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Use of Synthetic Oligopeptides in Identification and Characterization of Immunological Functions in the Amino Acid Sequence of the Envelope Protein of HIV-1

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Summary: Following computer-assisted analysis of the amino acid sequence of various HIV-1 isolates, we synthesized a series of oligopeptides derived from variable and conserved regions of the envelope protein complex gp120/gp41. The peptides were used in ELISA tests for their reactivity with human antisera from HIV-1 positive individuals; patients with clinically manifested AIDS showed only a rather limited reaction, predominantly with two peptides (p102-112, p316-326), which is in contrast to sera from HIV-1 positive asymptomatic individuals, whose sera were reactive with almost all peptides. Using consecutive sera of the same patients, decreasing antibody titers to defined epitopes could be shown to occur during the development of AIDS. Cellular immune response recognition was analyzed in T-cell proliferation assays by [^{3}H]thymidine incorporation. One peptide localized in a conserved region clearly induced proliferation of T-cells. Those data were combined to a map of the functions localized in the various regions of the HIV-1 envelope proteins.
Key Words: Synthetic oligopeptides—Humoral immunity—Cellular immunity—HIV-1.

The envelope protein complex gp120/gp41 of the human immunodeficiency virus (HIV), the causative agent of the acquired immune deficiency syndrome (AIDS), is responsible for adsorption of the virus particle to the CD4 receptor on the surface of the T-helper cell population (1,2), for penetration and for the cell fusion activity of HIV-infected cells (3,4). Most virus-neutralizing antibodies are directed to this protein complex (5,6). A major potential problem for the use of the outer membrane protein gp120 as a vaccine is the extensive heterogenicity of HIV isolates, especially in the most antigenic sites (7–9), which may result in the induction of predominantly virus type-specific antibodies (10). In addition to humoral immune response, helper T- and cytotoxic T-cells have been shown to be directed to gp120 (11–13). To locate the diverse functions in the amino acid sequence of gp120/gp41, we synthesized a series of oligopeptides from highly variable and conserved protein regions and tested those for their capacity to be reactive with the humoral and cellular immune system. In these tests, the preferred continuous epitopes for antibody recognition and T-cell activation were defined; both sites differ in their location and structural conditions in the gp120 molecule. The peptide that showed preferential activation of T-cells has been

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shown to be involved in the association of gp120 to the surface of T-cells (2). Since this epitope is located in a highly conserved region, it may be a good candidate for the elicitation of cellular immunity against the region involved in virus adsorption and should be included in subunit vaccines.

MATERIALS 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 (8,14). Highly glycosylated amino acid regions were not used as antigen for peptide synthesis since carbohydrate modification may result in a highly changed structure leading to the formation of discontinuous epitopes that cannot be simulated by synthetic oligopeptides. The selected peptides had a length of 12–14 amino acid residues.

Peptide Synthesis

Peptide synthesis was done in an 430A peptide synthesizer (Applied Biosystems, Weiterstadt, F.R.G.) using Fmoc (9-fluorenylmethyloxycarbonyl)-protected amino acids (15). The first residue coupled to *p*-alkoxybenzylalcohol-substituted polystyrene was purchased by Bachem AG (Bubendorf, Switzerland). In order to complete the coupling reactions, double couple cycles were used for each amino acid. *t*-Butyl (Ser, Thr, Tyr, Cys, Asp, Glu, His), *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; to avoid sterical problems in the availability of the terminal amino group, the coupling of each residue was repeated in dichloromethane. After the coupling reactions, the Fmoc groups were removed with 20% piperidine in dimethylformamide followed by a series of washes with dimethylformamide (all solvents were purchased from Merck AG, Darmstadt, F.R.G.; Fmoc-protected amino acids were from Bachem AG, Bubendorf, Switzerland; all chemicals from Aldrich, Steinheim, F.R.G.). After synthesis, side-chain protecting groups were removed by a 12 h treatment in trifluoroacetic acid containing 10% thioanisol and 10% metacresol at room temperature. The peptide was precipitated in an excess of ice-cold *t*-butyl ethyl ether, washed several times, and suspended in 1.5% ammonium bicarbonate. The peptide was lyophilized and purified by reversed phase high-performance liquid chromatography (HPLC) (TSK ODS 120 T, LKB, Gräfelfing, F.R.G.) using a gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. The sequences of the peptides were checked by amino acid analysis and the peptides were stored as lyophilized material at 4°C.

Immunization of Rabbits

For the use as immunogen in rabbits, the peptides were coupled at the amino terminal end to palmitic acid and used in an emulsion with Freund's adjuvant as described (16,17). In an alternate method, peptides were coupled via the sulfydryl group of cysteine to KLH (keyhole limpet hemocyanin, Calbiochem, Marburg, F.R.G.) (18). In peptides containing no natural occuring cysteine, this amino acid was included at the carboxy-terminal end.

ELISA Tests

Two hundred nanograms of uncoupled purified peptide per well were coupled overnight in 0.2 *M* sodium carbonate buffer, pH 9.5 to 96-well microtiter plates (Dynotech). Free protein binding sites were saturated by a 2 h incubation with gelatin solution (5 mg/ml, Sigma Chemicals, Munich, F.R.G.). Before and after the addition of the respective serum dilutions in PBS (phosphate buffered saline) plus 0.5% Tween 20 and 1% rabbit serum, the plates were washed several times with PBS/0.5% Tween 20. Rabbit anti-human IgG (Dako, Hamburg, F.R.G.) was added in a dilution of 1:1000 in PBS/0.5% Tween 20. Staining was done in 0.1 *M* phosphate buffer, pH 6.0 containing 0.5 mg/ml *o*-phenylenediamine and 0.1% H_2O_2 for 10 min, the reaction was stopped with 1 *M* H_2SO_4, and optical density was determined at 486 nm.

Lymphocyte Proliferation Assay

Microplates (Falcon 3040; Becton Dickinson Labware; Oxnard, CA, U.S.A.) with peptides in the solid phase were used for lymphocyte proliferation

assays. The peptides were used to coat wells at a concentration of 1 μg of protein per 100 μl. Lymphocytes from heparinized peripheral blood were separated by flotation on an Isopaque-Ficoll gradient (Pharmacia, Uppsala, Sweden). Cells were suspended in RPMI 1640 medium containing 10% heat-inactivated human serum (HIV antibody-negative AB+) to a concentration of 2×10^6 cells per ml. Then, 100 μl were plated in each well and incubated in a humidified atmosphere with 5% CO_2 in air at 37°C. At 24 h before harvest, 1 μCi of [^{3}H]-methylthymidine (^{3}HdT) with a specific activity of 20 Ci/mmol (Radiochemical Centre, Amersham, England) was added to each culture. Harvesting was performed with a cell-harvesting machine (Skatron AS, Lierbyen, Norway). Radioactivity was analyzed by determining the means of triplicate cultures. The data are given in terms of ratios: experimental counts divided by control counts. A net value of >3 was taken as a significant increase from that of the control.

RESULTS

Selected Peptides

According to computer analysis (8), the glycoprotein complex of HIV that is synthesized as a precursor protein of 160 kDa and subsequently cleaved into the outer membrane protein gp120 and the transmembrane part gp41 consists of highly variable, mostly antigenic, and conserved regions. These conserved regions are thought to form the inner core of the envelope protein. We selected amino acid regions mainly from conserved (C) and intermediately conserved (I) regions. Since almost all variable regions (V) that also show high indices to antigenicity have several recognition sites for N-linked glycosylation, only a part of the variable region 2 (V2) and most of the variable region 3 (V3), which has no potential N-glycosylation sequence, were synthesized in the form of oligopeptides, since sequential epitopes represented by short amino acid regions may be highly altered by the addition of carbohydrate groups and may not be represented by oligopeptides in in vitro experiments. The peptide synthesized from region V3 consists of two parts derived from the sequences of different strain isolates. Amino acids 319–326 were synthesized according to isolate HTLVIII/BH10, and these are relatively constant in all strains. The preceding four residues 315–318 represent a consensus sequence derived from all known virus strains. By this partially artificial epitope, we wanted to increase the reactivity with antibodies. For the sequence and location of the peptides synthesized here, see Table 1. The peptides derived from conserved and intermediately conserved regions were selected in such a way that they contained possibly occuring flexible, β-turn rich amino acid residues in the center of the synthesized region. We believe that these sequences represent a relatively good epitope with respect to the surrounding unflexible residues (e.g., see the location of peptides 80–92 and 100–112). Using this method of computer-based selection of antigenic amino acid regions as the basis for synthetic peptides, we presume to have a representative set of the sequential continuous antigenic epitopes. The numbers represent regions corresponding to isolate HTLV-III/BH10 (19) from the beginning of the open reading frame.

TABLE 1. *Amino acid sequences of synthesized peptides and localization on gp120/gp41 protein*

Localization on gp120/gp41 (AA–AA)	Amino acid sequence	Region
80–92	A–C–V–P–T–D–P–N–P–Q–E–V–V–G	C1 gp120
100–112	F–N–M–W–K–N–N–M–V–E–Q–M–C	C1 gp120
170–181	T–S–I–R–D–K–V–Q–K–E–Y–A–L–C	V2 gp120
213–225	P–K–V–S–F–E–P–I–P–I–H–Y–C	C2 gp120
254–266	C–T–H–G–I–R–P–V–V–S–T–Q–L–G	C2 gp120
315–326	S–I–Q–I–G–P–G–R–A–F–V–T–C	V3 gp120
349–361	L–K–Q–I–V–T–K–L–R–E–Q–F–G–C	I3 gp120
378–389	I–V–T–H–S–F–N–C–G–G–E–F–G	I3 gp120
435–446	Q–E–V–G–K–A–M–Y–A–P–P–I–G–C	C3 gp120
512–523	V–V–Q–R–E–K–R–A–V–G–I–G–C	C4/5 gp120/gp41
653–664	I–E–E–S–Q–N–Q–Q–E–K–N–E–C	C6 gp41

AA, amino acid.

Reactivity to Peptides by Human Sera

To test the reactivity of the peptides with the humoral immune system of HIV-infected persons, the peptides were adhered to the polystyrene walls of ELISA plates. The efficiency of the peptide binding to the plastic material was shown by the positive reaction of rabbit sera after immunization with the respective peptides; all peptides showed satisfactory adsorption onto the ELISA plates. Serum collections of the HIV-positive individuals with and without indications of the ongoing disease divided in AIDS patients, ARC patients, and those who were still without symptoms were used for testing. To avoid unspecific reactions that are relatively common in HIV-positive sera due to the frequent occurrence of opportunistic infections, sera were tested in dilutions of 1:500. Negative sera occasionally showed unspecific reactions up to serum dilutions of 1:100 with the exception of peptide 213–225, which showed positive reactivity with HIV-negative sera also in higher dilutions. In computer analysis, this peptide shows a high incidence for an amphipathic β-sheet and thus immunoglobulins may easily stick to it in a nonspecific way; the peptide also reacted with purified preparations of human monoclonal antibodies directed to gp41 (Desgranges and Modrow, unpublished results). Based on these experiments, the positive reactions of peptide 213–225 were assumed to be mainly unspecific. All other peptides reacted specifically to the dilution used in the experiments. A reaction was considered positive when the optical density exceeded 2.5 times the value of the panel of 10 HIV-negative sera used as controls. The reactivity of the peptides showed a very variable degree, probably based on the high amount of amino acid sequence variations, especially in gp120 (Table 2). None of the peptides used showed 100% reactivity. A relatively good reaction with 63% of AIDS patients' sera was shown by peptide 315–326, whose sequence was a combination of residues of various virus strains derived from most antigenic amino acid sequences of region V3 and peptide 100–112 from the constant region C1. All other peptides reacted only with 50% or less of sera tested. For most peptides, AIDS patients showed a lower degree of reactivity with respect to sera derived from HIV-positive individuals without symptoms (Table 2). When individual sera were checked for their reactivity, it could be shown that many of the symptom-free individuals reacted with almost all peptides in contrast to the AIDS patients,

TABLE 2. *Reaction of peptides with the immune system of HIV-positive individuals*

Peptide (AA–AA)	Humoral immunresponse (% of sera with positive reaction)			Cellular proliferative response (% of patients with positive reaction)
	Positive carrier	ARC	AIDS	
80–92	33	0	12	33
100–112	50	50	38	33
170–181	33	0	12	38
213–225	100	100	100	0
254–266	50	50	0	29
315–326	50	50	63	—
349–361	50	50	12	0
378–389	33	50	0	—
435–446	50	0	0	50
512–523	15	50	12	29
653–664	33	0	12	0

AA, amino acid.
Serum dilutions of 1:500 used.

who reacted preferentially with peptides 100–112 and 315–326 (Table 3). When tested in dilutions between 1:50 and 1:250, the percentage of reactive sera to peptide 315–326 could be increased. For further analysis of these results, we tested consecutive sera of the same patients, who were without symptoms when tested initially and developed AIDS subsequently. Sera were titrated up to dilutions of 1:800. Also, in these tests it could be shown that antibody titers to peptides 100–112 and 315–326 were almost constant during the progressive disease. In contrast, most of the other peptides showed decreasing reaction with antibodies (Table 4).

Reactivity of Peptides in T-Cell Proliferation Test

In order to test the recognition of the synthesized peptides by the cellular immune system, T-cell proliferation of 21 HIV-positive individuals was measured by the incorporation of [^{3}H]thymidine after incubation with peripheral lymphocytes whose surface was adsorbed with the purified peptide preparation. One of the peptides showed a relatively good reactivity with 50% (435–446) of the tested T-cell preparations of HIV-infected individuals. This peptide is located in highly conserved, rather hydrophobic regions of gp120 and show only a rather limited reactivity with antibodies (Table 2). These findings may reflect the different structural and hydrophilic conditions that have to be assumed for B- and T-cell-specific antigens.

TABLE 3. *Examples for the reaction of individual sera with peptides*

	Serum no.	Peptide										
		80–92	100–112	170–181	213–225	254–266	315–326	349–361	378–389	435–446	512–523	653–664
Without symptoms	1	−	+	−	+ +	(+)	+ +	(+)	−	+	−	−
	2	−	−	−	+ +	−	−	−	−	−	−	−
	3	−	−	−	+ +	−	−	−	−	−	−	+ +
	4	−	−	−	+ +	−	−	−	−	−	−	−
	5	+	+	+	+ +	+	+ + +	−	+	+	+	+
	6	+	+	+	+ +	+	+	+	+	+	−	−
ARC	7	−	+	−	+ +	−	−	−	−	−	−	−
	8	−	+	−	+ +	+	+ +	+	+	−	+	−
AIDS	9	−	+	−	+ + +	−	−	−	−	−	+ + +	−
	10	−	−	−	+	−	+	−	−	−	−	−
	11	−	−	−	+ +	−	−	−	−	−	−	−
	12	−	−	−	+	−	−	−	−	−	−	−
	13	−	−	−	+ +	−	+	−	−	−	−	−
	14	−	+ + +	+	+ + +	−	+ + +	−	−	−	−	−
	15	+	+	−	+ + +	−	+ + +	+	−	−	−	−
	16	−	−	−	+ +	−	+	−	−	−	−	+

+: positive reaction, optical density two times the mean value of negative sera. + +: optical density between 2–3 times the mean value of negative sera. + + +: optical density exceeding three times the mean value of negative sera.
Serum dilutions of 1:500 used.

DISCUSSION

The envelope protein complex gp120/gp41 of HIV-1 contributes many of the pathological and functional properties, which become relevant during the course of the viral infection and the progression of the disease, ending in ARC and/or AIDS. Using a series of peptides with predicted antigenic epitopes from conserved or variable amino acid regions, we tried to locate a part of the known functions. Within the sequence of this protein complex are segments for B-cell-specific antibody recognition, cellular immunity, and cell surface adsorption.

When used as antigen in ELISA assays, no amino acid region could be identified in gp120 by the methods used here that is recognized by all tested sera of HIV-positive individuals. This may be due to the fact that the amino acid sequence of the envelope proteins is highly variable among the individual isolates; in addition, gp120 is a highly glycosylated protein: about 60% of the molecular weight is due to carbohydrate groups. The epitopes showing the best conditions for sequential antigenicity were not used in our approach for peptide synthesis, since they contain a high number of potential N-linked glycosylation sites, which may alter the amino acid structure and mask great amounts of the primary sequence, which consequently are not accessible for antibody binding. The best recognized peptide 315–326 was the only one located in a nonglycosylated antigenic region. Peptides from this region have been shown to induce virus type-specific neutralizing antibodies (20), likely due to the high variability; particularly in this amino acid sequence, a 100% recognition could not be found despite our efforts to construct a consensus peptide of this region. In addition, it has to be assumed that the major part of antibodies in patients is directed against discontinuous and structural epitopes. One peptide, p213–225, showed unspecific reaction with HIV-negative sera. These unspecific reactions may occur occasionally when peptide sequences are used as antigen naturally not exposed to protein surfaces when presented without the surrounding regions of the native protein.

Testing consecutive sera, we have observed that in AIDS patients the humoral immune reaction was mainly directed to the peptide 315–326 of gp120. In symptom-free HIV-positives, antibodies against almost the entire series of peptides were observed. However, there were major differences in titers and reactivity in the individual samples. The decreased diversity of antibody formation in AIDS could be based on the specific loss of T-memory cells due to the ongoing HIV infection and concomitant destruction of the T-cell population. It seems unlikely that the decreasing recognition by antibodies would be due to the selection of an alternate HIV strain in

TABLE 4. *Antibody titers against peptides in consecutive sera of the same patients*

Patient	Date of sample (month/year)	Peptides 80–92	100–112	170–182	213–225	254–266	316–326	349–361	378–389	435–446	512–523	653–664	Clinical symptoms
1	10/85	—	—	—		—	—	—	—	—	—	—	HIV-negative
	6/86	1:100	1:100	1:100		1:200	1:400	—	—	1:100	—	—	HIV-positive
	1/88	1:50	1:100	1:100		1:100	1:200	—	—	1:100	—	—	without symptoms
2	5/87	1:100	1:100	1:100		1:100	1:800	—	—	—	—	—	HIV-positive
	10/87	—	1:100	1:100		1:100	1:800	—	—	—	—	—	without
	5/88	—	1:100	1:100		1:100	1:800	—	—	1:100	—	—	symptoms
3	8/86	1:400	1:200	1:400		—	1:800	1:400	—	1:100	1:800	1:400	HIV-positive
	8/87	1:50	1:200	1:400		—	1:400	1:200	—	1:100	1:400	1:400	LAS
	1/88	1:100	1:200	1:200		—	1:400	1:400	—	1:100	1:400	1:400	
	2/88	1:200	1:200	1:50		—	1:200	1:400	—	1:100	1:200	1:400	
	5/88	1:200	1:200	1:200		—	1:400	1:400	—	1:100	—	1:400	
4	9/86	—	—	—		1:100	1:200	—	—	—	—	—	HIV-positive
	3/87	1:200	1:100	1:200		1:200	1:100	—	—	1:400	1:400	1:400	Hemophilic
	12/87	1:100	1:400	1:400		1:200	1:100	—	—	1:400	1:100	—	ARC
	3/88	1:100	1:200	1:200		1:100	1:200	—	—	1:400	—	—	
5	9/86	1:200	1:200	1:100		1:50	1:800	—	—	1:200	—	—	HIV-positive
	2/87	—	1:100	1:100		—	1:800	—	—	—	—	—	AIDS
	8/87	—	1:200	—		1:100	1:400	—	—	—	—	—	
	1/88	—	1:100	—		1:50	1:800	—	—	—	—	—	
	4/88	—	1:100	—		—	1:800	—	—	—	—	—	
6	8/87	1:400	1:400	—		1:200	1:800	—	1:100	—	1:800	1:800	HIV-positive
	12/87	1:100	1:400	—		—	1:400	—	1:400	—	1:800	1:400	AIDS
	4/88	—	1:400	—		—	1:800	—	—	—	1:200	1:400	
7	8/86	1:400	1:800	1:200		1:400	1:200	—	1:800	1:400	1:400	1:800	HIV-positive
	8/87	1:100	1:400	—		1:400	1:400	—	1:100	—	1:400	1:800	AIDS
	1/88	—	1:800	—		—	1:400	—	—	—	1:100	1:400	

the AIDS patients, since the highly variable region V3, from which peptide 315–326 was derived, should be least recognized; this peptide, however, is well recognized in all serum populations tested.

In contrast to B-cell epitopes, which are mostly located in hydrophilic, flexible, loop-like regions on the surface of a protein molecule, T-cell specific recognition sites are—due to the different mode of antigen presentation in combination with HLA molecules after intracellular processing of the antigen—preferentially located in inner parts of the protein and show rather often a high incidence of amphipathicity (21,22). The peptide (435–446) that best induced T-cell proliferation has this amphipathic character. Another peptide including also this sequence (431–445) was very potent in inducing T-cell proliferation when 15 amino acid peptides covering the C-terminal of gp120 were assayed (B. Wahren et al., in preparation). An in vivo T-cell proliferation-inducing peptide overlapping this region was also described (23). However, also in T-cell recognition, a 100% reactivity could not be shown. This may be due to the genetically based differences of the antigen presenting HLA antigens in the individual patients; thus, the same peptide may be either presented in a different way or may not be recognized at all in patients of certain HLA phenotypes.

The peptide 435–446 is derived from a region of the gp120 molecule, which has been identified as being involved in virus adsorption (2). Using iodinated peptide preparations of p435–446, we could show that this peptide binds preferentially to the surface of T-cell lines (Modrow et al., in preparation). The peptide is located in a highly constant region, the property of cell adsorption being anticipated as a function equal to all HIV isolates. Although antibody reaction against this peptide was very weak, T-cell recognition was clearly detectable. It may be assumed that this region is located in a cleft between two highly variable antigenic regions, and that it is not accessible for antibody binding. Virus adsorption might therefore occur in a

similar way as described for polio virus adsorption mediated by the specific binding of the amino acid residues located in a "canyon" of the viral protein VP1 (24).

In summary, using synthetic oligopeptides, a number of functions could be mapped to the various regions of the envelope protein and a first example for a functional map of that polypeptide is suggested.

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