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Development of antibodies to feline IFN- γ as tools to elucidate the cellular immune responses to FeLV

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Received 26 November 2002; received in revised form 14 April 2003; accepted 2 June 2003

Abstract

An understanding of the nature of immune protection and the role of immune effector products such as interferon- γ (IFN- γ) in the control of infectious disease is fundamental to the rational design of effective vaccines and immunotherapeutic reagents. Murine monoclonal and sheep polyclonal antibodies (mAbs and pAbs) to feline IFN- γ (fIFN- γ) were generated firstly to facilitate further research into the role of cellular immune responses in the control of feline infectious disease, and secondly to enable evaluation of the efficacy of novel immunotherapeutic approaches. A hybridoma clone, D9, secreting IgG1 antibodies was selected for expansion and the mAbs affinity purified *in vitro*. Polyclonal antibodies were raised in a sheep against recombinant fIFN- γ and affinity purified. The sensitivity of the D9 mAb and the sheep anti-fIFN- γ pAb was determined using an indirect fIFN- γ enzyme-linked immunosorbent assay (ELISA) and immunoblots. These antibodies were assessed for their ability to detect the production of fIFN- γ by specific feline T cell populations *ex vivo* following coculture with mitogen or feline leukaemia virus (FeLV) antigens for 4 h in the presence of the protein secretion inhibitor brefeldin A (BFA). Production of fIFN- γ was evaluated using flow cytometry to simultaneously detect PE-labelled surface molecules and fluorescein isothiocyanate (FITC)-labelled intracellular fIFN- γ . Using this approach, our initial studies revealed an upregulation in virus-specific fIFN- γ -secreting CD4⁺T cells in the lymph nodes of FeLV latently infected cats.

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Keywords: IFN- γ ; Antifeline antibodies; Feline cytokines; Feline leukaemia virus

1. Introduction

Interferon- γ (IFN- γ) is recognised as a critical component of the host immune response to viral, bacterial and parasitic pathogens of man and animals (Kemp et al., 1999; Shankar et al., 2000; Pathan et al., 2001; Bourreau et al., 2002). Interferon- γ exerts its antimicrobial action either directly through the inhibition of viral replication, or indirectly by upregulating MHC classes I and II gene

Abbreviations: AP, alkaline phosphatase; BFA, brefeldin A; Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; FCoV, feline coronavirus; FeLV, feline leukaemia virus; FITC, fluorescein isothiocyanate; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; IFN- γ , interferon- γ ; PE, phycoerythrin; RT, room temperature; SPF, specific pathogen free.

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products and activating antigen presenting cells (APC) (Farrar and Schreiber, 1993; Young and Hardy, 1995; Boehm et al., 1997). Definition of the role of immune effector mechanisms, including IFN- γ , is an important prerequisite to the rationale design and development of novel immunotherapeutic approaches to the prevention and control of infectious disease.

The domestic cat is an important companion animal species, and is the natural host for a number of significant pathogens, including feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV) and feline coronavirus (FCoV) (Pedersen et al., 1987; Addie et al., 1995; Jarrett, 2001). Feline immunodeficiency virus infection of domestic cats is also recognised as an important animal model for human immunodeficiency virus (HIV) vaccine development (Willett et al., 1997). Currently, effective vaccines are not available for the majority of these feline infections, and curative treatment is often not possible. A concerted effort to develop feline-specific reagents has facilitated an improved understanding of the immune mechanisms that determine the disease outcome following exposure to these feline pathogens (Beatty et al., 1996; Flynn et al., 1996; Paltrinieri et al., 1998; Flynn et al., 2002). However, progress in this area is slow. Thus, there remains a requirement for the continued development of sensitive, species-specific reagents to quantitatively define the important immune control mechanisms in the cat.

At the commencement of these studies no antibody reagents were available to detect feline IFN- γ (fIFN- γ). Furthermore, we have been unable to detect fIFN- γ using reagents from other species in a number of immunological assays. In this report, we describe the development of monoclonal and polyclonal antibodies (mAbs and pAbs) specific for fIFN- γ . Using these highly sensitive reagents in flow cytometry, intracellular fIFN- γ produced by feline mononuclear cells in response to mitogenic and virus-specific antigenic stimulation could be detected and fIFN- γ -secreting cells could be directly quantified *ex vivo*. These reagents have clear applications in assessing the efficacy of novel prophylactic and immunotherapeutic approaches to the control of feline infectious disease.

2. Materials and methods

2.1. Recombinant fIFN- γ

The gene encoding fIFN- γ was cloned and sequenced, inserted into a glutathione S-transferase (GST) gene fusion vector (pGEX-4T-1, Amersham Biosciences U.K., Buckinghamshire, UK), and expressed in bacterial cells (BL21 (DE3), Stratagene Europe, Amsterdam, The Netherlands) (Argyle et al., 1995). The recombinant fIFN- γ protein was purified from the bacterial lysate using a glutathione sepharose affinity column according to the manufacturer's instructions (Amersham).

2.2. Experimental animals and immunisations

Female BALB/c mice were inoculated intraperitoneally with 20 μ g recombinant fIFN- γ in a total volume of 200 μ l complete Freund's adjuvant (Diagnostics Scotland, Edinburgh, UK). Two booster injections, each containing 10 μ g fIFN- γ in a volume of 100 μ l incomplete Freund's adjuvant, were given at 28-day intervals. Seven days after the second booster, sera were recovered and the antibody titres measured by enzyme-linked immunosorbent assay (ELISA). After a further 8 days, the mice received a final intravenous booster inoculation of 10 μ g fIFN- γ in 100 μ l PBS; their spleens were harvested 4 days later.

Polyclonal antiserum to fIFN- γ was prepared by the inoculation of sheep subcutaneously (sc) and intramuscularly (im) with 200 μ g fIFN- γ in complete Freund's adjuvant in a total volume of 5 ml (Diagnostics Scotland). Three booster inoculations of 200 μ g fIFN- γ in 5 ml incomplete Freund's adjuvant were given sc and im at 4-week intervals; serum was harvested 7 days after each booster injection. Antibody titres were determined by ELISA.

Feline mononuclear cells were obtained from the peripheral blood of specific pathogen free (SPF) domestic cats by jugular venepuncture, or from cryopreserved lymphoid tissues. All animals described in this study were housed and maintained in accordance with UK Home Office guidelines.

2.3. Generation and purification of murine monoclonal antibodies

Murine mAbs were prepared according to the method originally described by Kohler and Milstein (1992). Briefly, splenic mononuclear cells were fused to murine myeloma (NS0) cells at a ratio of 2:1 using PEG-1500 (polyethylene glycol-1500, BDH Laboratory Supplies, Poole, UK). The wells of 96-well U-bottom culture plates (Becton Dickinson Labware Europe, Le Pont de Claix, France) were seeded with 1×10^5 cells in a final volume of 200 μ l RPMI 1640 medium containing 10% heat-inactivated foetal calf serum (FCS), 100 U/ml penicillin, 10 μ g/ml streptomycin, 2 mM glutamine and 5×10^{-5} M 2-mercaptoethanol (complete RPMI 1640 medium; GibcoBRL, Paisley, UK), supplemented with HAT (5 mM sodium hypoxanthine, 20 μ M aminopterin and 0.8 mM thymidine, GibcoBRL). After 14 days incubation at 37 °C in a humidified atmosphere containing 5% CO₂, culture supernatants from wells containing visible cell colonies were collected and the presence of mAbs to fIFN- γ determined by ELISA. Hybridomas secreting the highest levels of detectable mAbs were cloned by limiting dilution, subcloned twice and expanded in vitro (CI-1000, Integra Biosciences, Hertfordshire, UK). The mAbs were then purified using a protein G affinity column (HiTrap Protein G, Amersham). Purified mAbs were eluted from the column according to the manufacturer's protocol and transferred to a storage buffer (0.05 M Na₂HPO₄, 0.1% NaN₃, pH 7.0) using a PD-10 column (Amersham). Antibody isotyping was performed using a commercially available kit (Sigma-Aldrich, Dorset, UK).

2.4. Detection of anti-fIFN- γ antibodies using ELISA

The wells of 96-well high-binding plates (Greiner Laboratechnik, Gloucestershire, UK) were coated overnight at room temperature (RT) with 100 ng fIFN- γ in 100 μ l coupling buffer (0.1 M NaHCO₃, 0.001 M EGTA). The plates were washed three times with 0.05% Tween-20 (BDH) in tris-buffered saline (TBS-T). Any unreacted sites were subsequently blocked by incubation for 30 min at RT with TBS-T supplemented with 2% dried skimmed milk powder ("Marvel", Premier Brands U.K., Merseyside, UK). After three washes, 100 μ l of culture supernatant was

added to each well directly, and incubated for 2 h at RT. After six washes in TBS-T, the wells were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse Ig pAb (Bio-Rad Laboratories, Hertfordshire, UK), diluted 1:1000 in TBS-T, for 1 h at RT. Bound antibodies were visualised using *p*-nitrophenyl phosphate disodium (Sigma-Aldrich) in AP buffer (0.1 M NaCl, 0.1 M diethanolamine, 0.005 M MgCl₂, pH 9.5). Absorbance values were read at 405 nm after 1 h (Labsystems Multiskan Ascent, Process Analysis and Automation, Hampshire, UK). The same protocol was used when titrating the D9 mAb, except that doubling dilutions were made in 20% normal goat serum (NGS, Diagnostics Scotland). Normal mouse serum (NMS, Diagnostics Scotland), diluted in the same way as the primary mAb, was used to determine the end point of the ELISA.

To detect sheep anti-fIFN- γ pAbs, unreacted sites on the ELISA plates were blocked for 30 min with 20% normal rabbit serum (NRS, Diagnostics Scotland) in TBS-T. Polyclonal antibodies were diluted in 20% NRS and an AP-conjugated rabbit anti-sheep Ig pAb (Southern Biotechnology Associates, Alabama, USA), diluted 1:2000 in 20% NRS, was used to detect bound sheep antibodies. Normal goat serum, diluted in the same way as the primary pAb, was used to determine the end point of the ELISA.

2.5. Detection of fIFN- γ by immunoblotting

Recombinant fIFN- γ was diluted 1:1 in 4% sodium dodecyl sulphate (SDS) loading dye containing 0.01% bromophenol blue and electrophoresed on a 12% SDS-polyacrylamide gel (SDS-PAGE; Mini-protean II, Bio-Rad) at 200 V for approximately 30 min. The proteins were transferred onto a nitrocellulose membrane (Bio-Rad) using a semi-dry technique (Trans-Blot Semi-Dry Transfer Cell, Bio-Rad); any unreacted sites were blocked by overnight incubation at 4 °C with TBS-T containing 4% Marvel. All washes were conducted in duplicate at 5-min intervals using TBS-T, and all incubations proceeded at RT on a slowly rotating platform. The nitrocellulose membrane was cut longitudinally into strips and probed with 200 μ l of either D9 mAb, or sheep anti-fIFN- γ pAb, diluted in 20% NGS and 20% NRS in TBS-T, respectively, for 2 h. Alternatively, the blots were probed with either NMS or NGS, which acted as negative controls for the

mAb and pAb, respectively. The blots were then incubated with either AP-conjugated goat anti-mouse Ig pAb or AP-conjugated rabbit anti-sheep Ig pAb, diluted 1:1000 and 1:2000, respectively, for 1 h. Bound antibodies were visualised using Fast 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium tablets (BCIP/NBT AP substrate, Sigma-Aldrich), dissolved in 10 ml distilled water.

2.6. Fluorescein isothiocyanate conjugation of monoclonal and polyclonal antibodies

Prior to coupling to fluorescein isothiocyanate (FITC, Sigma-Aldrich), sheep anti-fIFN- γ pAb was affinity purified using HiTrap NHS-activated columns (Amersham), previously coated with up to 4 mg of recombinant fIFN- γ . Purified pAb was eluted from the column according to the manufacturer's instructions and stored in a similar manner to the mAb. For coupling to FITC, mouse and sheep antibodies were first transferred into 0.5 M carbonate buffer, pH 9.5 by overnight dialysis. A FITC-dimethylsulphoxide (DMSO, Sigma-Aldrich) solution was prepared (1 mg/ml) and added to antibody (45 μ g/mg), the solution was then stirred for 1 h at RT in the dark. Unconjugated FITC was removed using a PD-10 column, the FITC-conjugated antibody was collected in PBS and stored at 4 °C in the dark until used in the assays.

2.7. Intracellular cytokine staining and analysis by flow cytometry

Feline mononuclear cells were prepared from either fresh peripheral blood or from single cell suspensions of cryopreserved feline lymphoid tissues by centrifugation over Ficoll-Paque (Amersham). The cells were resuspended at 10^6 cells/ml in complete RPMI 1640 medium (without ME) and transferred to the wells of 24-well plates (Costar U.K., Buckinghamshire, UK). The cells were incubated for 4 h at 37 °C in the presence of the protein secretion inhibitor brefeldin A (BFA) (10 μ g/ 10^6 cells, Sigma-Aldrich), and stimulated with concanavalin A (5 μ g/ 10^6 cells, Con A, Sigma-Aldrich), recombinant FeLV capsid (CA) protein p27 (25 μ g/ 10^6 cells), recombinant FeLV surface envelope (SU) protein p45 (25 μ g/ 10^6 cells), or

inactivated FeLV particles (25 μ g/ 10^6 cells). Unstimulated cells were included as a negative control. The FeLV p27 and gp70 genes were cloned in our laboratory by Drs. N. Spibey and J. MacDonald; the recombinant proteins were expressed in *Escherichia coli* bacterial cells. Feline leukaemia virus particles were purified from F422 cell culture supernatants and heat-inactivated; F422 cells constitutively express FeLV proteins.

Following 4 h stimulation, feline mononuclear cells were harvested, washed in PBS and incubated with a phycoerythrin (PE)-labelled anti-fCD4 mAb (2.5 μ g/ 10^6 cells, Southern Biotechnology) for 30 min at RT in the dark. After two washes in PBS, cells were fixed in 2% formaldehyde before permeabilisation

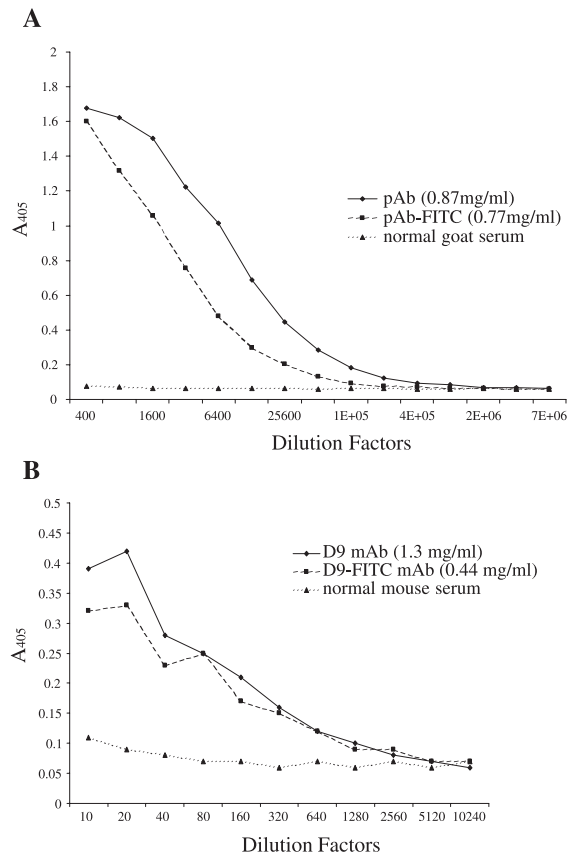


Fig. 1. Titration of sheep anti-fIFN- γ pAb (A), D9 mAb (B) and their respective FITC conjugates using an indirect fIFN- γ ELISA. The antibodies were diluted in 20% NRS (A) or 20% NGS (B) in TBS-T. NGS (A) and NMS (B) were used to determine the end-points of the ELISA.

with 1% saponin (Sigma-Aldrich) in fluorescence buffer (0.5% BSA, 1 mM NaN₃ in PBS). Permeabilised cells were incubated with D9-FITC mAb (2 µg / 10⁶ cells), or an isotype-matched control mAb FITC (6 µg/10⁶ cells, Southern Biotechnology), for 30 min in the dark at RT, or remained unstained. The cells were washed three times and stored in fluorescence buffer prior to analysis on a flow cytometer (EPICS XL-MCL™, Beckman Coulter, California, USA), using Expo 32 ADC software (Beckman Coulter). The small lymphocyte and lymphoblast populations were gated on the basis of cell size and granularity (forward and side scatter). For each sample 10,000 events were recorded, and the percentage of fIFN-γ-expressing CD4⁺T cells within the lymphoblast population was calculated.

3. Results

3.1. Selection of monoclonal antibody clones

Two hundred and sixty-five hybridomas were visible microscopically at 21 days post fusion out of 520 wells seeded. Subsequently, ELISA screening revealed that 31 were secreting anti-fIFN-γ mAbs. The five hybridomas secreting the highest levels of anti-fIFN-γ

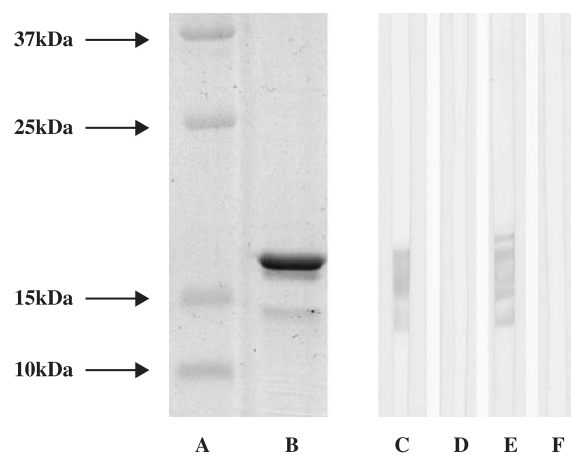


Fig. 2. Recombinant fIFN-γ (4 µg) stained with Coomassie Brilliant Blue on an SDS-PAGE gel (lane B) and probed with either D9 mAb diluted 1:1000 in 20% NGS (lane C), or sheep anti-fIFN-γ pAb diluted 1:100,000 in 20% NRS (lane E), in immunoblots. Negative control blots were probed with NMS (lane D) or NGS (lane F). Prestained markers were used to determine protein size (lane A).

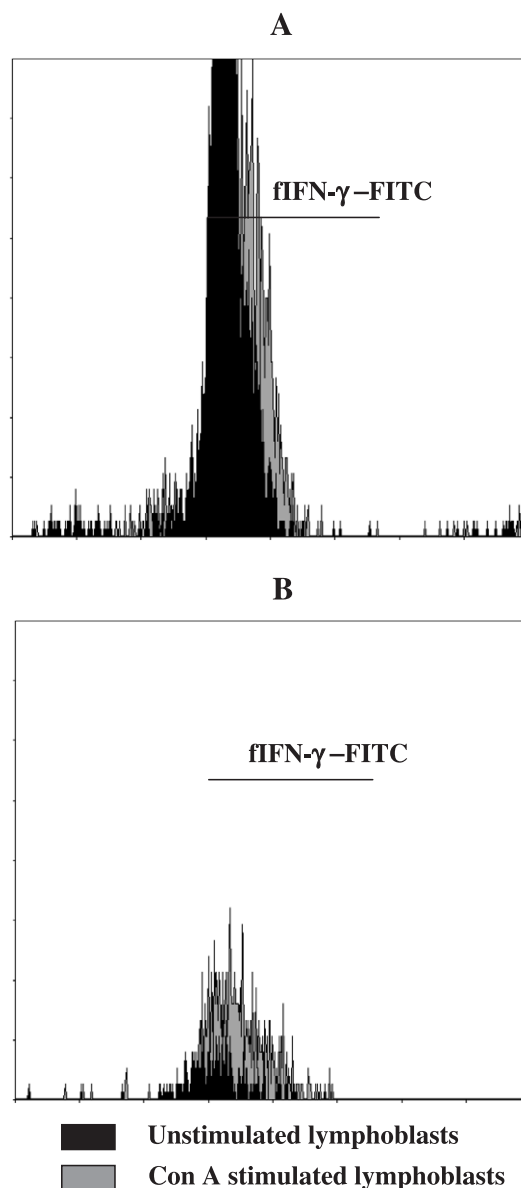


Fig. 3. Single parameter overlay plots showing fIFN-γ expression by feline mononuclear cells in the peripheral blood (A) and PLN tissues (B), detected using sheep anti-fIFN-γ-FITC pAb and D9-FITC mAb, respectively, following 4 h stimulation with Con A *in vitro*. Unstimulated cells were used as controls. To clarify, only the lymphoblast population was analysed for fIFN-γ production.

antibodies were selected for cloning and subcloning. This report describes the results obtained with clone 5B5.C5.D9 (referred to subsequently as D9) that was

selected for further expansion and affinity purification of the secreted antibodies. Using a commercially available isotyping kit, these antibodies were shown to be IgG1.

3.2. Titration of anti- γ antibodies using ELISA

The sensitivity of the affinity-purified D9 mAb and sheep anti- γ pAb, and their respective FITC conjugates, was established using γ -specific ELISA procedures. The antibodies were titrated against recombinant γ in an indirect ELISA. The affinity-purified sheep anti- γ pAb detected 100 ng γ at a titre of 1:1,638,400 (Fig. 1A) and the FITC conjugate recorded a titre of 1:819,200. The

D9 mAb demonstrated lower titres in ELISA, 1:5120 (Fig. 1B); these values were largely unaffected by conjugation with FITC.

3.3. Detection of γ using immunoblotting

Immunoblots provide an additional means of assessing antibody sensitivity. The recombinant protein γ characteristically displays polypeptide bands, approximately 16 kDa in size, in Coomassie Brilliant Blue-stained SDS-PAGE gels (Fig. 2, lane B). The distinctive double polypeptide bands were again in evidence when nitrocellulose-blotted γ was probed with the sheep anti- γ pAb generated in this study (Fig. 2, lane E). The sheep anti- γ

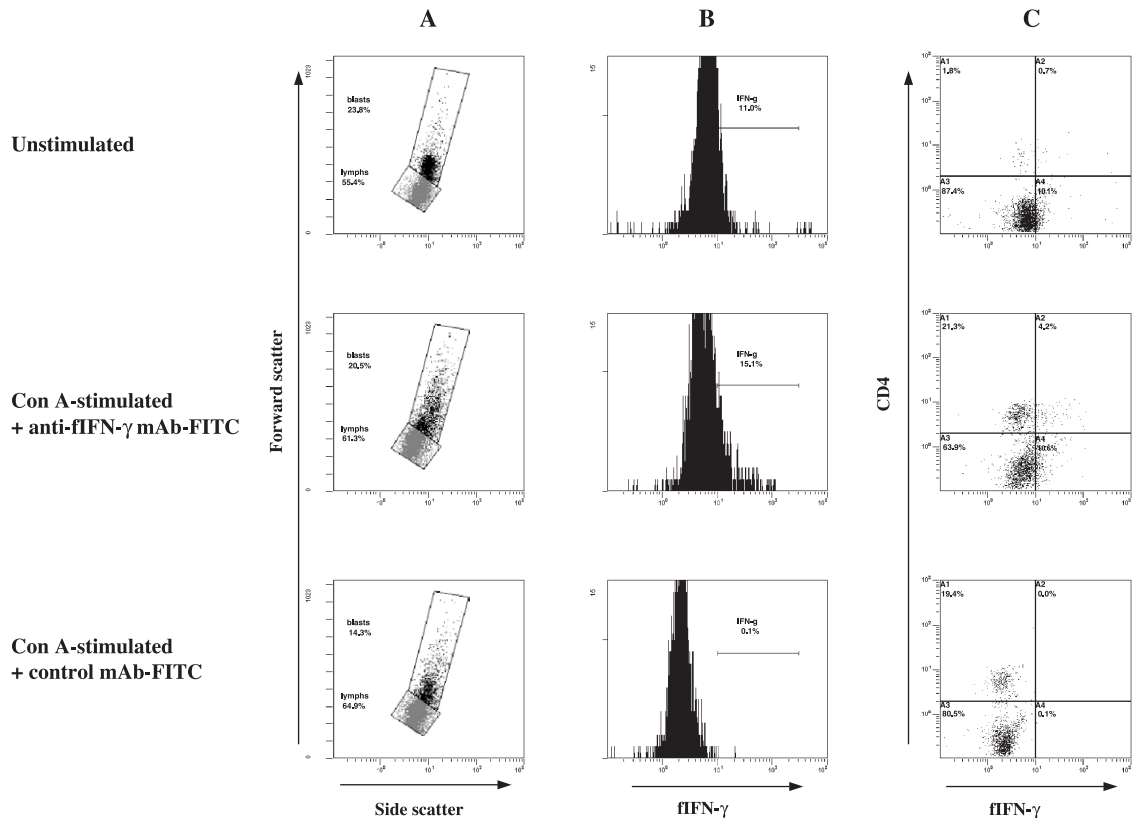


Fig. 4. Surface coexpression of fCD4 molecules in γ expressing T cells. Feline mononuclear cells from peripheral blood were stimulated with Con A for 4 h *in vitro*. Unstimulated cells served as controls. CD4 expression was detected using the anti-fCD4-PE mAb; γ expression was detected using either the D9-FITC mAb or the isotype-matched control FITC mAb. Forward versus side scatter dot plots illustrating lymphocyte ('lymphs') and lymphoblast ('blasts') populations in the peripheral blood (A); histograms illustrating γ expression in the lymphoblast population (B); two parameter dot-plots demonstrating CD4 and γ expression in the lymphoblast population (C); γ^+ CD4⁺T cells are shown in quadrant A2.

pAb detected 14 µg fIFN-γ at a titre of at least 1:100,000. The mAb D9 also detected 14 µg fIFN-γ at a high titre, 1:1000. No specific staining was visible on the negative control blots.

3.4. Intracellular cytokine staining and analysis by flow cytometry

Both D9 mAb and sheep anti-fIFN-γ pAb were assessed for their ability to detect the production of fIFN-γ by specific feline T cell populations in vitro

using flow cytometry, employing a combination of cell surface staining and intracellular cytokine staining. Feline mononuclear cells from the peripheral blood or lymphoid tissues were cocultured with mitogen or specific antigen for 4 h in the presence of a protein secretion inhibitor BFA to allow the accumulation of fIFN-γ within the cells (Fujiwara et al., 1988; Nylander and Kalies, 1999). Production of fIFN-γ was compared in unstimulated and stimulated cells using flow cytometry. Both D9-FITC mAb and sheep anti-fIFN-γ-FITC pAb detected a surge in fIFN-γ production in the

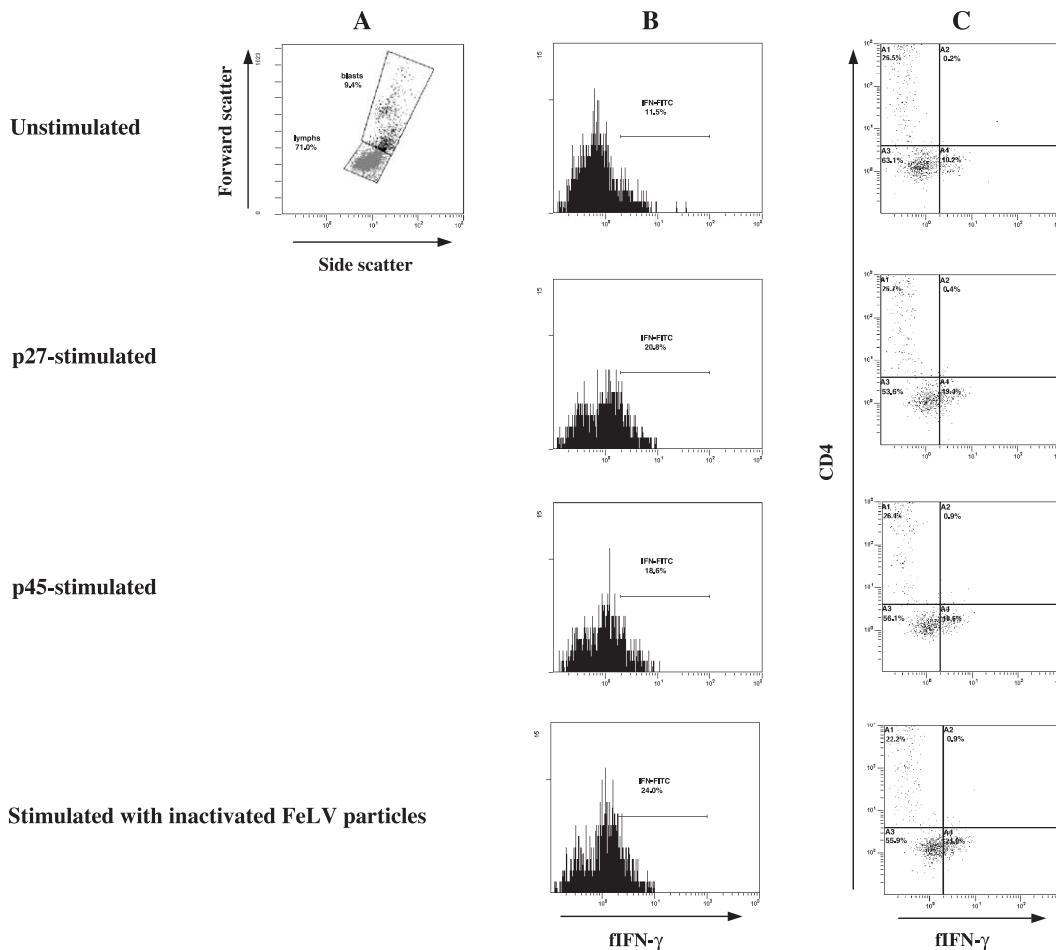


Fig. 5. FeLV antigen-specific upregulation of fIFN-γ expression in CD4⁺T cells. Peripheral lymph node cells were stimulated with either recombinant FeLV proteins p27 or p45, or with inactivated FeLV particles for 4 h in vitro. Unstimulated cells served as controls. CD4 expression was detected using the anti-fCD4-PE mAb; fIFN-γ expression was detected using the D9-FITC mAb. Forward versus side scatter dot-plot illustrating lymphocyte ('lymphs') and lymphoblast ('blasts') populations in the PLN tissues (A), histograms illustrating fIFN-γ expression in the lymphoblast population (B); two parameter dot-plots demonstrating CD4 and fIFN-γ expression in the lymphoblast population (C); fIFN-γ⁺CD4⁺T cells are shown in quadrant A2.

peripheral lymph node (PLN) tissues or in the peripheral blood, respectively, in response to Con A stimulation, compared to unstimulated cells (Fig. 3).

Using dual-colour flow cytometry, the proportion of fIFN- γ -producing cells coexpressing surface CD4 was determined. In these studies, we focussed on the use of the mAb to fIFN- γ , and used a combination of anti-fCD4-PE mAb and anti-fIFN- γ -FITC mAb to visualise the cells. The D9-FITC mAb detected fIFN- γ at higher levels in Con A-stimulated CD4⁺T cells (4.2%) than in unstimulated CD4⁺T cells (0.7%) (Fig. 4). No double staining was detected using a FITC-labelled isotype-matched control mAb.

The D9-FITC mAb was used to detect fIFN- γ production in CD4⁺T cells from PLN tissues in response to mitogenic or specific antigenic stimulation. Peripheral lymph node tissues were harvested from cats that had developed a latent FeLV infection following experimental exposure to FeLV. These cats developed a transient viraemia subsequent to oronasal exposure to FeLV, after which it was no longer possible to isolate FeLV from the blood. However, FeLV could be cultured from bone marrow cells harvested at post mortem, indicating the continued presence of FeLV proviral DNA within bone marrow cells. Peripheral lymph node cells from two latently infected cats were stimulated for 4 h in the presence of either recombinant p27 or p45 proteins, or inactivated FeLV particles. In one cat, stimulation with the recombinant p27 protein triggered increased fIFN- γ production in a proportion of CD4⁺T cells in PLN tissues (2.9%) compared to unstimulated CD4⁺T cells (1.7%) (data not shown). In a second latently infected cat, fIFN- γ -producing CD4⁺T cells were also detected following stimulation with the recombinant p27 protein (0.4%). Additionally, stimulation with recombinant p45 protein (0.9%) and inactivated FeLV particles (0.9%) caused an upregulation in fIFN- γ production by CD4⁺T cells compared to unstimulated cells (0.2%) (Fig. 5). Taken together, these findings indicate the presence of virus-specific CD4⁺T cells in the PLN tissues from latently infected cats.

4. Discussion

The lack of reagents suitable for feline immunological assays is a major limitation to the study of

feline disease and to the continued development of the cat as a model for the study of infectious diseases in other species. Although quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assays for many feline cytokines have been developed (Dean et al., 1998; Leutenegger et al., 1999), it is important to note that cytokines detected as mRNA may not ultimately be expressed as proteins. In the present report, we describe the generation of murine monoclonal and sheep polyclonal antibodies to fIFN- γ . We demonstrate that both the murine D9 monoclonal and the sheep anti-fIFN- γ antibodies are sensitive in ELISA and immunoblots, and that D9 can be used to detect the intracellular accumulation of fIFN- γ in feline cells stimulated with mitogens or viral antigens and then analysed by flow cytometry. We focussed on IFN- γ because it has an important role as an immune regulator and effector molecule and this role has been exploited to gain important information on the nature, specificity and efficacy of the immune response in various disease states.

The development of antibodies specific for fIFN- γ is a necessary prerequisite to the development of ELISAs, enzyme-linked immunospot (ELISpot) assays, immunocytochemical techniques and flow cytometric analysis of cytokine-secreting cells designed for use in domestic cats. In other species, ELISA procedures have been used to quantify the levels of soluble IFN- γ protein in blood, body fluids and tissue culture supernatants (Mateu de Antonio et al., 1998). The specificity of the immune response has been characterised using flow cytometry and ELISpot assays to enumerate IFN- γ -expressing cells following stimulation with cognate antigen (Czerkinsky et al., 1983; Jung et al., 1993; Pitcher et al., 1999). Furthermore, the efficacy of the immune response can be determined by examining the ability of antigen-specific effector cells to secrete IFN- γ (Shankar et al., 2000).

Recent studies in cats have attempted to evaluate the potential of feline cytokines, for example, IFN- γ , interleukin (IL)-12 and IL-18, as genetic vaccine adjuvants in vivo (Hosie et al., 1998; Flynn et al., 2000; Hanlon et al., 2001). An adjuvant effect has been demonstrated in certain studies as assessed by increases in virus-specific cell-mediated immune responses or by improved vaccine efficacy following challenge. However, the precise immune mechanisms

associated with these observations are poorly resolved. The anti-fIFN- γ antibodies described in this study have the potential to detect circulating fIFN- γ in plasma using a capture ELISA. Such an approach may also be a useful prognostic indicator when evaluating novel immunotherapies.

Likewise, feline-specific antibodies could be employed to identify and characterise fIFN- γ -expressing cells in tissue sections using immunohistochemistry. The demonstration of IFN- γ within tissues *in situ* may aid in the characterisation of the immune response during disease processes (Kakazu et al., 1999). Indeed, we have used the sheep anti-fIFN- γ pAb to identify fIFN- γ -expressing cells in cryostat sections of feline lymph node (data not shown). However, data from immunohistochemical studies should be subject to careful interpretation. It has been suggested that IFN- γ -expressing cells should be characterised in order to minimise spurious results (van Der Loos et al., 2001).

Perhaps the most important use of these novel reagents is to facilitate the quantitative analysis of antigen-specific T cells *ex vivo* using either intracellular cytokine staining techniques and flow cytometry, or ELISpot assays. These highly sensitive assays will enable the evolution of the immune response to be monitored following infection or following vaccination. Early Gag/Pro-specific CD8⁺CTL effector responses are associated with recovery from FeLV infection (Flynn et al., 2002). Therefore, the capacity of virus-specific CD4⁺T cells to activate CD8⁺T cells through the production of cytokines such as IFN- γ should be a key factor in the control of viraemia (Rosenberg et al., 1997). Our initial studies reveal that p27-specific and p45-specific CD4⁺T cells reside in the lymph nodes of latently infected cats. Stimulation with recombinant p27 and p45 proteins caused an upregulation of fIFN- γ production in a proportion of CD4⁺T cells compared to unstimulated CD4⁺T cells.

Interferon- γ is a key component of the Th1 cell-mediated immune response honed to combat microbial infection (Shtrichman and Samuel, 2001). Use of the reagents described here will further our understanding of the immunopathogenesis of feline infectious disease and allow evaluation of novel immunotherapies in the control of infectious disease in cats.

Acknowledgements

EMG is in receipt of a Travelling Studentship in Veterinary Medicine from the NUI.

JNF is a BBSRC Advanced Fellow. L. McMonagle and S. Taylor prepared the recombinant fIFN- γ protein for animal immunisations.

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