Properties of a neutralizing antibody that recognizes a conformational form of epitope ERDRD in the gp41 C-terminal tail of human immunodeficiency virus type 1

S. Matthew Cleveland,1† Tim D. Jones2 and Nigel J. Dimmock1

1 Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK
2 Axis Genetics plc, Babraham, Cambridge CB2 4AZ, UK

The possibility that epitopes from the C-terminal tail of the gp41 transmembrane protein of human immunodeficiency virus type 1 (HIV-1) are exposed the surface of the virion has long been contentious. Resolution of this has been hampered by the absence of any neutralizing monoclonal antibodies, but we have recently epitope-purified a neutralizing polyclonal IgG specific for one of the putative gp41 tail epitopes, 746ERDRD750. This was obtained from mice immunized parenterally with a plant virus chimera expressing residues 731–752 from the gp41 tail. The ERDRD epitope is highly conformational and is conserved in 81% of B clade viruses. Here, it is shown that this polyclonal ERDRD-specific IgG is highly potent, with an affinity of $2 \times 10^8 \text{ M}^{-1}$, and a neutralization rate constant ($-K_{\text{neut}}$) of $7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ that exceeds that of nearly all other known HIV-1-neutralizing antibodies. ERDRD-specific IgG gave 50% neutralization at 0.1–0.2 $\mu$g/ml and 90% neutralization at approximately 3 $\mu$g/ml. It also neutralized virus that was already attached to target cells, and this and other data suggest that it neutralized by inhibiting a virion event that precedes the fusion–entry process. Consistent with this conclusion was the finding that neutralizing amounts of ERDRD-specific IgG did not inhibit the attachment of free virus to target cells. ERDRD-specific IgG was also cross-reactive and neutralized all but one of six B clade T cell line-adapted strains tested.

Introduction

The presence of a neutralizing epitope situated in the cytoplasmic tail of the gp41 transmembrane envelope protein of human immunodeficiency virus type 1 (HIV-1) (Kennedy et al., 1986) has long been controversial and, until recently, there was no independent evidence that any part of the cytoplasmic tail was exposed on the outside of the virion at any stage of its existence. Indeed, one study of cell-associated gp41 concluded that the tail was not exposed on the surface of infected cells (Sattentau et al., 1995). The confusion was compounded by the unreproducible stimulation of neutralizing antibodies by sequences from the cytoplasmic tail of gp41. While several groups successfully obtained neutralizing antibody through immunization with conventional antigens or synthetic peptides (Charh et al., 1986; Dalgleish et al., 1988; Kennedy et al., 1986) or by using various antigen-presenting systems (Evans et al., 1989; McLain et al., 1995, 1996a, b; Newton et al., 1995), others were unsuccessful (Kalyan et al., 1994; Pincus et al., 1993), while still others were able to raise only weakly or non-neutralizing monoclonal antibodies (MAbs) (Dalgleish et al., 1988; Evans et al., 1989; Niedrig et al., 1992; Pincus et al., 1993; Vella et al., 1993). Furthermore, the antibody response to this region in infected individuals is weak or non-existent (Broliden et al., 1992; Davis et al., 1990; Goudsmit et al., 1990b; Niedrig et al., 1992; Pincus et al., 1993; Vella et al., 1991) and, lastly, although human and chimpanzee vaccinees immunized with gp160 responded strongly with antibodies against gp41 residues 731–752, these antibodies did not correlate with virus neutralization (Pincus et al., 1993, 1994). We suggested recently that the problem arises from the presence in the gp41 loop of a non-neutralizing epitope, 710IIEE712, that is immunogenically and antigenically domi-
nant over epitope \textsuperscript{74}ERDRD\textsuperscript{730} from which it is separated by just two glycine residues (see below) (Cleveland et al., 2000). The extent of the immunodominance of the IEEE sequence is host species-dependent. Rabbits immunized extensively with a cowpea mosaic virus chimera (CPMV–HIV\texttextsuperscript{1}) expressing a 22-residue peptide from the gp41 tail, \textsuperscript{731}PRGDRPEG–IEEEGGGERDRDR\textsuperscript{732}, made a strong response to both IEEE and the plant virus, but made no detectable neutralizing response or ERDRD-specific antibody. However, another plant virus chimera, CPMV–HIV\texttextsuperscript{29}, which expressed the \textsuperscript{745}GERDRD\textsuperscript{734} sequence, stimulated a strong neutralizing ERDRD-specific and ELISA response. In mice, the presumptive immunodominant effect of the IEEE sequence was quantitative and animals immunized with CPMV–HIV/29 made approximately 9-fold more neutralizing antibody than when immunized with CPMV–HIV/1. In addition to immunodominance at the level of antibody synthesis, there was antigenic neutralization with CPMV–HIV\textsuperscript{1} and animals immunized with CPMV–HIV\textsuperscript{29} had a higher ratio of IEEE-specific IgG to ERDRD-specific IgG than those that made a strong neutralizing response.

Here, we report on the properties of an ERDRD-specific neutralizing antibody. This antibody, made by immunization of mice with the CPMV–HIV/1 chimera, was epitope-purified by adsorption to and elution from a flocks house virus coat protein fused to the gp41 GERDRD sequence, which maintains its neutralizing conformation. We demonstrate that ERDRD-specific IgG is a high-affinity antibody that neutralizes with high specific activity and inhibited all but one of the B clad T cell line-adapted viruses tested. In addition, we show that it acts on a post-attachment step that rapidly becomes refractory to the effect of antibody once virus has attached to cells.

**Methods**

**Viruses.** Cowpea mosaic virus (CPMV) chimeras that express foreign peptides have been described by others (Porta et al., 1994; Dalsgaard et al., 1997). CPMV, a plant comovirus, has a genome consisting of two molecules of plus-sense, single-stranded RNA. Each has been cloned downstream of the cauliflower mosaic virus 35S promoter. Plasmid pCP2-0.51 contains RNA 2, which encodes the two virus coat protein molecules, S and L. The S protein is modified to contain a foreign peptide such that it is expressed on the outer surface of the CPMV chimeric virion. In the present study, chimera CPMV–HIV\textsuperscript{1} expresses the peptide \textsuperscript{731}PRGDRPEG–IEEEGGGERDRDR\textsuperscript{732}, from an external loop of the C-terminal tail of the gp41 transmembrane protein of HIV-1 strain IIIB (Cleveland et al., 2000), and CPMV–HIV/29 expresses \textsuperscript{745}GERDRD\textsuperscript{734}. Infectious virus was obtained by transfecting leaves of 10-day-old cowpea plants (Vigna unguiculata var. ‘Blackeye’) with a mixture of one of the linearized RNA 2 constructs with linearized cDNA of CPMV RNA 1 (pCP1). Virus was propagated and purified as described previously (Dalsgaard et al., 1997). There are 60 copies of foreign peptide per CPMV virion, and 1 ng of the CPMV–HIV/1 and CPMV–HIV/29 chimeras contains approximately 17 and 6 ng, respectively, of the expressed gp41 peptide. Virus concentrations were determined by the Bio-Rad assay.

For much of the neutralization work, we used HIV-1 strain IIIB. However, because this virus is specifically neutralized to about 50% by antibodies to wild-type (wt) CPMV (McLain et al., 1995), we prepared a neutralization escape mutant by passage of virus in the presence of anti-CPMV IgG, followed by limit-dilution cloning and screening for resistance to the selecting antibodies (S. M. Cleveland, T. D. Jones and N. J. Dimmock, unpublished). The escape mutant selected, HIV-\textsuperscript{1,cpmv/esc}\textsuperscript{1}, was completely resistant to neutralization by antisera to wt CPMV. A chronic infection of H9 T cells was set up with HIV-\textsuperscript{1,cpmv/esc} and stocks of virus were prepared in the usual way by co-cultivation with uninfected H9 cells. Cells were incubated for 3 days at 37 °C and placed in fresh medium 24 h before harvesting. Tissue culture fluids were clarified by low-speed centrifugation and stored in the vapour phase of liquid nitrogen. HIV-\textsuperscript{1,cpmv/esc} was used throughout this study. All of the HIV-1 B clade strains (IIIB, RF, MN, HXB-2D, a molecular clone of IIIB, and the candidate D clade virus CBL-4 (Goudsmit et al., 1990a)) were obtained from the AIDS Reagent Project, NIBSC, Potters Bar, UK. These were propagated as described above in H9 cells.

**Cells.** We used the human T cell lines H9 and C8166. Cells were grown in RPMI medium (Life Technologies) supplemented with 2 mM glutamine and 10% (v/v) heat-inactivated foetal calf serum (LabTech International), but without antibiotics.

**Immunization of mice with CPMV–HIV/1, CPMV–HIV/29 and wt CPMV.** C3H/He-mg mice (H-2\textsuperscript{b}), bred in house, were immunized at 6–8 weeks of age (McLain et al., 1995). Chimeras CPMV–HIV/1 and CPMV–HIV/29 or wt CPMV were filtered through a 0.2 µm membrane and dialysed against PBS, and then 100 µl containing 10 µg virus in aluminium hydroxide adjuvant (Inject Alum, Pierce & Warriner) was injected subcutaneously at multiple sites on days 0 and 28. Blood was obtained from the heart 14 days after the last injection. Antisera were stored at −70 °C and heated at 56 °C for 30 min before use.

**Flock house virus (FHV)-gp41 fusion proteins.** We used the FHV wt coat protein and fusion proteins FHV-L1-B, containing the HIV-1 gp41 sequence \textsuperscript{745}GERDRD\textsuperscript{734} and FHV-L2-A, containing the gp41 sequence \textsuperscript{110}IEEEGGGERDRDR\textsuperscript{734}. These were all cloned into pET vectors (Buratti et al., 1996) and expressed in Escherichia coli strain BL21. Proteins were separated on SDS–PAGE gels and extracted by electroelution as described previously. Both fusion proteins express ERDRD in its neutralizing conformation.

**Antibodies.** Polyclonal ERDRD-specific antibody from the serum of mice immunized with CPMV–HIV/1 was affinity-purified from nitrocellulose-immobilized FHV-L1-B (see above). Wt FHV coat protein alone yielded no HIV-1-neutralizing antibody (data not shown). IEEE-specific polyclonal antibody from the same antisera was purified in a similar way from a column of Sepharose to which the peptide SSPEGIEEGGSSS had been bound covalently. The isotype composition of purified antibody (mainly IgG; see Results) was determined by using the Stratagene IsoDetect kit. IgG was quantified by ELISA by using solid-phase goat anti-mouse IgG (Sigma) and a standard curve of mouse IgG. We used, in addition, MAbs 1575 specific for IEEE, MAb 1577 specific for ERDRD (Vella et al., 1993), MAb 2F5 specific for ELDKWA in the ectodomain of gp41 (Muster et al., 1993) and pooled human serum from HIV-infected (ARP510) and HIV-non-infected (ARP511) individuals. All were obtained from the AIDS Reagent Project, NIBSC, Potters Bar, UK. MAbs ICR39.13g to the CD4-binding site of gp120 (Cordell et al., 1991;
McKeating et al., 1992) was kindly provided by Jackie Cordell (Institute for Cancer Research, Sutton, UK).

**ELISA.** Wells of a microplate (Immulon 2; Dynex Laboratories) were incubated with protein overnight at 4 °C (0.1 µg/100 µl in 200 mM NaHCO₃, pH 9.6). After washing with Tris-buffered saline (TBS; 0.14 M NaCl, 0.02 M Tris–HCl, pH 7.6) and blocking with 2% BSA in TBS (Sigma) for 2 h at 20 °C, antibody (100 µl) in TBS containing 0.05% Tween 20 (TBST) and 0.5% BSA was added and plates were incubated overnight at 4 °C. Plates were washed with TBST and incubated with biotinylated anti-mouse, -rabbit or -human IgG (Amersham Life Science) for 2 h at room temperature. After washing with TBST, streptavidin–alkaline phosphatase (Amersham Life Science) was added for 1 h at room temperature. Wells were again washed and substrate (p-nitrophenyl phosphate, 1 mg/ml; Sigma) was added in buffer solution (diethanolamine, 0.5 M magnesium chloride, pH 9.8; Pierce & Warriner) at 37 °C. The resulting absorbance was measured at 405 nm.

**Assay of HIV-1-neutralizing antibody.** All virus strains used were syncytium inducers and the neutralization assay was based on the syncytium-inhibition assay of McLain & Dimmock (1994). Various amounts of antibody were incubated with 2000 syncytium-forming units (s.f.u.) per ml HIV-1, pmv/vesc for 1 h at the required temperature. Virus–antibody mixtures were then added to 2 × 10⁶ C8166 cells and incubated for 1 h at 37 °C to allow virus attachment. Cells were washed to remove excess virus and antibody and incubated for 3 days at 37 °C. The assay was linear up to 110 syncytia per well, and usually 50–100 syncytia in five replicate wells were counted under a microscope. The extent of neutralization was calculated as the percentage reduction in s.f.u. in wells containing antibody compared with the virus control.

**Kinetic neutralization assay.** HIV-1, pmv/vesc was incubated with 2 µg/ml ERDRD-specific affinity-purified IgG at 37 °C for various times and then inoculated onto C8166 cells as described above. Assay results were the means of five replicates. The rate of neutralization was derived from the slope of the line obtained by regression analysis, from a plot of logₐ [(V₀/Vₜ)] versus time, where V₀ is the infectivity titre at time zero and Vₜ is the titre after incubation at 37 °C for time t. The neutralization rate constant (−Kₙeut) was calculated from the equation:

\[ -K_{neut} = 2.3D \log_{10}(V₀/Vₜ)/t \]

where D is the reciprocal of the molar concentration of antibody.

**Post-attachment neutralization (PAN) assay.** This assay is based on that of Armstrong & Dimmock (1996). Briefly, C8166 cells (2 × 10⁸) were incubated with 2000 s.f.u. HIV-1 in medium (500 µl) for 2 h at 20 °C to allow virus attachment and then washed to remove unbound virus. These conditions gave maximum attachment and minimum fusion of virus to cells (S. J. Armstrong, personal communication). Cells were resuspended in dilutions of ERDRD-specific IgG in medium and incubated at 4, 21 or 37 °C for 1 h. After washing to remove antibody, cells were dispensed into five replicate wells in a 96-well plate and incubated at 37 °C for 3 days. Residual infectivity was determined by counting syncytia. A standard neutralization assay, under the same temperature conditions, was performed in parallel. In a separate study, the time taken for virus to become refractory to neutralization at 37 °C was determined. Virus was allowed to attach to cells at 37 °C at room temperature. Cells were then washed in medium at 4 °C and the temperature was raised to 37 °C. IgG was added at various times after the temperature increase. After 1 h, free antibody was removed by washing and the assay was continued as above.

**Measuring antibody affinity by surface plasmon resonance.** Affinities were determined by surface plasmon resonance with a BIACORE 2000 instrument (Biacore AB). The FHV-L2-A fusion protein (10 µg/ml) in acetate buffer, pH 5, was immobilized by succinate coupling to the carboxylated dextran polymer matrix of a CM-5 sensor chip, according to the manufacturer’s instructions, and the remaining active binding sites on the chip were blocked by the addition of 1 M ethanolamine. Approximately 3000 resonance units (3 µg) of FHV-L2-A was attached. Flowcell 1 with no antigen was used as a blank. Affinity measurements were made for at least four antibody concentrations at a flow rate of 20 µl/min. Buffer was then passed over the chip at 20 µl/min and the dissociation curve was followed for at least 600 s. After the dissociation phase, the captured antibody was removed with a 10 µl pulse of 0.1 M ammonium hydroxide (pH 11.5). The baseline was allowed to stabilize for at least 500 s before further use. Rates of association (kₐ) and dissociation (kₐ) were determined by using a software package (BIAevaluation 3.0) and the equilibrium association constant (Kₐ) was calculated from kₐ/kₐ퉓.

**Binding of ERDRD-specific affinity-purified IgG to soluble, recombinant (sr) gp160.** Various concentrations of srgp160 from baculovirus (sr gp160B; AIDS Reagent Project) were incubated with a critical amount (0.04 µg) of ERDRD-specific affinity-purified IgG at 20 °C for 2 h. Unbound IgG was then detected by binding to a 0.1 µg immobilized FHV-L1-B by ELISA as described above. MAB 2F5 to gp160 was used as a positive control and any unbound 2F5 was detected as before, but with immobilized sr gp160.

**Attachment of virus to C8166 target cells and the effect of neutralizing antibodies.** The assay has been described by Jackson et al. (1999). Briefly, virus (2 × 10⁴ s.f.u.) was purified on columns of Sephacryl S-1000 (Armstrong et al., 1996) and then allowed to attach to 3 × 10⁶ C8166 target cells for 2 h at 20 °C. Unattached virus was removed by washing. Cells were lysed with detergent and the attachment of virus was determined by measuring the amount of cell-associated virion p24 antigen by ELISA (see below). Virus controls typically gave λₐₙₐ readings of 0.5 with a background of 0.1. To determine the effect of antibodies on virus attachment, virus was incubated with dilutions of antibody for 1 h at 37 °C prior to the addition of cells. The assay then proceeded as described above. The extent of neutralization in each sample was determined by removing an aliquot of virus-inoculated cells and culturing these for 3 days at 37 °C as described above for the syncytium-inhibition assay.

**Assay of cell-associated p24 protein.** Cell pellets were lysed in 1% Empigen detergent (Calbiochem) in PBS at 56 °C for 1 h and virion p24 antigen was captured by incubation overnight at 20 °C with immobilized sheep anti-p24 polyclonal antibody (1 µg per well) (D7320; Aalto Bioreagents). After washing, non-specific binding sites were blocked as described above. Captured p24 was detected by incubation for 2 h with a biotinylated mouse anti-p24 MAb (AIDS Reagent Project) diluted 1:1000 in TBST plus 0.1% BSA. The assay was then developed as described above. Recombinant p24 (AIDS Reagent Project) was used as a standard when required.

**Results**

The functional affinities of neutralizing ERDRD-specific and other antibodies

Neutralizing ERDRD-specific polyclonal IgG and IEEE-specific antibody from the same antisera were purified as described above. These were mixtures of mainly IgG1 and G2a, although the former also contained G2b. Kappa light
chains predominated. We determined the on- and off-rate constants for bound FHV-L2-A protein, which contains the gp41 sequence \(7^{10}\text{IEEGGERDIRD}^{271}\), by surface plasmon resonance (Table 1). The equilibrium constant, \(K_A\), of affinity-purified ERDRD-specific polyclonal antibody was calculated as \(2.2 \times 10^7\text{ M}^{-1}\), a value considered respectable even for a MAb. This was approximately 30-fold greater than that of the ERDRD-specific MAb 1577, and resulted largely from a difference in their on-rates. The low affinity of MAb 1577 may contribute to its inability to neutralize HIV-1. The difference in their on-rates. The low affinity of MAb 1577 may contribute to its inability to neutralize HIV-1. The \(K_A\) of affinity-purified IEEE-specific polyclonal antibody was \(2.5 \times 10^7\text{ M}^{-1}\), about 9-fold lower than ERDRD-specific antibody made to the same immunogen in the same animals. This derived from lower values of both on- and off-rate constants.

Neutralization kinetics and rate constant
Neutralization followed essentially pseudo-first-order kinetics and was plotted as a two-component curve (Fig. 1). The initial neutralization component accounted for about 60% of infectious virus. The mean neutralization rate constant of the first component from two assays \((-K_{\text{neut}}\) was \(7.8 \times 10^4\text{ M}^{-1}\) s\(^{-1}\). This is at the high end of values for antibodies to HIV-1 IIIB, and second only to MAb b12 IgG (Krause et al., 1997). The mean neutralization rate constant of the second component was \(3.5 \times 10^4\text{ M}^{-1}\) s\(^{-1}\), about half the initial value.

Standard neutralization
ERDRD-specific antibody and virus were incubated together at various temperatures and then the efficacy of neutralization was determined by using C8166 cells and the syncytium-inhibition assay under standard conditions. At \(37\text{ °C}\), the \(N_{\text{sw}}\) was achieved at 0.12 \(\mu\text{g/ml}\) (Fig. 2a), while at 21 and 4 °C, it was 0.2 \(\mu\text{g/ml}\) (Fig. 2b, c). Thus, neutralization was essentially independent of temperature under these conditions.

Post-attachment neutralization (PAN)
PAN is the neutralization of virus that is already attached to its target cell. Individual HIV-1-neutralizing IgGs vary in their ability to cause PAN, some acting at any temperature up to \(37\text{ °C}\) up to the moment virus fuses with the cell, some only at temperatures of \(24\text{ °C}\) and below, while one was unable to give PAN at all (Armstrong & Dimmock, 1996; Armstrong et al., 1996; Jackson et al., 1999).

(i) Effect of temperature. Virus was allowed to attach to cells at \(20\text{ °C}\) for 2 h, during which time no detectable virus–cell fusion takes place. ERDRD-specific antibody was then added at the required temperature for 1 h. After washing to remove unbound IgG, cells were incubated at \(37\text{ °C}\) to develop syncytia. Fig. 2(a) shows that PAN at \(37\text{ °C}\) (\(N_{\text{sw}}\) 1 \(\mu\text{g/ml}\)) was reduced compared with standard neutralization (\(N_{\text{sw}}\) 0.12 \(\mu\text{g/ml}\)). At \(21\text{ °C}\) (Fig. 2b), standard neutralization was essentially unaffected (\(N_{\text{sw}}\) 0.2 \(\mu\text{g/ml}\)), but PAN now required \(~3\mu\text{g/ml}\) for \(N_{\text{sw}}\). At \(4\text{ °C}\), standard neutralization was again unaffected (\(N_{\text{sw}}\) 0.2 \(\mu\text{g/ml}\)), while PAN was reduced still further, with an extrapolated \(N_{\text{sw}}\) of approximately 10 \(\mu\text{g/ml}\) (Fig. 2c).

(ii) Time-dependence of PAN at \(37\text{ °C}\). The fusion–entry process of HIV-1 with the plasma membrane of the target cell is triggered by the binding of virion envelope proteins to the CD4 primary receptors and coreceptors. A complex multistage process is activated, in which the viral envelope proteins undergo major rearrangements preparatory to the fusion event itself. In this experiment, the PAN procedure above was modified by elevating the temperature of infected cells to \(37\text{ °C}\) after attachment of the virus and incubating for various times before adding ERDRD-specific IgG. Fig. 3(a) shows that 50% of virus became refractory to neutralization after 18 min, and 90% after approximately 50 min. Since there is minimal fusion after incubation of virus and cells for 120 min at \(37\text{ °C}\) in this system, it appears that the fusion event itself is not the

Table 1. Functional affinities of antibodies specific for the gp41 C-terminal tail determined by surface plasmon resonance with FHV-L2-A fusion protein
The polyclonal antibodies were epitope-purified (see Methods) from the same antiserum stimulated by immunization of mice with chimera CPMV–HIV/1. The MAbS are described elsewhere (Vella et al., 1993). FHV-L2-A expresses the gp41 sequence \(7^{10}\text{IEEGGERDIRD}^{271}\). \(K_A\) is calculated as \(k_{\text{on}}/k_{\text{off}}\). Numbers in parentheses denote the ranking order with respect to the property listed at the top of the column.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>(k_{\text{on}}) (M(^{-1}) s(^{-1}))</th>
<th>(k_{\text{off}}) (s(^{-1}))</th>
<th>(K_A) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal anti-ERDRD</td>
<td>8.43 \times 10^4 (1)</td>
<td>3.89 \times 10^{-4} (3)</td>
<td>2.2 \times 10^{6} (2)</td>
</tr>
<tr>
<td>Monoclonal anti-ERDRD (MAb 1577)</td>
<td>1.41 \times 10^4 (4)</td>
<td>1.98 \times 10^{-4} (2)</td>
<td>7.1 \times 10^{6} (4)</td>
</tr>
<tr>
<td>Polyclonal anti-IEEE</td>
<td>2.04 \times 10^4 (3)</td>
<td>8.12 \times 10^{-4} (4)</td>
<td>2.5 \times 10^{7} (3)</td>
</tr>
<tr>
<td>Monoclonal anti-IEEE (MAb 1575)</td>
<td>3.29 \times 10^4 (2)</td>
<td>6.54 \times 10^{-3} (1)</td>
<td>5.0 \times 10^{8} (1)</td>
</tr>
</tbody>
</table>
HIV-1 gp41-specific neutralizing antibody

![Graph](image)

**Fig. 1.** Neutralization kinetics of HIV-1cpmv/esc by ERDRD-specific affinity-purified IgG with C8166 cells. Virus (10^3 s.f.u./ml) was incubated with 2 µg/ml antibody at 37 °C for the times shown and the residual infectivity was assayed by the syncytium-reduction assay. A virus control held at 37 °C showed no loss of infectivity. A representative experiment is shown. Regression coefficients for the initial and second component curves were 0.96 and 0.94, respectively. The −K_neut value shown for each component is the mean from two assays.

Factor that limits PAN. It is more likely that the loss of PAN is the result of changes in conformation of the envelope protein that are initiated by the virus–cell interaction (see Discussion). For comparison, we used MAb ICR39.13g, which is specific to the CD4-binding site of gp120 (Fig. 3b). This gave entirely different kinetics, with a prolonged neutralization-sensitive phase, as described by others (Armstrong & Dimmock, 1996).

**Mechanism of neutralization:** ERDRD-specific IgG did not neutralize by inhibiting attachment of HIV-1 to target cells

Purified virus was neutralized with ERDRD-specific IgG and its attachment to C8166 cells was determined by assay of virion-associated p24 antigen. Fig. 4 shows that 3.3 µg/ml gave 87% neutralization but only 8% inhibition of attachment, not enough to account for the observed neutralization. With 1 µg/ml, there was 65% neutralization but < 5% inhibition of attachment. ERDRD-specific IgG thus behaves like MAb 2F5, which recognizes an epitope in the main ectodomain of gp41 and which also did not inhibit the attachment of neutralized virus to target cells (Fig. 4). The MAb 2F5 data agree with the findings of others (Mondor et al., 1998). The positive control for this experiment was an antisera pool from HIV-positive individuals, which inhibited attachment in proportion to the extent of neutralization. Serum from HIV-negative donors neither neutralized infectivity nor affected the attachment process (Fig. 4).

**Failure of ERDRD-specific IgG to bind to srgp160**

Since srgp160 contains all of the gp41 sequence and has been used as a putative vaccine, it was of interest to determine whether it possessed a functional ERDRD epitope as defined by binding of neutralizing, ERDRD-specific IgG. This reaction, using a critical amount of antibody (0.35 µg/ml), was carried out in solution to avoid the denaturation or masking of epitopes that can occur when antigen is bound to a solid-phase matrix. The control for the assay was MAb 2F5 (also...
0.35 μg/ml), which recognizes an epitope (ELDKWA) in the ectodomain of gp41. The amount of non-reacting ERDRD-specific IgG was then determined by reaction with immobilized FHV-L1-B and the amount of non-reacting 2F5 was determined by using immobilized srgp160, as described in Methods. Approximately 57% of MAb 2F5 bound srgp160, but there was no detectable binding of ERDRD-specific IgG (data not shown). In addition, there was no binding to srgp120 produced by baculovirus or CHO expression systems, as expected (AIDS Reagent Project; data not shown). Lack of binding of the ERDRD-specific IgG to srgp160 confirms the strong conformational nature of its epitope.

**Cross-neutralization of B and D clade viruses**

Fig. 5 shows the neutralization of five B clade viruses and one D clade virus by an ERDRD-specific antiserum made by immunizing mice with chimera CPMV–HIV/29. N_{50} titres derived from these and other data are summarized in Table 2. All B clade strains, with the exception of HXB-2D, were neutralized to within 4-fold of each other. This was surprising, as MN carries a T54R→T substitution that results in the loss of a positive charge. Presumably, T54R is not an essential part of the epitope. CBL-4, a candidate clade D strain (Goudsmit et al., 1990a), was also not neutralized significantly, possibly because of sequence changes at the centre of the epitope consensus sequence (Table 2).

**Discussion**

Affinity-purified polyclonal ERDRD-specific antibody neutralized HIV-1_{cpmv/esc} with an N_{50} of 0.1–0.2 μg/ml (Fig. 2). This compares favourably with two of the most potent HIV-1-neutralizing antibodies known: the N_{50} of human monoclonal IgG1 2F5, specific for the major ectodomain of gp41, is about 0.25 μg/ml for T cell-adapted virus in A3.01 cells (Ugolini et al., 1997) and that of b12 IgG, which recognizes the CD4-binding site of gp120, is 0.05 μg/ml in C8166 cells (McMenany et al., 1997). However, with increasing concentration, MAb b12 gave more neutralization than did our ERDRD-specific antibody. Their neutralization properties are consistent with the affinity data. ERDRD-specific IgG and 2F5 have similar equilibrium association constants (K_{A}: 2.17 × 10^8 M^{-1} for ERDRD-specific IgG and FHV-L2-A, and 2.4 × 10^8 M^{-1} for MAb 2F5 and recombinant baculovirus-expressed gp41 (Conlon et al., 1994), while the affinity of b12 IgG for
HIV-1 gp41-specific neutralizing antibody

**Fig. 5.** Neutralization of HIV-1 strains by ERDRD-specific antiserum prepared by immunization of mice with chimera CPMV–HIV/29. Virus and diluted antibody were incubated together for 1 h at 37 °C before determination of the extent of neutralization on C8166 cells by the syncytium-inhibition assay. Virus controls gave between 55 and 90 syncytia per well. Neutralization by pre-immune serum (-10%) has been subtracted. ■ Antiserum to CPMV–HIV/29; ▲ antiserum to wt CPMV. The viruses used were the B clade strains NL-4.3 and HXB2-D (both molecular clones of IIIB), RF and MN. CBL-4 is a candidate D clade virus (Goudsmit et al., 1990a). Data are the means of two separate experiments and bars represent ±1 SD.

srgp120 \((1.3 \times 10^9 \text{ M}^{-1})\); Burton et al., 1994) is only 6-fold higher than our value for the polyclonal ERDRD-specific antibody. Thus, considering that the ERDRD-specific antibody is a polyclonal preparation and that its \(-K_{\text{neut}}\) and affinity are the averages of those of all the ERDRD-specific antibodies present in the population, CPMV–HIV/1 appears to be a highly efficient immunogen. It is also notable that CPMV–HIV/1 did not appear to stimulate antibody that recognized the linear form of ERDRD (i.e. gp160) or solid-phase gp41 peptide 731–752 (Buratti et al., 1998; Cleveland et al., 2000). However, these data would also be explained if FHV-L2-B selected only neutralizing conformation-specific antibody. In contrast, a poliovirus chimera (Evans et al., 1989; Vella et al., 1993) that expressed essentially the same peptide stimulated poorly neutralizing antisera and ERDRD-specific MAbs (1577, 1583) that, in our hands, are not neutralizing, even as high titre ascitic fluids (Cleveland et al., 2000). As chimeric virus particles can be obtained in yields of 1–2 g/kg fresh leaf and can be purified in a relatively simple and cost-effective manner, these data attest further to their vaccine potential. Indeed, it has already been demonstrated that CPMV chimeras can stimulate immunity that protects animals against disease (Dalsgaard et al., 1997).

Different HIV-1-specific antibodies exhibit a diversity of neutralization kinetics (summarized in Krause et al., 1997). They can be pseudo-first order (e.g. b12 IgG; McLnerney et al., 1997) or multi-hit (e.g. MAbs ICR41.1i and ICR39.3b; McLain & Dimmock, 1994) and can comprise a single component curve (e.g. b12 IgG; McLnerney et al., 1997) or have a second, slower component (e.g. MAb F58; Jackson et al., 1999). Rate constants \((-K_{\text{neut}}\) for the neutralization of HIV-1 IIIB and closely related viruses range from \(3.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}\) for MAb b12 to \(6.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}\) for MAb ICR39.3b (Krause et al., 1997). ERDRD-specific IgG had a two-component curve, and its initial neutralization rate constant \((-K_{\text{neut}} = 7.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}\); Fig. 1) ranked it below only b12 IgG.

Our other recent work has demonstrated that a loop of the gp41 cytoplasmic tail is exposed on the surface of free virions
Table 2. Summary of the neutralization of HIV-1 strains by murine antiserum to chimera CPMV–HIV/29

Data were taken from Figs 3 and 6 and unpublished data. Sequence data are from the literature, where an upper-case letter indicates that this residue is 100% conserved and a lower-case letter that there is 50% conservation (Myers et al., 1993). —. Residue is the same as that shown for IIIB<sub>CPMV/ESC</sub>. CBL-4 is a candidate clade D virus (Goudsmidt et al., 1990a) and the consensus region for clade D viruses is given. Neutralization was determined by syncytium inhibition. NA. N<sub>50</sub> was not achieved.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence compared with the B clade Env protein consensus</th>
<th>1/N&lt;sub&gt;50&lt;/sub&gt; with antiserum to CPMV–HIV/29</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIIB&lt;sub&gt;CPMV/ESC&lt;/sub&gt;</td>
<td>74GERDRDR&lt;sup&gt;751&lt;/sup&gt;</td>
<td>890</td>
</tr>
<tr>
<td>RF</td>
<td>————t</td>
<td>316</td>
</tr>
<tr>
<td>MN</td>
<td>————t</td>
<td>250</td>
</tr>
<tr>
<td>NL-4.3</td>
<td>————t</td>
<td>200</td>
</tr>
<tr>
<td>HXB-2D</td>
<td>—rG—d—</td>
<td>NA</td>
</tr>
<tr>
<td>CBL-4</td>
<td>—rG—d—</td>
<td>NA</td>
</tr>
</tbody>
</table>

(S. M. Cleveland, T. D. Jones and N. J. Dimmock, unpublished). This evidence centres on the use of neutralizing antibody specific for a highly conformational epitope with the sequence 74<sup>ERDRDR<sup>751</sup></sup> (Buratti et al., 1998; McLain et al., 1996a, b) (according to the numbering system of Ratner et al., 1985). We showed that (i) ERDRD-specific IgG binds free HIV-1 particles in solution and that this virus remains neutralized after unbound antibody is separated from putative virus–antibody complexes by centrifugation (by using infectivity as the read-out, the problem of broken virus particles in which the epitope might be artefactually exposed was avoided); (ii) ERDRD-specific IgG failed to bind to protease-treated wt HIV-1 virions; (iii) a deletion mutant lacking most of the C-terminal gp41 tail was not neutralized, implying that the epitope was present in that location; and finally, (iv) neutralizing antibody escape mutants selected by growth of virus in the presence of ERDRD-specific antibody carried a single amino acid substitution adjacent to the epitope that eliminated the binding of the selecting antibody (S. M. Cleveland, L. McLain, T. D. Jones, C. Parry and N. J. Dimmock, unpublished data). Combined experimental and modelling data suggest that gp41 has a minimum of three transmembrane (tm) domains. The first two arise from the existing tm sequence (residues 691–712), which is interrupted by a predicted β-turn that carries the putative external loop of approximately 60–70 residues to the outer surface, while the third tm domain takes the remainder of gp41 C-terminal tail back into the interior of the virion (S. M. Cleveland, T. D. Jones and N. J. Dimmock, unpublished data).

Since our finding that the ERDRD neutralizing epitope was present on an externalized loop of the C-terminal tail of HIV-1 that had been considered previously to be inside the virion (S. M. Cleveland, T. D. Jones and N. J. Dimmock, unpublished), one of the key issues is to determine the function of this loop in the life-cycle of the virus. The fact that the virus could be neutralized without affecting its attachment to target cells (Fig. 4) indicated that the ERDRD sequence is not involved in attachment to the C8166 T cell line used in this study. In addition, we have presented data that show the ability of the ERDRD-specific IgG to give PAN (Figs 2 and 3), which demonstrates positively that it can inhibit a post-attachment step. PAN is useful, as it gives insights into the virus–cell interaction, and we have shown that the neutralization-sensitive step becomes 50% refractory to PAN after only 18 min at 37 °C (Fig. 3a). Thus, since 50% fusion takes about 90 min at 37 °C in this system (Armstrong et al., 1996), it is likely that virus–cell membrane fusion is the neutralization target. It may be that, during this time, conformational changes result in the ERDRD epitope becoming progressively less available to antibody or, alternatively, that an antibody-sensitive downstream function has been completed by the time antibody is added, so that neutralization can no longer take place. It is interesting and potentially important to protection in vivo that neutralizing IgGs vary greatly in their ability to give PAN. Of six neutralizing antibodies tested, two (MAbs ICR39.13g and b12) give PAN at 37 °C for a prolonged period and probably until fusion takes place, but the others (the ERDRD-specific IgG, MAbs ICR39.3b, ICR41.1i, F58 and the F58 MicroAb) enacts PAN only for a much shorter period of time (Fig. 3b; Armstrong & Dimmock, 1996; Armstrong et al., 1996; Jackson et al., 1999; unpublished data). Such antibodies provide the means to dissect out the complex events that constitute the fusion process, particularly as they recognize various different parts of the envelope protein, including the CD4-binding site region (ICR39.3b, MAbs ICR39.13g and b12), sequences within the V3 loop (ICR41.1i, F58 and its MicroAb) and the gp41 loop structure (ERDRD-specific IgG).

Finally, it is of interest that the ERDRD epitope is conserved within 81% of 31 B clade viruses (Myers et al., 1993) and that 4/5 B clade viruses that we tested were neutralized (Fig. 5; Table 2).

Virus strains IIIb, RF and NL-4.3 all have 74<sup>GERDRDR<sup>751</sup></sup>, the gp41 clade B consensus sequence, and were neutralized by antisera generated to CPMV–HIV/29. MN, which has a 751R→T residue change, is still neutralized, suggesting that residue 751 is not an essential part of the epitope and is not critical for maintaining its conformation. However, the N<sub>50</sub> titres of these viruses varied by over 4-fold (Table 2), suggesting that their epitopes were not all optimal. Unexpectedly, HXB-2D, a molecular clone of IIIb, was not neutralized. The reason for this is unclear, as we verified that the epitope and surrounding residues (amino acids 699–806) were unchanged (C. Parry and S. M. Cleveland, unpublished data), but might be explained by residue changes far away from the epitope that affect its conformation. Such changes were recorded when T→A in the gp41 transmembrane protein.
of HXB2 (Reitz et al., 1988) caused neutralization resistance to antibodies that bind the gp120 CD4-binding site (Klasse et al., 1993). Alternatively, it may be that the ERDRD epitope of HXB-2D is masked by gp120 or host cell proteins incorporated in the virion. The resistance of CBL-4, a candidate clade D virus, to neutralization is probably due to polymorphisms, notably $^{746}_G$ instead of $^{746}_D$ in the centre of the ERDRD epitope, that prevent recognition by ERDRD-specific antibody.

We thank Dr E. Buratti and Professor F. Baralle (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy) for flock house virus proteins and clones, Dr C. Vella (NIBSC) and Dr H. Holmes (AIDS Reagent Project, NIBSC) for MAbs 1575 and 1577 and Professor H. Kattinger and Dr M. Putsche (Institute of Applied Microbiology, University of Agriculture, Vienna, Austria) for MAb 2F5. We also thank Dr R. Desrosiers (Harvard Medical School, Southborough, MA, USA) and the NIH AIDS Research and Reference Reagent Program for HIV-1 NL-4.3. The other HIV-1 strains were provided by the AIDS Reagent Project. Ms Jackie Cordell (Institute for Cancer Research, Sutton, UK) kindly provided MAb ICR39.13g. Mrs Jean Westerman and her staff gave their usual high standard of technical support. We are also grateful to Dr W. D. O. Hamilton (Axis Genetics) for his continuing interest and critical input. S.M.C. was supported by a BBSRC CASE studentship in conjunction with Axis Genetics. Antibody work in the laboratory of N.J.D. is supported by grants from the National Heart, Lung and Blood Institute, NIH (5R01HL59726-02) and the WPH Charitable Trust. The BIACORE 2000 was purchased with a grant to N.J.D. from the Research Councils’ Joint Research Equipment Initiative.

References


Ala582 → Thr) decreases viral neutralization by monoclonal antibodies to the CD4-binding site. *Virology* **196**, 332–337.


Received 5 October 1999; Accepted 20 January 2000