Analysis of murine antibody responses to baculovirus-expressed human immunodeficiency virus type 1 envelope glycoproteins

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An analysis of the humoral immunogenicity of a candidate AIDS vaccine (VaxSyn) in a murine model system is presented. Sera taken from a panel of mice immunized with the immunogen were analysed for their ability to bind a panel of gp120-representing peptides and limited reactivity to known sites of immunological interest was observed. Monoclonal antibodies (MAbs) were characterized as binding to a more restricted variety of regions on gp120 including C1, V2, V4 and the C terminus. A tetrazolium-based cytocidicity assay was used and shown to be an effective and objective method for the screening of human immunodeficiency virus (HIV)-neutralizing activity in large numbers of samples. None of the MAbs characterized in this study neutralize HIV-1 reference strains. The significance of these findings in view of previous publications is discussed.

Human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins (gp160, gp120 and gp41) are found on external surfaces on both virions and infected cells (Gelderblom et al., 1987) and are involved in the initiation of infection (Dalgleish et al., 1984; Maddon et al., 1986) making them suitable targets for a protective immune response. There is controversy as to the significance of neutralizing antibodies in humans infected with HIV-1, although the role of neutralizing antibodies in containment of many other viruses is documented (for example Schlesinger et al., 1983). Cytotoxic T cells are of significance in the clearance of persistent viral infection, but the antigen processing pathway for such a response requires de novo antigen synthesis in infected cells (Morrison et al., 1986). It is therefore logical to examine the humoral immune response to assess a non-infectious immunogen.

Baculovirus-expressed recombinant gp160 (Bacrgp-160; HIV-1MILYSL43), extracted from cells using urea, was purchased from MicroGenSys (VaxSyn, lot 8947P) and Bacrgp120 was produced using a HIV-1LAI construct (kindly supplied by T. Wilkinson). Young adult BALB/c mice were inoculated intraperitoneally and boosted 6 weeks later with 40 μg doses of antigen in Freund's incomplete adjuvant. Three days post-boost, splenocytes were fused with the myeloma cell line Sp2/0-Ag14 (Shulman et al., 1978) according to the method of Fazekas de St Groth and Scheidegger (1980). Results of immunochemical screening of gp120- and gp41-reactive MAbs derived from fusions (B and D) of two murine spleens are summarized in Table 1. The prefixes B/ and D/ indicate MAbs of fusions B and D, derived from mice immunized with Bacrgp160 and Bacrgp120 respectively. The majority of MAbs produced were of IgG1 isotype with κ light chains as assessed by ELISA using biotinylated antisera specific to murine heavy and light chains (Southern Biotechnology). MAbs were screened for anti-HIV activity in triplicate ELISAs using 100 to 200 ng/well of specific antigen [Bacrgp160, Bacrgp120, Chinese hamster ovary cell-derived recombinant gp120 (CHOrgp120; ADP 604) or 8E5 cell (Folks et al., 1986) lysate] which was adsorbed onto polybrene-treated microtitre plates. All MAbs reacted with at least two of the HIV-1 glycoprotein-containing preparations. For Western blot analysis, 1011 8E5 cells were lysed isotonic by Dounce homogenization, and cytoplasmic membranes prepared by centrifugation at 20000 r.p.m. for 20 h in an SW28 rotor using discontinuous 35, 55 and 70% (w/v) sucrose density gradients. This was followed by harvesting and pelleting of the interfaces. Pellets were dissolved in 6 ml of buffer (Laemmli, 1970) and 10 μl aliquots were resolved by SDS-PAGE (8% gels). Proteins were transferred to Immobilon P membrane (Millipore) as described (Towbin et al., 1979) using a semi-dry electroblotting apparatus at a constant current of 300 mA for 90 min. After blocking with 10% (w/v) non-fat dried milk (Marvel), membranes were probed with 1:100 dilutions of ascitic fluids. Five MAbs from fusion B (Bacrgp160) were mapped by Western blotting to gp120 (MAbs 27, 221 and 242) and gp41 (127 and 270) but only one MAb (3G5) from fusion D (Bacrgp120) reacted in Western blotting. For radioimmunoprecipi-
**Table 1. Immunochemical profiles of MAbs**

<table>
<thead>
<tr>
<th>MAb</th>
<th>Bac gp160 Titre</th>
<th>Bac gp120</th>
<th>CHO gp120 lysate</th>
<th>Titre†</th>
<th>Western blot</th>
<th>Light chain</th>
<th>Isotype</th>
</tr>
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<tbody>
<tr>
<td>B/27</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>160</td>
<td>160/120×</td>
<td>ND</td>
</tr>
<tr>
<td>B/33</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>160</td>
<td>x</td>
<td>k</td>
</tr>
<tr>
<td>B/37</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>160</td>
<td>160/41</td>
<td>k</td>
</tr>
<tr>
<td>B/127</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>320</td>
<td>x</td>
<td>k</td>
</tr>
<tr>
<td>B/234</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>160</td>
<td>160/41</td>
<td>k</td>
</tr>
<tr>
<td>B/242</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>640</td>
<td>160/120</td>
<td>k</td>
</tr>
<tr>
<td>B/270</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>10240</td>
<td>160/41</td>
<td>ND</td>
</tr>
<tr>
<td>D/3G5</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>160</td>
<td>x</td>
<td>160/120</td>
</tr>
<tr>
<td>D/6D1</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>5120</td>
<td>x</td>
<td>k</td>
</tr>
<tr>
<td>D/6B2</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>160</td>
<td>x</td>
<td>k</td>
</tr>
<tr>
<td>D/5E12</td>
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<td>-</td>
<td>1</td>
<td>1</td>
<td>160</td>
<td>x</td>
<td>ND</td>
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<tr>
<td>D/6A11</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>80</td>
<td>x</td>
<td>ND</td>
</tr>
<tr>
<td>D/4B5</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>320</td>
<td>x</td>
<td>ND</td>
</tr>
<tr>
<td>D/5A11</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>80</td>
<td>x</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ELISA results were scored on a scale of 1 to 4, relative to the background A450; -, less than 150%; 1, 150 to 200%; 2, 201 to 300%; 3, 301 to 500%; 4, greater than 500%. Background A450 values were determined using Sf9 cell (Smith et al., 1985) and CEM cell (Foley et al., 1965) lysates, and averaged 0-130.

† Titres were determined as the reciprocal dilution at which 50% of the peak A450 was extinguished using Bacrgp160 as the antigen. For MAbs that scored 3 or 4 against Bacrgp160 in ELISA, the titre is the recommended working dilution of the ascitic fluid.

x, No reactivity.
§ Glycoprotein(s) recognized.
|| ND, Not determined.

**Peptides**

Peptides were obtained from the MRC-ADP Reagent Project [reference numbers ADP 740 (1 to 47), ADP 792 (2.1 to 2.3) and ADP 737 (V3)] and adsorbed onto microtitre plates at 4°C overnight (10 μg/ml peptide in 50 mM-NaHCO3, 100 μl/well). Bound antibody was detected with biotinylated anti-species conjugates (Sigma) and revealed with ExtrAvidin-Alkaline Phosphatase (Sigma) using a diethanolamine substrate. Each antibody–peptide combination was assayed twice in triplicate and the mean absorbance recorded. The MAbs that were mapped are shown in Table 2 and in the following text the peptide numbers are related to domains identified by Modrow et al. (1987). Antibody B/221 mapped to peptide 45 (gp120 C terminus). Antibody B/33 mapped to peptide 14 (V2 domain), with some overlap to peptide 13. Antibody D/6D1 mapped to peptide 37 (V4 domain), with some overlap to peptide 35/36. All other mapped antibodies recognized peptides 8 to 10 derived from the C1 domain. The preponderance of gp120-reactive MAbs mapping to linear epitopes in this study (three of five from fusion B and all from fusion D) may reflect the use of urea during purification of antigen for immunization.

**MAbs**

MAbs (as ascitic fluids and concentrated immunoglobulin) and sera were assayed for their ability to neutralize HIV-1_MYO, HIV-1_HXB2 and HIV-1_09 strains, with and without 10 units (50% haemolytic complement)/ml of guinea-pig complement. C8166 cells were used in syncytium inhibition assays in combination with a tetrazolium-based cytotoxicity assay (Mossman, 1983;...
Pauwels et al., 1988; Hansen et al., 1989). HIV-1 stocks were prepared by centrifugation of infected C8166 cell cultures and supernatants were stored over liquid nitrogen in 1 ml aliquots, which were thawed once only and quantified as s.f.u./ml on immobilized C8166 cells after thawing. Tenfold dilution series of virus stocks were prepared in RPMI 1640 medium to a final volume of 250 μl that contained either 1:10 dilutions of ascitic fluids, 10 to 40 μg of purified immunoglobulin or 1:50 dilutions of neutralizing sera and MAb (ADP 358). Following incubation at 4 °C for 60 min, duplicate 100 μl aliquots of antibody–virus samples were placed in 96-well flat-bottomed tissue culture plates, overlaid with a suspension of C8166 cells (100 μl, 10⁵ cells) and the plates were incubated at 37 °C for 1 to 7 days. Cells were examined twice daily for syncytia and at the end of the incubation each well received 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; 7.5 mg/ml). The plates were incubated for a further 45 min at 37 °C. After addition of solubilizing solution [100 μl/well; 20% SDS, 50% dimethyl formamide (DMF) pH 4.5; Hansen et al., 1989] plates were incubated at 37 °C overnight and absorbances determined at 546 nm. C8166 cells (10⁵) challenged in the absence of neutralizing antibody with 2 × 10⁴ s.f.u./ml of HIV-1, for 6 days gave an A₅₄₆ of 0.07 (s.d. of 0.012), and challenge with 2 × 10⁵ s.f.u./ml gave a reading of 0.418 (s.d. of 0.118; 200 samples). Absorbances obtained following treatment with representative MAbs (B/27, B/234, B/33 and B/242) are shown in Fig. 1 (a). None of the MAbs produced in this study neutralized the HIV-1 strains tested, but assays performed with neutralizing sera (Fig. 1 a) and MAb (Fig. 1 b) demonstrated the utility of the technique. A less refined version of this technique has been described for use with HIV (Neurath et al., 1990; Pitts et al., 1991) and respiratory syncytial virus (Rubino & Nicholas, 1992). The isopropanol-based solubilization used by these workers is susceptible to signal quenching, unlike the DMF-SDS system (Hansen et al., 1989).

Since infected humans mount a non-neutralizing response to the C terminus of gp120 (Palker et al., 1987; Krowka et al., 1991), the non-neutralizing nature of MAb B/221 is not surprising. The majority of MAbs mapped in this study react with peptides 8 to 10 within the C1 domain (Table 2). This region has been implicated in the processing of gp160 to gp120 and gp41, and the formation of a gp120–gp41 binding site (Kowalski et al., 1987), although recent evidence refutes the latter finding (Wyatt et al., 1993; Moore et al., 1993). HIV-1-neutralizing rabbit antisera, which may contain antibodies to more than one epitope, have been raised to a peptide similar to peptide 8 (Neurath et al., 1990). A neutralizing murine MAb has been mapped to the central portion of peptide 8 (Niedrig et al., 1992). No neutralization escape variant was described for this MAb and neutralization, monitored as a decrease in reverse transcriptase activity, was recorded as less than 0.5 log₁₀, which is at least two orders of magnitude lower than that
required for indisputable evidence of neutralization of HIV-1 (Drake, 1993). It is possible that our antibodies recognize slightly different epitopes to the MAb of Niedrig et al. (1992) or that parameters such as relative concentrations account for the discrepancy in results. Strain-specific, strongly neutralizing antibodies may not correlate with in vivo protection and indeed the converse may be true.

MAb D/6D1 was mapped to the V4 domain but reacted only with Bacrgp160 and 8E5 cell lysates (Table 1). This indicates that the epitope is conformationally disrupted in some of the antigens used. The antibody may have been raised against a denatured, minor component of the Bacrgp120 preparation, for example one with decreased glycosylation compared to the bulk of recombinant gp120 (the V4 domain has three potential glycosylation sequences), or a denatured form produced during immunogen preparation.

Prior to mapping of MAb B/33 to the V2 domain, its non-neutralizing nature was identified (J. McKeaning, personal communication). This result was confirmed by independent workers (Moore et al., 1993). Two neutralizing murine MAbs, raised against gp120, have been mapped to different epitopes in the V2 domain (Fung et al., 1992; Moore et al., 1993) and V2 peptides have been used to raise neutralizing antisera in monkeys (Vahl et al., 1991) and rabbits (Neurath et al., 1990). More recently, four neutralizing murine MAbs, which mapped with the same peptide specificity as B/33, have been reported (McKeating et al., 1993). These MAbs and B/33 may recognize slightly different epitopes which are affected to different extents by glycosylation of the antigen, as there are two N-linked glycosylation sites in this region. In this context, the four neutralizing antibodies were raised against HIV-1, recombinant gp120 produced in CHO cells whereas B/33 was raised against Bacrgp160. The CHOgp120 structure may resemble more closely the gp120–gp41 complex on infectious virus than does Bacrgp160 by virtue of it having been processed effectively (from gp160 to gp120 and gp41) and containing carbohydrates of mammalian cell origin. These effects have been demonstrated for influenza virus haemagglutinin produced in the baculovirus system which, compared to haemagglutinin produced in mammalian cells, contained truncated oligosaccharides and had undergone incomplete oligomerization and processing (Kuroda et al., 1990, 1991).

Sera derived from eight mice of each major haplotype, BALB/c (H-2b), CBA/Ca (H-2k), and C57BL/6 (H-2d), immunized with Bacgp160, were analysed by pepscan. Twenty-four statistically significant responses were seen (Table 3). Of these, 14 (58%) were to C1 (peptides 8 to 10), two (8%) were to the C terminus of gp120 (peptides 40 and 44) and three (13%) were to the V3 domain (peptides 2.2, 2.3 and V3). None of the three latter responses occurred in BALB/c mice. The remaining five responses (21%) were to C1 (peptides 5 and 6), of which four (17%) were in BALB/c mice. Statistical analyses (of S.E.M.) demonstrated that anti-peptide responses varied...
between haplotypes of mice immunized with Bacrgpl60 (data not shown). This suggests that the use of mice of different haplotypes may facilitate the production of MAbs of chosen specificities. The serum from a BALB/c mouse immunized with Bacrgpl20 (M/120) showed significant responses to peptides 4, 12, 19, 20, 2.2 and 29 (Table 3). The sample size prevented us from confirming statistically that responses to the V3 domain, or other regions, are more frequent in Bacrgpl20- than Bacrgpl60-immunized mice. However, in chimpanzees, gp120 induces stronger anti-V3 domain responses than gp160 (Berman et al., 1990). Sera raised in rabbits, R1/87 (CHOrgpl20) and R2/87 (Bacrgpl60), showed broader ranges of anti-peptide responses than any mouse serum and both reacted with V3 domain peptides (Table 3), but R1/87 showed a stronger HIV-1-neutralizing response than R2/87 (Fig. 1a).

No MAb and only three polyclonal sera produced in mice (CBA-6, C57-5 and C57-6), using Bacrgpl60, showed reactivity with peptides representing the V3 domain (Table 3). The paucity of such responses concurs with reports on humans immunized with Bacrgpl60 (Viscidi et al., 1990; Redfield et al., 1991). In contrast, immunization of humans with CHOgp120imm (Genentech) induced antibodies that recognized CHOgp120 and Bacrgpl60, bound peptides representing the V3 domain, blocked CD4 interaction, inhibited syncytium formation and neutralized homologous (HIV-1imm) and heterologous (HIV-1spf) viruses in the majority of individuals (Schwartz et al., 1993). Immunization of baboons with CHOgp120spf (Chiron) induced strong responses to the V1, V2 and V3 domains and weaker responses to the V4 and V5 domains (Stephens et al., 1992).

The MAbs characterized in this study were derived from BALB/c mice and there were discrepancies between their specificities and those observed for polyvalent sera (Tables 2 and 3). Studies on the antibody response to hen egg-white lysozyme showed that the hybridoma repertoire may reveal a broader spectrum of responses than serum antibodies from a given individual or inbred strain (Metzger et al., 1984) and that there is an ordered progression in the recognition of different determinants on the protein (Harvey et al., 1979; Wicker et al., 1982). Sera were prepared from mice that had received two boosting inoculations, as opposed to one for MAb production, and this may have allowed changes in the repertoires to occur.

Rabbit sera R1/87 and R2/87 recognized the long peptides V3 and 2.3, but none of the shorter peptides representing the V3 domain. Further assays using all permutations of shorter peptides from this region also failed to induce measurable binding of the rabbit sera. These results suggested that the epitope(s) recognized by the sera were highly conformation-dependent. Binding of the sera to peptides V3 and 2.3 was also observed to be unaffected by the presence of excess DTT, suggesting that the serum-binding conformation of the peptides was not mediated by disulphide linkage of the peptide termini. This strong conformational requirement of antibody binding to V3 is not typical of previously described antibodies to this region, and may be species-specific. Removal of the V3-reactive component of R1/87 by affinity chromatography, using the V3 peptide, abolished binding of the serum to Bacrgpl60, but not to other peptides or to CHOgp120. This indicated that no other epitopes recognized by R1/87 on CHOgp120 were present in Bacrgpl60 and that at least a significant component of the Bacrgpl60 preparation contained an uncleaved V3 loop. Removal of the V3-reactive component of R2/87 did not prevent binding of the serum to Bacrgpl60 or CHOgp120, indicating that there were other epitopes in both preparations that were recognized by R2/87. However, as virus-neutralizing activity was absent from the V3-adsorbed sera, the significance of the non-V3 component of the response to Bacrgpl60 in R2/87 must be questioned.

This report provides evidence that HIV glycoproteins produced using baculovirus expression systems are poor antigens in that none of the MAbs neutralized the HIV-1 strains tested and some MAbs (for example B/27; Fig. 1a) appeared to enhance infection. It also shows that the use of mice of different haplotypes may produce MAbs directed to different domains of the glycoproteins and that the MTT cytocidicity assay is an objective method for the study of HIV-1 neutralizing activity.

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References


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