Epitope mapping of the V3 domain of feline immunodeficiency virus envelope glycoprotein by monoclonal antibodies

Stefania Lombardi, Claudia Massi, Franco Tozzini, Lucia Zaccaro, Agostino Bazzichi, Patrizia Bandecchi, Corinna La Rosa, Mauro Bendinelli and Carlo Garzelli

1 Retrovirus Center, Department of Biomedicine and 2 Department of Animal Pathology, University of Pisa, 56127 Pisa, and 3 Department of Cell Biology, University of Camerino, 62032 Camerino, Italy

A panel of six IgG monoclonal antibodies (MAbs) was produced by immunizing mice with a 22 amino acid synthetic peptide, designated V3.3, of the third variable region of feline immunodeficiency virus (FIV) envelope glycoprotein. This peptide is known to induce neutralizing antibodies in cats. In ELISA all MAbs reacted with purified SDS-disrupted FIV and in flow cytometry all MAbs stained permeated, persistently infected FL4 cells but not unfixed FL4 cells; this indicated that the MAbs recognize essentially cryptic epitopes of the gpl00 V3 loop. By direct ELISA using partially overlapping synthetic peptides and by competition binding studies, the anti-V3.3 MAbs were shown to detect at least four distinct epitopes, two located in the amino-terminal half and two in the carboxy-terminal half of the sequence. When tested for neutralizing activity by the syncytium inhibition assay in Crandell feline kidney cells, all anti-V3.3 MAbs neutralized FIV at high dilution. However, at low dilution two MAbs exhibited much less neutralizing activity. These results indicate that the V3 region of FIV contains multiple epitopes involved in neutralization.

Introduction

Feline immunodeficiency virus (FIV) is a widespread lentivirus of domestic cats that shares numerous biological and pathogenetic features with the human immunodeficiency virus (HIV). FIV infection in cats has therefore been proposed as an animal model for AIDS studies with respect to pathogenesis, chemotherapy and vaccine development (Pedersen, 1993; Bendinelli et al., 1995).

Similar to HIV, FIV infection induces both antibodies that neutralize different FIV isolates and type-specific neutralizing antibodies (NA). Broadly reactive NA can be assayed by inhibition of FIV infectivity for the Crandell feline kidney fibroblastoid (CrFK) cell line (Tozzini et al., 1992, 1993), whereas type-specific NA are demonstrable using lymphoid cells (Baldinotti et al., 1994). The FIV surface protein (SU) gpl00, which is encoded by the env gene, appears to contain antigenic domains involved in virus neutralization. As in HIV and other lentiviruses, the env gene among different isolates shows sequence diversity that clusters in discrete segments known as variable (V) regions. Eight or possibly nine such V regions have been identified in FIV (Phillips et al., 1990; Morikawa et al., 1991; Siebelink et al., 1992; Rigby et al., 1993; Pancino et al., 1993a, b). Three possible sites directly or indirectly involved in FIV neutralization have been described: two map in the regions V4 and V5 of gpl00, as shown by the fact that a single amino acid substitution in these domains (483 and 560) confers resistance to virus neutralization (Siebelink et al., 1993, 1994), while the third neutralization site is located in the V3 region [Cys373–Cys424 according to Phillips et al. (1990) or Cys348–Cys397 according to Talbot et al. (1989); the latter numbering is used in the present paper]. The neutralization site on V3 was revealed by showing that a 22 amino acid synthetic peptide designated V3.3, spanning the sequence Gly285–Phe406 of the Petaluma isolate of FIV (FIV-P), reduced the FIV-neutralizing activity of pooled immune cat sera. Furthermore, when injected into specific pathogen-free (SPF) cats, V3.3 elicited NA that effectively neutralized the infectivity of the homologous and a heterologous FIV isolate for CrFK cells (Lombardi et al., 1993).

In this paper we report on the fine mapping of the FIV V3 neutralization domain using a panel of monoclonal antibodies (MAbs) raised by immunizing mice with the V3.3 peptide.

Methods

Virus. FIV-P was propagated in persistently infected CrFK cells or FL4 cells (kind gift of Dr J. Yamamoto, University of Florida, Fla., USA) as previously described (Yamamoto et al., 1988, 1991). Virus was
concentrated from tissue culture supernatants by ultrafiltration (Minital; Millipore) and purified by density gradient centrifugation (Montelaro et al., 1982). For immunochemical assays, purified FIV was disrupted in PBS containing 0.25% SDS at 4°C for 2 h with gentle stirring.

**Synthetic peptides.** V3-based synthetic peptides were synthesized as already described (Lombardi et al., 1993). In brief, on the basis of the published sequence of the 34TF10 clone of FIV-P (Talbot et al., 1989) three partially overlapping 22 or 23 amino acid peptides of the variable V3 domain were manually synthesized using N-FMOC-protected amino acids and a p-alkoxybenzyl alcohol resin as solid phase. The purity of peptide preparations, assessed by HPLC, exceeded 90%. The amino acid sequence (one letter code) of the three synthetic peptides named V3.2, V3.3 and V3.4 spanning residues 375 to 417 of the deduced env V3 domain (Talbot et al., 1989) are:

- **V3.2**
  - FHCQRTQSQPGRWFRAISSWKQ

- **V3.3**
  - GSWFRAISSWKQRNRWEWRPDF

- **V3.4**
  - QKRNRWEWRPDFSKKVKISLPC

**MAb production.** BALB/c mice (2 months old) were immunized subcutaneously with 0.2 ml of an emulsion of 100 μg of V3.3 peptide in Freund’s complete adjuvant (1:1). Mice were boosted intravenously with 100 μg of V3.3 peptide on day 25. Four days following the last booster, splenic lymphocytes were fused with P3.X63.AG8.653 mouse myeloma cells using 50% polyethylene glycol at neutral pH. Fused cells were plated in 96-well tissue culture plates and hybrids were selected in HAT medium. Hybrid supernatants were tested for V3.3-specific antibody by ELISA (see below). Hybridomas producing IgG antibodies to V3.3 peptide were cloned twice by limiting dilution and finally produced into ascites in BALB/c mice. Anti-V3.3 MAbs were purified by FPLC using a Protein G column (Pharmacia).

The MAbs isotypes were determined by ELISA using goat anti sera to mouse IgG1, IgG2a, IgG2b, and IgG3 (Sigma) according to the manufacturer’s protocol.

**ELISA for hybridoma screening.** ELISA plates (Probind; Falcon) were coated overnight with 1 μg/well of V3.3 peptide in carbonate buffer (pH 9.6). The plates were subsequently post-coated for 1 h with PBS containing 1% BSA (PBS-BSA) to block available sites for non-specific binding. Hybridoma supernatants, diluted 1:2 in PBS containing 40% Tween 20 (PBS-Tw), were added to the plates and incubated for 1 h. Bound IgG antibodies were identified by a horseradish peroxidase (HRPO)-conjugated goat anti-mouse IgG (Sigma) solution diluted 1:5000 in PBS-BSA-Tw. The enzyme reaction was carried out with tetramethylbenzidine (KPL, Denmark) and stopped with 0.05 M H2SO4; the absorbance was measured at 450 nm. All steps were performed at room temperature (rt). Post-coating was done with 150 μl/well; peptide, samples, conjugate, substrate and H2SO4 were added in volumes of 100 μl/well.

**FIV NA assay.** Purified MAbs were tested for neutralizing activity using a recently described assay based on inhibition of FIV-induced syncytium formation (Tozzini et al., 1992). Briefly, 100 μl of twofold dilutions of MAbs in PBS at a concentration of 1 mg/ml were mixed with an equal volume of FIV-P containing approximately 50 s.f.u. in 24-well plates. After 1 h at rt, 1 ml of medium containing 2 x 10^4 CrFK cells adapted to grow in 0.5% fetal bovine serum (FBS) was added to each well. Six days later the cultures were stained and the syncytia counted under a microscope. As already reported (Tozzini et al., 1993), FIV-induced syncytium formation parallels p24 production, as determined by a p24-specific MAb-based assay (Lombardi et al., 1994b).

**Biotinylation of MAbs.** Protein G-purified MAbs were dialysed overnight against 0.1 M NaHCO3, pH 8.4 at 4°C; biotinylation was performed by incubating 60 μl of biotin-N-hydroxysuccinamide ester at a concentration of 1 mg/ml in DMSO with 1 ml of purified MAb diluted at 1 mg/ml in NaHCO3 for 2 h at rt. The biotinylated MAbs were then extensively dialysed against PBS. Biotinylated MAbs maintained binding activity to V3.3 peptide.

**Peptide inhibition of anti-V3.3 MAb reactivity.** For evaluating the ability of the V3-based peptides to inhibit the reactivity of anti-V3.3 MAbs, 50 μl of unconjugated MAb (2 μg/ml) together with 50 μl of serially diluted V3.2, V3.3 or V3.4 synthetic peptide were added to V3.3-coated ELISA wells. After 1 h and four washes in PBS-Tw, 100 μl of goat HRPO-labelled anti-mouse IgG (Sigma) diluted 1:2000 in PBS-BSA-Tw were added to each well. Bound IgG MAbs were revealed as described above.

**Competition studies.** Competition experiments between anti-V3.3 MAbs were performed by ELISA in which plates were coated with 1 μg of V3.3 peptide in 100 μl of carbonate buffer pH 9.6 at rt. Wells were post-coated as described above and 50 μl of biotinylated MAb (2 μg/ml) together with 50 μl of serially diluted unlabelled homologous or heterologous MAbs were added. After 2 h at rt and four washes in PBS-Tw, 100 μl of goat HRPO-labelled anti-biotin serum (Sigma) diluted 1:1000 in PBS-BSA-Tw were added to each well. The plates were then developed as described above.

**Flow cytometry.** Flow cytometry analysis was performed using an Epics Elite cell analyzer (Coulter Electronics). To permeate cell membranes, 5 x 10^5 persistently infected FL4 cells were incubated for 10 min at 4°C with PBS containing 1% paraformaldehyde. After two washes with PBS containing 0.5% BSA, cells were incubated with absolute methanol at -20°C for 10 min, washed and then further incubated at 4°C for 1 h with an appropriate dilution of the anti-V3.3 MAbs in PBS containing 5% FBS and 0.1% sodium azide. After further washing, an FITC-conjugated goat anti-mouse IgG antibody (Sigma) was added for 30 min. Finally, cells were fixed in PBS containing 1% paraformaldehyde, 2% glucose and 0.1% sodium azide. Data were first collected in a two parameter histogram of size versus granularity and then, after removing from analysis any debris by selective gating, transferred to a single parameter log fluorescence histogram. Data from approximately 10000 cells were collected for each experimental condition.

**Results and Discussion**

Anti-V3.3 MAbs were prepared by fusing spleen cells from BALB/c mice immunized against the V3.3 synthetic peptide with P3.X63.AG8.653 mouse myeloma cells. One successful fusion (384 wells) yielded a total of 29 outgrowing hybridomas, which produced IgG antibody that reacted with V3.3 by ELISA. Six V3.3-

### Table 1. Reactivity of anti-V3.3 MAbs to V3-based synthetic peptides and to purified FIV

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>G7</th>
<th>F6</th>
<th>A10</th>
<th>D11</th>
<th>C7</th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3.2</td>
<td>0.044</td>
<td>0.05</td>
<td>0.02</td>
<td>0.06</td>
<td>0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>V3.3</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
<td>1.77</td>
<td>2.50</td>
<td>1.45</td>
</tr>
<tr>
<td>V3.4</td>
<td>0.08</td>
<td>0.08</td>
<td>0.03</td>
<td>0.06</td>
<td>1.70</td>
<td>1.23</td>
</tr>
<tr>
<td>FIV</td>
<td>0.40</td>
<td>0.42</td>
<td>0.44</td>
<td>0.37</td>
<td>1.10</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* ELISA plates were coated with 1 μg of peptide in 100 μl of carbonate buffer.
† MAbs were tested at a final concentration of 1 μg/ml.
‡ Data is expressed in A₄₅₀.
specific hybridomas were cloned twice by limiting dilution and finally produced into ascites in BALB/c mice. The secreted MAbs designated G7, A10, F6, C7 and A3 were isotype IgG1; MAb D11 was isotype IgG2b.

When tested by ELISA against detergent-disrupted purified FIV, only MAbs C7 and A3 reacted strongly, while MAbs G7, A10, F6 and D11 showed a weaker reactivity (Table 1). By flow cytometry using persistently infected FL4 cells, all the anti-V3.3 MAbs stained permeated cells, while an unrelated MAb did not (Fig. 1). In FL4 suspensions, the proportion of stained cells ranged between 13% (MAb G7) and 91% (MAb C7); none of the anti-V3.3 MAbs, however, stained unpermeated FL4 cells, as well as permeated peripheral blood lymphocytes from uninfected SPF cats (data not shown). These results indicate that the gp100 V3 loop epitopes recognized by the anti-V3.3 MAbs are exposed on mature virions and inside the infected cells during specific phases of virus biosynthesis; however, the epitopes are not exposed, or are not accessible to antibodies, on the surface of persistently infected cells. The virus envelope glycoproteins are in fact known to be

Fig. 1. Binding activity of anti-V3.3 MAbs to FIV-infected cells as assessed by flow cytometry. Permeated FL4 cells persistently infected with FIV-P were incubated with the indicated anti-V3.3 MAbs (solid line) or unrelated MAb (dotted line).

Fig. 2. Inhibition of anti-V3.3 MAb reactivity by V3-based synthetic peptides. The binding of a constant concentration (2 μg/ml) of MAb to V3.3-coated microtitre wells was measured in the absence (−) or in the presence of the indicated concentrations of synthetic peptides. (△), V3.2; (■), V3.3; (●), V3.4. Two control unrelated synthetic peptides derived from FIV V5 were also tested in each experiment and no inhibition was observed (data not shown).
endowed with great plasticity; oligomerization of these proteins and possibly other changes occurring during their synthesis and maturation can influence their quaternary structure with resultant changes in epitope exposure (Earl et al., 1994; Bou-Habib et al., 1994). It has recently been reported that certain epitopes are more exposed on monomers than on the native oligomeric form of the HIV-1 envelope (Hanson, 1994).

In subsequent experiments, the epitope(s) defined by the six MAbs were mapped using different approaches. First, the MAbs, purified by Protein G affinity chromatography, were analysed for reactivity to the V3.2 and V3.4 synthetic peptides coated to ELISA wells. Although, as expected, all the MAbs reacted strongly with the immunizing peptide V3.3, none reacted with peptide V3.2 and only two, C7 and A3, reacted with peptide V3.4 (Table 1). This suggested that the epitope(s) recognized by the latter MAbs is located in the region common to the peptides V3.3 and V3.4. Secondly, as antigen coating to ELISA wells can obscure immunoreactive sites of molecules, thus influencing antibody binding (Bolton et al., 1991; Seligman, 1994), the reactivity of the MAbs was studied using the target peptides in liquid phase. Fig. 2 shows the ability of solutions of peptides V3.2, V3.3 and V3.4 to inhibit the binding of the six MAbs to V3.3 peptide-coated wells. Binding of G7, A10, F6 and D11 MAbs was inhibited by the V3.3 peptide as well as by the V3.2 peptide in a dose-dependent manner, but not by peptide V3.4. As the V3.2 and V3.3 peptides share a 12 amino acid sequence, G7, A10, F6 and D11 MAbs most likely detect epitope(s) on the overlapping region of V3.3 and V3.2. In the same assay, the reactivity of MAbs C7 and A3 was inhibited in a dose-dependent manner by V3.3 and V3.4, thus confirming the above results that these MAbs are directed against epitope(s) of the V3.3–V3.4 overlapping region. Finally, the epitopes detected by the six MAbs were further characterized by competition ELISAs in which a constant amount of biotin-labelled MAb was allowed to compete with increasing concentrations of the unconjugated homologous or heterologous MAbs for binding to V3.3. As can be seen in Fig. 3, binding of biotinylated MAb G7 was inhibited by MAbs A10 and F6, as well as by homologous unlabelled G7, in a dose-dependent manner, but not by MAbs C7, A3 and D11; furthermore, unlabelled MAbs G7, F6 and A10 but not MAbs C7, A3 and D11 competed with biotin-conjugated A10 and F6. These results indicate that G7, A10 and F6 recognize the same or a closely adjacent epitope. The binding of A3, C7 and D11 was inhibited only by the unlabelled homologous antibody, thus indicating that these MAbs react with three different epitopes, which also differ from that recognized by the other MAbs.

As immunization of cats with the V3.3 peptide induces antibodies that neutralize FIV infectivity (Lombardi et al., 1993), Protein G-purified anti-V3.3 MAbs were also studied for neutralizing activity. All the anti-V3.3 MAbs reduced FIV-induced syncytium formation in CrFK
Mapping of FIV V3 domain by MAbs

Fig. 4. Neutralizing activity of anti-V3.3 MAbs, as assessed by inhibition of FIV-induced syncytium formation in CrFK cells. Twofold dilutions of Protein G-purified anti-V3.3 MAbs, diluted in PBS at a concentration of 1 mg/ml, were mixed with 50 s.f.u. of FIV-P in 24-well plates. After 1 h at rt, 10^6 CrFK cells adapted to grow in 0.5% FBS were added to each well. Six days later, the cultures were stained and the syncytia were counted under the microscope. Each line represents an individual MAb; the horizontal line represents the number of FIV-induced syncytia in the absence of antibody. (■), G7; (▲), A3; (●), C7; (▼), F6; (○), A10; (□), D11.

cells, while a control unrelated MAb did not. Similar to what has already been reported for sera of infected cats (Tozzini et al., 1993), inhibition of syncytium formation by MAbs correlated in repeated tests with inhibition of virus production, as judged from the levels of p24 antigen accumulated in culture fluids (data not shown). Moreover, as shown in Fig. 4, at high dilutions all the anti-V3.3 MAbs inhibited syncytium formation by 90% to 100%; at low dilutions, however, two of the six MAbs, C7 and F6, exhibited much less neutralizing activity. Interestingly, this paradoxical behaviour has also been observed with sera of cats hyperimmunized with V3.3 (Lombardi et al., 1994 a). The phenomenon is vaguely reminiscent of the antibody-dependent enhancement of infectivity (ADE) that has been seen at subneutralizing concentrations of immune sera with a number of viruses including HIV-1, simian immunodeficiency virus and, more recently, FIV (Robinson et al., 1989; Montefiori et al., 1990; Baldinotti et al., 1994). ADE is thought to involve Fc and/or complement receptors present on target cells, but the precise mechanisms involved remain largely elusive. With lentiviruses the occurrence of neutralization or ADE appears to be determined by a number of poorly defined factors pertaining to the antibody, the virus and the host cell (Montefiori, 1993; Kliks et al., 1993). As the paradoxical phenomenon described here occurred only at high concentrations of antibody, the underlying mechanism(s) is most likely different from those responsible for ADE. In any case, since it was observed with MAbs directed against two distinct epitopes, heterogeneity of the antibody population cannot be the cause. Experiments are under way to elucidate the basis of this phenomenon.

Taken together, our data show that the V3 loop of the FIV envelope glycoprotein contains several distinct antibody-binding sites. The panel of anti-V3.3 MAbs described here (Table 2) detected at least four: two were located in the amino-terminal half of the sequence (one recognized by MAbs G7, A10 and F6, the other by MAb D11); the other two epitopes, located in the carboxy-terminal half of the sequence, were detected by MAbs A3 and C7, respectively. The latter two epitopes, especially, seem to be well expressed on mature FIV virions and in the cytoplasm, but not on the surface of FIV-infected cells. They most likely represent conserved sequences because the corresponding MAbs were found to bind the recombinant Env protein of a heterologous FIV (FIV Bangston strain, kindly provided by Dr E. Young, Cambridge Biotech, Worcester, Mass., USA). The fact that, irrespective of their fine epitope specificity, all the MAbs neutralized FIV infectivity for CrFK cells indicates that in the V3 region of FIV there is an important neutralization domain. Potentially, the four distinct

<table>
<thead>
<tr>
<th>MAb</th>
<th>Isotype</th>
<th>CrFK</th>
<th>Lymphoid cells</th>
<th>Purified FIV</th>
<th>Permeabilized FIV-infected cells</th>
<th>V3.2</th>
<th>V3.3</th>
<th>V3.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>G7</td>
<td>IgG2a</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ (13%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A10</td>
<td>IgG2a</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ (74%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F6</td>
<td>IgG2a</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ (45%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D11</td>
<td>IgG1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ (53%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C7</td>
<td>IgG2a</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ (91%)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A3</td>
<td>IgG2a</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+ (82%)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Assessed on CrFK cells by syncytium inhibition assay (Tozzini et al., 1992) and on lymphoid MBM cells by inhibition of p24 production (Baldinotti et al., 1994).
† Assessed by ELISA for purified FIV and synthetic peptides and by flow cytometry on FIV-infected FL4 cells (the percentage of stained cells is given in parentheses).
binding sites defined on FIV V3 domain by our MAbs might interact and form a discontinuous neutralization epitope according to the secondary structure of the peptide. That a principal neutralization domain exists within the third hypervariable region of the envelope protein of HIV-1 and HIV-2 is well established (for review see Wolf et al., 1993) and there are data indicating that such regions consist of multiple and/or discontinuous epitopes (White-Scharf et al., 1993; Seligman, 1994; Björling et al., 1994). Recently, using overlapping fragments expressed in Escherichia coli and by Pepsan analysis, the V3 region of FIV SU glycoprotein has been shown to contain a highly immunogenic neutralization domain localized at amino acid positions 390-411 and consisting of linear as well as conformational epitope(s) (de Ronde et al., 1994; Egberink et al., 1994). The V3.3 domain has therefore been proposed as a suitable component for candidate subunit anti-FIV vaccines (Lombardi et al., 1993; de Ronde et al., 1994; Egberink et al., 1994), but recent data from our laboratory have clearly demonstrated that V3.3 immunization of cats fails to prevent FIV infection in spite of the induction of high NA levels (Lombardi et al., 1993a). However, since previous studies have shown that sera of infected cats may neutralize the infectivity of FIV for CrFK much more efficiently than the infectivity for lymphoid cells and cats (Baldinotti et al., 1994), a clear possibility is that the V3.3 region contains neutralization epitopes that are active solely on specific cell substrates such as the CrFK cell line; in fact, at least with the currently available assays, both the sera of cats immunized with V3.3 (Lombardi et al., 1993) and the anti-V3.3 MAbs described herein failed to neutralize FIV on lymphoid cells when tested at dilutions ranging from 1:4 to 1:1024 (Table 2).

In conclusion, although one major neutralization domain has been identified on FIV Env protein and the epitopes involved have been localized, several questions remain to be addressed. These include the identification and characterization of other neutralization domains, the type of neutralization assay that might parallel the in vivo situation, the possible synergy of neutralizing antibodies with different epitope specificities, and the role of antibodies (broadly reactive versus type-specific) in protection of the host. Anti-FIV humoral immunity might protect against the systemic spread of virus, but have little influence on virus-infected cells and on cell-to-cell virus spread in lymphoid organs. Both neutralizing antibody and cell-mediated immunity are probably required for effective immunity against FIV infection.

This work was supported by grants from Ministero della Sanità-Istituto Superiore di Sanità, ‘Progetto Allestimento Modelli Animali per l’AIDS’, Rome, Italy. We thank Dr E. Young for providing recombinant Env protein of FIV Bangstock strain.

References


(Received 13 March 1995; Accepted 11 April 1995)