14	NT	NT	P30	1.11	<10	<10
3	NT	NT	P31	0.35	20	<10
4	18.4	<0.3	P31	0.66	40	<10
2	14.5	2.0	P35	0.51	63	<10
1, 5, 6, 7, 16, 22, and 25	10 < c[g] < 18.5	1.7 < c < 4.7	None	<0.3		

[a] PX, immunoreactive peptide (i.e., for serum specimen 12, the PX is P18).
[b] IR, index of reactivity. The reactivity of a serum specimen with a peptide was considered positive when the index of reactivity was greater than 0.3. The different indices of reactivity were calculated for each individual peptide and indicated when positive (i.e., the index of reactivity of serum specimen 12 with P18 is 0.31).
[c] The P15 peptide was used as a control for all inhibition experiments.
[d] NT, not tested.
[e] Inhibition below 10% was considered negative.
[f] NA, not available; this serum specimen was no longer available when the experiment with the peptides was done.
[g] c, Ig concentration.

utive homologous amino acids (considered as a minimal putative antibody-binding site) was performed, and the results of that analysis are summarized in Table 3. We found that the 83/8309 and 91/8309 sequences showed homologies with the P31 and P35 peptides, respectively. Among these sequences, several (9/83 and 4/91, respectively) belonged to antigens of infectious agents, including human viruses, bacteria, and parasites. It was established thereafter that serum specimen 3 was obtained from a woman who had two acute infections with streptococcus in the 12 months prior to the discovery of her 24-kDa band reactivity in the WB test. Accordingly, an antibody response to a streptococcal antigen containing the KTILK sequence could explain the reactivities of serum specimens 3 and 4 (obtained from a mother and her neonate, respectively) with HIV-1-Bru $p24^{gag}$. No clinical information was available concerning subject 2. This analysis shows some evidence of conservation between HIV-1-Bru $p24^{gag}$ epitopes and epitopes of proteins from a number of other pathogens and indicates that antigenic cross-reactions can sometimes give rise to an indeterminate HIV-1 WB test result.

In the study described here, we observed that 32% of the sera classified as indeterminate for HIV-1 did not have detectable reactivity against the HIV-1-Bru $p24^{gag}$ recombinant protein. Failure of the sera to react with the HIV-1-Bru $p24^{gag}$ recombinant protein could be related to differences in folding between the recombinant and the viral $p24^{gag}$ proteins (8); it also remains possible that HIV-1 infection of cells used for viral propagation would result in viral transactivation of cellular genes (1); finally, these sera could react with

TABLE 3. Sequence comparison

Peptide PX (PX sequence)[a]	Homology[b]		Sequence of putative antigen[c]	Antigen origin[d]
	B/A	C/A		
P31 (ETLLVQNANPDCKTILKAL)	83/8309	9/8309	LIPTLLVQGA	Varicella-zoster virus
			CAWLLVQNFT	Herpesvirus
			LIGLLVQNPD	*Salmonella* spp.
			QFNLLVQNIQ	Papillomavirus
			TSYQNANPGE	Poliovirus
			PADNANPDAD	*Plasmodium* spp.
			SLVKTILKWE	*Streptococcus* spp.
			RREILKALQP	Cytomegalovirus
			DPYILKALRA	Varicella-zoster virus
P35 (GHKARVLAEAMSQVTN)	91/8309	4/8309	VFYARVLAPP	Epstein-Barr virus
			PYLARVLAVT	Epstein-Barr virus
			TATVLAEAIY	*Chlamydia* spp.
			PTYAEAMSDH	Herpesvirus

[a] PX, immunoreactive peptide.
[b] Among all sequences (8309) from the National Biomedical Research Foundation data bank (A), we searched for proteins that share five consecutive amino acids homologous to the P31 or P35 sequence (B) and for human antigens known to be targets for autoantibodies and for antigens of human infectious agents (C).
[c] Boldface letters delineate homologous amino acids.
[d] Origin of the proteins defined as C in the homology column.

a 24-kDa molecule that is constitutively expressed by CEM cells. We also observed that 12% of sera with an indeterminate WB test result reacted with cellular antigens. Altogether, our data corroborate those obtained by Povolotsky et al. (18), who found that 30% of the sera with an indeterminate WB test result that they studied had no apparent reactivity with a recombinant protein containing $p24^{gag}$.

The majority of peptides that were recognized by the serum specimens with an indeterminate WB test result were not those predicted to contain the major antigenic sequences of $p24^{gag}$. We and other investigators (8, 11, 22) have previously predicted, by computer analysis and/or demonstrated by using murine monoclonal antibodies, that the P21, P28, P29, and P30 peptides contain the major antigenic sequences of the $p24^{gag}$ protein. This immunodominance has also been verified with sera obtained from HIV-infected patients (12, 15, 16). In our blind tests of a series of sera with an indeterminate WB test result, only 3 of 25 serum specimens (serum specimens 9, 14, and 21) were found to react with one of the peptides that contain the major HIV-1-Bru $p24^{gag}$ epitopes. Interestingly, all of these sera were expected to contain antibodies to HIV-1; one (serum specimen 9) was obtained from a child born to an infected mother, and this child seroreverted at 12 months of age (indicating that the anti-HIV-1 antibodies found in this sample were of maternal origin); another (serum specimen 21) was obtained from a subject who had sexual contacts with an HIV-1-infected individual and who seroconverted (appearance of antibodies to HIV-1 glycoproteins on the next WB test), and the last (serum specimen 14) originated from a patient with no known behaviors that would put the individual at risk for HIV-1 infection but who later seroconverted to HIV-1 seropositivity. Therefore, reactivity with peptide P21, P28, P29, or P30 could be of predictive value for the diagnosis of HIV-1 infection. This confirms and extends a similar observation reported by Janvier et al. (12). However, we found no reactivity of serum specimen 1 with the HIV-1-Bru $p24^{gag}$-derived peptides, even though subject 1 was later shown to be infected with HIV-2. Although epitopes shared by HIV-1 and HIV-2 have previously been identified within the P28 and P29 peptides by using murine monoclonal antibodies (21), use of this panel of peptides might be appropriate for confirming infections in individuals suspected of being infected with HIV-1 but not HIV-2.

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