

Identification of Regions of HIV-1 p24 Reactive with Sera Which Give "Indeterminate" Results in Electrophoretic Immunoblots with the Help of Long Synthetic Peptides

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ABSTRACT

We analyzed nine sera from persons unlikely to be HIV infected which had an IgG reactivity directed against HIV-1 p24, and in two cases also to its precursor p55, but to no other HIV proteins, nor to proteins of the H9 host cell, in electrophoretic immunoblots (EIB). These sera are also referred to as having an indeterminate HIV EIB pattern or as HIV antibody false positive sera. Seven of nine sera reacted with longer (61–77 amino acids) and none with shorter (17–25 amino acids) p24-derived peptides in enzyme immunoassays (EIAs). This is compatible with a conformational (discontinuous) nature of the epitopes involved in many false positive HIV-1 p24 antibody reactions. Four sera reacted with an N-terminal, one with an internal, and two with a C-terminal fragment. Each of the seven sera thus only reacted with one of the long p24 peptides. The specificity and singularity of the reaction was further demonstrated by competition and/or absorption experiments with synthetic peptides. In contrast, 18 of 20 confirmed HIV-1⁺ sera with p24 reactivity in EIB reacted with at least one and often several of the longer peptides, most frequently the C-terminal one. Thus, the distribution of peptide reactivity of true HIV-1 antibody-positive sera was different from that of the falsely reactive sera. According to two of several explanations, these antibodies may have arisen because of (1) molecular mimicry by chance or by functional selection, (2) immunization by activation, noninfectious exposure, or infection involving non-HIV endogenous or exogenous retroviral antigens. The latter gains some support from our finding of antibody reactions with capsid proteins of the simian viruses, simian sarcoma-associated virus (SSAV), and Mason-Pfizer monkey retrovirus in some of the p24 ± p55 reactive sera.

INTRODUCTION

AFTER THE INTRODUCTION OF LARGE-SCALE human immunodeficiency virus type 1 (HIV-1) antibody testing, it was observed that a small portion of sera from humans unlikely to be HIV-1 infected contained antibodies to one or a few HIV-1 proteins, mostly derived from *gag*.^{1–8} Such reactivities can give rise to "indeterminate" HIV-1 electrophoretic immunoblot reactions. These are a constant cause of concern in laboratories responsible for confirmation of HIV-1 antibody screening results, and their origin should be elucidated. One of the most

common types of false positive HIV EIB reactivity is characterized by a reactivity with p24 with or without reactivity with its precursor protein, p55, but with no other HIV protein. We have studied the reactivity with p24 peptides in sera from healthy Swedish subjects who had the "p24 ± p55" pattern of serological reactivity. We were able to localize these antigenic determinants only by use of long synthetic peptides. The concentration of the p24 reactivity in false HIV antibody-positive sera to three different relatively narrow portions of the molecule indicates that these p24 antibodies can arise by different mechanisms. All three regions contain evolutionarily conserved sequences which could participate in interspecies serological cross-reactions.

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MATERIALS AND METHODS

Sera

Nine HIV-1 a strong sera with p24 \pm p55 pattern of reactivity were found during EIB testing of 785 sera which had tested repeatedly positive in an HIV-1 antibody screening test were sent to our laboratory for confirmatory testing. The 785 sera corresponded to around 150,000 HIV-1 screening tests conducted in southern Sweden, from primary test sites that regularly send sera for confirmatory analyses to our laboratory. Blood donors, pregnant women, and heterosexuals concerned about HIV infection, but essentially not at risk of being HIV infected, provided the great majority of these samples. Among the donors of the nine sera were 5 women, 3 men, and 1 of unknown gender. Ages were 22, 3 \times 24, 25, 33, 36, and 57. Age was unknown for one person. Reasons for making the HIV antibody test as stated on the request form were: blood donor 4, pregnancy 3, unknown 2. We did not contact the individuals deciding it was not necessary at this stage of our investigation of the nature of the false positive HIV serological reactions. Controls were 20 confirmed HIV-1 antibody positive (HIV-1⁺) sera and 17 seronegative unselected Swedish blood donors. Two additional sera that gave a p24 and a very weak gp120 band in EIB were initially included among the false-positive sera in error. They reacted with either three or two (HIV-1 *gag* 318–378 oxidized and unoxidized + 262–338) of the long p24 derived peptides and were therefore reinvestigated. These proved to be early sera from patients who shortly afterward seroconverted to full EIB positivity. These sera were consequently not included in the study.

Immunoassays

Four commercial assays were used: A whole virus HIV-1 antibody test, Organon Vironostica anti-HIV (Organon Technica, Turnhout, The Netherlands), the Abbott (Abbott Diagnostics, Chicago, IL) HIV-1 antibody EIA where recombinant antigen has been prepared from bacteria, the Abbott ENVA-CORE confirmatory HIV-1 antibody competition antibody EIA, and the Du Pont Biotech (Du Pont, Billerica, NJ) HIV-1 electrophoretic immunoblot (EIB) system. According to our information, the EIB test is based on the IIB isolate of HIV-1 grown on H9 cells. The commercial tests were used according to the instructions of the manufacturers. We always checked the reactivity of the p24 \pm p55 positive sera with EIB strips coated with H9 proteins (Du Pont). None reacted. For studies of competition between solid-phase retroviral antigen and synthetic peptide in solution, sera were diluted in the respective dilution fluids at the standard concentration together with varying concentrations of synthetic peptide or without peptide for 1 h at room temperature. The commercial EIAs were then performed according to the standard procedure. EIB strips were measured with a Bio-Rad 620 Video Densitometer (Bio-Rad, Richmond, CA).^{9,10} Peptide EIAs were performed approximately as described.^{11,12} Briefly, 100 μ l of 20 μ g/ml of peptide in PBS-M (PBS: 8.0 g NaCl, 0.2 g KH₂PO₄, 1.4 g Na₂HPO₄ · 2H₂O, 0.2 g KCl/l) (PBS-M: phosphate-buffered saline with 10 μ g/ml of sodium merthiolate) was allowed to bind first for 2–6 h at room temperature, then overnight at 4°C. Next, 200 μ l of blocking solution (4% bovine serum albumin (BSA), 0.2%

gelatin, 0.05% Tween-20) was added, allowed to bind for 2–6 h at room temperature, then overnight at 4°C, and frozen until use. Plates were thawed and washed three times in washing fluid (freshly made 0.05% Tween-20 in PBS). Aliquots of 100 μ l of appropriately diluted control sera and test sera diluted 1:50 in diluent I (3% BSA, 0.2% gelatin, 0.05% Tween-20 in PBS-M) were then added and the plates were incubated with shaking at room temperature for 1 h. After another wash, 100 μ l biotinylated affinity-purified goat antihuman IgG (Sigma) diluted 1:1500 in diluent II (0.2% gelatin in PBS-M) were added, and incubated with shaking for 1 h at room temperature. After washing, 100 μ l avidin-peroxidase (Sigma) diluted 1:300 in diluent II were added, and the plates were incubated with shaking for 1 h at room temperature. The plates were washed thoroughly and beaten dry. Then 100 μ l substrate solution [20 mg *o*-phenylenediamine + 10 μ l 30% H₂O₂ in color buffer (34.7 mM citric acid, 66.7 mM Na₂HPO₄, pH 5.0), made fresh each day] was incubated in the dark at room temperature for 30 min and read at 450 nm in a microplate photometer. Absorbance of peptide-coated wells were always subtracted with the absorbance of a well mock-coated with peptide dilution fluid and incubated with the same serum. We stipulated a cut-off absorbance differential value of 0.4, close to two standard deviations (2 \times 0.145) above the average absorbance differential (0.096) of the blood donor control sera with the six long peptides.

We followed internationally accepted criteria for HIV-1 seropositivity.^{13,14} Sera which reacted with at least one *env* and one *gag* band were classified as "confirmed HIV-1 antibody positive," also referred to as "true" positive. Sera which reacted only with *gag* proteins (p17 \pm p55, p24 \pm p55, or p55) were classified as having an "indeterminate" HIV-1 antibody reactivity or being "false positive" with respect to HIV-1 antibodies.

Electrophoretic immunoblot with simian sarcoma-associated virus (SSAV) and Mason-Pfizer monkey virus (MPMV) whole virus antigens was performed as described⁽¹⁰⁾ with 200 μ g (per polyacrylamide gel) virus purified by ultracentrifuge banding in sucrose density gradients twice (purchased from Viral Resources Program, National Cancer Institute, Bethesda, MD).

Synthetic peptides

The peptides used are presented in Table 1. We used both short and long peptides (17–77 aa) in an attempt to detect as much antibody reactivity as possible. The HIV-1 hxb2 sequence (*cf.*, Ref. 15) was used. Due to the evolutionary conservation of most of the *gag* proteins, it is reasonable to assume that peptides based on this sequence will react in a representative way with antibodies directed to many different HIV-1 strains. The most C-terminal p24-derived peptide (HIV-1 *gag* 318–378) also contained a short stretch of amino acids belonging to the p15 protein. Peptides were synthesized by a solid phase method with the 9-fluorenyl-methoxycarbonyl (Fmoc) strategy using a continuous flow automated synthesizer (Milligen 9050). Coupling efficiencies were controlled by qualitative monitoring of the Fmoc group and were in the range 95–100%. The peptides were subsequently purified by reverse-phase high-performance liquid chromatography (HPLC) on a C18 column. Purity was around 95% according to an analytical HPLC procedure. We used both the unoxidized and oxidized forms of HIV-1 *gag* 318–378. The

TABLE 1. SYNTHETIC PEPTIDES EMPLOYED IN THE PRESENT STUDY

HIV-1 hxb2 gag	133-193	PIVQNIQGQMVHQVHQAISPRTLN	AVKVVVEEKAFSPEVPMFSA	LSSEGATPQDLN	TMLNTVGG
HIV-1 hxb2 gag	143-156	VHQVHQAISPRTLN			
HIV-1 hxb2 gag	173-233	SALSEGATPQDLN			
HIV-1 hxb2 gag	203-218	TMLNTVGGHQAA			
HIV-1 hxb2 gag	210-230	MQLKETINEEAAEWDRVHPVHAGPIAPGQMREPRG			
		ETINEEAAEWDRVHPV			
		AEWDRVHPVHAGPIAPGQMRE			
HIV-1 hxb2 gag	213-273	DRVHPVHAGPIAPGQMREPRGSDIAGTTSTLQEQIGWMTNPP			
HIV-1 hxb2 gag	223-243	IAPGQMREPRGSDIAGTTSTL			
HIV-1 hxb2 gag	260-276	EIYKRWILGLN			
		KIVRM			
HIV-1 hxb2 gag	262-338	YKRWILGLN			
HIV-1 hxb2 gag	284-300	KIVRMYSPTSILDIRQGPKEPFRDYVDRFYKTLRAEQASQEVKNWMTETLLVQ			
		NANPDCKTILKALG			
		DIRQGPKEPFRDYVDRF			
		↓		↓	
HIV-1 hxb2 gag	318-378	TETLLVQ			
HIV-1 hxb2 gag	335-351	NANPDCKTILKALGPAATLEEMMTACQGVGGPGHKARVLAEAMSQVTNSATIMM			
HIV-1 hxb2 gag	348-373	KALGPAATLEEMMTACQ			
HIV-1 hxb2 gag	359-382	TACQGVGGPGHKARVLAEAMSQVTN			
		KARVLAEAMSQVTNSATIMMQRGN			

Arrows point to the cysteine residues which were joined by oxidation.

latter was produced by reaction with potassium hexacyanoferrate. The completeness of the oxidation was ascertained by titration of free thiol groups.

Absorption of sera with immobilized peptides

p24 antibody-positive sera were diluted in EIA dilution fluid (3% BSA, 0.2% gelatin in PBS-M) to yield an absorbance increment versus the negative control well of 0.5–1.0 with the reactive peptide. Sera were incubated in wells precoated with p24 peptides for 1 h with gentle shaking. The supernatant was then transferred to a neighboring well, and incubated for another hour. This was repeated another time. A total of three 1-h rounds of absorptions were thus made. The supernatant was then diluted 1:2 in blotting solution, transferred to an EIB well, and allowed to react in an HIV-1 EIB (DuPont) under otherwise standard conditions. In most experiments we used wells coated with the EIA-reactive p24 peptide together with the following controls: wells coated with an unreactive p24 peptide, a non-HIV peptide (HTLV-I gag 111–130), and wells mock-coated with PBS in parallel. The intensity of the p24 band was measured by reflectance densitometry on the EIB strips.^{9,10}

Amino acid sequence comparisons

Retroviral gag amino acid sequences were obtained from the SWISSPROT or Los Alamos¹⁵ databases. Alignments were made with the help of the following computer programs: AALIGN (in the DNASTAR package, Madison, WI) and PALIGN and SCANSIM (both from the PC/Gene program package; Genofit, Geneva, Switzerland). Through the courtesy of Dr. RF Doolittle (*cf.*, Ref. 16), we had available to us an unpublished multiple progressive alignment of the sequences of the major gag proteins of several retroviruses to serve as a guide when we made final manual adjustments of our retrovirus capsid protein alignment. The alignment was intended to demonstrate as many potentially conserved, matching, residues as possible.

RESULTS

Reactivity of p24 ± p55 reactive sera in commercial HIV-1 antibody tests

Three commercial antibody EIAs were used. The distribution of reactivities in the nine sera with p24 bands in HIV-1 EIB can be seen in Table 2. Five sera reacted only in the whole virus EIA, while two reacted only in the recombinant HIV-1 antibody EIA. The remaining two had been reactive in the whole virus lysate test at the remitting test site, but were not positive in our laboratory. None reacted in the commercial competition EIA (data not shown). Sera that were reactive in the whole virus test came from test sites that used the same test, and those who reacted in the recombinant test came from sites which employed that test. Three of five whole virus reactive sera were positive in EIA with peptide gag 133–193 and one with 262–338. These four sera were negative in the HIV-1 recombinant EIA, whereas the two recombinant reactive sera were positive in EIA with gag 318–378 and negative in the whole virus EIA. Thus, the pattern of reactivity in the commercial anti-HIV tests indicated to which portion of the p24 molecule the anti-p24 antibodies were directed.

Reactivity of p24 ± p55 positive sera with synthetic peptides derived from p24. Comparison with confirmed HIV-1 antibody positive and negative blood donor sera

The distribution of IgG reactivities of "p24 ± p55" antibody-positive sera on the five long and nine shorter p24-derived synthetic peptides is shown in Figure 1. Four p24 ± p55-positive sera reacted, two strongly, with the N-terminal long peptide HIV-1 gag 133–193, one serum with HIV-1 gag 262–358, and two with the C-terminal HIV-1 gag 318–378 in unoxidized form. One serum had a reactivity with gag 133–193 just below the stipulated cut-off absorbance differential value of

TABLE 2. CHARACTERISTICS OF THE 9 HIV-1 p24-REACTIVE SERA ANALYZED, AND THE PATIENTS DONATING THEM

Serum No.	Reactive in EIA with Long Peptide	Organon Whole Virus HIV-1 EIA (A492) ^a	Abbott Recombinant HIV-1 EIA (A492)	EIB pattern	Sex ^b	Age	Reason for Testing	Evidence for Specificity of Peptide Reactivity ^c
A	262-338	0.594/0.194	0.061/0.122	p24	F	25	Pregnancy	P,A
B	133-193	0.123/0.162	0.064/0.308	p24	F	24	Blood donor	P
C	133-193	0.141/0.130	0.178/0.357	p24	M	22	Blood donor	P,C
D	133-193	0.279/0.130	0.040/0.234	p24	F	24	Blood donor	P,C
E	133-193	0.209/0.137	0.109/0.244	p24 + p55	M	?	Unknown	P,C
F	318-378	0.056/0.109	0.448/0.216	p24 + p55	F	33	Pregnancy	P,C
G	None	0.052/0.123	0.158/0.396	p24	M	24	Blood donor	—
H	318-378	0.090/0.154	0.282/0.251	p24	F	36	Pregnancy	P,A
I	None	0.165/0.129	0.048/0.320	p24	F	57	Unknown	—

^a Absorbance and cut-off values are shown.

^b F = Female, M = Male.

^c P = Absorbance value difference above 0.4 in EIA with the respective peptide. A = Absorption of EIB p24 reactivity with peptide demonstrated. C = Inhibition of reactivity in commercial HIV-1 antibody EIA by peptide shown. — = Localization of reactivity to a certain portion of p24 not achieved.

0.4. None of the p24 ± p55-positive sera reacted with the shorter HIV-1 gag peptides. In contrast, 15 of 20 confirmed HIV-1⁺ sera reacted strongly with peptide 318-378 both in unoxidized and oxidized form (Fig. 1b), and to lesser extents with the other long p24-derived peptides. The sera from HIV-1 seronegative Swedish blood donors were largely unreactive with all the peptides (Fig. 1c). However, peptides 213-273 and 262-338 reacted significantly also with a few blood donor sera, and results obtained with them thus had to be interpreted with caution.

Neither the false HIV antibody positive nor the blood donor control sera reacted with the nine shorter p24-derived peptides. Five of the confirmed HIV antibody-positive sera reacted with HIV-1 gag 335-351, three with HIV-1 gag 260-276, two with HIV-1 gag 143-156, and one with HIV-1 gag 203-218 (Table 3). Thus the shorter peptides were not useful for demonstration of false positive HIV antibodies, but were useful for further delineation of some of the p24 epitopes recognized by HIV-1-infected persons.

The frequencies of reactivity with long synthetic p24 derived peptides are shown in Tables 3 and 4. Besides the expected significantly higher reactivity of confirmed HIV⁺ sera versus blood donor or false positive sera, a significant difference between false positive sera and blood donors was evident with peptide HIV-1 gag 133-193 ($p = 0.008$). The difference between false positive and blood donor sera for peptides HIV-1 gag 318-378 (unoxidized) and HIV-1 gag 262-338 only reached a weak significance ($p = 0.11$) or was not significant at all, respectively. The pattern of reactivity with long synthetic peptides is shown in Fig. 4. False positive sera reacted with only one p24 peptide per serum.

Absorption of p24 ± p55 positive sera with immobilized peptides

To ascertain that the peptide EIA reactivities really were directed against p24 of HIV-1, we performed absorption exper-

iments. Six of the eight peptide-reactive "p24 ± p55" reactive sera (nr A-E and H) were incubated with p24-derived and control peptides in wells of EIA microplates in three successive 1 h absorptions. We ascertained that an absorption of peptide-reactive antibody had taken place by running an EIA in the same wells after the completion of the absorptions. The remaining antibodies were then studied in a standard overnight HIV-1 EIB. In two (nr A and H) of the six sera the anti-p24 activity was visibly diminished by absorption with the immobilized peptide which had reacted strongly in EIA. One of these two sera were reactive with and absorbed by peptide 262-338 and one with peptide 318-378. The anti-p24 EIB bands were not diminished after absorption with another, HIV or HTLV-derived, immobilized peptide or with mock-coated wells. The results with one serum are shown in Figure 2. Thus, the argument for a localization of the antibody reactivity to these two portions of p24 was strengthened in two of six peptide-reactive p24 ± p55 positive sera subjected to peptide absorption.

Inhibition of reactivity in commercial EIAs by long synthetic peptides in solution

As the absorption experiments yielded conclusive results with only two sera, we tried to inhibit the weak reactions in the commercial whole virus lysate and recombinant protein EIAs. After the extensive testing, only three sera (C, D, E; Table 2) which reacted with the N-terminal peptide HIV-1 gag 133-193 and one serum reactive with the C-terminal peptide HIV-1 gag 318-378 (F) remained. The reactions of the three in the Organon whole virus lysate EIA were completely inhibited at 250 µg/ml and almost completely at 100 µg/ml of 133-193, but not by the control peptide HIV-1 gag 262-338 at the same concentrations (Fig. 3). Similarly, the reaction of the single remaining C-terminally reactive serum (F) in the Abbott recombinant EIA was completely inhibited by the peptide HIV-1 gag 318-378 in unoxidized form at 100 µg/ml (not shown).

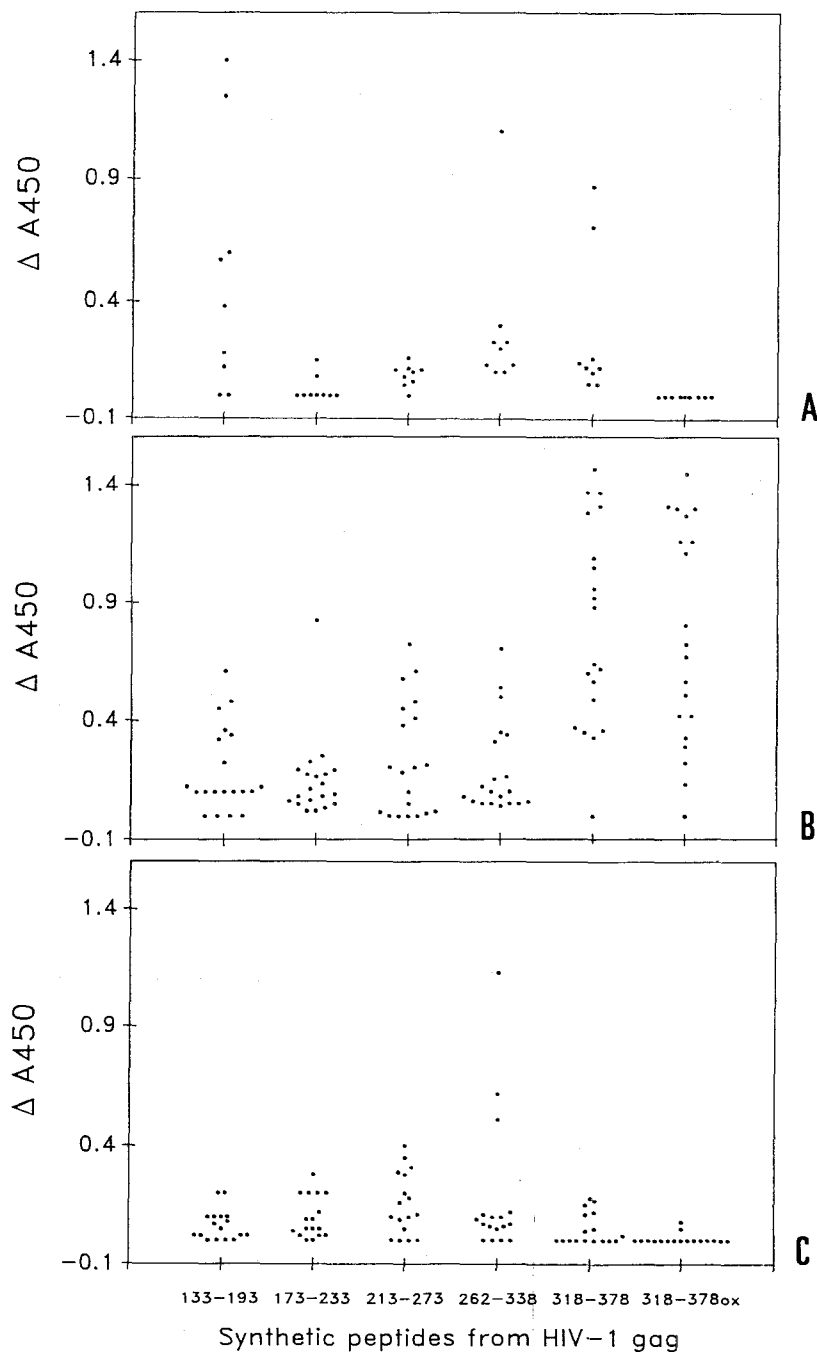


FIG. 1. Distribution of absorbance differences in EIA with synthetic peptides derived from HIV-1 *gag* of (a) the nine p24 \pm p55-reactive sera, (b) 20 confirmed HIV-1 antibody-positive sera, and (c) 17 HIV seronegative blood donors.

Amino acid sequence conservation in the three regions which reacted with p24 \pm p55 reactive sera.
Cross-reactivity with other primate retroviruses

The regions of HIV-1 p24 which reacted with the false positive sera contain evolutionarily conserved sequences (Fig. 5). These are potential sources of cross-reactions with other retroviruses which could be the reason for the p24 antibody

bands seen in EIBs with an indeterminate result. Eight of the nine p24-reactive sera were also analyzed in EIB with SSAV, which is relatively closely related to murine leukemia virus, and nine of nine with MPMV (a type D virus) virus antigens (Fig. 5). A weak band in the 30 kD region band in SSAV EIB was seen with five and a relatively strong band with one of nine sera in MPMV EIB. A weak band of the same molecular weight was seen in MPMV EIB with most human sera tested. Weak SSAV

TABLE 3. FREQUENCY OF REACTIVITY WITH LONG SYNTHETIC PEPTIDES DERIVED FROM HIV-1 p24

Peptide HIV gag	Group			Probability (Fisher Exact Test)		
	<i>p24 ± p55</i> Positive (FP)	Confirmed HIV-ab Positive (TP)	Seronegative Blood Donors (BD)	FP/TP	FP/BD	TP/BD
133-193	4/9	3/20	0/17	0.10	0.008	NS
173-233	0/9	1/20	0/17	NS	NS	NS
213-273	0/9	5/20	1/17	0.13	NS	0.13
262-338	1/9	3/20	3/17	NS	NS	NS
318-378	2/9	15/20	0/17	0.01	0.11	0.000001
318-378 ox	0/9	15/20	0/17	0.0002	NS	0.000001

and strong MPMV p30 bands were not observed in 10 blood donor control sera (three of which are represented in Fig. 4). Nine p17⁺/-p55 reactive sera were negative in SSAV EIB and gave a weak p30 band with MPMV. The strongly MPMV-reactive serum and three of the weakly SSAV reactive sera reacted with the long N-terminal peptide 133-193. Of the remaining two weakly SSAV reactive sera one bound to peptide 262-338 and one to none of the peptides.

DISCUSSION

Localization of anti-p24 activity in HIV-1 p24-positive sera of indeterminate specificity. Comparison with confirmed HIV-1 antibody-positive sera

Long peptides from three regions of p24 displayed binding activity with seven of nine sera with the p24 ± p55 reactivity pattern. The majority reacted with the N-terminal long peptide 133-193. A statistically significant higher frequency than in blood donor controls was notable with it. In contrast, true

positive sera reacted much less with this peptide but much more strongly with the C-terminal 318-378. This peptide overlaps the p2-p7 portion of the most C-terminal gag protein p15. It is not known to what extent its serological reactivity is dependent on this short non-p24 derived portion. The two 318-378 reactive false positive sera reacted relatively less with the oxidized form of the peptide than the confirmed HIV-1⁺ sera did.

We could diminish the EIB p24 reactivity in two p24 ± p55-positive sera reactivity by absorption with an EIA-reactive synthetic peptide. Probably, the amount or mode of presentation of the peptide antigen was not favorable for absorption with the other sera. However, in competition experiments we showed conclusively that the reactions in commercial HIV-1 EIAs noted with three N- and one C-terminally reactive serum were due to antibodies directed against the sequences 133-193 or 318-378 of HIV-1 gag, respectively. Thus, the localization of anti-HIV-1 p24 reactivity to only the portion of p24 included in the respective long peptide was ascertained in six of nine sera (Table 2).

The shorter peptides were not reactive with the false positive

TABLE 4. FREQUENCY OF REACTIVITY (ABSORBANCE DIFFERENCE > 0.4) WITH SYNTHETIC PEPTIDES DERIVED FROM THE HIV p24 SEQUENCE

	Frequency with Swedish p24 ± p55- Reactive Sera (N = 9)	Frequency with Swedish Confirmed HIV-1-Positive Sera (N = 20)	Frequency with Swedish Seronegative Blood Donor Sera (N = 17)
HIV-1 hxb2 gag 133-193	4	3	0
HIV-1 hxb2 gag 143-156	0	2	0
HIV-1 hxb2 gag 173-233	0	1	0
HIV-1 hxb2 gag 203-218	0	1	0
HIV-1 hxb2 gag 210-230	0	0	0
HIV-1 hxb2 gag 213-273	0	6	1
HIV-1 hxb2 gag 223-243	0	0	0
HIV-1 hxb2 gag 260-276	0	3	0
HIV-1 hxb2 gag 262-338	1	3	3
HIV-1 hxb2 gag 284-300	0	0	0
HIV-1 hxb2 gag 318-378	2	15	0
same, in oxidized form	0	15	0
HIV-1 hxb2 gag 335-351	0	5	0
HIV-1 hxb2 gag 348-373	0	0	0
HIV-1 hxb2 gag 359-382	0	0	0

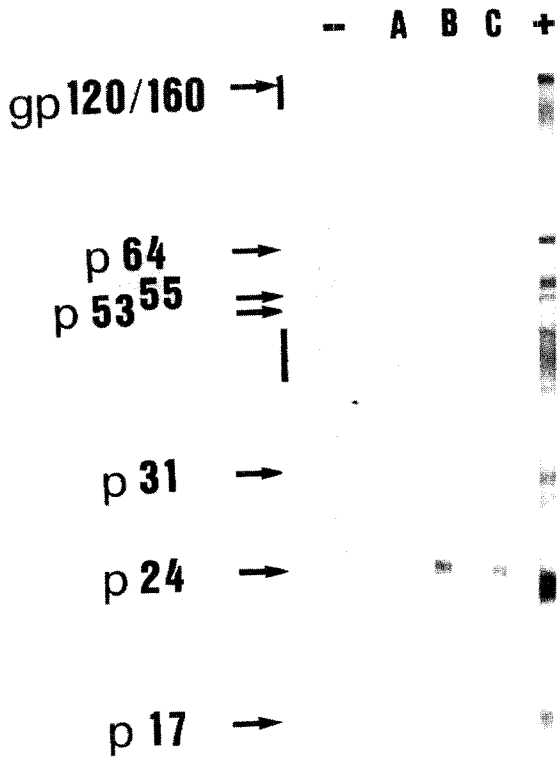


FIG. 2. Electrophoretic immunoblotting with HIV-1 antigen of one p24 ± p55 EIB and HIV-1 gag 318-378 reactive human serum (number 1, Table 3) before and after threefold absorption "in-well" with solid-phase-bound HIV-1 gag 318-378 (A), HTLV-1 gag 111-130 (B), or wells mock-coated with PBS (C). "+", Confirmed HIV antibody-positive control. "-", HIV antibody-negative serum control. Arrows indicate positions of HIV-1 protein.

sera. Thus some of the epitopes involved in p24 ± p55 reactions may be discontinuous. The reactions with peptide 318-378 were both abolished after oxidation of this peptide. This should create a loop involving cysteines 330 and 350 which is likely to affect both conformation and antigenicity of the evolutionarily conserved QNANPDCKTILKALG sequence (cf., Fig. 5). In contrast, confirmed Swedish HIV-1 antibody-positive sera on average changed their degree of reactivity only slightly. This is another indication that the epitopes recognized on p24 by false positive sera differ from those recognized by sera from HIV-1-infected persons.

Frequency of false-positive p24 reactions in HIV-1 EIB. Relation to results in screening tests

In our laboratory, about a half of the indeterminate HIV-1 serological reactions are of the p24 ± p55 type (unpublished observations). Two combinations of screening, EIB and long p24 peptide results were evident: (A) Five of nine sera were

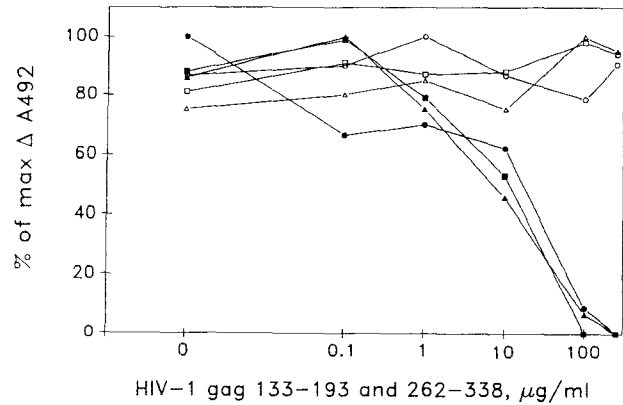


FIG. 3. Competition of synthetic peptide in solution with immobilized whole virus lysate antigen for binding to antibodies in three p24 ± p55 antibody-positive sera. Sera (Serum C, Circles, D, triangles, and E, squares) were preincubated with either HIV-1 gag 133-193 (filled symbols) or HIV-1 gag 262-338 (open symbols) at increasing concentrations, and then subjected to the Organon Vironostica EIA. X-axis: Log of peptide concentration, Y-axis: Percent of the average maximum difference of absorbance at 492 nm obtained with serum and the cut-off value (0.234) calculated according to the manufacturer. In one case, a difference of -0.012 was shown as 0%.

PATTERN OF REACTIVITY WITH LONG p24 PEPTIDES

PATTERN OF REACTIVITY WITH LONG p24 PEPTIDES						SERA	
133	173	213	262	318	318 _{ox}	No	GROUP
+	-	-	-	-	-	4	FP (9)
+	-	-	+	+	-	2	
+	-	-	+	+	-	1	
+	-	-	+	+	-	2	
+	-	-	+	+	-	4	TP (20)
+	-	-	+	+	-	3	
+	-	-	+	+	-	3	
+	-	-	+	+	-	2	
+	-	-	+	+	-	1	
+	-	-	+	+	-	1	
+	-	-	+	+	-	1	
+	-	-	+	+	-	1	
+	-	-	+	+	-	1	
+	-	-	+	+	-	2	
+	-	-	+	+	-	3	BD (17)
+	-	-	+	+	-	1	
+	-	-	+	+	-	13	

FIG. 4. Patterns of reactivity with the six long synthetic peptides derived from HIV-1 p24. The hatched areas show an absorbance differential greater than 0.4. The number of sera which displayed a certain reactivity pattern is shown under "No." FP, p24 ± p55 reactive (false-positive) sera. TP, confirmed HIV-1 antibody-positive sera. BD, HIV-1 seronegative blood donor sera. Peptide number codes are evident from Table 1.

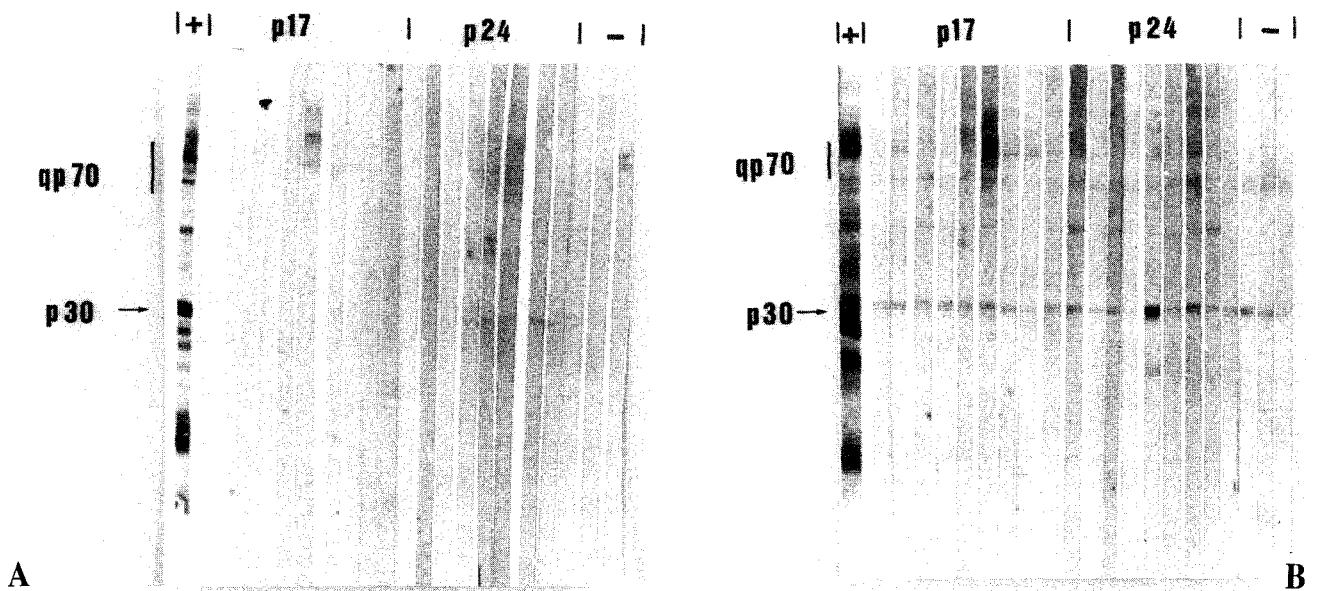


FIG. 5. Electrophoretic immunoblots of p24 \pm p55-reactive sera with primate retrovirus antigens. (A) EIB with simian sarcoma associated virus (SSAV). (B) EIB with Mason-Pfizer monkey retrovirus (MPMV). Numbers denote the position of the 30 kD molecular weight marker and the area around to be +, Positive control (goat hyperimmune serum). p17 = EIB with nine p17 \pm -p55-reactive human sera. p24 = EIB with p24 \pm p55-reactive human sera. p24 = EIB with p24 \pm p55-reactive sera. -, Sera from three HIV-1 seronegative blood donors.

weakly positive in a whole virus HIV antibody EIA, reacted with a peptide either from the N-terminus or in the middle of p24, but were negative in an HIV EIA with recombinant p24. (B) Two of nine sera did not react in a whole virus EIA, but did react with a C-terminal p24 peptide and in an EIA with recombinant p24 antigen. Thus, the selection of screening test influences the type of p24 false positive reaction found.

The five whole virus-reactive sera were derived from a base of approximately 75,000 screening tests, giving a frequency of around 1/10,000 strongly p24 EIB positive found by the whole virus antibody test. Of the remaining 75,000, around 50,000 were performed with the recombinant HIV-1 antibody EIA and 25,000 with other antibody tests (competition and peptide EIAs). As the latter tests would not be expected to pick up most of the false positive p24 EIB antibody reactions, the two sera which reacted only in the recombinant HIV-1 EIA also roughly correspond to a frequency of 1/10,000.

Structural predisposition for antigenic cross-reactivity in major gag proteins. Comparison of the three types of false anti-HIV-1 p24 reactivities with what is known regarding cross-reactivity on gag within retroviridae

Gag means group specific antigen.^{17,18} Much of the serological cross-reactivity in *retroviridae* appears to take place on the major gag (capsid) protein,¹⁹ which has a molecular weight of between 24 and 30 kD. Versteegen et al.²⁰ found that interspecies reactivity resided mainly in conformational epitopes at the N-terminus of the major gag protein of several retroviruses, whereas group-specific antigenic determinants were localized at its C-terminus. As demonstrated in Figure 6, the three regions of p24 which bound antibodies from most of the indeterminate

p24-reactive sera contain several stretches of moderate to high evolutionary conservation, cross-reactions with which could give rise to the p24 bands in EIB. Several of the peptide-reactive sera also reacted somewhat with the major gag proteins of other retroviruses (SSAV: five weak; and MPMV: one relatively strong). This supports the theory that some of the anti-p24 reactions have a rather broad specificity range.

Biological and medical interpretation of the phenomenon of false HIV-1 antibody positivity

Cross-reactions with nonretroviral antigens which could be caused by molecular mimicry (*cf.*, Ref. 21), inadvertent immunization with alimentary animal retroviruses such as bovine immunodeficiency virus,⁷ or putative new human retroviruses could be the basis of the described p24 \pm p55 reactions. If the latter is the case, such virus(es) should be relatively apathogenic. Judging from the reasons for HIV antibody testing (blood donation, pregnancy), the persons who donated the indeterminate HIV-1⁺ sera probably were healthy. This is in accord with the observations of others.²² The phenomenon of false positive HIV p24 antibodies is not confined to humans. They also occur in rabbits and goats.¹⁰ The responsible antigen(s) thus may be rather widespread.

Advantages of using long synthetic peptides for retrovirus serology

We found it crucial to use long (61–77 amino acids) peptides to detect anti-p24 activity in most of the false and true HIV-1 antibody-positive sera. Although the coupling efficiency for each amino acid was high, the accumulated risk for miscoupling

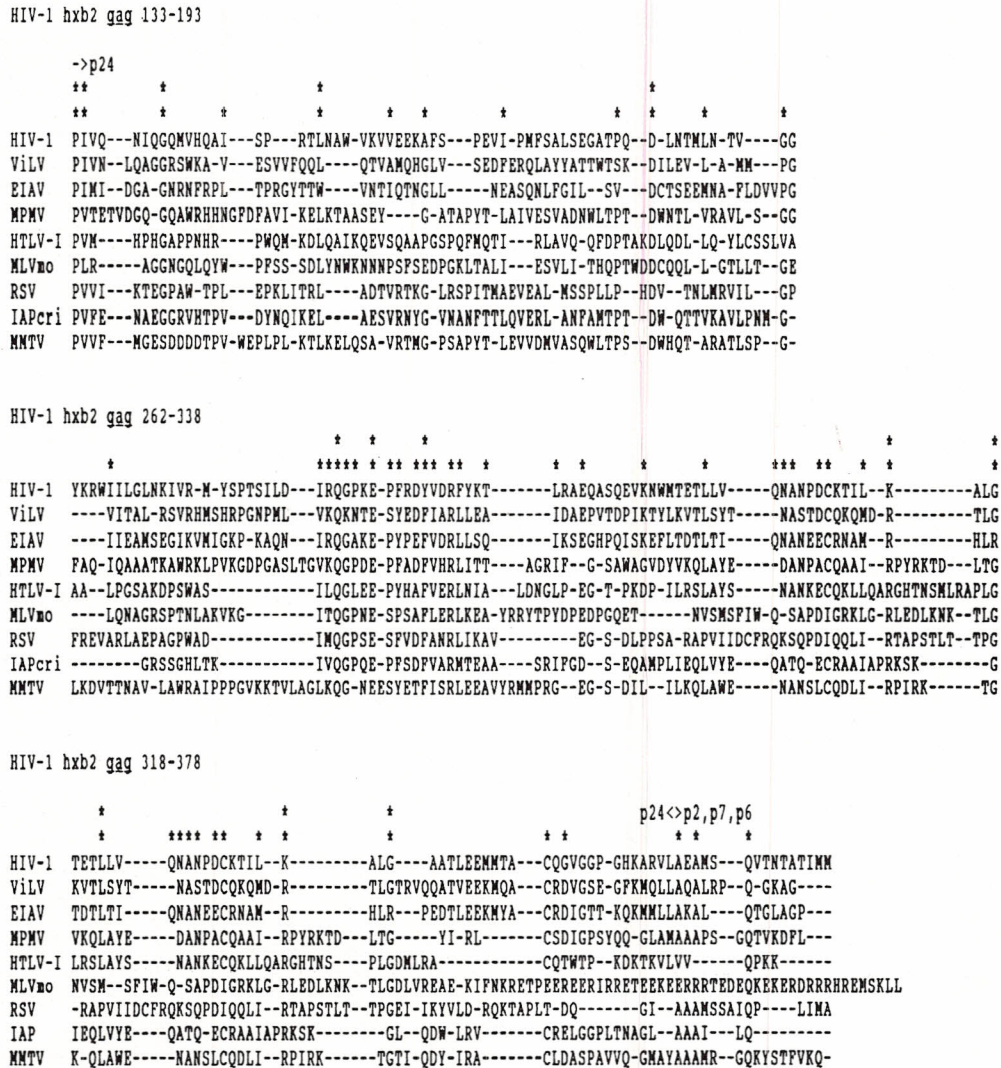


FIG. 6. Alignment of the three regions of p24 which were reactive in seven of nine sera with false positive p24 EIB antibody activity. Positions which have the same amino acid in more than 90% of the nine retroviral species examined are denoted by two asterisks. Positions which have the same or a similar amino acid in more than 50% of species are denoted by one asterisk.

becomes quite high in a long synthetic peptide. However, the degree of reactivity with unselected HIV-antibody-negative blood donor sera was low. This can be due to a strong tendency to form a certain tertiary structure by these peptides irrespective of minor errors in the sequence. The high frequencies of reactivity in the confirmed HIV-1 antibody-positive sera (e.g., with peptide 318-378: 15 of 20) is to our knowledge the highest reported for gag-derived synthetic peptides.

Principles and utility of a molecular dissection of HIV antibody specificities with a battery of synthetic peptides

A single anti-p24 band can be a sign of an early HIV-1 infection (cf., Refs. 13,14), be due to an HIV-2 infection (cf.,

Refs. 13,14), be the consequence of an abortive anti-HIV serological reaction,²³ or be a false positive reaction in a person who never encountered HIV. We have demonstrated that many confirmed HIV antibody-positive sera react with several p24-derived peptides, that the false positive sera studied reacted with only one of these peptides, and in the cases of HIV-1 gag 133-193 and, due to the differences between reactivities with unoxidized and oxidized peptide, HIV-1 gag 318-378, that the false positive sera react with epitopes infrequently recognized by true positive sera. Anti-p24 reactions are a major diagnostic problem in both developed and developing^{2,24} nations. It is costly to run several confirmatory tests on each of these sera. "Peptide reactivity spectra" with the long peptides used in the present study could provide valuable supplemental diagnostic information.

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