

Monoclonal antibodies to immunodominant and neutralizing domains of the envelope surface protein of feline immunodeficiency virus

Herman Egberink,^{1*} Lian Keldermans,¹ Nancy Schuurman,¹ Jeanette Stam,¹ Wim Hesselink,² Arno van Vliet,¹ Ernst Verschoor,¹ Marian Horzinek¹ and Anthony de Ronde¹

¹Virology Division, Department of Infectious Diseases and Immunology, Veterinary Faculty, Utrecht University, Yalelaan 1, 3584 CL Utrecht and ²Intervet International BV, Boxmeer, The Netherlands

Hybridomas secreting monoclonal antibodies (MAbs) specific for the surface protein (SU) of feline immunodeficiency virus were generated. Four MAbs were obtained which could be assigned to two groups based on their neutralization and competition behaviour. Using SU protein fragments expressed in *Escherichia coli* the antigenic site recognized by one of the MAbs (2H11) could be mapped to the c terminus. The neutralizing MAb 1E1 did not bind to any of the SU protein

fragments and was directed to a conformational epitope. Binding of the MAb 1E1 to native SU protein could be blocked with a rabbit serum raised against the SU3 fragment (amino acids 361 to 445). These data indicate that at least part of the epitope is located on this SU3 domain. In competition experiments most sera of naturally infected cats were able to inhibit binding of the MAbs. This shows the conserved and immunodominant nature of the epitopes involved.

Feline immunodeficiency virus (FIV) is a lentivirus that causes a disease in cats characterized by the development of opportunistic infections as a consequence of an acquired immunodeficiency; this disease syndrome is known as feline AIDS (Pedersen *et al.*, 1987). The pathogenesis, dynamics of the host immune response and nature of the clinical symptoms resemble infection with human immunodeficiency virus type 1 (HIV-1), the cause of AIDS in man (Ackley *et al.*, 1990; Barlough *et al.*, 1991; Egberink *et al.*, 1992; Ishida & Tomoda, 1990; Siebelink *et al.*, 1990). Therefore FIV infection in cats has been proposed as an animal model for AIDS with respect to the pathogenesis, development of vaccines and antiviral chemotherapy (Egberink *et al.*, 1990*a, b*; Jarrett *et al.*, 1990). FIV is also an important pathogen in feline medicine, necessitating the development of efficacious vaccines and reliable diagnostic assays.

A detailed analysis of the structure and function of the FIV surface protein (SU), gp100, is important for several reasons. The SU protein is most likely to contain the site involved in binding of the virus to the cell receptor as well as antigenic domains eliciting a protective immune response. In HIV-1 various regions of the SU protein have been identified that evoke type-specific or more broadly neutralizing antibodies (Weiss *et al.*, 1986; Berkower *et al.*, 1989; Prince *et al.*, 1987). Neutralizing antibodies can also be detected in the sera of FIV-infected cats (Tozzini *et al.*, 1993). In a previous study we have shown that an anti-SU protein response could be

detected in all seropositive cats tested (Egberink *et al.*, 1992). In some cats anti-SU protein antibodies were the only ones found and it is therefore important to include the SU protein in assays used for routine diagnosis.

The SU protein shows a high degree of amino acid sequence variation (Rigby *et al.*, 1993); study of conserved and variable domains is necessary for vaccine development and also for the development of second generation diagnostic assays based on recombinant DNA products or peptides. To define antigenic domains on the SU protein we generated SU protein-specific monoclonal antibodies (MAbs). In this report we describe the characterization of MAbs specific for two antigenic domains on the SU protein of FIV.

To generate an SU protein-specific antibody response, outbred Swiss mice were immunized three times at 0, 10 and 20 weeks with an inactivated whole virus preparation in alum/oil adjuvant. Swiss mice were used since they showed a superior anti-SU protein immune response compared to BALB/c mice (E. Verschoor, unpublished results). A final booster immunization was given at 27 weeks with a SU protein-enriched preparation consisting of matrix cellulose sulphate (MCS) affinity chromatography-purified FIV from the culture supernatant of CRFK cells persistently infected with the isolate FIV-UT113. Three days after the final boost, spleen cells of a Swiss mouse were fused with P3X63-AG8.653 mouse myeloma cells. The screening of the hybridomas was performed using a concanavalin A

Table 1. Properties of the MAbs and surface protein fragment-specific rabbit sera

	Virus neutralizing titre*	Competition of†	
		1E1	2H11
Hybridoma			
1E1 sup	90	+	-
1E1 asc	3000	+	-
7E1 sup	30	+	-
2H11 sup	< 2	-	+
7E4 sup	< 2	-	+
Rabbit serum			
Anti-SU1	< 10	-	-
Anti-SU2	< 10	-	-
Anti-SU3	320	+	-
Anti-SU4	< 10	-	-
Anti-SU5	< 10	-	-
Anti-SU6	< 10	-	+
Cat serum			
Anti-FIV	320	+	+
Neg. contr.	< 10	-	-

* Reciprocal dilution at which serum still shows neutralizing activity.

† A '+' indicates competition between labelled MAbs and hybridoma supernatant (sup), ascitic fluid (asc) or serum as reflected by a sigmoidal curve of A_{150} versus the MAb or serum dilution. Neg. contr., negative control.

(Con A)-env ELISA. This ELISA was performed essentially as described by Robinson *et al.* (1990). Greiner high binding microtitre plates were coated with 5 µg Con A per well to bind the glycosylated SU protein. Triton X-100-lysed culture supernatants of FIV-UT113-infected CRFK cells, containing the SU protein, and mock-infected CRFK cells were used as an antigen source. The plates were washed and treated with 10% fetal calf serum (FCS) in DMEM (blocking buffer). Undiluted hybridoma supernatants were added and binding of antibodies was detected by using horseradish peroxidase (HRPO)-labelled goat anti-mouse immunoglobulins in blocking buffer. Subsequently, hydrogen peroxide and the chromogen tetramethyl benzidine (TMB; Sigma) were added. The reaction was stopped by adding 2 M-H₂SO₄ and the absorbance was measured at 450 nm. A supernatant was considered positive if the absorbance of the FIV-coated wells was more than twice that of the mock-infected wells.

Positive hybridomas were selected and subcloned twice. Four gp100-specific clones were obtained, as confirmed by a Western blot (WB) using the MCS-purified FIV protein (Egberink *et al.*, 1992). Three of the MAbs were of the IgG1 isotype and one of the IgG2b (1E1) isotype as determined by Ouchterlony double diffusion tests (Table 1). The MAbs were also tested in a neutralization assay. Hybridoma supernatants and ascitic fluids (1E1), inactivated for 30 min at 56 °C and diluted in DMEM containing 2% FCS and antibiotics (maintenance medium), were mixed with 100 TCID₅₀ of

FIV-UT113. Positive and negative cat anti-FIV sera and culture medium were included as controls. The mixture was incubated for 1 h at 37 °C and subsequently added to subconfluent monolayers of CRFK cells in 96-well plates. After overnight incubation the wells were rinsed three times with PBS and maintenance medium was added. After 4 days of culturing the supernatants were collected and assayed for viral p24 production by an antigen capture ELISA similar to the assay described by Tilton *et al.* (1990). The ELISA was developed using our own p24-specific MAbs and was shown to be as sensitive as a reverse transcriptase assay (L. Keldermans, unpublished results). Neutralization was scored positive at > 90% inhibition of p24 production. The MAbs 1E1 and 7E1 were able to neutralize FIV-113 (Table 1).

To test the MAbs in a competition ELISA, 1E1 and 2H11 were purified by sodium sulphate precipitation and labelled with peroxidase or biotin respectively. Microtitre plates were coated overnight at 4 °C with 250 ng purified cat anti-FIV antibodies in PBS. After washing with PBS containing 0.05% Tween 20, the plates were blocked with PBS containing 5% newborn calf serum and 0.05% Tween 20 for 1 h at 37 °C. Culture supernatants of FIV-infected CRFK cells were lysed in 0.1% Triton X-100, diluted in blocking buffer and added to the plates, followed by an incubation of 1.5 h at 37 °C. Different dilutions of hybridoma supernatant were mixed with a standard dilution of MAb 1E1 and 2H11 that had been conjugated with HRPO or biotin respectively and added to the plates. The correct MAb concentration had been determined on the same plates without the addition of the competing antibodies. After 1 h of incubation with the HRPO-conjugated antibody the plates were washed and incubated with the substrate and TMB. The biotin-labelled antibody required an additional incubation with streptavidin-HRPO. Only the competition of the binding of MAb 1E1 and 2H11 was determined. In the competition ELISAs MAb 7E1 competed with the binding of 1E1 and MAb 7E4 with 2H11, in addition to the homologous reaction (Table 1). These competition experiments indicate that the MAbs 1E1 and 7E1 recognize an epitope different from the one recognized by 7E4 and 2H11. Although recognition of overlapping epitopes or induction of allosteric changes by the competing antibody cannot be excluded, the neutralization and competition experiments probably indicate that the MAbs recognize at least two different epitopes on the SU protein.

To localize the antigenic domains recognized by the MAbs two approaches were used. First the antigenic sites were mapped using SU protein fragments of FIV-UT113 expressed in *Escherichia coli*. Overlapping SU protein fragments expressed in *E. coli* downstream from and in frame with the N-terminal part of galactokinase

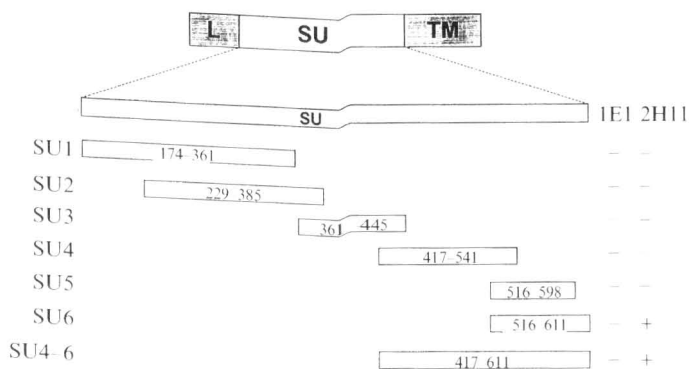


Fig. 1. Map of the envelope surface protein fragments. Nomenclature of the fragments runs from the N terminus of the envelope surface protein (SU-1) to the C terminus (SU-6). The reaction of the MAb in WBs as illustrated in Fig. 2 is indicated on the right.

(galK) were examined (Fig. 1). The cloning, expression and purification of the latter proteins was performed following standard procedures (Chiang *et al.*, 1989; Krone *et al.*, 1988). As shown in Fig. 2, 2H11 bound only to the expression products SU6 and SU4-6. The protein SU6 differs from SU5 only by the presence of 13 extra C-terminal amino acids, indicating that the epitope is located at the extreme C terminus, near the cleavage site between the SU and transmembrane proteins. MAb 1E1 showed no specific reaction with any of the expression products. By using WB with SU protein fragments expressed in *E. coli*, linear rather than conformational epitopes should be detected.

To investigate the possible specificity of 1E1 for a conformational epitope, a dot immunoblot was performed using native and denatured proteins of MCS-purified FIV-UT113 (Fig. 3). The proteins were de-



Fig. 3. Dot immunoblot using MAb to FIV proteins in their native (N) and denatured (D) state. The proteins were spotted undiluted (upper row) and at three further fivefold dilutions. The reactions of a positive cat serum (pos FIV), of MAb (4D2) not specific for FIV and of the MAb 1E1 and 2H11 are shown. The MAb were added as undiluted hybridoma supernatants.

natured by boiling for 2 min in a solution containing 2% SDS and 5% 2-mercaptoethanol and were spotted onto nitrocellulose strips (0.45 µm pore size; Schleicher & Schuell) together with the native protein. The strips were dried, saturated in phosphate gelatin buffer (PBS containing 0.1% Triton X-100 and 0.05% pig skin gelatin) and subsequently incubated for 1.5 h with the undiluted hybridoma culture supernatant of 1E1, 2H11, a hybridoma 4D2 which does not recognize FIV, and with an anti-FIV cat serum. Strips were washed in gelatin buffer and goat anti-cat HRPO or goat anti-mouse HRPO was added. After another hour of incubation and subsequent washing of the strips, hydrogen peroxide and chromogen 4-chloro-1-naphthol (Sigma) were added. The reaction was stopped by rinsing the strips in water. The feline anti-FIV serum bound to both preparations although the strongest reaction was seen with native protein. As expected, MAb 2H11 reacted with both the

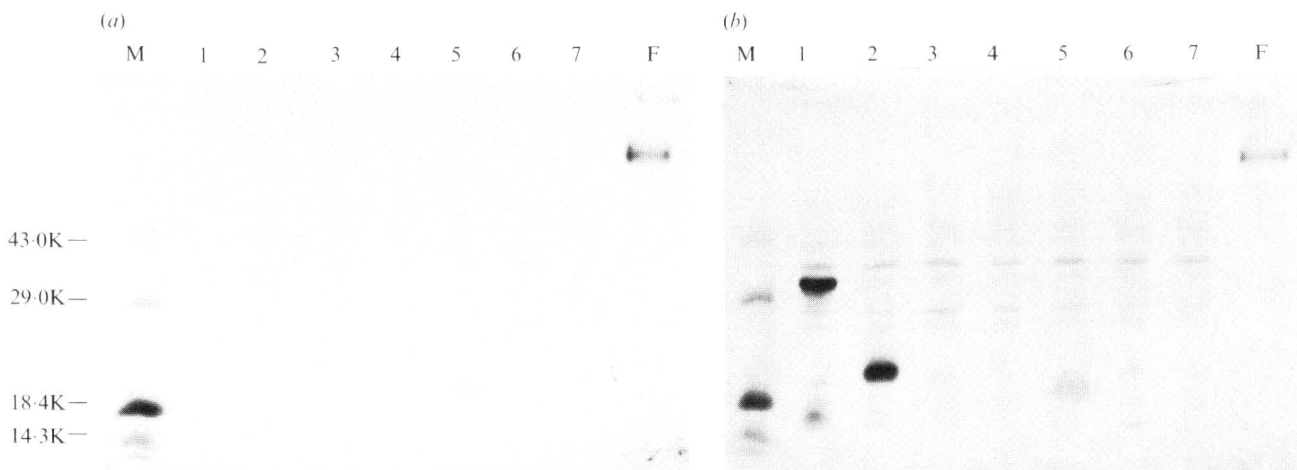


Fig. 2. SDS PAGE of purified galK-SU fusion proteins. The reaction of MAb (a) 1E1 and (b) 2H11 against seven different fusion proteins of the N-terminal part of galK and part of the SU envelope protein of FIV-UT113 is shown. Lane M contains prestained low M_r protein markers (M_r s are shown to the left; BRL). Lane 1, SU4-6; lane 2, SU6; lane 3, SU5; lane 4, SU4; lane 5, SU3; lane 6, SU2; lane 7, SU1; lane F, MCS-purified FIV. For nomenclature of the fragments see Fig. 1.

native and denatured protein whereas MAb 1E1 showed a more specific binding to the native protein (Fig. 3). This indicates that the epitope recognized by 1E1 is probably conformational rather than linear, although the SU protein from MCS-purified virus was also recognized on WB. However, this is probably due to a partial renaturation during the WB procedure and incubation of the nitrocellulose strips. MAb 1E1 also reacted with the SU protein that had been deglycosylated by endoglycosidase H treatment, which indicates that it recognizes the protein backbone rather than the sugar moiety (data not shown).

The second approach to identify the epitopes involved competition experiments using rabbit sera that had been generated against the overlapping SU protein fragments. The binding of both conjugated MAbs was assessed in the presence of different dilutions of the rabbit serum in an ELISA system. The ELISA was performed as described for the competition of the MAbs. As expected, binding of 2H11 was blocked by a rabbit serum specific for SU6 but not for SU5. Interestingly, binding of the neutralizing monoclonal 1E1 was inhibited by a rabbit serum specific for SU3. This rabbit serum also neutralizes FIV-UT113 (Table 1; De Ronde *et al.*, 1994). These results indicate that at least part of the epitope is located on this SU3 domain and suggest that the SU3 domain contains neutralizing antibody-inducing epitopes. Recently, both linear and conformational neutralization epitopes have been located in this region (Lombardi *et al.*, 1993; De Ronde *et al.*, 1994). However, inhibition of binding of 1E1 due to steric hindrance or by a change in conformation of the epitope cannot be completely ruled out.

The competition ELISA was also used to investigate whether cats mount an antibody response against the epitopes recognized by 2H11 and 1E1. In sequential serum samples of experimentally infected cats, antibodies competing for 1E1 and 2H11 binding were found as early as 2 to 3 weeks after infection. In a previous study these cats had been shown to seroconvert against the SU protein of FIV 2 to 4 weeks after infection (Egberink *et al.*, 1992). Sera of naturally FIV-infected cats were also screened: 46 out of 53 positive sera, as shown by WB, blocked binding of 1E1 and all positive serum samples competed with the binding of 2H11 (Table 2). In contrast, none of the 29 FIV-negative serum samples inhibited binding of either MAb. These results support the notion of a conserved and immunodominant epitope that is recognized by 2H11. Moreover the 13 C-terminal amino acids were shown to be recognized by cat sera as a major antigenic site in an ELISA using galK-SU-env fusion proteins as antigen (De Ronde *et al.*, 1994). An antigenic site at a similar position has also been identified using synthetic peptides (Avrameas *et al.*, 1992). In HIV-1 the

Table 2. Results of competition ELISA with sera of field FIV-infected cats

Number of sera tested	Competition of*					
	1E1			2H11		
	+	+/-	-	+	+/-	-
FIV-positive 53	46	2	5	53	0	0
FIV-negative 29	0	1	28	0	0	29

* A '+' indicates >70% inhibition of the A_{450} of a negative control; '+/-' , $\geq 30\%$ and $\leq 70\%$ inhibition; '-' , <30% inhibition.

C-terminal part of the SU protein was also shown to be antigenic (Palker *et al.*, 1987).

Our results indicate that the SU protein carries two antigenically conserved and immunodominant domains. The use of these domains in serological assays can facilitate the detection of cats that show only an anti-SU protein response. Furthermore, the epitope recognized by the neutralizing MAb could be important as a component of an efficacious vaccine against FIV.

References

- ACKLEY, C. D., YAMAMOTO, J. K., LEVY, N., PEDERSEN, N. C. & COOPER, M. D. (1990). Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *Journal of Virology* **64**, 5652-5655.
- AVRAMEAS, A., GUILLET, J.-G., CHOUCANE, L., MORAILLON, A., SONIGO, P. & STROBERG, A. D. (1992). Localisation of three epitopes of the env protein of feline immunodeficiency virus. *Molecular Immunology* **29**, 565-572.
- BARLOUGH, J. E., ACKLEY, C. D., GEORGE, J. W., LEVY, N., ACEVEDO, R., MOORE, P. F., RIDEOUT, B. A., COOPER, M. D. & PEDERSEN, N. C. (1991). Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections. *Journal of Acquired Immune Deficiency Syndromes* **4**, 219-227.
- BERKOWER, I., SMITH, G. E., GIRI, C. & MURPHEY, D. (1989). Human immunodeficiency virus 1: predominance of a group specific neutralizing epitope that persists despite genetic variation. *Journal of Experimental Medicine* **170**, 1681-1695.
- CHIANG, C. S., GROVE, T., COOPER, M., CUAN, J., KOWALSKI, A., PARCELLS, K., TSUNOKAWA, M., ROSENBERG, M., ARCURI, E., FRANKLIN, S., SMITH, T. & DEBOUCK, C. (1989). Development of a confirmatory enzyme-linked immunosorbent assay for HIV-1 antibodies. *Clinical Chemistry* **35**, 946-952.
- DE RONDE, A., STAM, J. G., BOERS, P., LANGEDIJK, H., MELOEN, R., HESSELINK, W., KELDERMANS, C. E. J. M., VAN VLIET, A., VERSCHOOR, E. J., HORZINEK, M. C. & EGBERINK, H. F. (1994). Antibody response in cats to the envelope proteins of feline immunodeficiency virus: identification of an immunodominant neutralization domain. *Virology* **198**, 257-264.
- EGBERINK, H. F., BORST, M., NIPHUIS, H., BALZARINI, J., NEU, H., SCHELLEKENS, H., DE CLERCQ, E., HORZINEK, M. & KOOLEN, M. (1990a). Suppression of feline immunodeficiency virus infection in vivo by 9-(2-phosphonomethoxyethyl)adenine. *Proceedings of the National Academy of Sciences, U.S.A.* **87**, 3087-3091.
- EGBERINK, H. F., EDERVEEN, J., MONTELARO, R. C., PEDERSEN, N. C., HORZINEK, M. C. & KOOLEN, M. J. M. (1990b). Intracellular proteins of feline immunodeficiency virus and their antigenic

- relationship with equine infectious anaemia virus proteins. *Journal of General Virology* **71**, 739–743.
- EGBERINK, H. F., KELDERMANS, C. J. E. M., KOOLEN, M. J. M. & HORZINEK, M. C. (1992). Humoral immune response to feline immunodeficiency virus in cats with experimentally induced and naturally acquired infections. *American Journal of Veterinary Research* **53**, 1133–1138.
- ISHIDA, T. & TOMODA, I. (1990). Clinical staging of feline immunodeficiency virus infection. *Japanese Journal of Veterinary Science* **52**, 645–648.
- JARRETT, O., YAMAMOTO, J. K. & NEIL, J. C. (1990). Feline immunodeficiency virus as a model for AIDS vaccination. *AIDS* **4**, supplement 1, S163–S165.
- KRONE, W. J. A., DEBOUCK, C., EPSTEIN, I. G., HEUTINK, P., MELOEN, R. & GOUDSMIT, J. (1988). Natural antibodies to HIV-tat epitopes and expression of HIV-1 genes in vivo. *Journal of Medical Virology* **26**, 261–270.
- LOMBARDI, S., GARCELLI, C., LA ROSA, C., ZACCARO, L., SPECTER, S., MALVALDI, G., TOZZINI, F., ESPOSITO, F. & BENDINELLI, M. (1993). Identification of a linear neutralization site within the third variable region of the feline immunodeficiency virus envelope. *Journal of Virology* **67**, 4742–4749.
- PALKER, T. J., MATTHEWS, T. J., CLARK, M. E., CIANCIOLO, G. J., RANDALL, R. R., LANGLOIS, A. J., WHITE, G. C., SAFAI, B., SNYDERMAN, R., BOLOGNESI, D. P. & HAYNES, B. F. (1987). A conserved region at the COOH terminus of human immunodeficiency virus gp120 envelope protein contains an immunodominant epitope. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 2479–2483.
- PEDERSEN, N. C., HO, E. W., BROWN, M. L. & YAMAMOTO, J. K. (1987). Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* **235**, 790–793.
- PRINCE, A. M., PASCUAL, D., KOSOLAPOV, L. B., KUROKAWA, D., BAKER, L. & RUBINSTEIN, P. (1987). Prevalence, clinical significance, and strain specificity of neutralizing antibody to the human immunodeficiency virus. *Journal of Infectious Diseases* **156**, 268–272.
- RIGBY, M. A., HOLMES, E. C., PISTELLO, M., MACKAY, A., LEIGH BROWN, A. J. & NEIL, J. C. (1993). Evolution of structural proteins of feline immunodeficiency virus: molecular epidemiology and evidence of selection for change. *Journal of General Virology* **74**, 425–436.
- ROBINSON, J. E., HOLTON, D., LIU, J., MCMURDO, H., MURCIANO, A. & GOHD, R. (1990). A novel enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to HIV-1 envelope glycoproteins based on immobilization of viral glycoproteins in microtiter plates. *Journal of Immunological Methods* **132**, 63–71.
- SIEBELINK, K. H. J., CHU, I., RIMMELZWAAN, G. F., WEJER, K., VAN HERWIJNEN, R., KNELL, P., EGBERINK, H. F., BOSCH, M. L. & OSTERHAUS, A. D. M. E. (1990). Feline immunodeficiency virus (FIV) infection in the cat as a model for HIV infection in man: FIV-induced impairment of immune function. *AIDS Research and Human Retroviruses* **6**, 1373–1378.
- TILTON, G. K., O'CONNOR, T. P. JR., SEYMOUR, C. L., LAWRENCE, K. L., COHEN, N. D., ANDERSEN, P. R. & TONELLI, Q. J. (1990). Immunoassay for detection of feline immunodeficiency virus core antigen. *Journal of Clinical Microbiology* **28**, 898–904.
- TOZZINI, F., MATTEUCCI, D., BANDECCHI, P., BALDINOTTI, F., SIEBELINK, K., OSTERHAUS, A. & BENDINELLI, M. (1993). Neutralizing antibodies in cats infected with feline immunodeficiency virus. *Journal of Clinical Microbiology* **31**, 1626–1629.
- WEISS, R., CLAPHAM, P., WEBER, J., DALGLEISH, A., LASKEY, L. & BERMAN, P. (1986). Variable and conserved neutralization antigens of the human immunodeficiency virus (HIV-1). *Nature, London* **324**, 572–595.

(Received 12 August 1993; Accepted 22 November 1993)