

# Carrier Bound Synthetic Oligopeptides in ELISA Test Systems for Distinction Between HIV-1 and HIV-2 Infection

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**Summary:** A series of synthetic carrier bound oligopeptides derived from corresponding regions of the core and envelope proteins of HIV-1 and HIV-2 were used in enzyme-linked immunoabsorbent assays (ELISA) for serodiagnosis of HIV-1 and HIV-2 infected individuals. The combination of peptides from regions either conserved or highly variable between the two virus types allowed the identification of HIV infection in general and the differentiation between HIV-1 and HIV-2. No specific reaction was found in seronegative individuals. The use of peptides bound to the same polystyrene carrier as in peptide synthesis allowed the establishment of a highly specific and sensitive test system without the risk of unspecific cross-reaction due to contamination with bacterial or cellular protein material. **Key Words:** Synthetic peptides—HIV-1/HIV-2 distinction—ELISA tests.

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Infections with human immunodeficiency virus type 1 (HIV-1), the retrovirus first shown to cause the acquired immune deficiency syndrome (AIDS) (1,2), are now known as an epidemic of universal dimensions with the main distribution in central Africa (3). Another pathogenic human retrovirus, HIV type 2, associated with a clinically indistinguishable immune deficiency syndrome, has been isolated from West African AIDS patients (4); also, HIV-2 infections show spreading to other parts inside and outside of Africa.

The serodiagnosis of both infections is mainly done with viral antigen preparations or isolated structural proteins produced by recombinant DNA technology (5-7). To avoid false-positive results in ELISA assays due to contamination with cellular or

bacterial cross-reacting proteins, confirmation by Western blot or radioimmunoprecipitation experiments is necessary (8,9). In most cases, these tests do not allow distinction between HIV-1 and HIV-2 infections. The use of synthetic oligopeptide preparations, which are free of contaminating proteins, has been shown to be a tool for specific and sensitive ELISA test systems (10,11). Especially, the diagnosis of HIV-1 and HIV-2, which both show a high degree of amino acid variation in the antigenic epitopes of the individual isolates mostly located in the envelope protein complex (12-15), but also present to a limited degree in the sequences of the core proteins, makes the application of a set of oligopeptides necessary in order to minimize false-negative results. In addition, it has to be anticipated that due to the different genetic basis of the immune system of each individual, antibodies in sera derived from different persons may not be directed to the same epitope and may differ in quality and quantity.

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Therefore, we synthesized a set of corresponding peptides from either conserved or variable regions of the core and envelope proteins of HIV-1 and HIV-2. We selected a constant region of the large core proteins p24/p26, two epitopes of the transmembrane proteins (gp41/gp36) each containing about 50% identical or similar residues and a highly different sequence region of p17/p16. By that combination of peptides, we were able to diagnose HIV infections in general and to distinguish between HIV-1 and HIV-2 infections.

By the use of the same polystyrene carrier in peptide synthesis and the antibody test (16), a specific, sensitive, and fast ELISA test could be established, which also readily allows the inclusion of additional epitopes if new HIV types are discovered.

## MATERIAL AND METHODS

### Selection of Peptide Sequences

Antigenic amino acid sequences were selected using a computer program written for a Digital Equipment VAX 750 as described previously (14,17). Highly glycosylated amino acid regions of gp41/gp36 were not used as an antigen for peptide synthesis since carbohydrate modification may result in an highly changed structure, leading to the formation of discontinuous epitopes that cannot be simulated by synthetic oligopeptides. The selected peptides had a length of 12–18 amino acid residues; the sequence and location on the proteins are shown in Table 1.

### Peptide Synthesis

Peptide synthesis was done in an 430A peptide synthesizer (Applied Biosystems, Weiterstadt, F.R.G.) using Fmoc (9-fluorenylmethyloxycarbonyl)-protected amino acids (18) and polystyrene resin with the first residue coupled in ester linkage

(16). In order to complete the coupling reactions, double-couple cycles were used for each amino acid. *t*-Butyl-(Ser,Asp), *t*-butoxycarbonyl-(Lys), and 4-methoxy-2,3,6-trimethylbenzenesulfonyl-(Arg) groups were used for side-chain protection. Fmoc-protected amino acids were converted to hydroxybenzotriazol-activated esters by treatment with 1.5 mmol of hydroxybenzotriazol and 1.2 mmol of diisopropylcarbodiimide per mmol of amino acid for 30 min in dimethylformamide; the subsequent coupling reaction was performed in dimethylformamide. After the coupling reaction, Fmoc groups were removed with 20% piperidine in dimethylformamide followed by a series of washes with dimethylformamide. (All amino acids were purchased from Bachem AG, Bubendorf, Switzerland; chemicals from Aldrich, Steinheim, F.R.G.; and solvents from Merck AG, Darmstadt, F.R.G.) After synthesis, side-chain-protecting groups were removed in trifluoroacetic acid (TFA) with 10% thioanisole and 10% *m*-cresol as scavengers. The resin-bound peptides were washed in series with an excess of dichloromethane, ethyldiisopropylamide, and dichloromethane and dried. For confirmation of the amino acid sequence, the carrier-bound peptides were sequenced using an Applied Biosystems (Weiterstadt, F.R.G.) gas phase sequencer.

### ELISA Tests

The ELISA tests were conducted in a Millipore filtration system using 96-well plates sealed on the bottom with hydrophilic membrane (GV, pore diameter of 0.2  $\mu$ m), which has a very low protein binding capacity (Millitersystem, Millipore, Eschborn, F.R.G.). The resin-bound peptides were suspended in PBS (phosphate-buffered saline) containing 0.1% Tween 20. The beads were washed four times with 100  $\mu$ l of PBS/0.1% Tween 20 and incubated 1 h in a solution of 5 mg of gelatin in H<sub>2</sub>O and washed again. HIV-1- and HIV-2-positive and

TABLE 1. Sequences of the synthesized peptides; identical residues in corresponding peptides are indicated

gp41-A: AA 562-575	HIV-1 Arg-Ala-Ile-Glu-Ala-Gln-Gln-His-Leu-Leu-Gln-Leu-Thr-Val-Ala
gp36-A: AA 556-569	HIV-2 Asp-Val-Val-Lys-Arg-Gln-Gln-Glu-Leu-Leu-Arg-Leu-Thr-Val-Ala
gp41-B: AA 588-604	HIV-1 Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-Cys-Ser-Ala
gp36-B: AA 582-598	HIV-2 Ile-Glu-Lys-Tyr-Leu-Gln-Asp-Gln-Ala-Arg-Leu-Asn-Ser-Trp-Gly-Cys-Ala
p24: AA 226-237	HIV-1 Gly-Gln-Met-Arg-Glu-Pro-Arg-Gly-Ser-Asp-Ile-Ala
p26: AA 228-242	HIV-2 Gln-Met-Arg-Glu-Pro-Arg-Gly-Ser-Asp-Ile-Ala-Gly-Thr-Thr-Ser-Ala
p17: AA 109-123	HIV-1 Asn-Lys-Ser-Lys-Lys-Lys-Ala-Gln-Gln-Ala-Ala-Ala-Asp-Thr-Gly-Ala
p16: AA 111-126	HIV-2 Glu-Thr-Gly-Thr-Ala-Glu-Lys-Met-Pro-Ser-Thr-Ser-Arg-Pro-Thr-Ala

-negative human sera were diluted in PBS/0.1% Tween 20/1% rabbit serum. One hundred microliters of each dilution was added and incubated with the resin-bound peptide for 1 h. The beads were washed four times with PBS/0.1% Tween 20, incubated with rabbit anti-human IgG (Dako, Hamburg, F.R.G., diluted 1:1,000 in PBS/0.1% Tween 20) for one additional hour followed by four washes in PBS/0.1% Tween 20. For staining, 100  $\mu$ l of sodium phosphate buffer at pH 6.0 containing 0.5 mg/ml of *o*-phenylenediamine and 0.1% H<sub>2</sub>O<sub>2</sub> were added for 2 min; the solution was then transferred to a 96-well ELISA plate (Dynatech), each well containing 100  $\mu$ l of 1M H<sub>2</sub>SO<sub>4</sub> to stop the staining reaction. The optical density was determined at 486 nm.

### Sera and Routine Diagnostics

HIV-1 sera were obtained from German patients in 1987 and negative sera were collected from German students in 1980. Almost all HIV-2 sera were obtained from West Africans or from their respective sexual partners. Serum I (19) and VI are from German women who were the sexual partners of a Senegalese man. Virus has been isolated and characterized from patient I (20). Serum II was collected from a German homosexual man who had no known African contacts; serum V was derived from his partner. Serum II was obtained from a German woman infected by a Ghanaese man, serum IV is derived from a German woman infected in West Africa, and serum VII from a German man infected in Mali by medical treatment with injections (21). Diagnostics were conducted using HIV-1 ELISA tests (Abbott Laboratories) and HIV-2 ELISA tests using viral proteins of LAV-ROD-2 (15) as antigen. HIV-2 infection was assumed when the reaction was negative or weak on HIV-1 ELISA tests, but showed an extinction of  $>1.0$  with HIV-2 antigen. Immunofluorescence was done by titrating sera using HIV-1- and HIV-2-infected H9 cells as antigen. HIV-2 infection was indicated by elevated titers on HIV-2 antigen. Competitive Western blots (19,21) were carried out using HIV-1 and HIV-2 antigen grown in H9 cells, separated on polyacrylamide gels and transferred to nitrocellulose. Sera were tested in parallel on HIV-1 and HIV-2 antigen strips in a dilution of 1:400; washing was done in Tris-buffered saline containing 0.1% Tween 20. HIV-2 infection was diagnosed by specific recognition of p16, gp36, p57, p68, and gp130 on HIV-2 antigens. On HIV-1 blots, only the cross-reacting bands of the *gag* com-

plex (p24 and p55) were detected. Final diagnosis was achieved by combining the results of the three independent test systems.

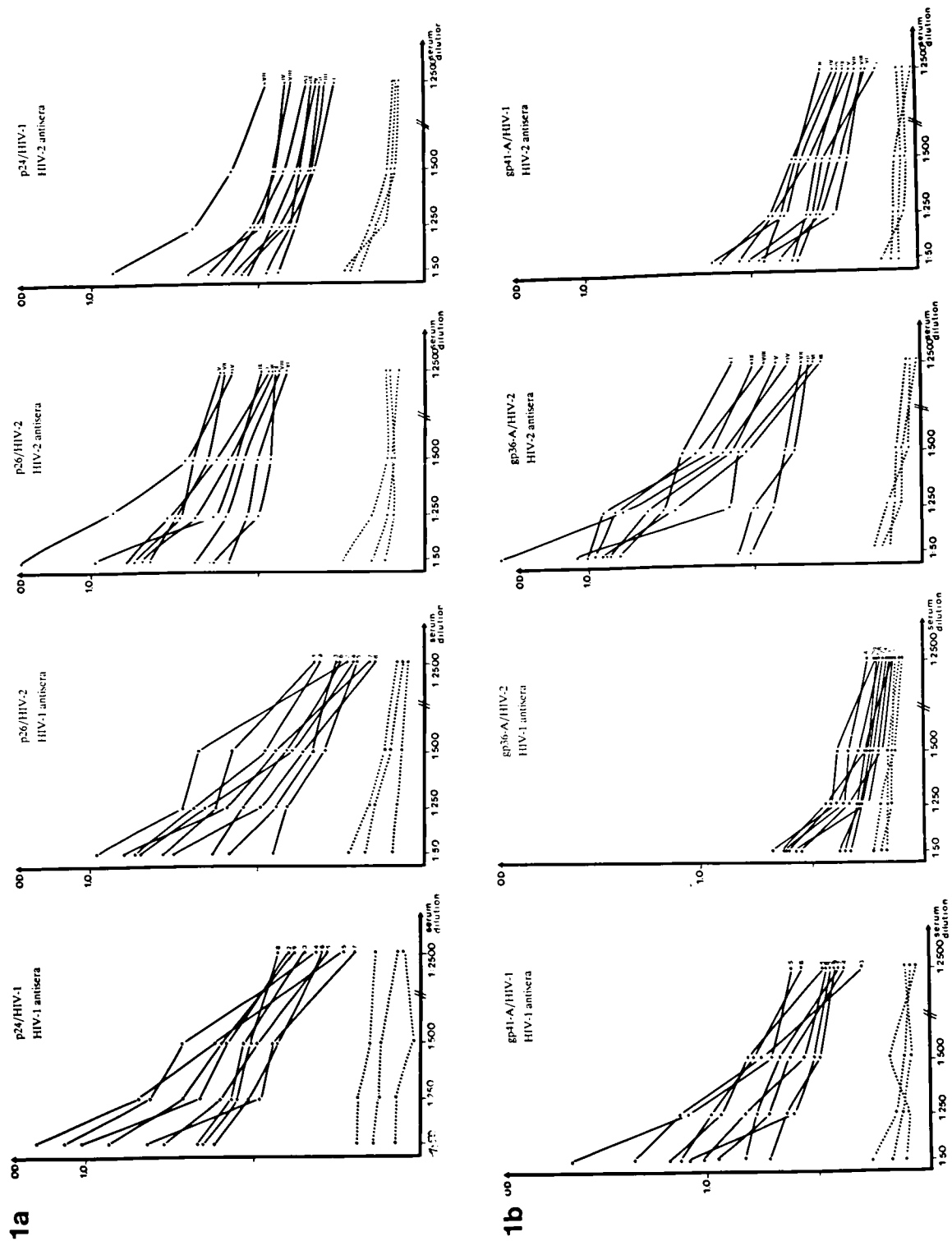
## RESULTS

### Epitope Selection and Peptide Synthesis

The synthesized epitopes were selected from the amino acid sequences of HIV-1 [strain HTLV-III/BH-10 (22)] and HIV-2 (23) according to their potential antigenicity using a computer program for amino acid analysis, which combines parameters for secondary structure with values for local hydrophilicity, flexibility, and surface probability (17); in the transmembrane proteins gp41/gp36, regions were selected that are located in the protein part, which is probably positioned outside the membrane (14), and that contain none of the four potential N-glycosylation sites. From this external loop region, two corresponding antigenic sites were selected, which contain the lowest possible content of similar amino acid residues in combination with potential good antigenicity. About 50% of the amino acids were equal, and for both peptides a certain degree of cross-reactivity was expected, also because a remarkable degree of amino acid variation in individual isolates of HIV-1 has been found, which should be expected for HIV-2 in a similar way.

In the core protein p24/p26, the antigenic epitope is highly conserved in HIV-1 and HIV-2. These sites were synthesized as a conserved sequence region in order to identify HIV-1- and HIV-2-specific antibodies and thus should allow the diagnosis of an HIV infection in general. The most variable epitope between HIV-1 and HIV-2 was selected from an antigenic region located near the carboxy terminus of p17/p16. A similar high degree of variability is only found in the external glycoproteins (gp120) of both virus strains; due to the high amount of carbohydrate modification, antibodies to these proteins, however, are mainly directed to structural or discontinuous epitopes and synthetic peptides may not be reactive with patient antibodies. The sequence of all peptides synthesized is shown in Table 1.

The peptides were synthesized in a way that allowed the use of the same polystyrene carrier during peptide synthesis and immunoabsorbent assay (16). The first amino acid residue of each peptide was covalently bound via an acid- and base-stable ester linkage to the resin. Amino acid sequencing of the peptides showed the material to be highly homo-



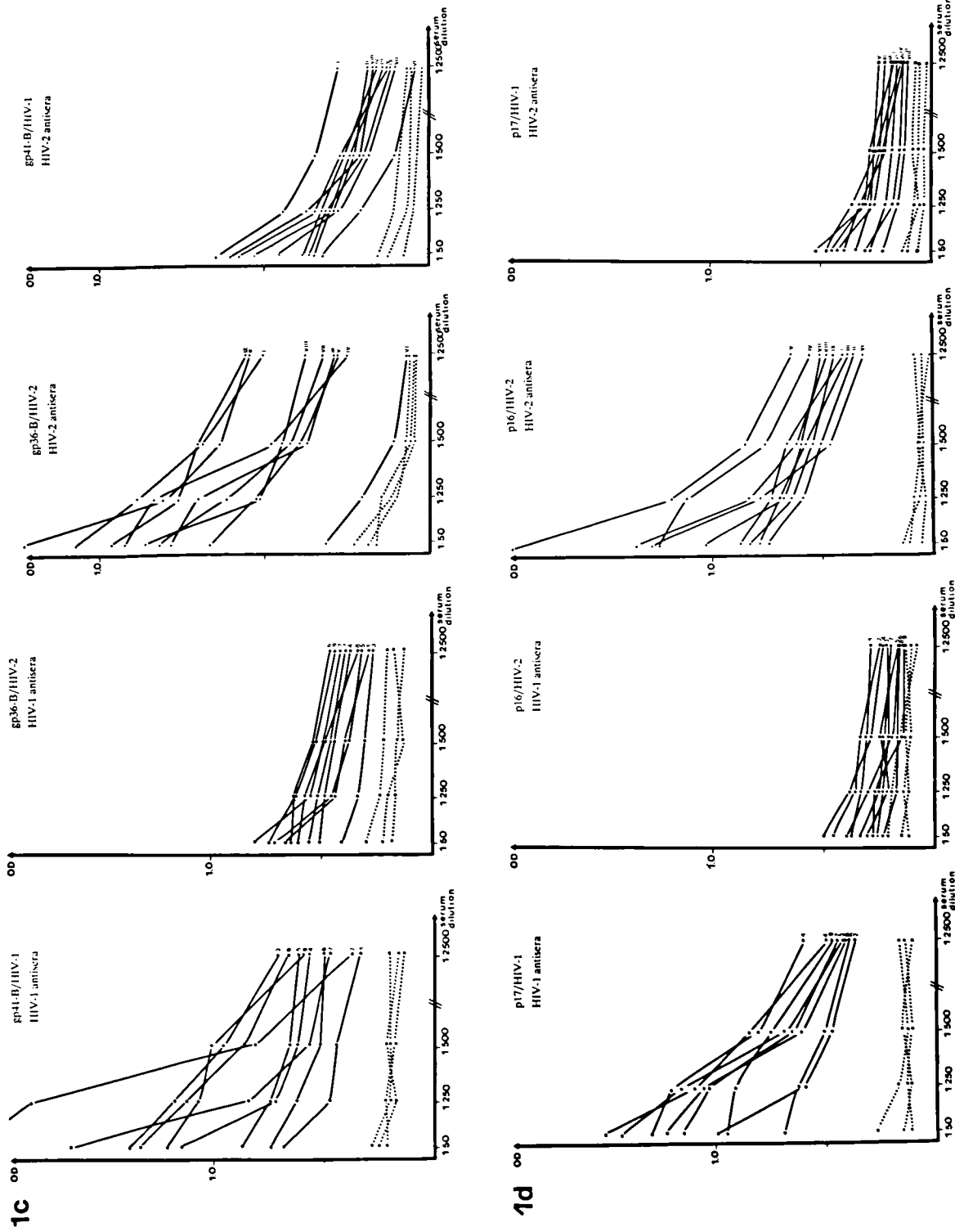


FIG. 1. Reaction of HIV-1-positive (1-9) and HIV-2-specific peptide (1-IX) sera on HIV-1- and HIV-2-specific peptide antigens. Reaction of negative sera is shown by hatched lines. (a) Reaction with p24/p26 derived peptide antigens. (b) Reaction with gp41A/gp36A peptide antigens. (c) Reaction with gp41B/gp36B peptide antigens. (d) Reaction with p17/p16-derived peptide antigens.

genic, which is probably due to the relatively mild cleavage conditions for permanent side-chain-protection groups and to the use of double-couple cycles during synthesis. Those beads have been shown to be highly substituted with the specific peptide in an optimal antigen presentation, which allows the reduction of peptide antigen used per test up to 5 ng of peptide. In contrast, free purified peptides have to be recoupled to plastic support at high pH values, a procedure that often results in artificial antigenic structures, which are the basis for nonspecific reactions. The peptides here are covalently coupled via their carboxy terminal ends and thus allow optimal presentation of the antigen and the use of defined equal amounts of peptide, since influences in antigen quantity by different peptide adsorption properties to polystyrene are avoided.

### ELISA Tests

The ELISA tests were performed in Millilitre plates (Millipore, Eschborn, F.R.G.), sealed at the bottom with a membrane with low protein binding capacity, using 500 ng of carrier-bound peptide as antigen, which represents about 200 ng of peptide antigen. Sera of HIV-1- and HIV-2-positive and -negative individuals were tested in dilutions up to 1:2,500. All tests were done using HIV-1- and HIV-2-specific peptide antigens in parallel experiments. All sera had been characterized for the presence of HIV-1- and HIV-2-specific antibodies in routinely used ELISA, immunoblot, and immunofluorescence tests.

Using p24/p26 derived peptides, all HIV-1- and HIV-2-positive sera showed a high degree of cross-reactivity. All sera showed positive reactions when tested on HIV-1 and HIV-2 peptides; on the corresponding peptide antigens (HIV-1-positive sera tested on HIV-1-specific peptides), the reaction was slightly elevated with respect to the values obtained, when HIV-1-positive sera were tested on HIV-2-specific antigen and vice versa (Fig. 1a). Positive reaction was assumed when values for optical density exceeded 2.1 times the values of negative sera in the respective dilution. Based on these criteria, all sera showed positive reaction in a dilution of 1:500, and could be partly diluted up to 1:2,500. All HIV-2 sera were shown to be reactive with p24 on HIV-1-specific Western blots; both ELISA tests and immunoblots showed the same amount of cross-reaction (Table 2).

Using gp41/gp36 peptides as antigen, sera showed an elevated positive reaction, when tested on the strain-specific corresponding antigens (HIV-1 sera to HIV-1 peptides and HIV-2 sera to HIV-2 peptides). However, some degree of cross-reactivity was observed when HIV-1 sera were used with HIV-2 antigens and vice versa (Fig. 1b,c); this is due to the content of similar or equal amino acid residues in the peptides in combination with isolate-specific sequence variation, which has to be anticipated in different individuals. The reaction of the individual sera on both gp41/gp36 peptide antigens A and B was not equal. One HIV-2 serum did not react at all on gp36/B; the reactivity against peptide gp36/A, however, was rather good (Fig. 1b,c). From our tests, we cannot decide whether the non-reactivity to gp36/B is due to the genetic constellation in the immune reaction of this patient, or to the fact that the HIV-2 isolate that led to infection has some amino acid sequence variation in this epitope.

The cross-reaction of the HIV-2 sera to HIV-1 antigens on immunoblots was compared with the values for optical density obtained in the ELISA tests using HIV-1- and HIV-2-specific peptide antigens (Table 2). For this comparison, values obtained in a dilution of 1:250 are shown, since those reflect specific cross-reactivity. The highest cross-reaction in both gp41A and B peptides was seen in serum IV, which also showed unspecific reaction on immunoblots resulting in multiple bands. Serum II showed cross-reactivity in gp41A; titers, however, were very low, so that a similar reaction with gp41 on HIV-1 blots was not observed. Serum VII showed cross-reactivity on Western blots and relatively high optical density values in ELISA tests with both gp41/HIV-1 peptides. In conclusion, cross-reactivity to the transmembrane part of both viral proteins (gp41 and gp36) may at least partly be based in the epitopes used in the test described here; in immunoblots, however, other epitopes may contribute to the amount of the observed reactions.

The best distinction between HIV-1 and HIV-2 infection was provided by the ELISA tests done with p17/p16 peptides. Reaction was almost only observed on the strain-specific peptides, especially when sera were used in dilutions of 1:250–1:500 (Fig. 1d). This is probably based on the highly different amino acid sequence in HIV-1 and HIV-2, localized near the carboxy terminal end of p17/p16. All sera had been screened for the presence of anti-p17/p16 antibodies on Western blots, since not all patients develop immune reaction to this protein

TABLE 2. Comparison of the extent of cross-reaction of HIV-2 positive sera obtained in peptide ELISA-tests in a dilution of 1:250 and on Western blots; ELISA test values are given by the optical density measured at 486 nm

Serum (HIV-2)	Peptide ELISA		Western blot		Peptide ELISA		Western blot		Peptide ELISA				Western blot	
	HIV-1 p17	HIV-2 p16	HIV-1 p17	HIV-2 p16	HIV-1 p24	HIV-2 p26	HIV-1 p24	HIV-2 p26	HIV-1 gp41A	HIV-2 gp36A	HIV-1 gp41B	HIV-2 gp36B	HIV-1 gp41	HIV-2 gp36
I	0.25	0.8	-	+	0.45	0.75	+	+	0.25	0.8	0.45	0.9	-	+
II	0.15	0.7	-	+	0.45	0.5	+	+	0.45	0.55	0.3	0.8	-	+
III	0.25	0.8	-	+	0.45	0.7	?	+	0.4	0.8	0.3	0.8	?	+
IV	0.2	1.2	-	(+)	0.5	0.9	+	+	0.55	0.9	0.40	0.8	+ <sup>a</sup>	+
V	0.2	1.3	-	+	0.5	0.7	?	+	0.4	0.8	0.3	0.6	?	+
VI	0.2	0.9	-	+	0.55	0.55	+	+	0.25	0.8	0.25	0.25	-	+
VII	0.25	0.8	-	+	0.75	0.8	+	+	0.25	0.65	0.35	0.65	(+)	+

?, highly unspecific reactions could not be correlated to HIV-1-specific protein bands.

<sup>a</sup> Multiple bands on HIV-1 Western blots.

during the course of infection. This is also the reason why these epitopes cannot solely be used for distinction between the two virus types.

## DISCUSSION

Synthetic peptides derived from the presumed externally exposed region of gp41 of HIV-1 have been shown to be an excellent tool for the diagnostic antigen in ELISA assays (10,11,24). The presence of HIV-2 infection in the same geographic areas as HIV-1, however, made it necessary not only to have a safe and quick test for the detection of virus-specific antibodies, but also for the distinction between the two HIV strains. Due to the high strain variability, especially in the region of potential antigenic epitopes, which has been shown for HIV-1 (12-14) and has to be anticipated also for HIV-2 (15) in combination with the genetically based differences in antibody production in individual patients—in quantity as well as in quality—we believe that determination of HIV-1- and HIV-2-specific antibodies and the distinction between these two viruses, which are very similar, both in amino acid sequence and function of their encoded proteins, have to be based on a diagnostic test system, which consists of a combination of antigenic sites of both virus types and allows the fast and easy determination of antibody titers.

Diagnosis of HIV infection, particularly in Europe, is often associated with the problem that the individual patients are infected with virus isolates originating in different parts of the world. Therefore, they exhibit a very high degree of sequence and antigen variation, resulting in a considerably elevated degree of cross-reaction between HIV-1

and HIV-2 proteins. This variation has been reported for HIV-1 isolates from patients in different regions of Africa (12) and contrasts with the relatively homogenous isolates found in the United States. Antigenic cross-reaction was also noticed by Gnann et al. (25) when African HIV-1 sera were used for testing the reaction to gp41/gp36 peptides generally useful in the distinction between HIV-1 and HIV-2 infection. Based on the large diversity among individual virus isolates, diagnosis of HIV-1 and HIV-2 infection in Europe by testing only one epitope seems highly questionable.

Carrier-bound synthetic peptides have been shown to be an excellent tool for a specific and quick HIV-1 ELISA test system (16). By selecting different epitopes, which are either conserved or variable between HIV-1 and HIV-2, and producing them as carrier-bound synthetic peptides, we have shown in this paper that it is possible to distinguish clearly between HIV-1- and HIV-2-specific antibodies by combining the values obtained from gp41/gp36 and p17/p16 epitopes. Due to the selected presentation of the peptide antigens, which are covalently bound to the polystyrene carrier via the carboxy terminal ends, and therefore avoiding coupling artefacts by unspecific adsorption to plastic support at high pH values, we could establish a very quick and specific ELISA test system for site-directed serology. The simultaneous testing for antibodies directed to the set of peptides derived from various proteins allows a clear and easy distinction, and also when the serum fails to react with one of the epitopes (see Fig. 1b,c).

In addition, this system allows the inclusion of further peptides and thus a very quick reaction to amino acid sequence variations of new virus isolates, which may be detected in the future.

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