Specificity and Function of the Individual Amino Acids of an Important Determinant of Human Immunodeficiency Virus Type 1 that Induces Neutralizing Activity

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SUMMARY

An important antigenic determinant of human immunodeficiency virus type 1 that induces neutralizing activity in infected humans and chimpanzees was previously mapped with nonapeptides between amino acids 307 and 320 on the external envelope glycoprotein (gp120) of strain HTLV-IIIB (molecular clone BH10) and amino acids 320 to 330 of strain HTLV-IIIRF. Using different sera we found different reactive nonapeptides that overlapped and shared a tetrapeptide, GPGR. This tetrapeptide, which is the same in HTLV-IIIB and HTLV-IIIRF, is flanked by amino acids that vary between virus strains. Because GPGR is predicted to form a β-turn and is flanked by two cysteine residues that may form a disulphide bridge, a hairpin-like structure is suggested for this part of gp120. The tetrapeptide GPGR and the reactive peptides are located on top of this structure, well exposed to antibodies. We determined the role of the individual amino acids in antibody binding using three sets of peptide analogues derived from three reactive nonapeptides (two of strain HTLV-IIIB which overlapped and one of strain HTLV-IIIRF). Each set contained peptide analogues in which each amino acid was replaced, one at a time, by all genetically encoded amino acids. At least five consecutive amino acids in each nonapeptide were essential for antibody binding. They include amino acids of GPGR and potentially provide the virus with ample opportunity to escape immune surveillance.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) induces the acquired immunodeficiency syndrome (AIDS). Soon after infection, when neutralizing antibodies appear, free virus antigens disappear from the serum (Goudsmit et al., 1986). After an asymptomatic period of variable duration, HIV-1 antigenaemia may reappear even in the presence of high neutralizing antibody activity (Goudsmit et al., 1988d). A similar sequence of events occurred when sheep were infected with visna virus (Narayan et al., 1981), a related virus of the lentivirus subfamily. During a visna virus infection neutralizing antibodies were induced that have an increasing neutralization range. Eventually, new pathogenic virus mutants occurred that evaded neutralizing antibodies. Similar events occur during an HIV-1 infection. It is suggested that HIV-1 might be able to ‘outrun’ the immune response by rapidly diverging to a population of related but distinct virus mutants (Coffin, 1986).

As treatment and prevention of AIDS may depend on our understanding of the inability of neutralizing antibodies to stop the reappearance of virus, we studied in detail an important antigenic determinant of HIV-1, which induces neutralizing activity. This determinant resides on the C-terminal portion of the external envelope glycoprotein (gp120) (Putney et al., 1986; Rusche et al., 1987, 1988; Goudsmit et al., 1988a, d). Using sera from HIV-1-infected humans
and chimpanzees we identified reactive nonapeptides between amino acids 307 and 320 of strain HTLV-IIIB (molecular clone BH10) and between amino acids 320 and 330 of strain HTLV-IIIRF with the PEPSCAN method (Geysen et al., 1984, 1985). Polyclonal and monoclonal antibodies raised against whole or parts of gp120 or raised against gp160 reacted also with these nonapeptides. The polyclonal antibodies were raised in goats with purified gp120, with baculovirus-expressed gp160, with C-terminal gp120 expressed in Escherichia coli and in rabbits with C-terminal gp120 expressed in E. coli (Goudsmit et al., 1988a, d, e). The monoclonal antibodies were raised with viral lysate (Bahravoi et al., 1988; Thomas et al., 1988). The reactive nonapeptides were located in an area that had been predicted to be antigenic, and named V3 (Modrow et al., 1987). Although both strains share the tetrapeptide GPGR in this immunodominant area, antibodies induced by strains of HIV-1 related to BH10 (HTLV-IIIB and LAV-1) react with nonapeptides from the 307 to 320 amino acid region of BH10 but do not cross-react with nonapeptides of the corresponding 320 to 330 region of HIV-1 strain HTLV-IIIRF and vice versa (Goudsmit et al., 1988a, b, c). Thus the strain specificities of the antibodies induced by this part of gp120 are defined by the amino acids that directly flank the tetrapeptide GPGR. Antibodies raised against the nonapeptides from this area, or against larger peptides that overlap this area, neutralize the virus and show cell fusion-inhibiting activities (Ho et al., 1987; Goudsmit et al., 1988d; Palker et al., 1988).

As the tetrapeptide GPGR is predicted to form a \( \beta \)-turn and because it is flanked by two cysteine residues (amino acids 296 and 331 of BH10 and amino acids 309 and 343 of RF) which may form a disulphide bridge, we suggested a hairpin-like structure for this part of gp120; GPGR is at the head of the loop and the arms are linked by a disulphide bridge. We studied the role of the individual amino acids in antibody binding with three sets of peptide analogues derived from three reactive nonapeptides (two of strain BH10 that overlapped and one of strain RF). Each set was made of peptide analogues in which each amino acid, one at a time, was replaced by all other genetically encoded amino acids.

**METHODS**

Sera. Serum samples (Gajdusek et al., 1985a, b; Goudsmit et al., 1987) were taken from three chimpanzees (A3D, A86B and A251) inoculated with HTLV-IIIB and from one chimpanzee (A22) inoculated with LAV-1. Two chimpanzees (A3A and A243B) were inoculated with whole blood taken from chimpanzee A22. Chimpanzee A304 was inoculated with whole blood taken from chimpanzee A243B. Chimpanzee A233 was inoculated with HTLV-IIIRF and chimpanzee A3 was inoculated with brain tissue from an AIDS patient with progressive encephalopathy. Sera from humans originated from homosexual males participating in a prospective study (De Wolf et al., 1987).

**HIV cell fusion inhibition assay.** Sup-T1 cells (1.5 \( \times 10^5 \) to 2 \( \times 10^5 \) cells/well) were attached to 96-well microtitre plates as described before (Goudsmit et al., 1988c). The medium was removed and B24\(^+\) cells (5 \( \times 10^5 \) to 25 \( \times 10^5 \) cells/well), washed twice with phosphate-buffered saline, were added to each well in 100 \( \mu l \) of Iscove's modified medium plus 10% foetal bovine serum and threefold dilutions of heat-inactivated (for 30 min at 56 °C) human serum. Reciprocal dilutions were 20, 60, 180 and 540. After incubation at 37 °C for 20 min the plates were centrifuged at 1000 r.p.m. for 10 min at 37 °C. All experiments were done in duplicate and each plate included immune and negative control sera. Syncytia were counted after 24 h of incubation at 37 °C. Ninety percent reduction of syncytia formation at a certain serum dilution after 24 h was taken as the titre of the syncytium formation-inhibiting antibody in the particular serum. A titre of 1:20 was considered significant.

**PEPSCAN.** Peptides were synthesized on solid supports and tested as described previously (Geysen et al., 1984, 1985). To prepare a set of replacement peptides (RNET) for RGPGRAFVT, IQRGPGRAF or ITKGPGRVI [these peptides start at positions 312 and 310 of gp120 of strain HTLV-IIIB (molecular clone BH10) and at position 320 of gp120 of strain HTLV-IIIRF], each of the nine amino acids in the appropriate peptide was replaced, one at a time, by the other 19 natural amino acids, while the rest of the sequence remained the same. An RNET consisted of 180 peptides, including nine copies of the parent sequence as controls. The scans were tested at a 100-fold dilution: a peroxidase-labelled anti-antibody was used as the second antibody at a 1000-fold dilution.

**RESULTS**

The reactive nonapeptides that we studied in detail were located previously on gp120 of HIV-1 between the two cysteine residues at positions 296 and 331 of strain HTLV-IIIB and at positions 309 and 343 of strain HTLV-IIIRF (Fig. 1; Goudsmit et al., 1988e), and were found always to contain the amino acids of the tetrapeptide GPRP. In other words this tetrapeptide is conserved between both strains.
Neutralizing determinant of HIV-1

Fig. 1. Structure of the immunodominant area on gp120 of HIV-1 strains HTLV-IIIB (to the left) and HTLV-IIIRF (to the right). (a) Schematic illustration of the area under investigation in each HIV-1 strain. We assumed that the cysteine residues which flank this area form a disulphide bridge between the arms of the hairpin-like structure. At the head of the hairpin-like structure the locations are shown of the nonapeptides of HTLV-IIIB maximally reactive with chimpanzee serum A3A (RIQRGPGRA) and with human serum 172/12 (RGPGRAFVT) and of the nonapeptide of HTLV-IIIRF maximally reactive with chimpanzee A233 serum (KGPGRVIYA). (b) Molecular models (without the amino acid side chain) of the immunodominant area. The structure of HTLV-IIIB (amino acids 293 to 334) and HTLV-IIIRF (amino acids 306 to 343) were generated with Macromodel (R. M. Liskamp, unpublished results). The amino acids important for immunological recognition are displayed with van der Waals’ dot surface.
Table 1. Serological reactivities of sera of one human and several chimpanzees infected with HIV-1

<table>
<thead>
<tr>
<th>Serum</th>
<th>HIV-1 inoculum</th>
<th>HTLV-IIIb titre*</th>
<th>Maximally reactive nonapeptide†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Unknown</td>
<td>60</td>
<td>RGPGRAFVT</td>
</tr>
<tr>
<td>172/12</td>
<td>Unknown</td>
<td>540</td>
<td>RGPGRAFVT</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td></td>
<td>180</td>
<td>RIQRGGRG</td>
</tr>
<tr>
<td>A304</td>
<td>LAV-1</td>
<td>65</td>
<td>IRIQRGPGR</td>
</tr>
<tr>
<td>A3A</td>
<td>LAV-1</td>
<td>60</td>
<td>IQRGPGRA</td>
</tr>
<tr>
<td>A3D</td>
<td>HTLV-IIIIB</td>
<td>60</td>
<td>IQRGPGR</td>
</tr>
<tr>
<td>A243B</td>
<td>LAV-1</td>
<td>60</td>
<td>PGRAFVTIG</td>
</tr>
<tr>
<td>A233</td>
<td>HTLV-IIIIRF</td>
<td></td>
<td>KGPGVR1A</td>
</tr>
</tbody>
</table>

* Titre is expressed as reciprocal dilution at which 90% of syncytia occur; – indicates titre <20.
† Sequence according to Myers et al. (1987); IRIQIGKPGGR of HTLV-IIIb starts at position 307 of gp120 and KGPGVR1A starts at position 322 of gp120 of HTLV-IIIIRF.

Table 1 shows that the serum of a human, infected with an unknown strain of HIV-1 (172/12), reacted with nonapeptides of strain HTLV-IIIb but not with those of strain HTLV-IIIIRF. The cell fusion inhibition titres underestimated the actual neutralizing activities, because the cell fusion inhibition test, although directly related to neutralizing activity, is less sensitive than a test measuring neutralizing activity. Thus values in the range of 60 must be regarded as intermediate titres and those in the range of 540 as high titres (see Palker et al., 1988). Sera of chimpanzees (A304, A3A and A243B) infected with strain LAV-1 or with strain HTLV-IIIb (A3D) reacted with nonapeptides of strain HTLV-IIIb but not with those of strain HTLV-IIIIRF. The serum from a chimpanzee (A233) infected with the latter strain reacted only with the nonapeptides of that strain and not with those of strain HTLV-IIIb. One serum sample, of a chimpanzee (A3) infected with brain tissue of a human who died of AIDS, did not neutralize the virus nor did it react with the nonapeptides of either strain.

We determined the role of the individual amino acids in antibody binding using three sets of peptide analogues derived from three reactive nonapeptides (two of strain HTLV-IIIb that overlapped and one of strain HTLV-IIIIRF). Each set was made of peptide analogues in which each amino acid, one at a time, was replaced by all other genetically encoded amino acids; a set of replacement peptides is named RNET. In the PEPSCAN high densities of peptides are used. Antibody binding with high densities of epitopes is thought to be independent of affinity (Nimmo et al., 1984; Griswold, 1987). This could lead to an overestimation of the low affinity bindings in the PEPSCAN as compared with other (affinity-dependent) methods. Therefore the absence of reactivities found with this method and with peptides substituted at certain positions seems rather significant.

One RNET was made for the sequence RGPGRAFVT which reacted maximally with human serum 172/12. The peptides of this RNET were tested with sera 172/12, A304 and A3 (Fig. 2). The results obtained using serum 172/12 show that four out of nine amino acids cannot be replaced; three irreplaceable amino acids, PGR, are from the conserved tetrapeptide GPGR, the fourth irreplaceable amino acid is F. A fifth amino acid, A, can be replaced by amino acids T, S or N only. The results obtained using serum A304 show that six out of nine amino acids are irreplaceable. A seventh amino acid, T, can be replaced by S only.

Another RNET was made for the sequence IQRGPGR which reacted maximally with serum A243B. The peptides of this RNET were tested with sera A3A, A3D and A243B (Fig. 2). The results with serum A3A show that five consecutive amino acids are irreplaceable: Q, R, G, P and G. The results with serum A3D are the same (not shown). The results with serum A243B show that six amino acids are irreplaceable: I, the first R, G, P, the second R and F. A seventh amino acid, the second G, can be replaced only by M.
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The last RNET was made for the sequence ITKGPGRVI of strain HTLV-IIIRF which reacted maximally with serum A233. The results with the peptides of this RNET show that of six consecutive amino acids, five are irreplaceable: K, G, P, G and V. Amino acid R, located between the second G and V, can be replaced only with K which has properties similar to those of R.

**DISCUSSION**

In this paper we studied an antigenic determinant of HIV-1 which induces neutralizing activity. Although we do not claim that it is the sole one (Ho *et al.*, 1988), it appears to play a major role in the induction of neutralizing activity after infection with HIV-1 or vaccination.
with gp120 or gp160 (Putney et al., 1986; Rusche et al., 1987, 1988; Goudsmit et al., 1988a, d, e). The determinant induces antibodies in most if not all sera of infected humans and chimpanzees which neutralize the virus or cause cell fusion inhibition (Goudsmit et al., 1988a). Peptides that span this determinant completely inhibit the fusion of HIV-infected cells (Rusche et al., 1988) and readily induce neutralizing activity when used to vaccinate animals (unpublished results) whereas a nonapeptide spanning GPGR is able to adsorb a substantial portion of the neutralizing activity from sera of infected chimpanzees as well as from human sera (J. Goudsmit & J. McKeating, unpublished results).

The reactive nonapeptides that we studied in detail were located previously on gp120 of HIV-1 between the two cysteine residues, at positions 296 and 331 of strain HTLV-IIIB (molecular clone BH10) and at positions 309 and 343 of strain HTLV-IIIRF (Fig. 1; Goudsmit et al., 1988a). The peptides form an antigenic determinant which induces neutralizing activity in the sera of primates. The determinant is located in an area on gp120 designated V3 by Modrow et al. (1987). The determinant spans the tetrapeptide GPGR which is conserved between European and American strains of HIV-1 and is predicted to be a β-turn. The amino acids that flank GPGR vary between strains of HIV-1. We assumed that the two cysteine residues located approximately 10 amino acids to the left and to the right of the antigenic determinant are linked by a disulfide bridge. Fig. 1(b) shows molecular models based on this assumption and on the assumption that GPGR forms a β-turn (R. M. Liskamp, unpublished results). The models for strains HTLV-IIIB and HTLV-IIIRF are very similar. They have a hairpin-like structure in which the arms are linked with a disulfide bridge and the head is the antigenic determinant.

The results of the RNET experiments (Fig. 2) show, in general, similar patterns irrespective of the species, human or chimpanzee, or of the HIV-1 strain used. Five or more consecutive amino acids appear essential for antibody binding; the sequence always includes amino acids of the tetrapeptide GPGR, the predicted β-turn. Geysen et al. (1988) made the assumption that if a certain amino acid can be replaced by a limited number of other amino acids, it too is essential for antibody binding. In the case of human serum 172/12, we found that A can be replaced in the RGPGRAFVT sequence by three other amino acids, T, S or N. Therefore this amino acid is regarded as essential for antibody binding. The RNET results show minor differences between the human and chimpanzee sera. This agrees with observations in other systems (Geysen et al., 1986; R. H. Meloen, unpublished results) and may reflect species-dependent differences in the B cell repertoire.

As the amino acids of the tetrapeptide GPGR are the same in strains HTLV-IIIB and HTLV-IIIRF of HIV-1, and that the antibodies raised against either strain react with homologous but not heterologous peptides, we suggest that the amino acids of GPGR do not interact directly with the antibody but play an indirect, although essential, role in antibody binding perhaps by maintaining the hairpin-like structure.

There are several reasons to argue that peptides reacting with antibodies may not properly represent the native structures reacting with antibodies. First, in the PEPSCAN, amino acid substitutions allowed at a given position may not be allowed in the native structure because they are lethal to the virus. This we cannot rule out and if it is the case, it emphasizes even more the irreplaceability of the amino acids of this determinant. The second argument is that amino acid substitutions not allowed in the PEPSCAN are allowed in the native structure. In general the assumption is that free peptides have a fixed structure (PEPSCAN peptides sitting at the end of long polycrylic strands surrounded by water). Available data suggest that this assumption may be wrong because small free peptides do not usually have fixed or strongly favoured conformations and are expected to adapt to the more structured antibody binding site (Getzoff et al., 1987). To what extent the substitutions reported here will be matched by substitutions of virus variants that escape the action of neutralizing antibodies remains to be seen. However, limited data obtained with escape variants of another virus (foot-and-mouth disease virus) and replacement studies did not show any incongruities (unpublished results). Thus we feel confident that the strict structural requirements of the determinant suggested by the RNET data are real.

RNA viruses mutate easily and because a single amino acid change is sufficient to mask the
neutralizing determinant, the presence in the determinant of five or more amino acids which are essential for antibody binding gives the virus ample opportunity to escape immunosurveillance.

Strain specificity of the neutralizing activity in serum of HIV-1-infected individuals broadens when the infection progresses (Goudsmit et al., 1988c) and is a general phenomenon for lentiviruses (Kono et al., 1973; Henson & McGuire, 1974; Ishii & Ishitani, 1975; Narayan et al., 1981). It has been suggested that during infection with a lentivirus mutants arise that escape the action of the existing antibodies. The new virus mutants may induce additional antibodies and thus broaden the strain specificity of the antisera. However antibody activity against the HIV-1 determinant, described in this report, remains strain-specific as infection progresses (J. Goudsmit, C. Thiriart & R. H. Meloen, unpublished observation); the broadening of the neutralizing activity is not paralleled by more extensive recognition of peptides in the V3 area. This indicates that the broadened neutralizing activity is not induced by peptides in the V3 area.

We speculate that the determinant in the V3 area is important for protection and that the immune system produces antibodies that recognize only the determinant on the virus that initiated the infection. When subsequent virus mutants arise the immune system is unable to produce changed antibodies that protect against the changed determinant. This could explain why AIDS occurs in the presence of neutralizing activity.

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