B cell epitope mapping of human immunodeficiency virus envelope glycoproteins with long (19- to 36-residue) synthetic peptides

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Envelope glycoproteins, gp120 and gp41, of the human immunodeficiency virus type 1 (HIV-1) elicit immune responses, including virus-neutralizing antibodies, which are expected to play a role in the defence against HIV-1 infection. Subregions of the gp120/gp41 sequence have immunosuppressive effects or may be implicated in autoimmune responses. Some of the immunodominant epitopes of gp120/gp41 do not contribute to protective immunity and act as immunological decoys. These circumstances emphasize the need to select from gp120/gp41 regions inducing protective responses. Towards this goal, 30 peptides covering approximately 87% of the HIV-I strain BH10 gp120/gp41 sequence were synthesized. Antibodies in rabbit and human anti-HIV-1 sera recognized 28 and nine of the peptides, respectively, indicating that most of the gp120/gp41 sequence is immunogenic and secondly, that the antibody response to HIV-1 is restricted in infected humans. Most of the peptides, without conjugation to carriers, elicited high levels of anti-peptide (endpoints 1: > 10⁴) and anti-gp120/gp41 (endpoints 1: > 10⁵) antibodies. The highest levels of virus-neutralizing antibodies were elicited by peptide 306 to 338 from a hypervariable loop of gp120. Additional peptides from the full-length hypervariable loop (303 to 338) of HIV-1 BH10 and from 20 additional HIV-1 isolates were recognized differentially by human anti-HIV, suggesting that success of passive immunization may depend on a match between administered antibodies and the challenging HIV-I strain, and also that active immunization with selected peptides from a hypervariable region of distinct HIV-1 isolates should be explored further as a method for prophylaxis against infection.

Introduction

The envelope (env) glycoproteins gp120 and gp41 of the human immunodeficiency virus type 1 (HIV-1) elicit antibodies which neutralize the infectivity of the virus and inhibit fusion of the virus with host cells (Lasky et al., 1986; Robey et al., 1986; Matthews et al., 1986; Weiss et al., 1986; Berman et al., 1988; Krohn et al., 1987; Rusche et al., 1987; Arthur et al., 1987; Zagury et al., 1988; Nara et al., 1988; Thomas et al., 1988). Antibodies with anti-gp120 specificity mediate antibody-dependent complement-mediated cytotoxicity (ACC) against HIV-1-infected target cells (Nara et al., 1987b; Lyerly et al., 1987b). Anti-gp120 and anti-gp41 antibodies also mediate antibody-dependent cell-mediated cytotoxicity (ADCC) against HIV-1-infected cells (Blumberg et al., 1987; Ojo-Amaize et al., 1987; Lyerly et al., 1987a; Shepp et al., 1988; Koup et al., 1989). This suggests that antibodies with gp120 or gp41 specificity play a role in modifying HIV-1 infection transmitted either by free virus or by virus-infected cells having exposed HIV-1 env glycoproteins. Cytotoxic T lymphocytes specific for env glycoproteins are elicited as a result of infection with HIV-1 or by immunization with gp120/gp41 (Zagury et al., 1988; Walker et al., 1987; Takahashi et al., 1988; Koenig et al., 1988; Tyler et al., 1989). Thus both antibody-mediated and cell-mediated env glycoprotein-specific immune responses may contribute to protection against HIV-1 infection and/or to a delay of disease symptoms after infection.

Partial sequence homology between distinct segments of HIV-1 env glycoproteins and domains of functionally important host proteins suggested that immunization with the entire gp120 + gp41 sequence may elicit harmful autoimmune responses (Maddon et al., 1986; Reiher et al., 1986; Golding et al., 1988; Brenneman et al., 1988). Selected regions from HIV-1 gp120 and gp41 glycoproteins have immunosuppressive effects, suppress lymphocyte blastogenic responses and inhibit the activity of natural killer cells (Klasse et al., 1988; Nair et al., 1988; Chanh et al., 1988; Cauda et al., 1988). Most HIV-1-infected individuals have an antibody response to an
immunodominant region of gp41. The corresponding antibodies are not virus-neutralizing and may not have any protective effect (Wang et al., 1986; Certa et al., 1986; Palker et al., 1987; Gnann et al., 1987a). For these reasons it may be an advantage to delete from a potential HIV-1 protective immunogen sequences that may be involved in eliciting autoimmune responses and induce immune responses not relevant to protection. One possible way to accomplish this is to design synthetic peptide immunogens.

Infection or immunization with HIV-1 resulted in a predominantly type-specific virus-neutralizing antibody response (Putney et al., 1986, 1988; Nara et al., 1987a; Cheng-Mayer et al., 1988b; Goudsmitt et al., 1988; Matsushita et al., 1988; Looney et al., 1988). Immunization with HIV-1 env glycoproteins failed to protect chimpanzees against HIV-1 challenge (Berman et al., 1988; Hu et al., 1987). The failure of active immunization may be explained by the presence of distinct HIV-1 variants in the challenge virus, by the great diversity of variants selected under immunological pressure (Hahn et al., 1986; Robert-Guroff et al., 1986; Cheng-Mayer et al., 1988a; Reitz et al., 1988; Fisher et al., 1988; Saag et al., 1988). Therefore, immunogenic components derived from several distinct HIV-1 isolates will have to be incorporated into a broadly effective anti-HIV-1 vaccine. This can be accomplished more easily by incorporating into such a vaccine dominant protective epitopes rather than entire env glycoprotein sequences corresponding to an array of distinct virus isolates. The most feasible way to accomplish this is by the chemical synthesis of dominant protective epitopes corresponding to HIV-1 env glycoproteins from distinct isolates. The feasibility of this approach is supported by the finding that glycosylation of envelope proteins is not required for the induction of virus-neutralizing antibodies (Putney et al., 1986).

Long synthetic peptides (> 20 amino acid residues) containing both B and T cell epitopes derived from the env glycoprotein of hepatitis B virus (HBV) were highly immunogenic and elicited virus-neutralizing and protective antibodies recognizing env proteins from distinct HBV subtypes differing in amino acid sequence (Neurath et al., 1987, 1989a; Neurath & Kent, 1988). Based on this, we decided to synthesize long synthetic peptides from the sequence of the HIV-1 env glycoproteins gp120 and gp41 and to search for HIV-1 peptide immunogens with properties similar to selected HBV peptides.

Methods

Peptide synthesis. Fifty-one peptides derived from HIV-1 env glycoproteins gp120 and gp41 were synthesized (Table I). The

<table>
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<th>Sequence no.</th>
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* Entries 31 to 51 for peptide 303-338 from HIV-1 isolates: BH10 [1]; HBX2 [1]; HBX3 [1]; PY2 [1]; RF [1]; MN [0]; SC [1]; WMJ-1 [0]; WMJ-2 [0]; WMJ-3 [0]; ARV-2 [1]; NY-5 [1]; CDC-451 [1]; BR [1]; JY-1 [0]; MAL [1]; ELI [0]; Z-3 [0]; Z-6 [0]; LV-MA [1]; Z-321 [1]. The last seven isolates are from Africa. Numbers in brackets indicate number of N-glycosylation sites in the corresponding sequence of gp120. 'Daggers' (†) indicate that the source of peptides was F. All other peptides were from N.

numbering of amino acid residues corresponds to that described in Ratner et al. (1985). Peptides 1 to 30 are derived from the BH10 isolate of HIV-1. Peptides 31 to 51 are derived from a hypervariable loop in the gp120 sequence of distinct HIV-1 isolates. These loops are analogous to the loop in the sequence 303-338 of the BH10 isolate (Ratner et al., 1985). The sequences are derived from Myers et al. (1988) and Anand et al. (1989).

The peptides were obtained from three sources: Dr S. B. H. Kent, California Institute of Technology (K); Neo system Laboratories, Strasbourg, France (N); and Dr R. Fields, The New York Blood Center (F). Peptides from the first source (K) were synthesized as described by Kent & Clark-Lewis (1985). Peptides from the second source (N) were synthesized as follows: Boc-amino acids were purchased from Propeptide. Both Boc-Cys (4-MeBzl) PAM resin and Boc-Glu (OcHx) PAM resin were prepared as described (Plaue & Heissler, 1987). For trifunctional amino acids, the following side-chain protecting groups were used: tosyl for Arg, cyclohexyl for Asp and Glu, 4-methylbenzyl for Cys, benzyl for Thr, chlorobenzoxycarbonyl for Lys, 2,6-
tions were performed in dimethylformamide with a threefold excess of crude product was directly purified (without lyophilization) by medium purification procedures are described in more detail by Plaue & Heissler (1987) and Van Regenmortel & Heissler (1987) and Van Regenmortel (dimethylamino)-phosphonium hexafluorophosphate plus 1-hydroxy-benzotriazole activation in a 9-fluorenylmethoxycarbonyl-mediated synthesis (Biosearch Technical Bulletin No. 9000-03, Biosearch Incorporated). The sequence of each peptide was determined using an Applied Biosystems Protein Sequencer Model 477A with online HPLC analysis of PTH amino acids using an Applied Biosystems Model 120A PTH analyser.

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All peptides listed in Table 1, except 808-845, had either one or two cysteine residues in their sequence. If regions of HIV-1 envelope glycoproteins corresponding to the individual peptides did not contain cysteine residues, these residues were added either to the N or C termini of the peptide preceding by either Gly-Gly (GG) or Lys-Lys-Lys (KKK) spacers. The cysteine residues were utilized for the attachment of the peptides to protein carriers.

Control peptides from distinct regions of the HIV-1 envelope protein were S(105-120), S(121-151), preS(1-32), preS(72-94), preS(120-145) and preS(128-153) (Neurath & Kent, 1988; Neurath & Strick, 1989). S(105-120), S(121-151), preS(1-32), preS(72-94), preS(120-145) and preS(128-153) (Neurath & Kent, 1988; Neurath & Strick, 1989).

**Covalent linking of HIV-1 peptides to bovine serum albumin (BSA).** One mg of BSA (500 mg/ml in 0.1 M-phosphate–0.1 M-NaCl, pH 7.5) was mixed with N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP; 10 µl of a 10 mg/ml solution in ethanol) for 30 min at 25°C. The derivatized BSA was separated from excess SPDP by molecular exclusion chromatography on Sephadex G-25. Fractions corresponding to the void volume of the column were mixed with 1 mg of the respective peptides having cysteine residues, and reduced with 2-mercaptoethanol (10 mg/ml) in 0.1 M-ammonium bicarbonate and rapidly lyophilized before use. The mixture of SPDP-treated BSA and of reduced peptide was kept overnight at 25°C and then chromatographed on a column of Sephadex G-50. Pooled fractions corresponding to the void and retained volumes of the column were tested for the presence of the respective peptides.

For this purpose, polystyrene beads were coated with serial 10-fold dilutions of the pooled fractions in 0.1 M-Tris–HCl pH 8.8 overnight at 20°C. The beads were then coated with BSA (10 mg/ml) and gelatin (2.5 mg/ml). The peptide attached to the beads was detected by radioimmunoassays (RIA) as described below using antibodies to unconjugated homologous peptides. The efficiency of coupling was >90%.

**Double-antibody RIA.** Wells of 96-well polystyrene plates (Immulon II, Dynatech Laboratories) were coated with the respective synthetic peptides (200 µl; 20 µg/ml in 0.1 M-Tris–HCl pH 8.8) overnight at 20°C. The wells were then coated with BSA and gelatin (10 and 2.5 mg/ml, respectively). BSA was omitted from the second coating solution when antiserum to BSA–peptide conjugates were assayed for anti-peptide antibodies.

A pool of sera from 10 HIV-1-infected, apparently healthy individuals was used as a positive control in RIA assays and for mapping of B cell epitopes (Fig. 1b). The individual sera were selected on the basis of high levels of anti-HIV antibodies as determined by RIA. The dilution endpoint of anti-HIV-1 in the serum pool was 1:7 × 10^6 as determined using plates coated with HIV-1 proteins (DuPont).

For determination of anti-peptide antibodies, it was necessary to demonstrate that each of the synthetic peptides could effectively adsorb to wells of polystyrene plates. Each of the peptides was serially diluted (10 µg/ml to 10 µg/ml in 10-fold steps in 0.1 M-Tris–HCl pH 8.8). Each dilution was added to the wells and then dried with gelatin and used in a double antibody RIA as described below with 1:200 diluted homologous anti-peptide antiserum and control rabbit sera, respectively. The minimum concentration of peptide to obtain a significant difference (>2.5-fold) in RIA of anti-peptide sera in comparison with control sera was in each case between 100 and 200 µg/ml, i.e., by five orders of magnitude less than the actual concentration (20 µg/ml) of peptides used for coating wells in routine assays.

Wells of 96-well plates coated with recombinant gp160 (500 ng/ml; MicroGeneSys) were used to detect antibodies recognizing HIV-1 gp120 and gp41.

Control rabbit and human sera as well as anti-peptide antisera and sera from HIV-1-infected individuals were serially diluted (starting dilution exceeded 1:20) in a mixture of foetal bovine serum and goat serum (9:1; 0.1% Tween 20, adjusted to pH 8.0). The quantity of attached rabbit or human IgG was determined from the subsequent attachment of 123I-labelled second antibodies as described previously (Neurath et al., 1987). Dilution endpoints were calculated as described by Ritchie et al. (1983).

**Western immunoblots.** All rabbit antiseras (diluted 1:40) were assayed by immunoblotting using nitrocellulose strips with bound and electrophoretically separated HIV-1 proteins. These strips were from a commercial test kit for detection of anti-HIV antibodies (DuPont). The attachment of rabbit antibodies to the separated HIV-1 gp120 or gp41 was detected by biotinylated goat-anti-rabbit IgG antibodies (Bethesda Research Laboratories) followed by avidin-conjugated horseradish peroxidase from the DuPont HIV Western blot kit. Procedures recommended by the manufacturer of the kit were followed. Pooled human anti-HIV-1 was assayed at a dilution of 1:100.

**Immunization of rabbits.** NZW rabbits were immunized with 200 µg of the respective peptides in combination with complete Freund’s adjuvant. Two rabbits were used for each peptide. The rabbits were boosted with 200 µg doses of peptides in combination with incomplete Freund’s adjuvant at intervals of two weeks. Two weeks after each immunization, blood samples were taken and analysed for antibodies by RIA. Ten weeks after the initial immunization, the rabbits were sacrificed after the collection of blood by cardiac puncture. In some cases peptides linked to BSA were used to hyperimmunize rabbits after five doses of unconjugated peptides. The immunizing dose contained 200 µg of peptide. Antibody titres elicited by the same peptide in two rabbits differed by fourfold or less. Geometric mean titres are reported in the Results section.

To obtain antiseras to gp120 and gp41, rabbits were immunized with a preparation of membrane proteins obtained from 109-infected cells which are a richer source for HIV-1 envelope glycoproteins than are purified virus particles (Robey et al., 1986; Zweig et al., 1988). Cell membranes were isolated from the infected cells as described by Lodish et al. (1983). Rats were immunized with a membrane preparation obtained from 100 cells using complete Freund’s adjuvant. The rabbits were boosted with four additional two-weekly doses of equal amounts of cell membrane preparations in incomplete Freund’s adjuvant. The resulting antiserum reacted with cell extracts from both HIV-1-infected and uninfected cells (data not shown). In order to remove antibodies reacting with cellular components, the antiserum was adsorbed twice onto a column of insolubilized proteins from uninfected cells. A cell lysate from uninfected H9 cells was prepared, the nuclei were removed by centrifugation. Thirty-eight mg of IgG protein was attached to 1 g of CNBr–Sepharose. One ml of the antiserum was adsorbed onto a 2 ml
column of the derivatized H9-Sepharose for 30 min at 37 °C. The column was washed with Tris-buffered saline pH 7.2 (TS), the void volume was collected and the procedure was repeated using the same column which had been regenerated by elution with 4 M-MgCl₂ followed by washing with TS. The adsorbed antisera recognized HIV-1 proteins in Western immunoblots (Fig. 2; right lane; dilution 1:50) and was positive by double antibody RIA using HIV-1-coated wells (endpoint dilution 1:9000) and recombinant gp160-coated wells (endpoint dilution 1:2000). The serum before and after adsorption was assayed for anti-H9 antibodies by RIA using wells coated with H9 cell lysates (4 μg protein per well). The results of assays revealed that 98% of anti-H9 antibodies were removed by adsorption onto H9-Sepharose.

Virus neutralization assays. Anti-peptide antisera obtained after final bleedings of rabbits, as well as sera collected before immunization, used as controls, were tested. Immunoglobulins were precipitated by ammonium sulphate (40% saturation), redissolved in a volume of TS corresponding to the original volume of sera, and filtered through 0.45 μm pore filters. The presence of antibodies neutralizing HIV-1 was determined colorimetrically.

For the first assay, samples were serially diluted in RPMI 1640 medium containing 10% foetal calf serum (FCS) and 1% glutamine. Each dilution was filtered through 0.2 μm Centrex cellulose acetate discs. Filtered aliquots were added to wells of 96-well plates and mixed with an equal volume of diluted HIV-1 (m.o.i. of 0.003). After incubation for 1 h at 37 °C, MT-2 cells (Harada et al., 1985) were added to each well. After incubation for 1 h at 37 °C, the medium was removed and replaced by fresh medium. After incubation for 4 to 5 days at 37 °C the medium from each well was assayed for P24 using a kit from Coulter.

For the colorimetric assay, diluted and filtered samples in 96-well plates were mixed with an equal volume of diluted HIV-1 (m.o.i. of 0.003). After incubation for 1 h at 37 °C, MT-2 cells (Harada et al., 1985) were added to each well. After incubation for 1 h at 37 °C, the medium was removed and replaced by fresh medium. After incubation for 4 to 5 days at 37 °C the medium from each well was assayed for P24 using a kit from Coulter.

The titres of virus-neutralizing antibodies in human anti-HIV-1-positive sera (Fig. 1b). These peptides were 61-90, 138-164, 164-187, 219-245, 306-338, 477-508, 579-605, 771-802 and 845-862. This indicates that the human antibody response to gp120/gp41 is

Mapping of anti-protein antibodies is expected to identify sites which elicit anti-peptide antibodies recognizing the native protein antigen (Getzoff et al., 1988). All peptides, except 386-417 and 411-455, were recognized by rabbit anti-HIV-1 (Fig. 1a). This indicates that nearly the entire sequence of gp120/gp41 is immunogenic. Six control peptides from distinct regions of the HBV envelope protein did not react with this antisera. Antibodies recognizing distinct epitopes on gp120/gp41 were not equally represented in the anti-HIV-1 antisera, since the dilution endpoints of the distinct antibodies measured by double antibody RIA on peptide-coated plates varied by two orders of magnitude (range 1:100 to 1:10000). On the other hand, only nine of the 30 peptides synthesized were recognized by a pool of human anti-HIV-1-positive sera (Fig. 1b). These peptides were 61-90, 138-164, 164-187, 219-245, 306-338, 477-508, 579-605, 771-802 and 845-862. This indicates that the human antibody response to gp120/gp41 is

**Results**

Antigenicity of peptides from the gp120 and gp41 sequence of HIV-1 env glycoproteins

Long peptides (19 to 36 amino acid residues) were synthesized for the purpose of mimicking epitopes on native gp120 and gp41, because they have more chance of having a conformation similar to the corresponding portion of the native protein antigen (Van Regenmortel et al., 1988a). The reactivity of these peptides with anti-HIV-1 antibodies was first assessed, since peptide

Fig. 1. Antigenicity and immunogenicity of synthetic peptides from HIV-1 gp120 and gp41. Recognition of peptides by serially diluted rabbit anti-HIV-1 (a) and by a pool of anti-HIV-1-positive human sera (b). Results concerning the immunogenicity of the synthetic peptides are shown in (c) and (d). Endpoint dilutions of the anti-peptide antisera measured by RIA tests with homologous peptides (c) and recombinant HIV-1 gp160 (d). Dashed columns in (c) and (d) correspond to results obtained with peptides linked to BSA. Open columns correspond to results obtained with unconjugated peptides. Summary of results of Western blots and of virus neutralization tests is shown in (e). Open boxes correspond to peptides elicitating antibodies recognizing HIV-1 gp120 and gp41 in Western blots. Closed boxes correspond to peptides eliciting both Western blot-positive and virus-neutralizing antibodies.
restricted to limited regions of the HIV-1 envelope glycoproteins. Recognition of these regions varies according to the HIV-1-infected individual and changes during the course of disease (see Discussion). The peptide preferentially recognized by antibodies in human anti-HIV-1 was 579-605, derived from gp41 and overlapping peptides which have been recommended by others as reagents for diagnosis of HIV-1 infection (Wang et al., 1986; Gnann et al., 1987a).

Immunogenicity of synthetic peptides from HIV-1 gp120 and gp41

All peptides, except 458-488, elicited in rabbits in unconjugated form, i.e. without any carrier, and in combination with complete and incomplete Freund's adjuvant, anti-peptide antibody responses to the homologous peptides (Fig. 1c) but not to the six control peptides from the HBV envelope protein. The lowest levels of anti-peptide antibodies were elicited by peptide 518-542. Peptide 477-508 was the most immunogenic (antibody dilution endpoint titre > 1:10^6). In some cases, when peptides elicited low or intermediate levels of anti-peptide antibodies but not an anti-HIV-1 response, rabbits were further immunized with peptides conjugated to BSA after completion of an immunization schedule with free peptides. This resulted in an increased anti-peptide (Fig. 1c) and anti-HIV-1 (Fig. 1d) response. This suggests that linking of some of the peptides to a carrier changes the specificity of elicited antibodies and favours the production of antibodies recognizing the HIV-1 envelope glycoproteins, although most of the peptides elicited anti-HIV-1 antibodies without the need to be linked to a carrier protein (Fig. 1d).

To detect in anti-peptide antisera antibodies specific for denatured HIV-1 glycoproteins, all antisera were screened by immunoblotting. Antiserum to peptides 38-61, 138-164, 238-261, 331-361, 386-417, 411-445, 438-466, 547-571, 611-637, 683-707, 713-734 and 771-802, although positive in double antibody RIA, were negative in immunoblots (Fig. 1e, Fig. 2). All other anti-peptide antisera positive by double antibody RIA also gave positive results in immunoblots (Fig. 1e, Fig. 2). Among these, antisera to peptides from gp120 recognized gp120, in agreement with the observation that polyclonal antibodies to recombinant gp120 recognize in Western blots exclusively gp120 (Pinter et al., 1989), and antisera to peptides from gp41 recognized either gp41 [anti-(518-542) and anti-(639-666)] or both gp41 and gp160. The 'gp160' band in immunoblots corresponds to a tetramer of gp41 (Pinter et al., 1989), and anti-gp41 with distinct specificities may recognize either gp41 or 'gp160' preferentially. Additional unexpected bands were observed with anti-(164-187), anti-(518-542) and anti-(808-845). The reason why these three antisera recognized HIV-1 components other than 'gp160', gp120 and gp41 remains unexplained. The corresponding preimmune sera did not recognize any HIV-1 components by immunoblotting (data not shown).

Since all peptides except 458-488 and 518-542 were highly immunogenic without being linked to a protein carrier, the peptides must have recruited T cell help to produce antibodies, and consequently are presumed to have T helper (Th) cell epitopes. Th cell epitopes for native gp120 were identified within residues 410 to 443 (Cease et al., 1987; Berzofsky et al., 1988; Siliciano et al., 1988). To investigate whether covalent linking of a peptide encompassing a Th cell epitope to another peptide would enhance the antibody response to the latter peptide, peptide 411-445, encompassing a major Th cell epitope in humans and mice, was covalently linked to the peptide 306-338, representing a candidate component for synthetic HIV-1 vaccines (see below). The resulting dipeptide elicited an antibody response to both peptide components [the antibody dilution endpoints were 1:14200 and 1:173000 for anti-(306-338) and anti-(411-445), respectively], and to HIV gp160 (endpoint dilution 1:15000). Thus, the covalent linking resulted in a diminished antibody response (for comparison see Fig. 1c and d).

Virus-neutralizing activity was detected in antisera against 10 of the synthetic peptides either by inhibition of P24 synthesis or colorimetrically (Fig. 1e, Table 2).
Table 2. Neutralization of HIV-1 infectivity by anti-peptide antisera.

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<tr>
<td>808-845</td>
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</tbody>
</table>

However antisera to only seven of the peptides elicited antibodies which neutralized the infectivity of HIV, as measured by both methods. Complete inhibition of P24 synthesis occurred only with antisera to the peptide 306-338 and to the hybrid peptide (306-338)-(411-445) (Table 2). Virus-neutralizing antibodies were also elicited by a (306-338)-HBV surface antigen (HBsAg) conjugate (Neurath et al., 1989b) administered with alum as adjuvant. The virus-neutralizing activity of the anti-peptide antisera reflects the specificity of antibodies rather than the titre of antibodies recognizing the homologous peptides or HIV-1 gp120/gp41 (compare black boxes in Fig. 1e with Fig. 1c, d).

Antigenicity of peptides from a hypervariable region (303-338) of gp120 from 21 distinct HIV-1 isolates

Based on the results of virus neutralization tests, peptide 306-338 can be recommended as a potential immunogenic component of a synthetic HIV-1 vaccine. Since this peptide is derived from a hypervariable region of the gp120 sequence, analogous peptides from 21 distinct HIV-1 isolates were synthesized. These peptides in the region 303-338 contained three additional residues 303, 304 and 305, and thus had cysteine residues at both the N and C termini. Results presented in Fig. 3 show that antibodies present in a pool of anti-HIV-1 sera (from infected individuals in the New York area) differentiate between analogous peptides derived from distinct HIV-1 isolates. The peptides derived from isolates ARV-2, RF and HIV-1-BR were recognized optimally by these antibodies. Sequences corresponding to the related isolates BH10, HXB2, HXB3 and PY22, and to African isolates (JY-1, MAL, ELI, Z-3, Z-6) were less reactive with antibodies from this serum pool, as indicated by lower antibody dilution endpoints (Fig. 3). Rabbit anti-HIV-1, raised against the BH10 isolate, recognized the majority of peptides; peptides from the CD451 isolate and from some African isolates (Z-6, JY-1) were the least cross-reactive (Fig. 4).

Discussion

Many algorithms predicting the localization of B cell epitopes on proteins have been developed. These algorithms are limited to sequential determinants which often represent only portions of conformational sites. The reliability of predictions is limited. Therefore systematic empirical B cell epitope mapping using
synthetic peptides derived from the entire sequence of a protein is justified in vaccine design, despite the considerable amount of work involved (Getzoff et al., 1988). Polyclonal sera rather than a series of monoclonal antibodies are most suitable for this purpose. The entirely exposed protein surface accessible to immunoglobulins is expected to be antigenic. In agreement with this, rabbit anti-HIV-1 recognized synthetic peptides derived from nearly the entire length of the env glycoproteins gp120 and gp41. Portions of the gp41 sequence are not accessible on the surface of virus particles and represent either transmembrane spanning regions or cytoplasmic anchor sequences (Modrow et al., 1987). However all peptides derived from gp41 were recognized by rabbit anti-HIV-1. This full spectrum of anti-gp41 antibodies must either be a consequence of in vivo processing of this glycoprotein or the result of immunization with purified cell membranes from HIV-1-infected H9 cells. On the other hand, a pool of human anti-HIV-1-positive sera [preselected on the basis of high anti-HIV-1 antibody levels as measured by RIA on wells coated with disrupted HIV-1 (endpoint dilution titre 1:7 x 10⁶)] recognized only peptides derived from restricted portions of gp120 and gp41 sequences. This observation is in agreement with the general conclusion that responses of different species to sequential epitopes vary considerably (Getzoff et al., 1988). This bias of human anti-HIV-1 towards recognition of peptides from the HIV-1 gp120 and gp41 sequences probably cannot be ascribed to regions of homology between the virus glycoproteins and human host proteins. For example, peptide 808-845, sharing a region of homology with the human major histocompatibility complex class II peptide β-domain (Golding et al., 1988) and peptides 411-445 and 38-61, sharing homology with the human immunoglobulin heavy chain constant region (Maddon et al., 1986) were not recognized by human anti-HIV-1. On the other hand, peptide 845-862 sharing homology with human interleukin 2 (Reiher et al., 1986) and peptide 61-90 sharing homology with the human immunoglobulin heavy chain constant region (Maddon et al., 1986) are recognized by human anti-HIV-1.

Using shorter peptides, other investigators have identified regions in HIV-1 gp120 and gp41 recognized by antibodies from HIV-1-infected individuals. Some of these peptides overlap with longer peptides that were shown to be reactive with a pool of human anti-HIV-positive sera (Fig. 1b) (Klasse et al., 1988; Goudsmit et al., 1988; Modrow et al., 1989; Gnann et al., 1987a, b). Other peptides, synthesized by our group, and overlapping shorter peptides reported by others to react with human anti-HIV-1, were found to be non-reactive with antibodies from the anti-HIV-1-positive serum pool. The reason for this discrepancy is not known but may be ascribed to distinct anti-HIV antibody spectra in different individuals and to changes in antibody levels to distinct epitopes during the course of HIV-1 infection (Modrow et al., 1989). The major gp120 B cell epitope located within residues 504 to 518 (Palker et al., 1987) did not overlap with any of the peptides synthesized by us; we did not synthesize this peptide because the corresponding anti-peptide antibodies are not virus-neutralizing (Palker et al., 1987).

All peptides, except 386-417 and 411-445, were recognized by rabbit anti-HIV-1 and, except 458-488, elicited in rabbits antibodies recognizing HIV-1 gp120 and gp41 as measured by RIA and immunoblotting. Some of the anti-peptide antisera recognized gp120/gp41 as measured by both methods. However discrepancies between the two assay results were noticed in the case of other peptides (Fig. 1d and e). These discrepancies may be ascribed to distinct sensitivities using RIA and immunoblotting. Peptides eliciting virus-neutralizing antibodies (Fig. 1e, Table 2) overlapped the sequence of peptides reported by others to elicit such antibodies (Chanh et al., 1986; Ho et al., 1987; Dalgleish et al., 1988; Linsley et al., 1988; Palker et al., 1988; Rusche et al., 1988). However peptide 254-281 overlapping peptide 254-274, reported to elicit virus-neutralizing antibodies (Ho et al., 1988), failed to elicit antibodies with such properties. This discrepancy could be ascribed to either the increased length of the peptide or, more probably, to the use of different carriers (BSA instead of keyhole limpet haemocyanin) and different methods for coupling peptides to carriers. It has been reported that the carrier and the method of linking peptides to carriers may affect the specificity of anti-peptide antibodies (Dyrberg & Oldstone, 1986; Schaaper et al., 1989).

The highest level of virus-neutralizing antibodies was elicited by the unconjugated peptide 306-338, by the same peptide linked to another peptide representing a Tₘ cell epitope, or to HBsAg (Table 2). On the basis of the results of virus neutralization tests, peptide 306-338 or related peptides among the synthesized series should therefore be considered preferentially for inclusion into a potential anti-HIV-1 vaccine. This is in agreement with the finding that the immunodominant virus-neutralizing epitope on gp120 is located within this region (Goudsmit, 1988: Palker et al., 1988). Additional studies concerning ACC, ADCC, the elicitation of Tₘ cell immunity and of cytotoxic T cells are needed to determine which additional peptides should be included in a multicomponent HIV-1 immunogen.

It is of interest that peptide 306-338 elicited high levels of anti-peptide antibodies with virus-neutralizing properties, although the Tₘ cell epitopes on gp120 were reported to be located on distinct regions of the env glycoprotein (Cease et al., 1987; Siliciano et al., 1988).
Thus peptide 306-338 must have contained Th cell epitopes. Further studies are needed to determine whether or not the latter epitopes cross-react at the T cell level with native gp120. The attachment of peptide 306-338 to another peptide encompassing the reported Th cell epitope on gp120 (411-445) resulted in a decreased antibody response to 306-338 as compared with that elicited by the unconjugated peptide. Similar observations were made with synthetic peptides derived from the preS region of the HBV env protein. In that case, it was observed that linking of peptides corresponding to B cell epitopes to peptides corresponding to non-adjacent Th cell epitopes resulted in a decreased immune response as compared with that elicited by peptides containing adjacent B and Th cell epitopes (Neurath et al., 1989c).

It is remarkable that peptide 102-126, encompassing a reported Th cell epitope (Cease et al., 1987; Zagury et al., 1988), elicited antibodies recognizing HIV-1 gp120 which were virus-neutralizing. Thus this peptide also includes a B cell epitope from the C1 'constant' region of gp120 (Modrow et al., 1987) potentially contributing to protective immunity against HIV-1.

In general, the reactivity of anti-peptide antibodies with glycoprotein antigens is influenced by the attached carbohydrate moieties (Alexander & Elder, 1984). Glycoproteins gp120 and gp41 of the HIV-1 (BH10) isolate have 29 N-glycosylation sites; 22 of these are located on gp120. Carbohydrates represent approximately half of the Mr of gp120 (Geyer et al., 1988). Therefore both the recognition of gp120/gp41 by anti-peptide antibodies and the reactivity of anti-HIV-1 antisera with peptides from the gp120/gp41 sequence may be influenced by carbohydrate chains attached to the gp120 and gp41 proteins. In this respect, it is of interest that peptides 386-417 and 411-445 not recognized by rabbit anti-HIV are derived from regions containing four and one N-glycosylation sites, respectively (Table 1; Myers et al., 1988). Among the 30 peptides synthesized, 18 were derived from regions having N-glycosylation sites and 12 were from regions without such sites (Table 1). Peptides from regions without N-glycosylation sites were more frequently recognized by human anti-HIV-1 (five of 12) than peptides derived from regions with N-glycosylation sites (four of 18). Peptides eliciting virus-neutralizing antibodies (Table 2) were from both glycosylated (five) and non-glycosylated (five) regions. Peptide 306-338, the most effective in eliciting virus-neutralizing antibodies, is derived from a region of gp120 containing a single N-glycosylation site. Analogous regions of gp120 corresponding to HIV-1 isolates distinct from BH10 contain either one glycosylation site or no glycosylation sites (Table 1).

Virus-neutralizing antibodies directed towards epitopes located within residues 303 and 338 are subtype-specific (Goudsmid et al., 1988; Palker et al., 1988; Rusche et al., 1988). Therefore an HIV-1 immunogen expected to contain dominant epitopes involved in virus neutralization, must contain peptides derived from hyper-variable loops (sequences corresponding to residues 303-338 of the BH10 isolate) of several HIV-1 isolates in order to elicit broad immunity against HIV-1 infection. Towards this goal, peptides corresponding to hyper-variable loops of 21 HIV-1 isolates were synthesized which were differentially recognized by human (Fig. 3) and rabbit anti-HIV-1 (Fig. 4). However recognition of several of these peptides by the human and rabbit antibodies suggests substantial immunological cross-reactivity between peptides corresponding to subgroups of HIV-1 isolates. Of special interest is the finding that the BH10 as well as the closely related HXB2, HXB3 and PV22 peptides were recognized by anti-HIV-1 from a pool of human sera at much lower dilutions than were other non-African virus isolates (Fig. 3). Similar findings were made with an immunoglobulin preparation used for passive immunization of chimpanzees (data not shown). The lack of protection of these chimpanzees (Prince et al., 1988) may be explained by the inappropriate selection of the BH10 isolate as challenge virus. More attention should be devoted to appropriately matching the challenge virus subtype with the specificity of passively administered antibodies and/or with the subtype of HIV-1-derived antigens used for active immunization.

Further studies on the immunological cross-reactivity between antibodies elicited by synthetic peptides derived from the gp120 hypervariable loops (303-338) from distinct HIV-isolates, including studies on cross-neutralization of infectivity of distinct HIV-1 isolates, should provide a rationale for the selection of peptide components for HIV-1 immunogens eliciting broad protection against HIV-1 infection.

We thank Dr S. B. H. Kent, Dr R. Fields, J. P. Salley, and Neosystem, Strasbourg, France for peptide synthesis; Dr A. Hellman and Dr A. K. Fowler, S.R.A. Technologies Incorporated, Alexandria, Va., U.S.A. for help in virus neutralization assays; T. Huima for photography; N. Harrison and C. Abraham for typing; and Dr C. E. Stevens, Dr P. Ruhinstein and Dr P. Taylor for anti-HIV-1-positive human sera. This study was supported by grant CA43315 from the National Institute of Health.

References


Peptides from HIV-1 envelope glycoproteins


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(Received 2 May 1989; Accepted 12 September 1989)