Characterization of two monoclonal antibodies against feline immunodeficiency virus \textit{gag} gene products and their application in an assay to evaluate neutralizing antibody activity

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Monoclonal antibodies (MAbs) 3B7 and 1C11 were produced against the \textit{gag} gene products of feline immunodeficiency virus (FIV). These MAbs reacted strongly with FIV p24 in Western blots (immunoblots) and recognized p50 with a lower intensity. They specifically bound antigens in the cytoplasm of FIV-infected cells as determined by indirect immunofluorescence and immunocytochemistry. Although neither MAb inhibited viral replication \textit{in vitro}, they were useful in a simple assay for the detection and quantification of infectious virus and neutralizing antibody activity. The assay utilizes Crandell feline kidney cells and requires 4 days for completion. Neutralizing antibodies in cats were detected 3 to 4 weeks after experimental infection with FIV. Antibody titres progressively increased during the first year of infection reaching high titres which were maintained 2.5 years post-infection. The MAbs produced should be valuable reagents for the monitoring of viral replication in cells or tissues from FIV-infected cats and for other \textit{in vitro} applications.

Introduction

Feline immunodeficiency virus (FIV) was isolated in 1986 from immunodeficient domestic cats in California (Pedersen \textit{et al.}, 1987) and has since been isolated in a number of different countries (Harbour \textit{et al.}, 1988; Ishida \textit{et al.}, 1988). There is no statistical linkage between FIV and feline leukaemia virus (FeLV) (Yamamoto \textit{et al.}, 1989), a retrovirus that also causes signs of immunosuppression in cats. FIV has a tropism for T lymphocytes (Pedersen \textit{et al.}, 1987) and has been propagated in Crandell feline kidney (CrFK) cells (Yamamoto \textit{et al.}, 1989) and the permanent feline T cell lines LSA-1 (Yamamoto \textit{et al.}, 1986) and MVA-1 (Miyazawa \textit{et al.}, 1989). \textit{In vivo} and \textit{in vitro} infection of peritoneal macrophages of cats was reported by Brunner & Pedersen (1989). Several proteins encoded by FIV \textit{gag}, \textit{pol} and \textit{env} genes have been identified; the \textit{gag} gene encodes a polypeptide precursor with an apparent $M_r$ of 50K (Talbott \textit{et al.}, 1989; Egberink \textit{et al.}, 1990; Steinman \textit{et al.}, 1990). When this protein is cleaved by the protease encoded by the \textit{pol} gene, the major core protein p24 and at least two more proteins, p15 and p10, are produced (Egberink \textit{et al.}, 1990; Steinman \textit{et al.}, 1990). Quantitative measurement of FIV has been conducted by immunofluorescence or by detection of reverse transcriptase activity in tissue culture supernatants. However, for many studies a plaque assay using susceptible cells adherent to plastic would be more convenient. In this report we describe the production and characterization of two monoclonal antibodies (MAbs) specific for FIV \textit{gag} gene products and their use in a simple assay to titrate infectious virus doses and neutralizing antibody activity.

Methods

\textit{Virus and cell lines}. The FIV Petaluma strain was propagated in persistently infected CrFK cells as previously described (Pedersen \textit{et al.}, 1987; Yamamoto \textit{et al.}, 1988). This cell line is free of feline syncytium-forming virus (FeSFV) and feline infectious peritonitis virus (FIPV) as determined by an indirect immunofluorescence assay (IFA) and by immunoblot analysis using cat sera for FeSFV (Merieux) and FIPV-1 and -II (American Biorsearch), respectively. A microcarrier technique was employed to increase virus production by FIV-infected CrFK cells (FIV/CrFK). Briefly, 1-5 g of preswollen Cytodex 1 microcarrier beads (Pharmacia) were seeded with $6 	imes 10^7$ FIV/CrFK cells in 100 ml of Dulbecco's modified Eagle's medium (DMEM), 10% heat-inactivated foetal bovine serum (FBS) and 1% antibiotic–antimycotic (Gibco), and incubated at 37°C with stirring (20 r.p.m.) for 2 min every 30 min for 4 to 5 h in a spinner vessel (Techne MCS-104 S). The culture volume was later increased to 500 ml. Virus was concentrated from tissue culture supernatants by precipitation with polyethylene glycol as described (Bishop \textit{et al.}, 1971). The precipitated
proteins and viruses were layered over a double cushion of 20 and 80% (w/v)sucrose and centrifuged for 120 min at 25000 r.p.m. in a Beckman SW27 rotor. The band of material between the sucrose layers was collected and diluted in 0.15 M-NaCl, 0.01 M-Tris-HCl buffer pH 7.5. After a two-step differential centrifugation at 6000 r.p.m. for 20 min and 11000 r.p.m. for 10 min in a Beckman 30 rotor, the supernatant was filtered through a 0.45 µm Millipore filter and the virus pellet was finally centrifuged at 30000 r.p.m. for 30 min. All procedures were carried out at 4 °C. The other viruses used as antigens were human immunodeficiency virus type 1 (HIV-1), human T cell leukaemia virus type 1 (HTLV-I), the Rickard strain of FeLV, FeSFV, endogenous cat virus RD114, equine infectious anaemia virus (EIAV) and visna virus (VV). FeLV was produced in the FL-74 feline T lymphoblastoid cell line and purified as previously described (Noronha et al., 1977); RD114 virus was prepared from RD114B cell extracts and FeSFV (Cornell and ATCC isolates) was cultivated in CrFK cells. VV was grown in lamb testicle primary cultures. EIAV was propagated in the equine dermal cell line CCL57.

**Virus isolation.** FIV was isolated from peripheral blood mononuclear cell (PBMC) cultures of experimentally FIV-infected cats as previously described (Pedersen et al., 1987). Virus replication was monitored by a standard reverse transcriptase assay, IFA and immunocytochemistry.

**MAb production.** BALB/c mice (2 months old) were immunized intraperitoneally with a 0.2 ml emulsion of 50 µg of purified FIV in phosphate-buffered saline (PBS) and Freund's complete adjuvant (1:1). Inoculations were repeated with the same amount of virus in Freund's incomplete adjuvant after 2 weeks, and in PBS after 4 weeks. The mice were given intraperitoneal and intravenous inoculations with disrupted virus in PBS 30 days later. Four days following the final booster, the spleens were removed and desegregated and red blood cells were lysed in 0.16 M-MN4Cl, 0.017 M-Tris buffer pH 7.65. Fusion and cloring were done as described previously (Youngren et al., 1984). Viable hybridoma clones were tested for virus-specific antibody by ELISA, with purified FIV and normal CrFK cell lysates as coating antigens. Cell lines producing antibodies were cloned three times by limiting dilution and expanded in spinner vessels (20 r.p.m.) in a 3% CO2 humidified atmosphere. Medium was removed and serial dilutions of the virus in DMEM were added to the plates (three per dilution; 0.2 ml/plate). After a 1 h adsorption period at 37 °C, the monolayers were washed and fresh medium was added. The plates were incubated for 4 days at 37 °C and then washed and fixed with 10% buffered formalin. Undiluted cell-free supernatant (2 ml) from hybridoma cell line 3B7 (or 1C11) was added for a period of 30 to 45 min at 37 °C. The plates were washed with PBS and a biotinylated rabbit anti-mouse antibody was added for 10 min at 37 °C. They were then rinsed and incubated for an additional 10 min with streptavidin-peroxidase (Zymed Laboratories) diluted 1:20 in PBS. The substrate solution (H2O2 and amionetoxy1 carbazole) was added and the reaction was stopped after 5 to 15 min with distilled water. After staining with haemafoxin the foci were counted using a microscope at ×5 magnification. A virus stock obtained from cell-free supernatants of a 5 day culture of FIV-infected CrFK cells was titrated, and kept in aliquots in liquid nitrogen for use in neutralization assays.

**Neutralization assay.** Filter-sterilized MAbs (0-1 mg/ml) were preincubated with a equal volume of virus (100 f.f.u.) for 1 h at RT, then virus-MAb mixtures were added (0.2 ml/plate) and incubated for 1 h at 37 °C. The plates were further incubated for 4 days at 37 °C and the number of foci was scored as described above. Three plates were used for each MAb tested and three other plates with a MAb of irrelevant specificity served as virus controls. The neutralization titres were determined by using the 50% (V50/V0 = 0.5) neutralizing point, where V0 is the number of f.f.u. and V50 is the total number of f.f.u. in the control to which the MAb of irrelevant specificity was added. MAbs causing a 50% or greater reduction of the average focus number compared to control plates were considered to be positive.

For seroconversion studies, eight specific pathogen-free (SPF) kittens of both sexes at 6 to 10 weeks of age were infected by a subcutaneous (s.c.) inoculation of 3 x 105 f.f.u. of FIV in 2 ml DMEM. In addition, cat 642 was inoculated twice and cat 671 once with their own virus isolates at 47 and 49 weeks, and at 50 weeks after the first inoculation, respectively. Inoculations were performed s.c. with 3 x 106 infected PBMCs in 2 ml RPMI 1640. The cats were bled regularly for virus isolation and to determine antibody specificity. Sera from 13 cats with non-specific illness were also tested. All samples were heat-inactivated for 30 min at 56 °C before testing. Two serial dilutions of the samples in DMEM were incubated in duplicate with 100 f.f.u. of FIV for 60 min at RT prior to inoculation onto CrFK cells; pooled normal SPF cat sera (diluted 1:10) were included as virus controls.

**Kinetics of serum neutralization.** Dilutions of an immune serum (cat 633 at 1:5000, 1:10000 and 1:20000) were incubated with FIV (100 f.f.u.) for 30 min at room temperature (RT). The cells were washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) for an additional 30 min at RT. The cells were then washed, resuspended in PBS, mounted on slides and examined with a fluorescence microscope.

FIV/CrFK cells were trypsinized, resuspended in culture medium (4 x 105 cells/ml) and spotted into six wells of a 12-well Teflon-coated slide; adjacent wells were seeded with an equal number of uninfected negative control cells. After an adsorption period of 1 to 2 h at 37 °C, the cells were fixed with cold acetone for 10 min, air-dried and stored at −20 °C. The incubation steps with the MAbs and FITC-conjugated goat anti-mouse IgG were done at 37 °C for 30 min.

**Virus titration using MAbs 3B7 and 1C11.** CrFK cells (2.5 x 105/5 ml) were seeded into 2 mm gridded, 60 mm diameter Petri dishes in DMEM supplemented with 10% heat-inactivated FBS, 2 µg/ml polybrene (Aldrich) and antibiotics. The plates were incubated at 37 °C in a 5% CO2 humidified atmosphere overnight. Medium was removed and serial dilutions of the virus in DMEM were added to the plates (three per dilution; 0.2 ml/plate). After a 1 h adsorption period at 37 °C, the monolayers were washed and fresh medium was added. The plates were incubated for 4 days at 37 °C and then washed and fixed with 10% buffered formalin. Undiluted cell-free supernatant (2 ml) from hybridoma cell line 3B7 (or 1C11) was added for a period of 30 to 45 min at 37 °C. The plates were washed with PBS and a biotinylated rabbit anti-mouse antibody was added for 10 min at 37 °C. They were then rinsed and incubated for an additional 10 min with streptavidin-peroxidase (Zymed Laboratories) diluted 1:20 in PBS. The substrate solution (H2O2 and aminonitroxy1 carbazole) was added and the reaction was stopped after 5 to 15 min with distilled water. After staining with haemafoxin the foci were counted using a microscope at ×5 magnification. A virus stock obtained from cell-free supernatants of a 5 day culture of FIV-infected CrFK cells was titrated, and kept in aliquots in liquid nitrogen for use in neutralization assays.
measured at increasing times of incubation of the mixture on CrFK cells (Fig. 4). The dishes were scored 4 days later as described above.

**SDS–PAGE and Western blot procedure.** For Western blot (immunoblot) analysis, purified viral pellets and lysates of normal cells were separated by SDS–PAGE through a 12% gel with the buffer system described by Laemmli (1970). After electrophoresis the proteins were transferred to nitrocellulose sheets (Towbin et al., 1979) and the sheets were placed in protein blocking solution [20 mM-Tris base, 500 mM-NaCl pH 7.4 (TBS), containing 2% bovine serum albumin and 0.02% Tween 20]. The blots were then incubated with undiluted hybridoma supernatants at 4 °C overnight and, after extensive washing in TBS–0.02% Tween 20, the bound antibodies were visualized by enzyme immunoassay using a biotinylated sheep anti-mouse and a streptavidin–biotinylated horseradish peroxidase complex (Amersham) diluted at 1:1000 in TBS-0.02% Tween 20 containing 2% FBS. The reaction was developed with a 4-chloro-1-naphthol/TBS, hydrogen peroxide substrate system until dark blue bands of the desired intensity appeared. Blots were also probed with a 1:100 dilution of polyspecific cat sera for FIV as a positive control.

To confirm that the proteins recognized by MAbs 3B7 and 1C11 were the same as those recognized by antibodies from FIV-infected cats, an affinity column was made. Protein A-purified MAb 3B7 was coupled to CNBr-activated Sepharose 4B (Pharmacia). Heat-inactivated, Triton X-100-disrupted virus was incubated at 4 °C overnight with 3B7-Sepharose. The column was extensively washed with PBS and bound protein(s) eluted with 1 M-glycine buffer pH 2.5. Collected fractions were lyophilized and subjected to 13% SDS–PAGE, transferred to nitrocellulose paper and tested with a cat serum for FIV.

**Biotinylation of MAbs and competition studies.** Protein A-purified MAbs were dialysed overnight against 0.1 M-NaHCO3 pH 8-4 at 4 °C and labelled with biotin-N-hydroxysuccinimide ester (BRL), according to the manufacturer's protocol. Estimates of antibody avidity and competition binding studies were performed (Shaw et al., 1986). Competition was defined as significant if the concentration of an unlabelled heterologous MAb required to achieve 70% binding inhibition was less than 2.5-fold that of the concentration of the unlabelled homologous biotinylated MAb necessary to produce an equivalent degree of binding inhibition.

**Isotype determination.** The Ig class of antibodies secreted by the established hybridoma cell lines was determined by ELISA as described above, using goat antisera to mouse IgG1, IgG2a, IgG2b, IgA and IgM, followed by horseradish peroxidase-labelled swine antiserum to goat Ig (Boehringer Mannheim). These MAbs were isotype IgG1 and both have κ light chains.

**Results**

**IFA and immunocytochemistry**

The fluorescent labelling of virus-positive cells appeared to be within the cytoplasm as only fixed cells could be labelled (Fig. 1a). The same location of FIV antigens was shown by immunocytochemistry. None of the MAbs reacted with normal cat PBMC or CrFK cells by these methods. FIV foci on CrFK cells consist of both syncytia and single cells (Fig. 1b).

**Western blot analysis**

The specificities of the MAbs for viral proteins were characterized using a standard Western blot technique.

MAbs 3B7 and 1C11 bound strongly to a protein band with an Mr of 24K and, with a lower intensity, to a band of 50K (Fig. 2). Occasionally, the MAbs also reacted with two other bands at 15K and 10K (data not shown); the antibodies did not react with uninfected cells. None
Serology on FIV-infected cats

All experimentally FIV-infected cats developed neutralizing antibodies which were first detected at 3 to 4 weeks. These antibody titres progressively increased during the first year of infection, reaching titres of 1:5120 to 1:81960 which were maintained at 2-5 years (Table 1). The virus was reisolated several times in PBMC cultures from all cats and was detected after 2 to 4 weeks of cultivation as measured by reverse transcriptase activity and by IFA. During this study period all but two of the eight experimentally infected cats remained asymptomatic. Cats 642 and 671 showed persistent loose stools for more than 6 months and cat 642 lost weight. As shown in Table 2, nine of the 13 clinical samples reacted with FIV proteins in Western blots. These sera showed neutralizing titres ranging from 1:1000 to greater than 1:2000. The other four non-reactive samples (6, 9, 10 and 13) did not have neutralizing activity at a 1:10 dilution.

As shown in Fig. 4, neutralization of FIV was a linear function of the incubation time of virus with immune serum and the rate of neutralization changed with serum dilution.

Discussion

MAbs 3B7 and 1C11 have been described which react specifically with FIV p24 in ELISA, IFA, immunocytochemistry and Western blotting. MAbs 3B7 and 1C11 reacted strongly with a 24K protein and faintly labelled other bands at 50K, 15K and 10K. The relative size of these proteins is consistent with previously published data for FIV gag products (O'Connor et al., 1989; Olmsted et al., 1989; Egberink et al., 1990; Steinman et al., 1990). This pattern could be attributed to recognition of both precursor and mature forms of the same gag proteins. These two MAbs showed identical avidities and, since they mutually inhibited each other's binding to the virus, they were considered to be directed against the same or closely related epitopes.

Immunofluorescent labelling of virus-infected cells using MAbs 3B7 and 1C11 was restricted to the cytoplasm. Only fixed cells were labelled, supporting the idea that p24 is an internal FIV protein. Identical results were obtained by immunocytochemistry. The latter test enabled the development of a simple and rapid in vitro neutralization assay, in which FIV infection foci can easily be scored using the MAbs. This assay could also be applied to evaluate the efficacy of anti-retroviral compounds in vitro.

The lack of neutralizing activity of the MAbs was expected. As with other lentiviruses, neutralizing antibodies are most likely to be directed against the envelope glycoprotein.
Table 1. Neutralizing antibody titres and virus isolation in cats after FIV infection

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* Reciprocal of the highest dilution of sera causing a 50% reduction in foci compared to a negative sera control.
† NT, Not tested.

Table 2. Western blot specificity and neutralizing antibody activity in samples from cats suspected to be FIV-infected

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* As determined by Western blot (immunoblot).
† Reciprocal of the highest dilution tested of sera causing a 50% reduction in foci compared to a negative sera control.
‡ –, No reaction.

Serological studies in cats after experimental inoculation with FIV showed that they rapidly develop neutralizing antibodies. The maintenance of high levels of neutralizing antibodies for a long time period (2-5 years) and the concurrent isolation of the virus from these animals demonstrate the persistent nature of FIV infection. Although these antibodies are highly effective in binding cell-free virus in vitro, they are unable to eliminate viral infection as shown by repeated virus isolation from these cats. This is similar to other lentivirus infections in which a cell-mediated immune response appears to be required for virus clearance (Kannagi et al., 1988; Chenciner et al., 1989; Riviere et al., 1989). The majority of the sera from the experimental group of cats did not react or reacted weakly with FIV envelope proteins in Western blots, but still showed neutralizing activity; the loss of viral glycoproteins during virus purification is a possible explanation for this discrepancy. Like others (O'Connor et al., 1989), we found that sera reacting solely with FIV core proteins in Western blots were able to precipitate viral envelope proteins when tested by radioimmunoprecipitation (data not shown). The MAbs described in this report provide a useful reagent for monitoring in vitro infection, for the detection of viral antigens in various pathological conditions and to study viral latency. They are essential for the detection of new FIV isolates in primary cultures of cat PBMCs and in cultures of bone marrow cells from experimentally FIV-infected cats.

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References


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