Journal of General Virology (1997), **78**, 611–618. Printed in Great Britain

Inhibition of feline immunodeficiency virus infection by CD9 antibody operates after virus entry and is independent of virus tropism

Brian Willett,[1] Margaret Hosie,[1] Andrew Shaw[2] and James Neil[1]

[1] Department of Veterinary Pathology, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK
[2] Department of Medical Oncology, Cross Cancer Institute, University Avenue, Edmonton, Alberta, Canada

A monoclonal antibody which blocks infection with feline immunodeficiency virus (FIV) was found previously to react with the cell surface molecule CD9, implicating CD9 in the process of virus entry. We report here that inhibition by anti-CD9 antibody does not operate at the level of virus entry but at a subsequent stage in the virus life-cycle. Moreover, inhibition of infection is independent of the passage history of the virus or the virus subtype. Inhibition of FIV infection by anti-CD9 antibody does not operate in 3201 cells, which do not express this surface antigen. However, ectopic expression of CD9 on 3201 cells enhances infection with FIV, suggesting that the role of CD9 may be direct rather than via cellular signalling pathways. These results suggest a novel control point in the lentivirus life-cycle which might be susceptible to modulation by natural antagonists.

Introduction

Infection by human immunodeficiency virus (HIV) is inhibited by monoclonal antibodies recognizing diverse cell surface antigens including CD4 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984), CD7 (Sato *et al.*, 1994), MHC class I and II (Corbeau *et al.*, 1990; Devaux *et al.*, 1990), LFA-1 (Hildreth & Orentas, 1989) and ICAM-3 (Sommerfelt & Åsjo, 1995). However, there appear to be several distinct mechanisms by which such antibodies achieve their inhibitory effect. Inhibition of virus entry may be mediated by direct neutralization of the virus, as with antibodies against MHC class II (Arthur *et al.*, 1995). This phenomenon is thought to be the result of the incorporation of cellular antigens into the viral envelope as the virus buds from the cells (Arthur *et al.*, 1992). An alternative mechanism by which antibodies against cell surface antigens may inhibit infection involves binding of the antibody to the virus receptor CD4 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984), co-receptors such as LESTR (Feng *et al.*, 1996), or to cell membrane proteins involved in the process of cell–cell fusion (Braun *et al.*, 1995). The interaction between the viral envelope glycoprotein gp120 and CD4 has been studied extensively and the gp120 binding site on CD4 mapped to the complementarity-determining region-2 (CDR2) of domain 1 (D1) (Peterson & Seed, 1988; Arthos *et al.*, 1989). Anti-CD4 antibodies recognizing this region, such as Leu3a, appear to inhibit infection by steric hindrance of the interaction between gp120 and CD4 (Ryu *et al.*, 1990). In contrast to the inhibition of virus entry and fusion by anti-CD4 or LESTR antibodies, antibodies recognizing cell surface antigens have been described which inhibit HIV infection without affecting the process of virus entry. Antibodies recognizing the CDR3 domain of CD4 D1, such as 13B8-2, inhibit infection at the transcriptional level, but prevent neither the initial interaction between gp120 and CD4, nor the subsequent stages of fusion and virus entry (Benkirane *et al.*, 1993). Similarly, while many anti-MHC class I antibodies inhibit HIV infection by direct neutralization of the virus (Devaux *et al.*, 1990), the anti-MHC class I antibody B1-1G6 inhibits HIV infection at a post-entry stage of the virus life-cycle by a mechanism similar to that of anti-CD4 CDR3 domain antibodies: suppression of virus transcription in antibody-treated cells (Briant *et al.*, 1996).

In a previous study, we demonstrated that the monoclonal antibody vpg15 recognizing the feline homologue of CD9 (Willett *et al.*, 1994*b*), a member of the four transmembrane domain (TM4) superfamily of molecules, could inhibit infection with feline immunodeficiency virus (FIV) (Hosie *et al.*, 1993). The anti-CD9 antibody displays no virus neutralizing activity and there is a good correlation between CD9 expression and susceptibility to infection with FIV both *in vitro* and *in vivo* (Willett & Hosie, 1995). However, the correlation is incomplete

Author for correspondence: Brian Willett.
Fax +44 141 330 5602. e-mail b.willett@vet.gla.ac.uk

0001-4416

since some CD9-expressing cells are refractory to infection. Moreover, a recent report has suggested that the apparently CD9-negative cell line 3201 is susceptible to infection with subtype A, but not subtype B isolates of FIV (Hohdatsu *et al.*, 1996).

In order to address the role of CD9 in FIV infection, we investigated whether infection is inhibited at virus entry or at a subsequent stage of the virus life-cycle. We demonstrate that the inhibitory effect of anti-CD9 antibody is independent of virus passage history, tropism or subtype. Finally, we provide evidence that CD9 expression directly enhances infection with FIV suggesting a potentially important accessory role for the molecule in the virus life-cycle.

Methods

■ **Viruses and cell lines.** The F14 molecular clone of FIV was obtained from P. Johnson, Georgetown University, Rockville, Md., USA, and the TM219 molecular clone from T. Miyazawa, University of Tokyo, Japan. The feline thymic lymphosarcoma line 3201 was obtained from J. Rojko, Ohio State University, Ohio, USA. The QN10 cell line was obtained from Oswald Jarrett, University of Glasgow, Glasgow, UK. The CrFK cell line (clone ID10) was obtained from P. Anderson, Idexx Laboratories, Portland, USA. Q201 cells are an antigen-specific, IL-2-dependent feline helper T cell line (Willett *et al.*, 1991). All cell culture media and reagents were obtained from Life Technologies. 3201 cells were cultured in RPMI 1640; 3T3 and ID10 cells were grown in Dulbecco's modification of Eagle's medium. All culture media were supplemented with 10% foetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin. In addition, RPMI 1640 medium was supplemented with 5×10^{-5} M 2-mercaptoethanol. Q201 and Mya-1 cultures were supplemented with 100 IU/ml rh-IL-2 (a gift of J. Nunberg, Cetus Corp.).

■ **Cloning and expression of feline CD9 and CD29.** cDNAs encoding the feline homologues of CD9 and CD29 were generated from mRNA from the IL-2-dependent feline T cell line Mya-1, which is highly susceptible to infection with both primary and CrFK-adapted isolates of FIV. The cDNA cloning and eukaryotic expression of feline CD9 have been described (Willett & Neil, 1995). Poly(A)$^+$ RNA was prepared from the Mya-1 cell line using a FastTrack mRNA isolation kit (Invitrogen). Double-stranded DNA was prepared using The Copy Kit (Invitrogen). Feline CD29 was then amplified by PCR and cloned into the pCRII vector (TA cloning kit, Invitrogen). The CD9 and CD29 cDNAs were subcloned into the pcDNA3 vector (Invitrogen).

■ **Monoclonal antibodies and flow cytometry.** Anti-feline CD9 (vpg15) has been described (Hosie *et al.*, 1993). The cross-species reactive anti-human CD29 antibody 4B4 was obtained from Coulter Immunology. Primary antibodies were detected using fluorescein isothiocyanate conjugated F(ab′)$_2$ fragment of sheep anti-mouse IgG whole molecule (Sigma). Cells were processed for flow cytometry as described previously (Willett & Neil, 1995). Analysis was performed on an EPICS Elite flow cytometer (Coulter Electronics). A total of 5000 events was collected in LIST mode for each sample. Analysis gates were established using a sample of each cell type stained with an isotype-matched control monoclonal antibody and set such that $< 1{\cdot}0\%$ of cells were positive.

■ **Electroporation and immunomagnetic purification.** Cells (5×10^6) of the 3201 cell line were suspended in 0·4 ml and added to a 4 mm electroporation cuvette. Plasmid DNA (20 μg) was added, mixed with the cell suspension and incubated on ice for 10 min. The cells were then pulsed with a single 960 μF, 250 V pulse using a Bio-Rad Gene Pulser. The pulsed cells were incubated for a further 10 min on ice and then seeded into a 75 cm^2 culture flask and incubated at 37 °C for 3 days to allow transient expression of the transfected cDNA. The cell population was then pelleted by centrifugation and incubated with either anti-feline CD9 or anti-human CD29 monoclonal antibody for 30 min at 4 °C. The cells were then washed by centrifugation through ice-cold culture medium and incubated with sheep anti-murine IgG (whole molecule) coated M450 Dynabeads (Dynal) at an approximate bead-to-cell ratio of 5:1. Antigen positive cells were purified using a cobalt–samarium magnet (MPC-1, Dynal) and reseeded in fresh culture medium. The process of immunomagnetic enrichment was repeated at weekly intervals until the cell population appeared to be almost entirely positive for expression of the antigen. Uncloned pools of cells which stably expressed the transfected cDNAs were used in the virus entry studies to avoid possible clonal variations. Previous studies suggested that cloning the transfected cells selected preferentially for cells which grew well whereas immunomagnetic separation selected for cells which expressed the antigen.

■ **Transfection and preparation of virus stocks.** Adherent cells were transfected by calcium phosphate DNA co-precipitation as described (Gorman, 1985). FIV_{F14} virus was harvested from a persistently infected culture of the CrFK cell line, the 'cell-culture adapted' stock. RD114-free stocks of the FIV_{F14} and FIV_{TM2} viruses were produced by transfection of the murine fibroblast cell line 3T3 with the F14 and TM219 molecular clones respectively, followed by recovery of virus into the IL-2-dependent feline T cell line Q201. The virus stocks were shown to be free from RD114 using a focal assay based on the S+L−QN10 feline fibroblast cell line, which harbours a defective murine sarcoma virus carrying the *mos* oncogene. Infection of the QN10 cell line with RD114 or feline leukaemia virus (FeLV) leads to morphological transformation/focus formation, apparently by rescue of the murine sarcoma virus by the infecting RD114 or FeLV. The virus stocks were titrated by incubating 2-fold dilutions of virus with Q201 cells in 24-well plates. Q201 cell cultures were maintained for approximately 3 weeks and passaged at weekly intervals. Supernatants from each well were then clarified and assayed for viral capsid protein p24. Stocks of the primary isolate FIV_{GL8} were prepared by infection of the Q201 T cell line with low passage virus. This cell line is non-transformed, antigen-specific, IL-2-dependent and highly sensitive to infection with primary isolates of FIV. Primary isolates grown in this cell line remain non-infectious for the CrFK cell line. CrFK-adapted isolates grown in the Q201 cell line retain their ability to infect CrFK cells.

■ **Blocking studies.** Cells were pre-incubated for 1 h at 4 °C with either anti-feline CD9 antibody vpg15 (Hosie *et al.*, 1993), anti-feline CD4 antibody vpg39 (Willett *et al.*, 1994*a*), 3′-azido-3′-deoxythymidine (AZT, Sigma), or with culture medium alone (control). The cells were then infected with the respective virus for a further hour at 4 °C. The cells were then washed twice by centrifugation in complete medium and re-seeded in 5 ml of complete medium supplemented with maintenance antibody at 1/5th of the blocking concentration. Supernatant samples (0·5 ml) were collected each day and replaced with 0·5 ml of fresh medium containing maintenance antibody.

■ **Western blotting.** Cultures of Q201 cells treated with anti-CD4 (vpg39), anti-MHC class II (vpg39), anti-CD29 (4B4), anti-CD9 (vpg15) or mock-treated were pelleted by centrifugation at day 5 post-infection with FIV_{GL8}. Western blots were then prepared as described previously (Willett *et al.*, 1991). Briefly, following transfer to nitrocellulose, blotted proteins were probed with a pooled FIV-infected cat serum and developed

with biotinylated protein A followed by streptavidin-conjugated alkaline phosphatase (Bio-Rad). Bound conjugate was detected using BCIP/NBT substrate (Sigma).

■ **Assay for virus entry.** Cells (1×10^6) of each cell line were seeded into Falcon 2054 tubes and incubated with DNase-treated virus at 37 °C for 1 h. Cells were then washed twice by centrifugation through culture medium, and either pelleted and frozen at −70 °C for use as a zero time-point control, or resuspended in fresh culture medium and incubated at 37 °C. Cells were cultured for the appropriate time period, harvested by centrifugation and cell pellets frozen at −70 °C. Total cellular DNA was then prepared from the cell pellets collected at each time-point simultaneously. Cell pellets were resuspended in lysis buffer consisting of 10 mM Tris–HCl pH 8·0, 100 mM NaCl, 10 mM EDTA and 0·5% SDS. RNase was added to 100 μg/ml and the cells were incubated at 37 °C for 1 h. Proteinase K was added to 300 μg/ml and the lysates were incubated for a further 15 h at 37 °C. Total DNA was purified by sequential extraction with phenol, phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1) and precipitated with ethanol. One μg of each DNA sample was used as template in nested PCRs as described previously (Sodora *et al.*, 1994) on a Perkin Elmer Thermal Cycler 2400. The primers used were designed to amplify the unspliced *env* gene from the virus corresponding to a product of 2·1 kb (first round primers 5′ ACT TCA TCA TTC CTC CTC TT, 5′ GCT CAG GTA GTA TGG AGA CT; second round primers 5′ TCA TCA TTC CTC CTC TTT TTC AGA, 5′ GCT CAG GTA GTA TGG AGA CTT CCA CCA TT). Products were analysed by agarose gel electrophoresis followed by ethidium bromide staining. High sensitivity detection of p24 utilized the PetChek Feline Immunodeficiency Virus Antigen Test Kit (Idexx Laboratories).

Results

Anti-CD9 antibody inhibition operates at a post-entry stage of infection

In order to examine the effect of vpg15 on immediate post-entry events in FIV infection, Q201 T cells were challenged with 5000 TCID of the primary isolate FIV_{GL8} in the presence or absence of anti-CD9 antibody at a concentration known to inhibit productive infection (25 μg/ml). A third culture was treated with 50 μM AZT to control for inhibition of reverse transcription. Cell pellets were collected at intervals post-infection and frozen at −70 °C for subsequent analysis by PCR. Viral reverse transcripts could be detected in both the control and anti-CD9 antibody treated cultures as early as 3 h post-infection confirming that reverse transcription and thus virus entry had occurred (Fig. 1). In contrast, reverse transcription was significantly inhibited in the AZT-treated culture. Supernatants were collected from the control, anti-CD9- and AZT-treated cultures and assayed for viral p24 by ELISA (Fig. 1). While p24 production was detected in the control culture by 48 h post-infection, p24 production was not detected in either the anti-CD9- or AZT-treated cultures confirming that anti-CD9 antibody inhibited productive infection despite allowing virus entry. In order to assess whether virus release (as measured by p24 ELISA) or virus production was inhibited by anti-CD9 antibody, cell pellets were collected from cultures of Q201 cells treated with either anti-CD4, anti-MHC class II, anti-CD29 or anti-CD9, or mock-treated, at day 5 post-infection with FIV_{GL8}, lysed, separated by PAGE, transferred to nitrocellulose and analysed by Western blot for virus-specific proteins. Virus-specific bands corresponding to capsid protein p24, Gag precursor p55 and envelope glycoprotein gp120 were evident in the mock-treated, and anti-CD4-, anti-CD29- and anti-MHC class II-treated cultures, but were absent from the anti-CD9-treated culture (Fig. 1*b*). The data suggest that virus production is suppressed in the CD9-treated culture.

CD9 antibodies inhibit infection of Q201 helper T cells by primary and laboratory adapted isolates, regardless of subtype and virus passage history

In previous studies we showed that the anti-CD9 monoclonal antibody vpg15 could inhibit productive infection of feline T cells with a primary isolate of FIV, FIV_{GL8} (Hosie *et al.*, 1993). We asked whether the inhibition of T cell infection by anti-CD9 antibody was restricted to primary subtype A isolates of FIV (FIV_{GL8}) or could be extended to cell culture adapted virus (FIV_{F14}) and virus of a distinct subtype (FIV_{TM2}, subtype B). Moreover, we investigated whether the passage history of the virus was critical for the blocking effect of anti-CD9 antibody by comparing Q201-grown FIV_{F14} with CrFK-grown FIV_{F14}. Q201 cells were incubated with either 25 μg/ml anti-CD4 or anti-CD9 antibody, or culture medium alone (control), and challenged with 500 TCID of the respective virus. A sharp rise in p24 production was detected in the control and anti-CD4-treated cultures infected with Q201-grown FIV_{GL8} (Fig. 2*a*), Q201-grown FIV_{F14} (Fig. 2*b*), CrFK-grown FIV_{F14} (Fig. 2*c*) and Q201-grown FIV_{TM2} (Fig. 2*d*). In contrast, incubation of Q201 T cells with anti-CD9 antibody inhibited productive infection with each of the viruses suggesting that the inhibition of FIV infection of T cells by anti-CD9 antibody is independent of cell culture adaptation, passage history or virus subtype. The data suggest the use of a common pathway of infection of Q201 T cells by both the primary subtype A virus FIV_{GL8}, the CrFK-adapted virus FIV_{F14} and the subtype B virus FIV_{TM2}, and that this pathway is sensitive to inhibition by anti-CD9 antibody.

Infection of 3201 T cells by FIV_{F14} is CD9 independent

Previous studies have described CD4-dependent infection of human cells expressing CD4 at levels below the sensitivity of flow cytometry (Jordan *et al.*, 1991). As 3201 cells appear ostensibly CD9-negative by flow cytometry (see Fig. 3), we investigated whether infection of 3201 cells with the cell culture adapted virus FIV_{F14} was susceptible to inhibition. 3201 cells were pre-incubated with either anti-CD9 or anti-CD4 antibody at 1, 10 or 25 μg/ml and then exposed to 200 TCID of FIV_{F14}. The cultures were maintained in the presence of antibody at 1/5th of the concentration used for blocking. In all cultures a sharp rise in p24 production was detected by day 5 post-infection (data not shown) irrespective of the type and

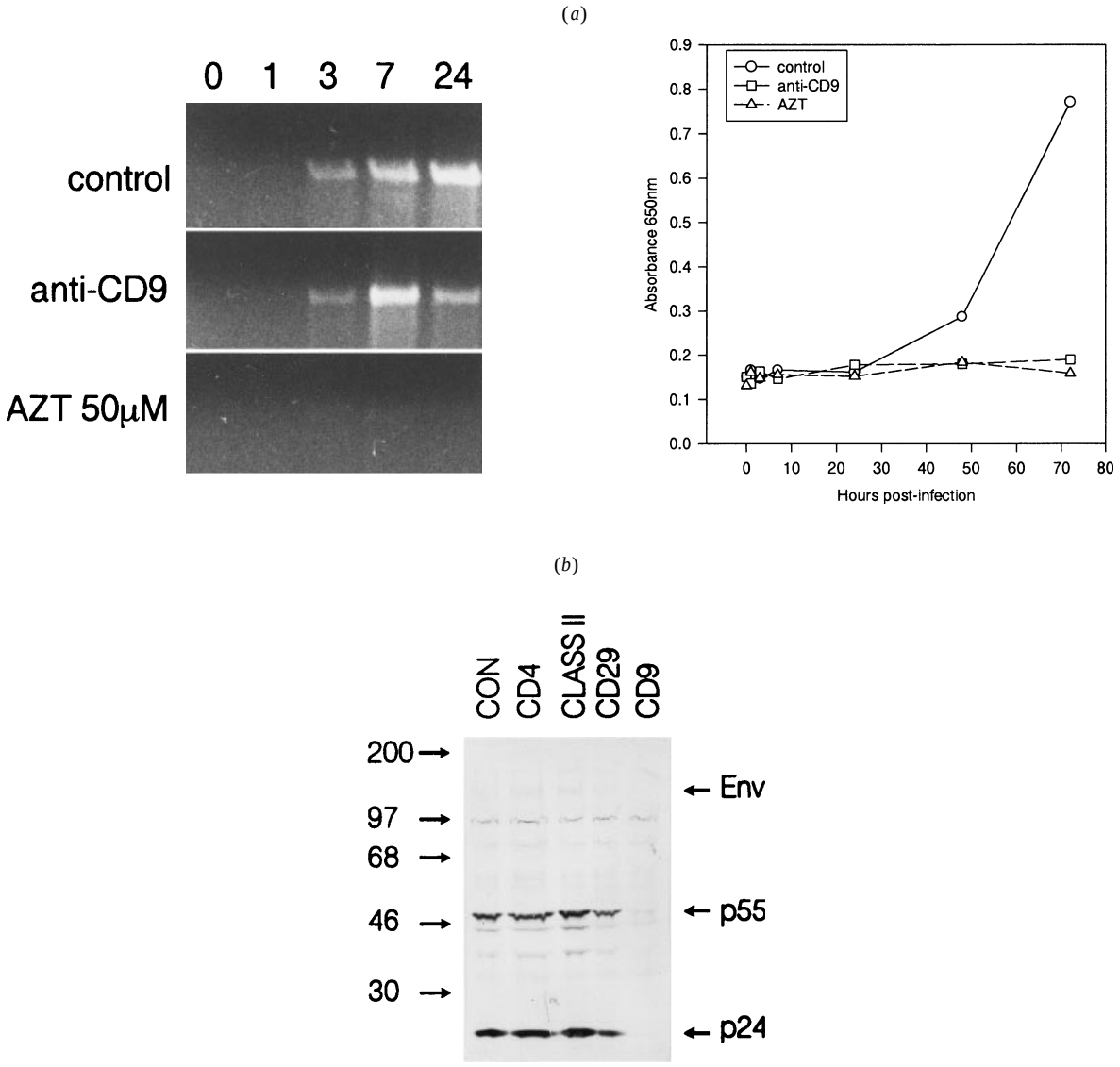

Fig. 1. (*a*) Anti-CD9 antibody inhibits infection with FIV at a post-entry stage of the virus life-cycle. Q201 cells were incubated at 4 °C with either 25 μg/ml anti-CD9 antibody, 50 μM AZT or culture medium alone (control). The cells were then challenged with 5000 TCID of FIV_{GL8}. Cell pellets were collected at intervals of 0, 1, 3, 7 and 24 h post-infection and analysed for viral DNA by PCR. Viral DNA was evident in the control and anti-CD9-treated cultures by 3 h post-infection whereas the AZT-treated culture remained negative. Supernatants were collected from each culture and analysed for p24 production by ELISA. p24 production was detected in supernatant from the control culture but not the anti-CD9- or AZT-treated cultures. (*b*) Cell pellets were collected from antibody-treated cells at 5 days post-infection with FIV_{GL8}, lysed and analysed by Western blot for virus-specific proteins. Following incubation with a serum from an FIV-infected cat, virus-specific bands corresponding to capsid protein p24, Gag precursor p55 and Env protein were evident in cultures treated with anti-CD4, anti-MHC class II or anti-CD29. However virus-specific bands could not be detected in the cultures treated with anti-CD9 antibody. Molecular mass markers are depicted (left) in kDa.

concentration of antibody used, confirming that infection of 3201 cells with CrFK-adapted FIV_{F14} is refractory to inhibition.

Ectopic expression of CD9 on 3201 T cells augments FIV infection

3201 cells were transfected with a eukaryotic expression vector encoding either feline CD9 or feline CD29. CD29 was chosen for comparison as it is known to associate with CD9 on the cell surface and is co-expressed with CD9 in several cell types. Cells expressing CD9 or CD29 stably were immunopurified using anti-CD9 or anti-CD29 antibody and immunomagnetic beads. Following three cycles of immunopurification the cells were analysed by flow cytometry (Fig. 3). CD9 and CD29 were expressed to a similar degree (CD9 98·1%, CD29 97·3%) and intensity (CD9 mean intensity $1{\cdot}62 \pm 1{\cdot}14$, CD29 mean intensity $1{\cdot}69 \pm 0{\cdot}75$) whereas untransfected control cells were negative for expression of both antigens [Northern blot analysis for CD9 mRNA suggested that CD9 expression

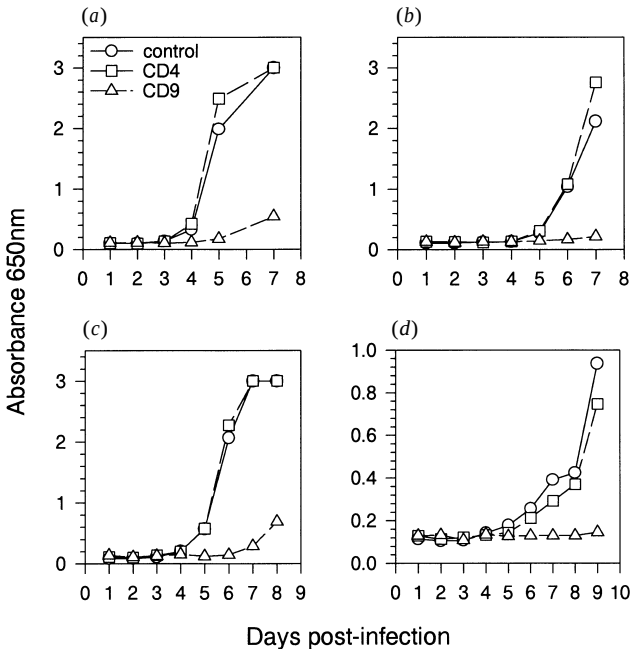

Fig. 2. Anti-CD9 antibody inhibits infection with FIV irrespective of passage history or subtype. Q201 cells were incubated with 25 μg/ml of anti-CD4 or anti-CD9 antibody for 1 h at 4 °C and then infected with either Q201-grown FIV_{GL8} (*a*), Q201-grown FIV_{F14} (*b*), CrFK-grown FIV_{F14} (*c*) or Q201-grown FIV_{TM2} (*d*) for 1 h at 4 °C. Cultures were monitored for p24 production by ELISA. Anti-CD9 antibody inhibited infection with FIV irrespective of passage history or subtype.

is inhibited at the transcriptional level (data not shown)]. The level of CD9 expression achieved on 3201 cells was approximately 10-fold less than that found on primary activated T cells (Hosie *et al.*, 1993). The CD9- and CD29-expressing 3201 cells, and the control 3201 cells, were then challenged with 500 TCID of FIV_{F14} and cell pellets were collected at days 0, 1 and 2 post-infection and analysed for viral reverse transcripts by PCR. The CD9-expressing 3201 cells were strongly positive for viral reverse transcripts by PCR at days 1 and 2 post-infection whereas the control and CD29-expressing cultures were weakly positive at day 1 post-infection, increasing by day 2 (Fig. 4*a*). Moreover, analysis of p24 levels in the culture supernatants from the three cultures suggested that p24 production was enhanced in the culture of CD9-expressing cells in comparison with either the control or CD29-expressing cells (Fig. 4*b*). In order to confirm that ectopic expression of CD9 enhanced virus entry the control (−CD9) and CD9-expressing (+CD9) 3201 cells were challenged with 10, 50, 125, 250, 500 or 1000 TCID of FIV_{F14} and cell pellets were collected at day 1 post-infection and analysed by PCR (Fig. 4*c*). Viral DNA could be detected by PCR at 500 TCID of input virus with the control 3201 cells. In contrast, the CD9-expressing 3201 cells were positive at 125 TCID of virus suggesting that the CD9-expressing cells were at least 4-fold more sensitive to infection with FIV_{F14}. Repeat experiments suggested some variability in the degree of enhancement between assays but that infection was consistently enhanced in the CD9-expressing cells. Despite prolonged culture (up to 1 month), FIV_{GL8} infection could not be established in either the control, CD9-expressing or CD29-expressing 3201 cells.

Discussion

In this study, we demonstrate that the inhibition of FIV infection of Q201 cells by anti-CD9 antibody operates after virus entry and is independent of virus tropism, subtype and passage history. Also, the CD9-negative 3201 cell line supported infection with the cell culture adapted virus FIV_{F14} and the infection process was not inhibited by anti-CD9 antibody, confirming that CD9 is not obligatory for FIV

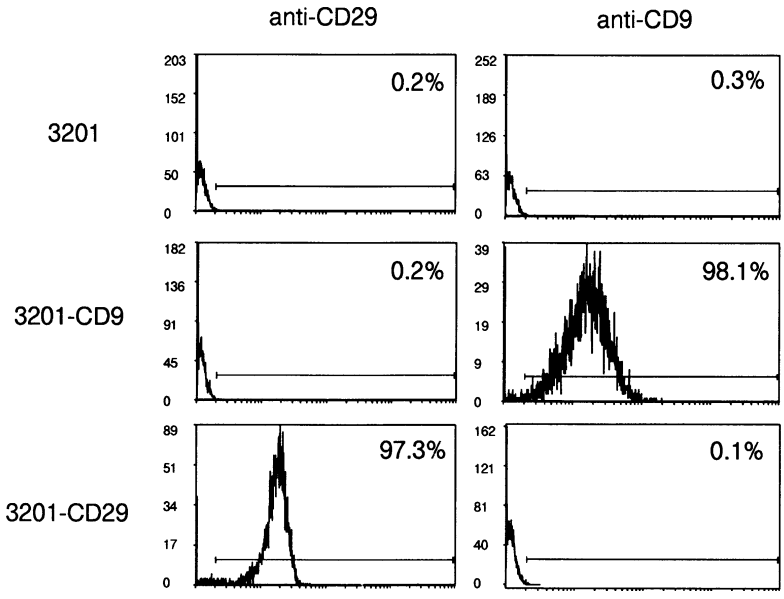

Fig. 3. Flow cytometric analysis of CD9 and CD29 expression on transfected 3201 cells. Cells (1×10^6) of control 3201, CD9-transfected 3201 or CD29-transfected 3201 were incubated with anti-feline CD9 (vpg15) or anti-CD29 (4B4) and processed for flow cytometry; 5000 events were collected in LIST mode for each sample. The histograms represent fluorescence intensity (abscissa) versus relative cell number (ordinate).

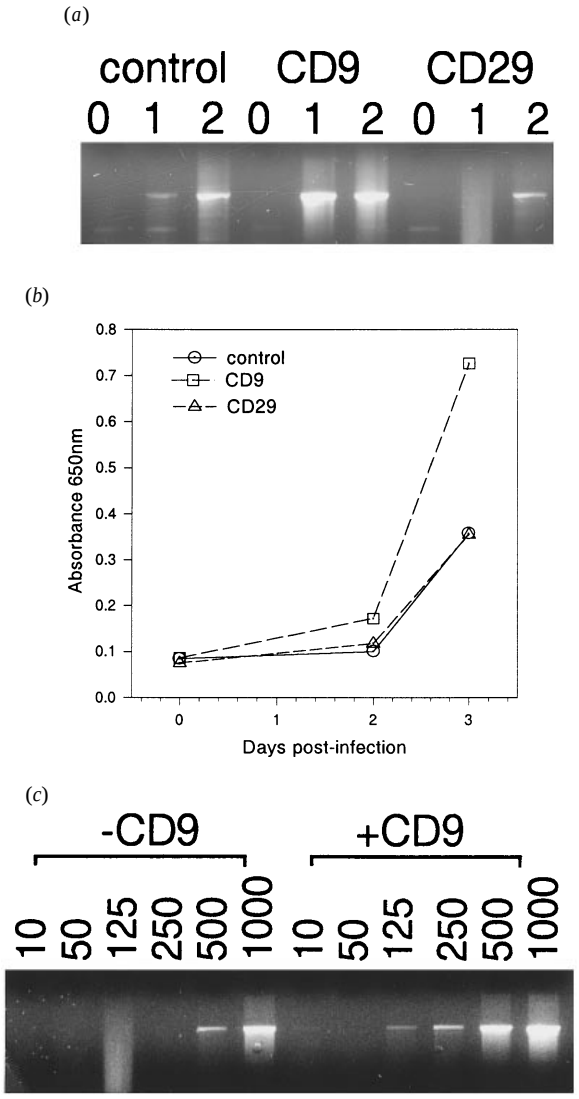

Fig. 4. Enhancement of FIV infection following ectopic expression of CD9. Control 3201 cells and 3201 cells transfected with CD9 or CD29 were exposed to 500 TCID FIV_{F14} and analysed by PCR for viral DNA at 0, 1 and 2 days post-infection (*a*). Higher levels of viral DNA appeared to be present in the CD9-expressing 3201 cells. Culture supernatants were collected and analysed by ELISA for p24 (*b*). p24 production was enhanced from the CD9-expressing 3201 cells at 2 and 3 days post-infection. The enhanced sensitivity of CD9-positive 3201 cells to FIV infection was quantified by exposing CD9-negative and CD9-positive 3201 cells to 10, 50, 125, 250, 500 and 1000 TCID of FIV_{F14} and analysing cell pellets collected at 1 day post-infection for viral DNA by PCR (*c*). Ectopic expression of CD9 on the 3201 cells rendered the cells at least 4-fold more susceptible to infection with FIV_{F14}.

infection. Ectopic expression of CD9 on 3201 cells enhanced infection with FIV suggesting that CD9 may act as a facilitator of virus replication and that the inhibition of FIV infection by anti-CD9 antibody reflects ablation of this function.

Inhibition of FIV infection by anti-CD9 antibody was independent of the strain of virus. This is in marked contrast to the strain-specific inhibition of HIV infection by blockade of β-chemokine receptors where an antiserum raised against LESTRinhibited infection with T cell-tropic, but not macrophage-tropic strains of HIV (Feng *et al.*, 1996). Conversely, MIP-1α, MIP-1β and RANTES, the ligands for CKR5, inhibit infection with macrophage-tropic isolates of HIV (Deng *et al.*, 1996; Dragic *et al.*, 1996). FIV resembles HIV in that virus strains can be subdivided on the basis of cell tropism. FIV strains adapted for growth in the CrFK cell line show characteristic mutations in the V3 loop of gp120 that increase the net charge of the loop (Verschoor *et al.*, 1995; Siebelink *et al.*, 1995), mirroring the increase in charge of the HIV V3 loop with the switch from NSI to SI variants (de Jong *et al.*, 1992; Shioda *et al.*, 1996). However, we have found no evidence for strain-specificity of inhibition by anti-CD9 antibody; infection was inhibited equally as potently between strains of FIV representative of distinct virus tropisms and subtypes.

The anti-CD9 antibody vpg15 inhibited FIV infection at a post-entry stage of the virus life-cycle. The detection of viral reverse transcripts by PCR in anti-CD9 antibody treated cultures confirmed that the stages of binding, fusion and entry were not inhibited. In comparison, AZT inhibited reverse transcription significantly, confirming the validity of the PCR assay. Despite failing to inhibit virus entry, the anti-CD9 antibody prevented productive infection with FIV. An alternative inhibitory mechanism is therefore implicated. It is possible that the inhibitory effects of anti-CD9 antibody are linked to the ability of TM4 superfamily molecules to form intimate associations with other molecules at the cell surface. CD9 has been shown to associate with a variety of cell surface molecules including the platelet glycoprotein complex GPIIb–IIIa (Slupsky *et al.*, 1989), β-integrin (Rubenstein *et al.*, 1994), and the TM4 superfamily molecules CD63 and CD81 (Radford *et al.*, 1996). It is possible that CD9 associates with viral proteins during transport or virus assembly and that anti-CD9 antibody prevents this association, perhaps by sequestering CD9 from its normal cellular function. CD9 may conceivably function as a 'molecular chaperone', assisting in the transport of FIV through the cell, both during virus entry and egress from the cell. Such a role for CD9 would explain the enhancement of FIV_{F14} entry by ectopic expression of CD9 on 3201 cells. Indeed, the degree of enhancement of FIV infection is similar to that of diphtheria toxin binding to its receptor on Vero cells following ectopic expression of CD9 (Iwamoto *et al.*, 1994). The envelope glycoproteins of vesicular stomatitis virus (Hammond & Helenius, 1994), cytomegalovirus (Yamashita *et al.*, 1996) and HIV (Otteken & Moss, 1996) have been shown to interact with the molecular chaperones BiP, calnexin and calareticulin; this interaction is required for protein folding, intramolecular disulphide bond formation and export from the endoplasmic reticulum. Inhibition of the interaction between chaperones and the viral glycoprotein (e.g. by glucosidase inhibitors) might conceivably lead to a build-up of inappropriately folded and functionally inactive gp120. Similarly, inhibitors of protein-disulphide isomerase (PDI) have

been shown to inhibit infection with HIV (Ryser *et al.*, 1994). Thus, there are several stages in the virus life-cycle that may involve CD9 and may hence be sensitive to inhibition by the treatment with anti-CD9 antibody.

Antibodies recognizing the CDR3 domain of CD4, the primary receptor for HIV, inhibit HIV infection at the level of transcription but do not inhibit virus entry (Benkirane *et al.*, 1993). While it seemed possible that the anti-CD9 antibody vpg15 might inhibit FIV infection by a similar mechanism, recent studies suggest that levels of spliced mRNAs in FIV-infected cells are unaffected by treatment with anti-CD9 antibody (A. De Parseval & J. E. Elder, unpublished observations). If, as we now believe, anti-CD9 antibody inhibits FIV infection at a post-transcriptional stage of the virus life-cycle, a novel mechanism of anti-viral activity is implicated. Moreover, as an antibody recognizing another member of the TM4 superfamily, C33, inhibits syncytium formation by human T-lymphotropic virus (Imai & Yoshie, 1993; Fukudome *et al.*, 1992), and TM4 superfamily molecules have been linked to integrin-mediated fusion by HIV gp120 (Ohta *et al.*, 1994), this observation may be indicative of a wider involvement of this gene family in retroviral replication.

To date, there has been no conclusive evidence for the existence of natural ligands for TM4 superfamily molecules although an interaction between purified CD9 and fibronectin on platelets has been described (Jennings *et al.*, 1994). The potent signalling function of CD9 and CD81 (Masellis-Smith *et al.*, 1990; Oren *et al.*, 1990; Arthos *et al.*, 1990) would suggest that such ligands may exist. If such a ligand can be identified for CD9, novel approaches to the treatment of FIV infection may be possible.

The authors thank Dr John Elder for helpful discussions. This study was funded by The Wellcome Trust and the Medical Research Council.

References

Arthos, J., Deen, K. C., Chaikin, M. A., Fornwald, J. A., Sathe, G., Sattentau, Q. J., Clapham, P. R., Weiss, R. A., McDougal, J. S., Pietropaolo, C., Axel, R., Truneh, A., Maddon, P. J. & Sweet, R. W. (1989). Identification of the residues in human CD4 critical for the binding of HIV. *Cell* **57**, 469–481.

Arthos, J., Deen, K. C., Chaiken, M. A., Sattentau, Q. J., Maddon, P. J., Axel, R., Rosenberg, M., Shatzman, A., Truneh, A. & Sweet, R. W. (1990). Structural analysis of the HIV gp120 binding domain of the CD4 receptor. *Aids Research and Human Retroviruses* **6**, 118.

Arthur, L. O., Bess, J. W. J., Sowder, R. C., Benveniste, R. E., Mann, D. L., Chermann, J. C. & Henderson, L. E. (1992). Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. *Science* **258**, 1935–1938.

Arthur, L. O., Bess, J. W., Urban, R. G., Strominger, J. L., Morton, W. R., Mann, D. L., Henderson, L. E. & Benveniste, R. E. (1995). Macaques immunized with HLA-DR are protected from challenge with simian immunodeficiency virus. *Journal of Virology* **69**, 3117–3124.

Benkirane, M., Corbeau, P., Housset, V. & Devaux, C. (1993). An antibody that binds the immunoglobulin CDR3-like region of the CD4 molecule inhibits provirus transcription in HIV-infected T cells. *EMBO Journal* **12**, 4909–4921.

Braun, J. M. A., Gras, G., Chapuis, F., Sommerfelt, M. A., Clapham, P. R., Weiss, R. A., Åsjo, B., Gluckman, J.-C., Dormont, D. & Barré-Sinoussi, F. (1995). In *Leucocyte Typing V*, pp. 465–468. Edited by S. F. Schlossman, L. Boumsell, W. Gilks, J. M. Harlan, T. Kishimoto, C. Morimoto, J. Ritz, S. Shaw, R. Silverstein T. Springer, T. F. Tedder & R. F. Todd. Oxford: Oxford University Press.

Briant, L., Benkirane, M., Girard, M., Hirn, M., Iosef, C. & Devaux, C. (1996). Inhibition of human immunodeficiency virus type 1 production in infected peripheral blood mononuclear cells by human leukocyte antigen class I-specific antibodies: evidence for a novel antiviral mechanism. *Journal of Virology* **70**, 5213–5220.

Corbeau, P., Devaux, C., Kourilsky, F. & Chermann, J. C. (1990). An early postinfection signal mediated by monoclonal anti-beta 2 microglobulin antibody is responsible for delayed production of human immunodeficiency virus type 1 in peripheral blood mononuclear cells. *Journal of Virology* **64**, 1459–1464.

Dalgleish, A. G., Beverley, P. C. L., Clapham, P. R., Crawford, D. H., Greaves, M. F. & Weiss, R. A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**, 763–767.

de Jong, J. J., de Ronde, A., Keulen, W., Tersmette, M. & Goudsmit, J. (1992). Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. *Journal of Virology* **66**, 6777–6780.

Deng, H., Liu, R., Ellmeir, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. & Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661–666.

Devaux, C., Boucraut, J., Poirier, G., Corbeau, P., Rey, F., Benkirane, M., Perarnau, B., Kourilsky, F. & Chermann, J. C. (1990). Anti-beta-2-microglobulin monoclonal antibodies mediate a delay in HIV1 cytopathic effect on MT4 cells. *Research in Immunology* **141**, 357–372.

Dragic, T., Litwin, T., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. & Paxton, W. A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR5. *Nature* **381**, 667–673.

Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. (1996). HIV-1 entry co-factor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872–877.

Fukudome, K., Furuse, M., Imai, T., Nishimura, M., Takagi, S., Hinuma, Y. & Yoshie, O. (1992). Identification of membrane antigen C33 recognized by monoclonal antibodies inhibitory to human T-cell leukemia virus type 1 (HTLV-1)-induced syncytium formation: altered glycosylation of C33 antigen in HTLV-1-positive T cells. *Journal of Virology* **66**, 1394–1401.

Gorman, C. (1985). In *DNA Cloning*, vol. 2, *A Practical Approach*, p. 143. Edited by D. M. Glover. Oxford: IRL Press.

Hammond, C. & Helenius, A. (1994). Folding of VSV G protein: sequential interaction with BiP and calnexin. *Science* **266**, 456–458.

Hildreth, J. E. K. & Orentas, R. J. (1989). Involvement of a leukocyte adhesion receptor (LFA-1) in HIV-induced syncytium formation. *Science* **244**, 1075–1078.

Hohdatsu, T., Hirabayashi, H., Motokawa, K. & Koyama, H. (1996). Comparative study of the cell tropism of feline immunodeficiency virus isolates of subtype A, subtype B and subtype D classified on the *env* gene V3–V5 sequence. *Journal of General Virology* **77**, 93–100.

Hosie, M. J., Willett, B. J., Dunsford, T. H., Jarrett, O. & Neil, J. C. (1993). A monoclonal antibody which blocks infection with feline immunodeficiency virus identifies a possible non-CD4 receptor. *Journal of Virology* **67**, 1667–1671.

Imai, T. & Yoshie, O. (1993). C33 antigen and M38 antigen recognized by monoclonal antibodies inhibitory to syncytium formation by human T cell leukemia virus type 1 are both members of the transmembrane 4 superfamily and associate with each other and with CD4 and CD8 in T cells. *Journal of Immunology* **151**, 6470–6481.

Iwamoto, R., Higashiyama, S., Mitamura, T., Taniguchi, N., Klagsburn, M. & Mekada, E. (1994). Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *EMBO Journal* **13**, 2322–2330.

Jennings, L. K., Orthofer, C. S. & Wilkinson, D. A. (1994). Platelet CD9 binds the adhesive protein fibronectin. *Blood* **84**, A471(abstract).

Jordan, C. A., Watkins, B. A., Kufta, C. & Dubois-Dalq, M. (1991). Infection of brain microglial cells by human immunodeficiency virus type 1 is CD4 dependent. *Journal of Virology* **65**, 736–742.

Klatzmann, D., Champagne, E., Chamaret, S., Guetard, D., Hercend, T., Gluckmann, J. C. & Montagnier, L. (1984). T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312**, 767–768.

Masellis-Smith, A., Jensen, G. S., Seehafer, J. G., Slupsky, J. R. & Shaw, A. R. E. (1990). Anti-CD9 monoclonal antibodies induce homotypic adhesion of pre-B cell lines by a novel mechanism. *Journal of Immunology* **144**, 1607–1613.

Ohta, H., Tsurudome, M., Matsumura, H., Koga, Y., Morikawa, S., Kawano, M., Kusugawa, S., Komoda, H., Nishio, M. & Ito, Y. (1994). Molecular and biological characterization of fusion regulatory proteins (FRPs): anti-FRP mAbs induced HIV-mediated cell fusion via an integrin system. *EMBO Journal* **13**, 2044–2055.

Oren, R., Takahashi, S., Doss, C., Levy, R. & Levy, S. (1990). TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. *Molecular and Cellular Biology* **10**, 4007.

Otteken, A. & Moss, B. (1996). Calareticulin interacts with newly synthesized human immunodeficiency virus envelope glycoprotein, suggesting a chaperone function similar to that of calnexin. *Journal of Biological Chemistry* **271**, 97–103.

Peterson, A. & Seed, B. (1988). Genetic analysis of monoclonal antibody and HIV binding sites on the human lymphocyte antigen CD4. *Cell* **54**, 65–72.

Radford, K. J., Thorne, R. F. & Hersey, P. (1996). CD63 associates with transmembrane 4 superfamily members, CD9 and CD81, and with β1 integrins in human melanoma. *Biochemical and Biophysical Research Communications* **222**, 13–18.

Rubenstein, E., Le Naour, F., Billard, M., Prenant, M. & Boucheix, C. (1994). CD9 antigen is an accessory subunit of the VLA integrin complexes. *European Journal of Immunology* **24**, 3005–3013.

Ryser, H. J.-P., Levy, E. M., Mandel, R. & DiSciullo, G. J. (1994). Inhibition of human immunodeficiency virus infection by agents that interfere with thiol-disulfide interchange upon virus–receptor interaction. *Proceedings of the National Academy of Sciences, USA* **91**, 4559–4563.

Ryu, S. E., Kwong, P. D., Truneh, A., Porter, T. G., Arthos, J., Rosenberg, M., Dai, X. P., Xuong, N. H., Axel, R., Sweet, R. W. & Hendrickson, W. A. (1990). Crystal structure of an HIV binding recombinant fragment of human CD4. *Nature* **348**, 419–426.

Sato, A. I., Balamuth, F. B., Ugen, K. E., Williams, W. V. & Weiner, D. B. (1994). Identification of CD7 glycoprotein as an accessory molecule in HIV-1-mediated syncytium formation and cellfree infection. *Journal of Immunology* **152**, 5142–5152.

Shioda, T., Levy, J. A. & Cheng-Mayer, C. (1996). Small amino-acid changes in the V3 hypervariable region of gp120 can affect the T-cell line and macrophage tropism of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences, USA* **89**, 9434–9438.

Siebelink, K. H. J., Karlas, J. A., Rimmelzwaan, G. F., Osterhaus, A. D. M. E. & Bosch, M. L. (1995). A determinant of feline immunodeficiency virus involved in CrFK tropism. *Veterinary Immunology and Immunopathology* **46**, 61–69.

Slupsky, J. R., Seehafer, J. G., Tang, S., Masellis-Smith, A. & Shaw, A. R. E. (1989). Evidence that monoclonal antibodies against CD9 antigen induce specific association between CD9 and the platelet glycoprotein IIb–IIIa complex. *Journal of Biological Chemistry* **264**, 12289–12293.

Sodora, D. L., Shpaer, E. G., Kitchell, B. E., Dow, S. W., Hoover, E. A. & Mullins, J. I. (1994). Identification of 3 feline immunodeficiency virus (FIV) *env* gene subtypes and comparison of the FIV and human immunodeficiency virus type-1 evolutionary patterns. *Journal of Virology* **68**, 2230–2238.

Sommerfelt, M. A. & Åsjo, B. (1995). Intercellular adhesion molecule 3, a candidate human immunodeficiency virus type 1 co-receptor on lymphoid and monocytoid cells. *Journal of General Virology* **76**, 1345–1352.

Verschoor, E. J., Boven, L. A., Blaak, H., van Vliet, A. R. W., Horzinek, M. C. & de Ronde, A. (1995). A single mutation within the V3 envelope neutralization domain of feline immunodeficiency virus determines its tropism for CRFK cells. *Journal of Virology* **69**, 4752–4757.

Willett, B. J. & Hosie, M. J. (1995). In *Feline Immunology and Immunodeficiency*, pp. 205–219. Edited by B. J. Willett & O. J. Jarrett. Oxford: Oxford University Press.

Willett, B. J. & Neil, J. C. (1995). cDNA cloning and eukaryotic expression of feline CD9. *Molecular Immunology* **32**, 417–423.

Willett, B. J., Hosie, M. J., Dunsford, T. H., Neil, J. C. & Jarrett, O. (1991). Productive infection of T-helper lymphocytes with feline immunodeficiency virus is accompanied by reduced expression of CD4. *AIDS* **5**, 1469–1475.

Willett, B. J., de Parseval, A., Peri, E., Rocchi, M., Hosie, M. J., Randall, R., Klatzmann, D., Neil, J. C. & Jarrett, O. (1994*a*). The generation of monoclonal antibodies recognising novel epitopes by immunisation with solid matrix antigen–antibody complexes reveals a polymorphic determinant on feline CD4. *Journal of Immunological Methods* **176**, 213–220.

Willett, B. J., Hosie, M. J., Jarrett, O. & Neil, J. C. (1994*b*). Identification of a putative cellular receptor for feline immunodeficiency virus as the feline homologue of CD9. *Immunology* **81**, 228–233.

Yamashita, Y., Shimokata, K., Mizuno, S., Daikoku, T., Tsurumi, T. & Nishiyama, Y. (1996). Calnexin acts as a molecular chaperone during the folding of glycoprotein B of human cytomegalovirus. *Journal of Virology* **70**, 2237–2246.

Received 18 September 1996; Accepted 26 November 1996