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Investigations into the cross-reactivity of rabbit antibodies raised against nonhomologous pairs of synthetic peptides derived from HIV-1 gp120 proteins

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Abstract: The immunological cross-reactivity of several peptides with specific pattern-property characteristics related to the epitopes of human immunodeficiency virus type 1 (HIV-1) gp160/120 envelope proteins has been investigated. Proteins with similar primary structures can be expected to show functional or topographic similarities, such as specific epitopes which may cross-react with antibodies derived from the immunisation of animals with other members of the same protein family. These structure-function characteristics may be revealed as periodicities derived from presentations based on the discrete Fourier transformation of the distributions of various physico-chemical amino acid descriptors, constituting the polypeptide backbone and amino acid side-chains of the protein molecule. Such approaches, for example, have permitted prediction of periodicities corresponding to secondary structural motifs, including amphipathic α-helices and β-sheets, within protein sequences, and have helped to clarify potential binding sites for ligands, substrates or cofactors with interacting macromolecules. Based on this approach, characteristic periodicities have been identified which represent common Fourier transform spectral properties of the envelope (ENV) gp160/120 glycoproteins from a range of HIV-1 isolates. In addition, similar periodicities have been detected as components of the discrete Fourier transform representation of the corresponding amino acid descriptors of the CD4 binding domain of gp120. Accordingly, we have synthesised several peptides having periodic characteristics in their discrete Fourier transform representations similar to these HIV-1 proteins. These nonhomologous synthetic peptides induced cross-reactive antibodies in New Zealand White rabbits. Polyclonal antibodies raised to one of these peptides reacted with HIV-1 ENV gp120-related proteins, as determined by enzyme-linked immunosorbent assay and Western blotting techniques. These findings provide further evidence for a role of immunological

cross-reactivity and molecular biomimicry in the development of peptide-based vaccines directed against viral or bacterial pathogens.

Abbreviations: ADCC, antibody-dependent cytotoxic cell response; BSA, bovine serum albumin; CTL, cytotoxic T-lymphocyte; ELISA, enzyme-linked immunosorbent assay; HIV-1, human immunodeficiency virus type 1; PND, principal neutralising determinant; RP-HPLC, reversed phase high-performance liquid chromatography.

A concept central to the development of peptide-based vaccines is the concurrent activation of several essential features of the immune system. The stimulation of specific cytotoxic T cells (CTLs), enhancement of helper T-cell responses, production of significant ADCC responses, as well as the generation of cross-reactive, ideally neutralising, humoral antibodies are all required for peptide-based vaccines to be effective. The induction of significant mucosal response as a consequence of the immunisation approach often also has been viewed as an important prerequisite of the presentation strategy with the peptide-based immunogen. Concurrent achievement of these different aspects of activation of the immune system would be of particular importance with peptide immunogens related to viral pathogens, such as the human immunodeficiency virus-1 (HIV-1), where the virus can undergo rapid mutation of genes coding for the surface coat proteins or other proteins associated with virus replication which have a high degree of homology with the cellular proteins of the host. These adventitious mutations will facilitate the ability of the virus to escape detection and elimination, and thus to infect and replicate within its host cells.

With the HIV-1 envelope protein, gp120, many investigations have been carried out to identify structural domains containing determinants responsible for cell infectivity and cytopathology. An important focus of earlier studies was the identification of domains containing contiguous or non-contiguous epitopes recognised by neutralising antibodies and cytotoxic lymphocytes (1–5). The available evidence indicates that synthetic peptides related to the third variable (V3) loop of gp120 satisfy some of the above-mentioned requirements as target compounds for use in peptide-based vaccine development, particularly regarding their ability to mimic the "principal neutralising determinant" (PND) and to bind to neutralising antibodies (1–3, 5–7). Despite encouraging results of investigations with V3 loop peptide analogues and peptide fragments related to other HIV-1 polyprotein sequences, the fundamental consideration still remains whether investigators, by focusing on the development of an optimised PND inadvertently have overlooked other equally important viral epitopic structures required for protective immune responses to viral challenges. Although the use of phage display combinatorial peptide libraries may overcome these limitations to some extent, the need still exists for synthetic peptides, which can induce antibodies that efficiently cross-react with viral components, yet have no or little sequence homology with these specific viral proteins.

Analyses of protein structure-function relationships in terms of pattern-property concepts and information theory considerations (8–12) or based on neural network theory (13–16), knowledge-based protein modeling tools such as Promod (17, 18) or template-based modeling methods (14, 19), have provided valuable insight into the characteristics of recognition motifs within proteins and have suggested possible procedures for the *de novo* design of protein analogues. These methods attempt to interpret inherent functional relationships of protein-ligand interactions in terms of various physico-chemical descriptors of the amino acid side-chains which contribute to the structure, and thus to the resultant biospecificity, of the protein. The approach pioneered by Eisenberg *et al.* (20, 21), Cornette *et al.* (22) and Finer-Moore *et al.* (23) uses discrete Fourier transformation of numerical representations of the primary structure in terms of the hydrophobicity parameters of the constituent amino acids (22, 24–29). This procedure provides a generic informational analysis method for the characterisation of periodic features of a polypeptide or protein sequence associated with specific secondary structures, e.g., amphipathic α-helices and β-sheets present as part of membrane proteins (30, 31) or protein-ligand biorecognition sites (30–32). Enhanced precision in the prediction of such secondary structural features of proteins using discrete Fourier transform procedures recently has been found in studies with randomly selected monomeric and multimeric proteins (Wirth and Hearn, unpublished results) using amino acid hydrophobicity parameters derived from the reversed phase HPLC behaviour of more than 1724 polypeptides (28).

Similar to all Fourier-based approaches, the amino acid sequence of the protein is converted into a series of integer numbers, assigned according to a characteristic feature of each amino acid residue. Cornette *et al.* (22), as well as our own investigations (28), used amino acid parameters related to the incremental free energy differences for the

partition of an amino acid residue in a polypeptide chain between an aqueous polar environment to an apolar, lipid-like environment. Various other parameter values can be selected from the more than 220 known amino acid physico-chemical descriptors, related mostly to incremental free energy, hydrophobicity or steri-molar parameters (22, 24–26, 28, 29). Extensions of this Fourier-based approach have been described with other types of steri-electronic amino acid descriptors (Wirth and Hearn, manuscript submitted), as well as with electron-ion interaction pseudopotential (EIIP) of the delocalised electrons (33, 34) of the individual amino acid residues which form the sequence of a protein. Collectively, these results suggest that the matching of the periodicities of the incremental free energy distributions of the amino acid residues in protein structures may be important for identifying structural features associated either with the intramolecular architecture of a protein or with recognition processes that occur between interacting biomacromolecules.

In the case of identifying the secondary structure periodicities for an α-helix or β-sheet within a polypeptide, the discrete Fourier transform values [usually expressed as a "spectral frequency" value (ν)] can be derived for each protein from analysis of its amino acid sequence and usually one set of amino acid hydrophobicity parameters in terms of a normalised reciprocal value of the periodicity within the notional range of ν = 0–0.5, or a rotational angle with a radian value between 0 and ± π. For example, for a polypeptide of *n*-residues, the value ν = 0.2770 is obtained for amino acid sequence region(s) with a high propensity for the individual amino acids to be arrayed with α-helical periodicity of high hydrophobic moment and amphipathic features with a translational step of 1.5 Å per amino acid residue (5.41 Å/turn) involving a 100° (or 2π/3.6 = 1.7453 radian) rotation along the polypeptide backbone. Similarly, the value ν = 0.5000 corresponds to a protein sequence region with a high propensity for the amino acids to be arrayed with a β-sheet periodicity of high hydrophobic moment and amphipathic features with a translational step of 3.2 Å or 3.4 Å per repeat unit of two amino acid residues (depending on whether a parallel or antiparallel β-sheet arrangement occurs) and involving a 180° (or π = 3.1415 radian) rotation along the axis of the polypeptide backbone. In contrast to the prediction of secondary structural periodicities for polypeptides or proteins, the so-called "biorecognition" periodicities associated with other ν values cannot be evaluated directly from the use of a single set of amino acid descriptors with a single amino acid sequence by Fourier transform methods, because these characteristics are screened by other periodic components of the polypeptide or protein structure. However, by examining the periodicities within the Fourier spectra of several other closely related proteins (or their target binding proteins or corresponding receptors), it may be possible to determine periodicity subsets which are associated with specific binding or biomimicry properties. Such extensions may then permit prediction of the position of specific amino acid residues or sequence regions within a protein's primary structure which may be essential for the derivation of these characteristic periodicities, some of which may be related to the biological function of the protein. Studies with several globular proteins (35, 36) have suggested that such methods and other procedures based on motif analysis and secondary structure prediction (8–12, 24, 25; Wirth and Hearn, manuscript submitted) may help locate amino acid sequence regions within the tertiary structure of a protein that are grouped spatially around a binding or active site, or alternatively aid the identification of subsets of non-homologous amino acid sequences, which can mimic some of the properties of the target protein.

The present work was based on the consideration that different members of a family of proteins can share common pattern-property characteristics that together embody information relevant to a specific binding phenomenon, such as antigenicity. Synthetic peptides exhibiting some of these characteristic pattern-property dependencies then would be anticipated to manifest some of the biological features of the parent protein(s), i.e., be able to mimic the antigenic and perhaps other functional properties of the protein(s). These peptides then could be considered to represent sets of topographic structures (or "structural images") which can mimic the surface features and pattern-property periodicities of the protein at or near its antibody binding sites. We have examined this concept by assessing the binding characteristics of a series of peptides which were selected on the basis of Fourier spectral representations of HIV-1 proteins. Here, we show that a set of synthetic peptides, distinct in amino acid sequences, can induce polyclonal antibodies, which cross-react with HIV-1 proteins, in New Zealand White rabbits.

Materials and Methods

Protein analysis

In these investigations, 25 amino acid sequences (Table 1) related to the envelope precursor glycoprotein (gp160) and the internal GAG precursor protein (p55) from different

isolates of HIV-1 from the NBRF database were used. The methods of protein analysis used in this study were based on the presentation of the protein primary structure as a numerical series by assigning to each amino acid a numerical value that describes a physico-chemical property involved in the characteristic interactive behaviour of the protein. In the present investigation the amino acid descriptors used were the EIIP values. In contrast to experimentally derived amino acid hydrophobicity descriptors used generally (20–25, 28, 32; Wirth and Hearn, manuscript submittted) for the identification of α-helical or β-sheet propensities of protein sequences, steri-electronic descriptors, of which the EIIP descriptors are but one empirical example (26, 28, 33), tend to emphasise other features within the Fourier transformation of a numerical representation of the primary structure because of differences in the relative ranking and value of the individual amino acid descriptors. Other procedures for the derivation of the Fourier spectral characteristics of protein sequences together with criteria for the selection of related peptide sequences have been described elsewhere (Wirth and Hearn, manuscript submitted; 35–38).

Peptide synthesis

The protected peptides, corresponding in amino acid sequence to those listed in Table 2, were synthesised using the *p*-methylbenzhydrylamine resin (100–200 mesh, 0.4–0.8 mequiv/g) and *N*-α-*tert*-butoxycarbonyl (*t*-Boc) amino acids (Bachem Inc., Torrance, CA) by simultaneous multiple peptide synthesis (39). The peptides were cleaved from the resin and deprotected by the conventional hydrogen fluoride/anisole procedure (40). After synthesis, the peptide products were assessed by fast protein liquid chromatography (FPLC) on reversed phase PepRPC HR5 column (Pharmacia, Uppsala, Sweden) and found to have an average purity of 85%. The analytical chromatograms were developed with a gradient of 0.1% aqueous CF_3COOH (trifluoroacetic acid) (buffer A) to 65% aqueous acetonitrile (CH_3CN) containing 0.1% CF_3COOH (buffer B) during 60 min. Peptides were purified by similar procedures using the same RP-HPLC system with semipreparative loading of the crude sample to yield the purified peptide of >95% purity with the required analytical characteristics, as assessed by amino acid composition, automated Edman sequencing and electron-spray (ES) and fast atom bombardment (FAB)-mass spectrometry.

Table 1. **List of HIV envelope gp160 polyproteins from NBRF data base**

1.	ENHXB2	Envelope polyprotein-HIV-1, isolate HXB2
2.	ENBRU	Envelope polyprotein-HIV-1, isolate BRU
3.	ENNL43	Envelope polyprotein-HIV-1, NY5/BRU (LAV-1) recCLONE NL43
4.	ENSF2 2	Envelope polyprotein-HIV-1, isolate SF2
5.	ENSC	Envelope polyprotein-HIV-1, isolate SC
6.	ENMN	Envelope polyprotein-HIV-1, isolate MN
7.	ENRF	Envelope polyprotein-HIV-1, isolate RF
8.	ENWMJ2	Envelope polyprotein-HIV-1, isolate WMJ2
9.	ENCDC451	Envelope polyprotein-HIV-1, isolate CDC451
10.	ENNY5	Envelope polyprotein-HIV-1, isolate NY5
11.	ENJH3	Envelope polyprotein-HIV-1, isolate JH3
12.	ENBRVA	Envelope polyprotein-HIV-1, isolate BRVA
13.	ENELI	Envelope polyprotein-HIV-1, isolate ELI
14.	ENMAL	Envelope polyprotein-HIV-1, isolate MAL
15.	ENZ6	Envelope polyprotein-HIV-1, isolate Z6
16.	ENZ2Z6	Envelope polyprotein-HIV-1, isolate Z2Z6
17.	ENZ3	Envelope polyprotein-HIV-1, isolate Z3
18.	ENS321	Envelope polyprotein-HIV-1, isolate Z321
19.	ENJY1	Envelope polyprotein-HIV-1, isolate JY1
20.	EN2ISY	Envelope polyprotein-HIV-1, isolate ISY
21.	ENNDK	Envelope polyprotein-HIV-1, isolate NDK
22.	ENOYI	Envelope polyprotein-HIV-1, isolate OYI
23.	VCLIBR	Envelope polyprotein-HIV-1, isolate BR
24.	VCLI3W	Envelope polyprotein precursor-HIV-1, isolate WMJ1
25.	VCLIZR	Envelope polyprotein precursor-HIV-1, isolate ZR6

Table 2. **Amino acid sequences of the peptides derived from the analysis of various HIV-1 gp160 protein sequences and the corresponding characteristic periodicity values derived from the discrete Fourier transform representations**

Peptide	Sequence	v1	v2
A1	KQQYYWYAWCQPPQDQLIMD	0.1855	0.2188
A2	LKRDQEPMDFHIWDDYLKRD	0.1855	–
B1	DDALYDDKNWDRAPQRCYYQ	0.1855	0.2188
B2	DFHIWDDYLKRDQEPMDFHI	0.1855	–

The v1 and/or v2 periodicities of the peptides A1 and A2 have phase(s) opposite to those exhibited by the v1 and/or v2 periodicities of the peptides B1 and B2 or the Lav-Bru gp120 protein. Peptide A2 and B2 contained two differently arranged partial sequences.

Immunization procedures

Two-month-old New Zeland white rabbits (n = 4/peptide immunogen) were immunised with the different peptides. No conjugation to a carrier protein was required with the examined peptide immunogens. The rabbits were injected subcutaneously on day 0 with 100 μg of peptide in complete Freund's adjuvant. Every 10 days thereafter, each rabbit received another 100 μg of peptide in incomplete Feund's adjuvant, with a total of six immunisations. Preimmune and immune sera (14 days after the last immunisation) were tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies against the immunising peptide and other peptide analogues.

Enzyme-linked immunosorbent assay

Standard 96-well microtiter plates were coated with the different synthetic peptides or with HIV-1_{BRU} recombinant gp120 glycoprotein (Neosystem Laboratoire, Strasbourg, France), using 100 μL per well of a 1 mg/mL solution of peptide in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Un-

occupied binding sites were blocked overnight at 4°C with a 1% (w/v) solution of bovine serum albumin (BSA) in 0.1 M phosphate buffer, pH 7.4, and the plates then were washed thoroughly with the 0.1 M PBS buffer. The immune and nonimmune rabbit sera, after being diluted serially with a solution consisting of 0.01 M PBS, pH 7.4, 0.15 M NaCl, 1% BSA and 0.1% Tween 20, were added as 100 µL aliquots to the wells. The plates were then incubated at 37°C for 1 h. After additional washes, a 1000-fold dilution of a horseradish peroxidase-conjugated goat anti-rabbit IgG serum was added and the plates were incubated at 37°C for another hour. The enzyme activity was determined by adding o-phenylenediamine-H_2O_2 as substrate to the wells followed by incubation for 30 min at room temperature in the dark. The reaction was stopped by adding 4 N H_2SO_4 (50 µL) and the optical absorbance was measured at 492 nm with a Titertek ELISA multiscan reader.

Immunoblot analysis

Sera from New Zeland White rabbits immunised with synthetic peptides, diluted in 0.1 M PBS, pH 7.4, were incubated with commercial (Sanofi-Pasteur Diagnostics Marnes-la-Coquette, Paris, France) immunoblot strips. The strips were then incubated with alkaline phosphatase-labeled goat anti-rabbit IgG and developed with a phosphatase substrate system (Sanofi-Pasteur Diagnostics Marnes-la-Coquette, Paris, France) using the protocols suggested by the vendor. The data shown in Fig. 3 are representative of the results obtained on the same day using the same assay strips.

Results

The sequences of the synthetic peptides investigated in this study and their relevant Fourier spectral values are listed in Table 2. These peptides, prepared by solid-phase peptide synthesis methods, were selected after analysis of the amino acid sequences of the envelope precursor glycoprotein (gp160) and the internal GAG precursor protein (p55) from 25 different isolates of HIV-1, the sequences of which are identified in Table 1. Based on this analysis, a characteristic Fourier transform periodicity at a value of v1 = 0.1885 was calculated for the gp160 envelope glycoproteins from these 25 isolates (Fig. 1a). This v1 value simply reflects a major periodicity, determined by the Fourier transform procedure, that is common to all 25 gp160 proteins when they are represented as numerical sequences with the EIIP amino acid descriptors. The same Fourier transform periodicity value, v1 = 0.1885, was evident as a spectral characteristic for the p55 GAG proteins (from 11 isolates) (Fig. 1b) and could be distinguished in the informational spectrum of gp160 and p55 proteins of single isolates such as HIV-1_{BRU} (Fig. 1, c and d). Similar analysis of the CD4-binding fragment (41) of the gp120 envelope glycoprotein corresponding to amino acid residues gp120$^{[418-461]}$ from eight HIV-1 isolates, revealed a major characteristic periodicity component at v1 = 0.1855 as well as an additional minor characteristic periodicity value corresponding to v2 = 0.2188 (Fig. 1, e and f). For example, the 44-mer gp120$^{[418-461]}$ sequence: TITLP^{5}CRIKQ^{10}FINMW^{15}QEVGK^{20}AMYAP^{25}PIGGQ^{30}-IRCES^{35}NITGL^{40}LLTR corresponding to the CD4-binding fragment identified in the HIV-1_{BRU} isolate (n143 variant) exhibited both characteristic periodicities v1 and v2 by this discrete Fourier transform analysis (Fig. 1f). In addition, peptide fragments related to HIV-1_{BRU} gp120, including gp120$^{[105-117]}$, gp120$^{[252-272]}$, gp120$^{[414-434]}$, gp120$^{[433-448]}$, gp120$^{[437-448]}$ and gp120$^{[449-469]}$ were examined also. These fragments previously attracted interest from various investigators [42–44] because of the ability of some of these synthetic peptides to generate immuno-neutralising antibodies. When compared with HIV-1_{BRU} ENV or GAG proteins, none of these peptide fragments related to HIV-1_{BRU} gp120 had a significant characteristic periodicity corresponding to v1 or v2 (Table 3). Application of similar procedures revealed the presence of the periodicity characteristics, v1 = 0.1855 and v2 = 0.2188, for the human and murine CD4 protein sequences. Based on these considerations, the periodicity characteristics, v1 = 0.1855 and v2 = 0.2188, thus may represent Fourier spectral features that are related to structural units associated with the gp120/160 interaction with the extracellular domain of CD4.

According to the findings above, a 20-amino-acid residue peptide, called A1 (KQQYYWYAWCQPPQDQLIMD), was synthesised based on the consideration that this peptide should exhibit Fourier transform spectral frequency characteristics of v1 and v2 (Fig. 2a). However, with A1 the spectral phases at these periodicities derived from the Fourier transformation analysis were selected to be opposite to the phases for the corresponding Fourier spectral characteristics of the gp120$^{[418-461]}$ fragment involved in CD4 binding. As such, A1 should represent a control peptide incapable of binding strongly to CD4 as a mimic of the gp120$^{[418-461]}$ binding sequence. A second control 20-amino-acid residue peptide (LKRDQEPMDFHIWDDYLKRD, referred to as peptide A2) was also synthesised based on the consideration that this peptide should contain only one of

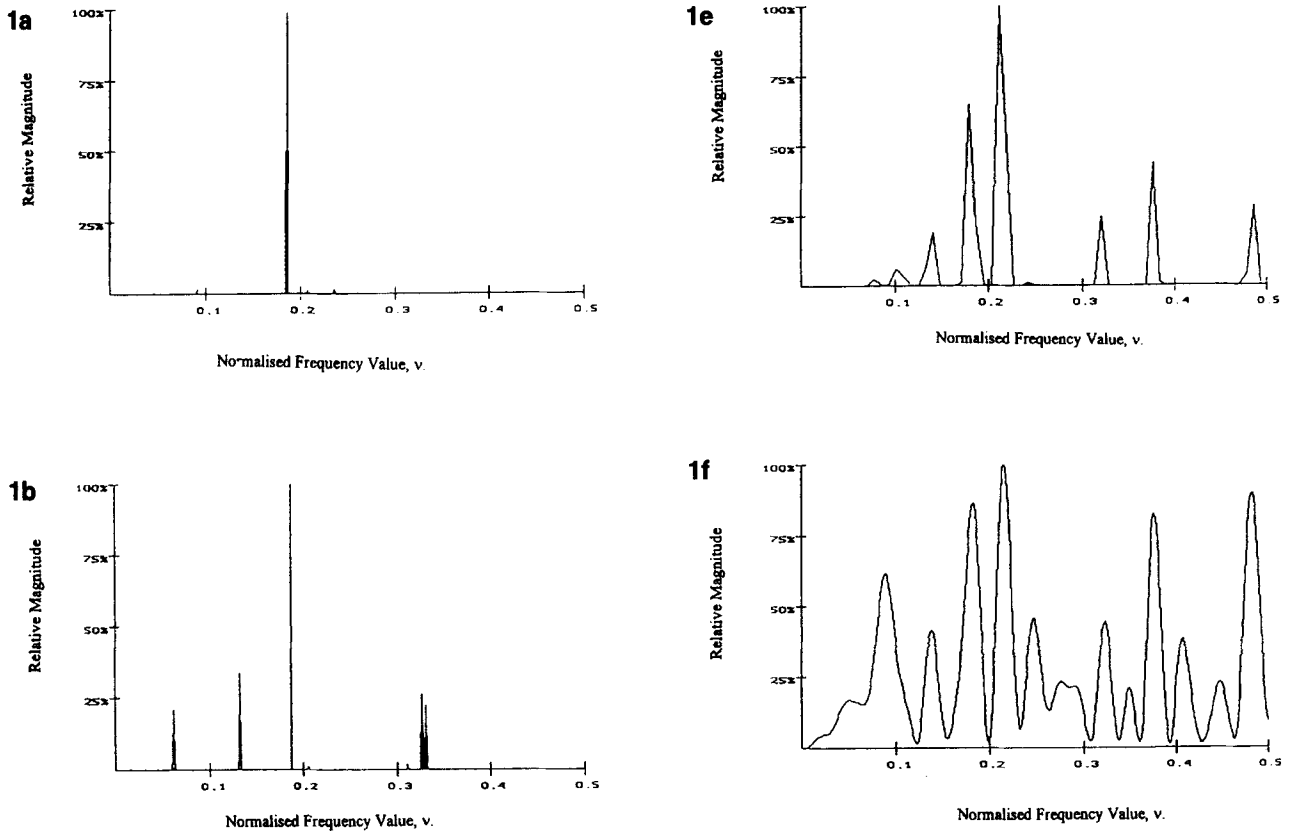

Figure 1. (**a**) The plot of the relative amplitude (expressed as a percentage of the amplitude of the dominant frequency) versus the frequency, v, for the Fourier cross-spectral representation of gp160 envelope proteins from 25 HIV isolates. The prominent peak is at v = 0.1855. (**b**) The plot of the relative amplitude (expressed as a percentage of the amplitude of the dominant frequency) versus the frequency, v, for the Fourier cross-spectral representation of p55 gag proteins from 11 HIV isolates. The major peak is at v = 0.1855. (**c**) The plot of the relative amplitude (expressed as a percentage of the amplitude of the dominant frequency) versus the frequency, v, for the Fourier single spectral representation of gp160 from HIV-1_{BRU} isolate. The major peak is at v = 0.1855. (**d**) The plot of the relative amplitude (expressed as a percentage of the amplitude of the dominant frequency) versus the frequency, v, for the Fourier single spectral representation of p55 from the HIV-1_{BRU} isolate. The major peak at at v = 0.1855. (**e**) The plot of the relative amplitude (expressed as a percentage of the amplitude of the dominant frequency) versus the frequency, v, for the Fourier cross-spectral representation of the CD_4 binding fragment of the envelope protein gp120 from 8 HIV-1 isolates. Two prominent peaks occur at v_1 = 0.1855 and v_2 = 0.2188. (**f**) The plot of the relative amplitude (expressed as a percentage of the amplitude of the dominant frequency) versus the frequency, v, for the Fourier single spectral representation of the CD_4 binding fragment of gp120 envelope protein from HIV-1_{BRU} isolate, which exhibits prominent peaks at the periodicities v_1 = 0.1855 and v_2 = 0.2188.

Table 3. **Fourier spectral characteristics of various gp120-related peptide fragments and synthetic peptides**

Peptide	S/N(v1)	ϕ(v1)	S/N(v2)	ϕ(v2)
gp120[418–461]	3.25	0.27	3.38	–0.92
gp120[105–117]	1.17	3.08	0.05	1.1
gp120[252–272]	3.12	0.13	2.07	–0.82
gp120[414–434]	1.91	2.99	1.23	0.78
gp120[433–448]	0.72	–0.72	0.27	0.58
gp120[437–448]	1.18	3.03	0.15	1.75
gp120[449–469]	3.47	–1.27	0.87	2.88
CD4 extracellular domain	1.46	–2.15	0.76	2.08
A1	>20	–0.3	>20	1.0
A2	>20	–0.3	–	–
B1	>20	0.3	>20	–1.0
B2	>20	0.3	–	–

The numerical values of the signal-to-noise (S/N) and the phase (ϕ) (expressed in radians) were derived for the periodicity components v1 = 0.1855 and v2 = 0.2188, respectively.

corresponding periodicity characteristic, i.e. v1 = 0.1855, with again a Fourier spectral phase opposite to that of the gp120[418–461] binding fragment (Fig. 2b). Peptides A1 and A2 do not share any sequence homology, although they have the same predicted common characteristic periodicity v1 and phase in their Fourier spectral analysis. We have raised in New Zealand White rabbits polyclonal antibodies (pAbs) directed against these synthetic, structurally unrelated peptides, to examine whether the derived pAbs would cross-react in ELISAs. In all cases, the preimmune sera from the New Zealand White rabbits did not show any significant reactivity with the target peptides, in contradistinction to the derived immune sera. Moreover, maximal and sustained titres of the immune sera were observed after four to six immunisations with the peptide alone, without the requirement for conjugation to a carrier protein. In subsequent studies, competitive ELISA assay procedures (data not shown) confirmed that the specific polyclonal antibodies reacted with the immunising peptide with apparent average distribution affinity constants at low nanomolar levels, i.e. approximately 5×10^{-9} M. In Table 4, the data show that the rabbits immunised with the peptide A1 generated pAbs that cross-reacted with peptide A2, and alternatively animals immunised with peptide A2 produced antibodies that cross-reacted with peptide A1. The results with the A1 and A2 peptides indicate that synthetic nonhomologous peptides, which show common periodic patterns within the Fourier transformations of their corresponding amino acid physico-chemical descriptor presentations, can generate cross-reactive antibodies. In addition, gp120[418–461] and other shorter gp120 peptide fragments that lack the common characteristic periodicity v1 did not show any cross-reactivity with the rabbit anti-A1 or anti-A2 sera within the limits of detection of the ELISA procedure (Table 4).

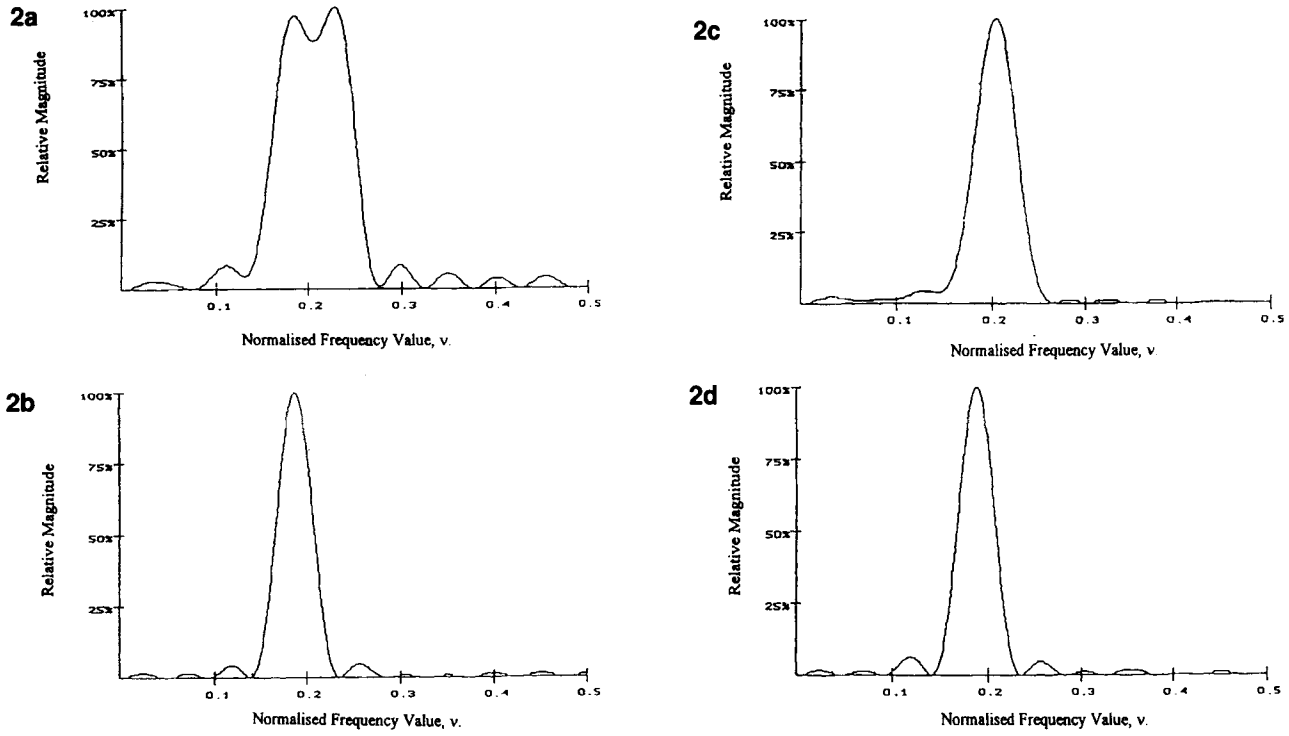

Figure 2. The plots of the relative amplitude (expressed as a percentage of the amplitude of the dominant frequency) versus the frequency, v, for the Fourier spectral representation of (**a**) peptide A1, (**b**) peptide A2, (**c**) peptide B1, (**d**) peptide B2.

Table 4. **ELISA cross-reactivity of rabbit polyclonal antibodies to synthetic peptides and HIV-1 gp120[a]**

Peptide	Anti-A1	Anti-A2	Anti-B1	Anti-B2
A1	5.1	3.3	2.4	0.9
A2[b]	3.6	5.1	2.1	2.7[b]
B1	0	0	3.6	2.4
B2[b]	0.9	3.6[b]	3.9	5.4
gp120	1.5	1.2	4.5	1.8

a. These data represent the $\log_{10}$ values of the end-point dilution titrations in the competitive ELISA assays.

b. The peptides A2 and B2 have the amino acid sequences LKRDQEPMDFHI and WDDYLKRD in common. The $\log_{10}$ values of the end-point dilution for the interaction of anti-B1 serum with the various gp120 peptide fragments were as follows: (a) gp120$^{[105-112]}$, 2.1; (b) gp120$^{[252-272]}$, 2.1; (c) gp120$^{[414-432]}$, 2.1; (d) gp120$^{[432-448]}$, 0.9; (e) gp120$^{[437-448]}$, 0.0; (f) gp120$^{[449-469]}$, 0.6; (g) gp120^{CD4} fragment, 0.0.

Based on these observations, a third 20-amino-acid residue peptide, referred to as peptide B1 (DDALYDDKNWDRAPQRCYYQ), predicted to exhibit the same major periodicities as the gp160/GAG proteins and the gp120$^{[418-461]}$ polypeptide (i.e. the same v1 and v2 values) and with the similar phases at these periodicities, was examined. The peptide B1 was not homologous with that of peptide A1 or peptide A2 and did not cross-react with anti-A1 and anti-A2 sera. In contrast, the peptides A1 and A2 showed low levels of cross-reactivity with the rabbit polyclonal anti-B1 antibodies in the ELISA procedure (Table 4). By analogy with peptides A1 and A2, a fourth 20-amino-acid residue peptide, called B2 (DFHIWDDYLKRDQEPMDFHI), but with only the characteristic periodicity of v1 = 0.1855 and the same phase at v1 as peptide B1 was synthesised. Anti-B1 serum cross-reacted significantly with the peptide B2, whereas anti-B2 serum exhibited a lower cross-reactivity with peptide B1.

The immunological relationship between polyclonal antibodies generated by these peptides and HIV-1 gp120 envelope glycoprotein was examined further. The ELISA results documenting that rabbit pAbs derived with peptide B1 can cross-react significantly with baculovirus-derived recombinant HIV-1 gp120 are shown in Table 4. The recombinant gp120 glycoprotein reacted poorly with the pAbs present in the anti-A1, anti-A2 and anti-B2 sera (Table 4). Moreover, only the anti-B1 rabbit serum cross-reacted with the individual HIV-1_{BRU} proteins using Western immunoblot analysis with commercial immunoblot strips containing the viral proteins HIV-1_{BRU}. When antisera directed against the A1, A2, B1 and B2 peptides were tested for their ability to react in Western immunoblot assays with individual HIV proteins, only the antibodies in the anti-B1 serum were captured significantly by the viral proteins.

As shown in the Western immunoblot (Fig. 3), the cross-reactivity of anti-B1 serum was evident with GAG proteins p18, p25, p40 and p55, as well as with a component of about 43 kDa localised in the area corresponding to the diffuse band of the viral transmembrane glycoprotein gp41. In addition, binding of the anti-B1 antibodies to the gp120 ENV protein was observed as a faint (low-intensity) band. The cross-reaction of the anti-B1 serum with the reverse transcriptase p68 was particularly evident. Several antisera raised against this peptide in different New Zealand White rabbits typically showed similar band patterns, although the relative intensity of the Western immunoblot patterns differed between sera from different rabbits regarding gp120/160, which typically did not generate a significant band. Consistent with these results, Fourier analysis of the POL-proteins from HIV-1_{BRU} strain also demonstrated the v1 characteristic (data not shown). Qualitative trends in the extent of cross-reactivity of anti-B1 sera derived from different rabbits could also be observed with gp120 ENV proteins. However, some variability in the capture of anti-B1 antibodies from a specific rabbit by the gp120/160 component in the ELISA or Western blotting format was noted from quantitative assessments. Although a high cross-reactivity of anti-B1 serum with gp120 in the ELISA assay was observed, the lower intensity in the Western immunoblot detected for the cross-reactivity of the anti-B1 serum with the intact gp120 may

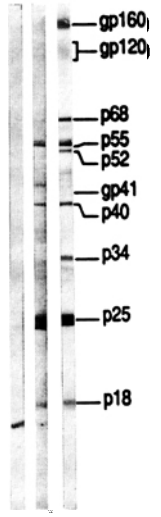

Figure 3. Immunoblot analysis with the HIV-1_{BRU} proteins with the rabbit anti-peptide B1 sera. (**a**) Preimmune rabbit serum; (**b**) serum from rabbit immunised with peptide B1; (**c**) HIV-1 positive human serum. Details of the experimental procedures and the assignment of the location of the cross-reactive protein bands in this Western immunoblot are given under "Materials and Methods" and described under "Results," respectively.

not be surprising when the mode of immobilisation of the recombinant gp120 to the immunoblot strips is taken into account, i.e. specific gp120 conformational epitopes recognised by the anti-B1 antibodies in the ELISA may become partially inaccessible because of the adsorption of the gp120 protein to the immunoblot support. The antisera directed against the remaining synthetic peptides (A1, A2 and B2) or preimmune sera did not show any specific cross-reactivity in these immunoblot experiments (data not shown), except for a weak cross-reactivity of the GAGp18 protein with some normal preimmune rabbit sera. These results confirm that peptide B1 can induce in New Zealand White rabbits pAbs which can be captured by the nonhomologous ENV, POL and GAG-related proteins of the HIV-1_{BRU} strain, but not the GAGp15 protein.

Discussion

Genetic variability between different isolate strains is one of the striking features of the HIV-1 gp160. However, the variability is not distributed randomly throughout the sequence but is located within regions of very high variability, flanked by well-conserved domains. Within these constant domains, sequence conservation is greater than 80% between different strain isolates of HIV-1. Ho et al. (42) have shown that a rabbit antiserum raised against a synthetic peptide related to the amino acid sequence gp120[254–274] within the second conserved domain of gp120 reacts strongly with gp120 and neutralises diverse isolates of HIV-1. However, this antiserum does not block the binding of HIV-1 to $CD4^+$ cells. Various studies (45–47) have indicated that the PND of HIV-1 ENV protein is within a disulphide loop in the hypervariable V3 domain. Other studies (44) have shown that synthetic peptides related to the V3 domain of gp120 can be used to elicit strain-specific HIV-1-neutralising antibodies and stimulate cytotoxic T lymphocytes. Although amino acid 325 in the V3 loop is critical for recognition by HIV-1 strain-specific cytotoxic T lymphocytes (48), the variability of the V3 loop sequences poses special problems for the design of V3 loop peptide vaccines. Consequently, identification of immunodominant peptide sequences within the primary structure of gp160, which permit induction of broadly cross-reactive cytotoxic T-cell responses or generation of cross-reactive, domain-specific antibodies, still presents significant challenges (8,43,44,49–51).

In the present investigation, an alternative strategy was used with peptide immunogens which can elicit antibodies that cross-react with HIV-1 proteins but which have no homology to the gp160 and GAG protein sequences. Similar to other knowledge-based procedures, the approach followed in this study was predicated on the ability of the Fourier transform-based pattern-property algorithm to determine the periodic characteristics of a family of proteins, in this case involving HIV-1-related proteins. The present study has documented that peptides so selected can present cross-reactive determinants recognised by pAbs. The ability of some of these peptides to elicit proliferative and cytotoxic T-cell responses will be described in a subsequent manuscript [Krsmanovic *et al.*, unpublished results].

Determination of common periodic characteristics for HIV-1 proteins from various HIV-1 isolates represents a possible approach to obtain similar patterns of biorecognition, despite the great sequence variability of these viral proteins. To determine whether such periodic characteristics can be reflected in a biological property such as the characteristics can be reflected in a biological property such as the induction of a specific immune response, we examined several peptide pairs expressing the same characteristic periodicities (as v1 and/or v2 values) in the Fourier transform spectra of numerical representations of the sequences of the HIV-1 ENV proteins. As shown in Table 3, pairs of nonhomologous peptides that have common Fourier spectral properties, i.e. peptides A1 and A2, were able to induce cross-reactive antibodies. This result shows that nonhomologous peptides (e.g. peptides A1 and A2 or B1 and B2) that have at least one characteristic periodicity in common may induce a cross-reactive immune response.

As shown in Table 4, the anti-A1 serum did not cross-react with peptide B1 nor was B2 antiserum able to cross-react significantly with peptide A1. The anti-B2 serum moderately cross-reacted with peptide A2, whereas the polyclonal antibodies within the anti-A2 serum reacted significantly with the peptide B2 in ELISA systems. This latter cross-reactivity apparently reflects the significant sequence homology between the peptides A2 and B2 resulting from these peptides sharing two common amino acid sequence motifs (e.g. LKRDQEPMDFHI and WDDYLKRD), although the relative position of the sequences are reversed in the two peptides. As also evident in Table 4, none of the other gp120 peptide fragments examined in this study were able to cross-react with anti-B1 serum in the ELISA assay, which is consistent with the observation that these gp120 peptide fragments do not have the periodicity v1 and/or v2.

If the periodicity v1 of the A1 and A2 peptide pair is one pattern-property characteristic relevant to the ability of these peptides to generate cross-reactive antibodies, does the change of phase at this value alter the cross-reactivity? The peptide B1 has the characteristic periodicities of v1 and v2, but with opposite phases at these v1 and v2 values as peptides A1 and A2. The ELISA results indicate that peptide B1 and anti-B1 serum have some of the binding features expected of a cross-reactive immunogen related to a HIV-1 gp120 epitope. Thus baculovirus-derived recombinant HIV-1 gp120 cross-reacted with the anti-B1 antiserum using both ELISA and Western blotting procedures. The ability of the anti-B1 serum, but not the anti-B2 serum, to cross-react with the gp120 viral protein and other proteins derived from gp160, suggests that the presence of a characteristic v2 periodicity, which is not exhibited by peptide B2, is also required for the cross-reactivity to occur with the gp120 ENV protein. Besides the characteristic v1 value detected as a major common periodicity in the cross-spectra of all tested isolates of HIV-1 GAG precursor p55, the characteristic periodicity v2 was found in some single isolates such as $HIV\text{-}1_{BRU}$. Furthermore, these two periodicities also were detected in the Fourier spectra of individual GAG proteins such as precursor p40, and proteins p24 and p18 from the same isolates, but not for GAG protein p15. The periodicity v1, the cryptic periodicity v2 and other as yet undetermined periodicity characteristics could thus be markers of the "ideal" nonhomologous polypeptide for the full functional requirements for mimicking the major epitopes of gp120 glycoproteins.

As noted above, the peptides A2 and B2 have a significant sequence homology, consistent with these two peptides exhibiting the common periodicity v1. However, what distinguishes these two peptides is their opposite phase values in the discrete Fourier transform spectra. In this respect, it should be noted that the algorithmic procedure used in this study leads to similar structures being generated by the displacement of block(s) of amino acid residues within the sequence of the peptide. The cross-reactivities observed in the ELISA experiments with the A2 and B2 peptides and their antisera suggest that for peptides with a high degree of sequence homology and the same number of amino acid residues, the Fourier spectral phase may not be a stringent criterion, provided the value of the periodicity characteristic(s) is conserved. Moreover, implicit to discrete Fourier transform procedures, cross-multiplication of the derived spectra results in abolition of the phase component in the composite Fourier spectra, irrespective of whether a first-order or a power function is used as part of the Fourier transform spectral cross-multiplication subroutine. Similar algorithmic procedures (8) have analogous constraints in not providing a single, unique prediction, possibly because of the limitations which exist with current computational methods and the imprecision in our knowledge on the physico-chemical relevance of the different numerical values of the amino acid descriptors that can be used.

Several conclusions can be drawn based on the results of the present investigation. First, as presented in this study, synthetic peptides, such as peptide B1, which have no sequence homology with the parent protein, can be used as immunogens to produce polyclonal antibodies that interact with HIV-1 viral proteins. These results suggest that further experimentation on the effectiveness of these antisera *in vitro* and *in vivo* HIV-1 challenge systems is warranted. Second, further refinement to predictive algorithms which permit assessment of the biomolecular structure-function behaviour of proteins is required if peptidic compounds with enhanced biological and immunological functions are to emerge, not only with retroviral pathogens of the HIV-1 family, but also with other classes of proteins. Third, the preliminary immunochemical findings reported in this manuscript raise the codicil on the extent of diversity in peptide structure which can be tolerated before the features of cross-reactivity become absent or nonspecific. Each of these issues is currently under investigation. What is evident from this and related studies is that knowledge-based approaches will become especially important in the future in circumstances in which the desired biological response cannot be replicated easily for a particular protein by current recombinant DNA procedures. One situation in which such considerations could have a significant impact relates to the development of peptide-based vaccines which prevent HIV-1 infectivity. Currently, no effective vaccine against HIV-1 has been produced, which is partly because of the structural variability of the viral proteins. The ability to produce more effective immunodominant peptides which mimic, in terms of their immunogenicity, HIV-1 proteins and which are capable of eliciting broadly cross-reactive immune responses would significantly advance vaccine research in this field.

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