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Polyclonal B-cell activation in cats infected with feline immunodeficiency virus

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SUMMARY

The specificity of the antibody response following natural or experimental infection of domestic cats with feline immunodeficiency virus (FIV) was examined. The antibody response to a range of non-viral antigens, including trinitrophenol (TNP), ovalbumin, β-galactosidase, deoxyribonucleic acid (DNA) and keyhole limpet haemocyanin (KLH), was measured in 220 cats naturally infected with FIV. Infected cats had higher antibody levels to these antigens, in particular TNP, KLH and β-galactosidase, than non-infected control cats. Competition binding studies demonstrated that this response was not due to the presence of cross-reacting epitopes on recombinant FIV p17 or p24 antigens, suggesting that the B-cell activation associated with infection was polyclonal rather than entirely virus specific. Studies on cats experimentally infected with FIV revealed a similar pattern, with infected cats developing an antibody response to heterologous non-viral antigens at 6–8 weeks post-infection. There were two discernible peaks of antibody activity, the first occurring 10–20 weeks post-infection and the second peak 40–60 weeks post-infection. The antibody response to KLH, DNA and β-galactosidase remained elevated throughout the 90-week study period, whereas the antibody levels to the other antigens declined to levels approaching those observed in normal cats.

INTRODUCTION

Feline immunodeficiency virus (FIV) is a lentivirus first isolated from domestic cats suffering from an immunodeficiency-like syndrome.[1] The impairment of immune function associated with FIV infection has been studied in naturally and experimentally infected cats and is remarkably similar to that observed in man following infection with the human immunodeficiency virus (HIV). In FIV infection there is a progressive depletion of $CD4^+$ T cells and an associated decrease in the $CD4^+$:$CD8^+$ T-cell ratio.[2] Proliferative responses of mononuclear cells isolated from symptomatic and asymptomatic cats after naturally or experimentally acquired infection with FIV, induced by *in vitro* activation with the mitogens concanavalin A (Con A), pokeweed mitogen (PWM), phytohaemagglutinin (PHA), or by stimulation with human interleukin (IL)-2, are all significantly lower than those observed with non-infected control cats.[3–5]

Despite these defects in T-cell function, infection induces a marked activation and expansion of B cells. Following infection there is generalized lymphadenopathy characterized by hyperplasia of B-cell areas with prominent and irregular expansion of lymphoid follicles accompanied by a paracortical hyperplasia.[6] A hypergammaglobulinaemia is often associated with this B-cell proliferation.[7] However, it is not clear if this response is entirely FIV-specific or whether it reflects a more generalized non-specific polyclonal activation of B cells following FIV infection. Polyclonal B-cell activation has been described in HIV-infected patients with hyperactive spontaneous B-cell responses[8] and refractoriness to T-cell-independent B-cell activation.[9] Such polyclonal B-cell activation would appear likely to be involved in the hypergammaglobulinaemia,[10] development of autoantibodies[11] and frequent development of B-cell malignancies observed in HIV-infected patients.[12]

In the present study, we have examined the antibody response of cats either naturally or experimentally infected with FIV to a range of heterologous non-viral antigens in an attempt to elucidate the specificity of the B-cell response. Competition binding studies were performed to determine if reactivity to heterologous non-viral antigens was associated with the development of antibodies to cross-reactive epitopes on viral proteins. The development of this phenomenon was also studied in a longitudinal experimental infection with the $Glasgow_8$

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Abbreviations: Con A, concanavalin A; FeLV, feline leukaemia virus; FIV, feline immunodeficiency virus; HEL, hen egg lysosyme; HIV, human immunodeficiency virus; IL, interleukin; KLH, keyhole limpet haemocyanin; LPS, lipopolysaccharide; PHA, phytohaemagglutinin; PWM, pokeweed mitogen, SPF, specific pathogen free; TBS, Tris-buffered saline; TNF, tumour necrosis factor; TNP, trinitrophenol.

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strain of FIV (FIV-GL8) to determine the onset and duration of this response.

MATERIALS AND METHODS

Animals and sample preparation

Heparinized blood samples from field cases of FIV were obtained from the Feline Virus Unit of the Department of Veterinary Pathology, University of Glasgow.

Ten 12-week-old specific pathogen-free (SPF) cats were inoculated intraperitoneally with 2×10^3 cat infectious doses of the $Glasgow_8$ strain of FIV (FIV-GL8). At week 8 after infection, cats were confirmed FIV antibody positive by ELISA (FIV antibody detection kit, IDEXX Laboratories, Slough, U.K.) and virus was isolated by methods described previously.[13] Five age-matched cats were kept as naive controls. Blood samples were collected before infection and at 2-week intervals post-infection into heparinized containers for the first 6 months post-infection. Subsequent samples were collected monthly. Plasma was stored at $-20°$ until use in the assays.

Cats were infected with feline leukaemia virus (FeLV) by oronasal administration of 1×10^6 focus-forming units of FeLV-A/Glasgow-1, 12 weeks prior to the collection of plasma. These cats became viraemic 3 weeks post-infection.

Detection of antibodies to FIV

Routine diagnosis of FIV infection was based on the detection of antibodies in cat plasma to recombinant FIV p17 using a microtitre plate-based ELISA as described previously.[14] Antibodies to FIV p24 were detected in the same manner.

Detection of antibodies to heterologous non-viral antigens

A microtitre plate-based ELISA was developed to detect plasma antibodies to ovalbumin, hen egg lysosyme (HEL), trinitrophenol (TNP), β-galactosidase, horse heart myoglobin, thyroglobulin and calf thymus DNA (all obtained from Sigma, Poole, U.K.). The antigens were coupled to the wells of a 96-well microtitre plate (Immulon 1; Dynatech Laboratories, Chantilly, VI) overnight at 4° at a concentration of 1 μg/ml in coupling buffer (10 mM $NaHCO_3$, 1 mM EGTA, pH 9·6). The plate was then washed three times in Tris-buffered saline (TBS; 100 mM NaCl, 50 mM Tris, plus 0·05% Tween 20, pH 7·6) and any unreacted sites were blocked by a further incubation in this buffer supplemented with 2% low-fat milk powder for 1 hr at room temperature. The plate was then washed three times with TBS, and cat plasma diuted 1:50 in TBS containing 20% normal goat serum (SAPU, Carluke, U.K.) was added to duplicate wells of the microtitre plates and incubated for 2 hr at room temperature with gentle agitation. The plate was washed six times with TBS. Bound antibodies were detected using a 1:500 dilution (100 μl/well) of goat anti-cat IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates Inc., Birmingham, AL) and developed with p-nitrophenyl phosphate (Sigma). The optical density (OD) of each well was measured at 405 nm using a multiscan spectrophotometer (Flow Laboratories, Irvine, U.K.) following overnight incubation at 4°. The result was recorded as significant when the mean OD_{405} value of duplicate samples was greater than twice the background value obtained with alkaline phosphatase-conjugated goat anti-cat IgG alone.

Table 1. Detection of antibodies in the plasma of cats naturally infected with FIV

Antigen	No. of reactive cats (%)*		
	FIV^+	FIV^-	$FeLV^+$
Ovalbumin	21 (9·5)	3 (6)	0
Hen egg lysozyme	14 (6·4)	0	0
Trinitrophenol	104 (47·3)	7 (14)	5 (33)
Keyhole limpet haemocyanin	141 (64·1)	13 (26)	3 (20)
β-galactosidase	51 (23)	0	1 (6·7)
Horse myoglobin	12 (5·5)	0	2 (13·3)
Calf thymus DNA	18 (8·2)	0	1 (6·7)

*Samples from naturally infected FIV seropositive cats ($n = 220$), experimentally infected FeLV seropositive cats ($n = 15$), or from normal seronegative cats ($n = 50$) were examined by ELISA for the presence of antibodies to a range of heterophile antigens.

Competition-binding studies

In the competition-binding ELISA, plasma from cats with known reactivity to β-galactosidase, TNP or keyhole limpet haemocyanin (KLH) were preincubated with a 1 mg/ml solution of recombinant FIV p24 or p17 for 1 hr at 37° before performing the assay as described in the preceding section. The reciprocal of this assay was also performed by attempting to competitively inhibit anti-p24 antibody activity by preincubation with β-galactosidase, TNP or KLH at 1 mg/ml for 1 hr at 37°.

Statistical analysis

The differences in the frequency of heterologous antigen recognition by FIV-infected and non-infected cats were tested with the general association Cochran Menton Henzel (CMH) statistic. Differences for individual antigens were then tested by Fisher's Exact Test. A conventional 5% level was used to define statistical significance and all programs were run on the SAS package (SAS Institute, Cary, NC).

RESULTS

Detection of heterophile antibodies in naturally FIV-infected cats

Plasma collected from 220 naturally infected cats was examined for the presence of antibodies to ovalbumin, HEL, TNP, KLH, β-galactosidase, horse heart myoglobin and calf thymus DNA (Table 1). When compared with non-infected control cats, 47%, 64% and 23% of infected cats recognized TNP, KLH and β-galactosidase, respectively. The other test antigens were also recognized by up to 10% of the FIV-infected cats, but not by the control cats.

To establish whether the greater number of cats producing antibodies to these heterologous antigens was a feature specific to FIV infection or was a phenomenon associated with retroviral infections in general, a group of FeLV-infected cats was included as an additional control. Although 33% and 20% of FeLV-infected cats had antibodies which recognized TNP and KLH, respectively, these values were similar to those observed in non-infected control cats and were lower than the values obtained with FIV-infected cats. A small number of FeLV-infected cats appeared to have an antibody response to β-

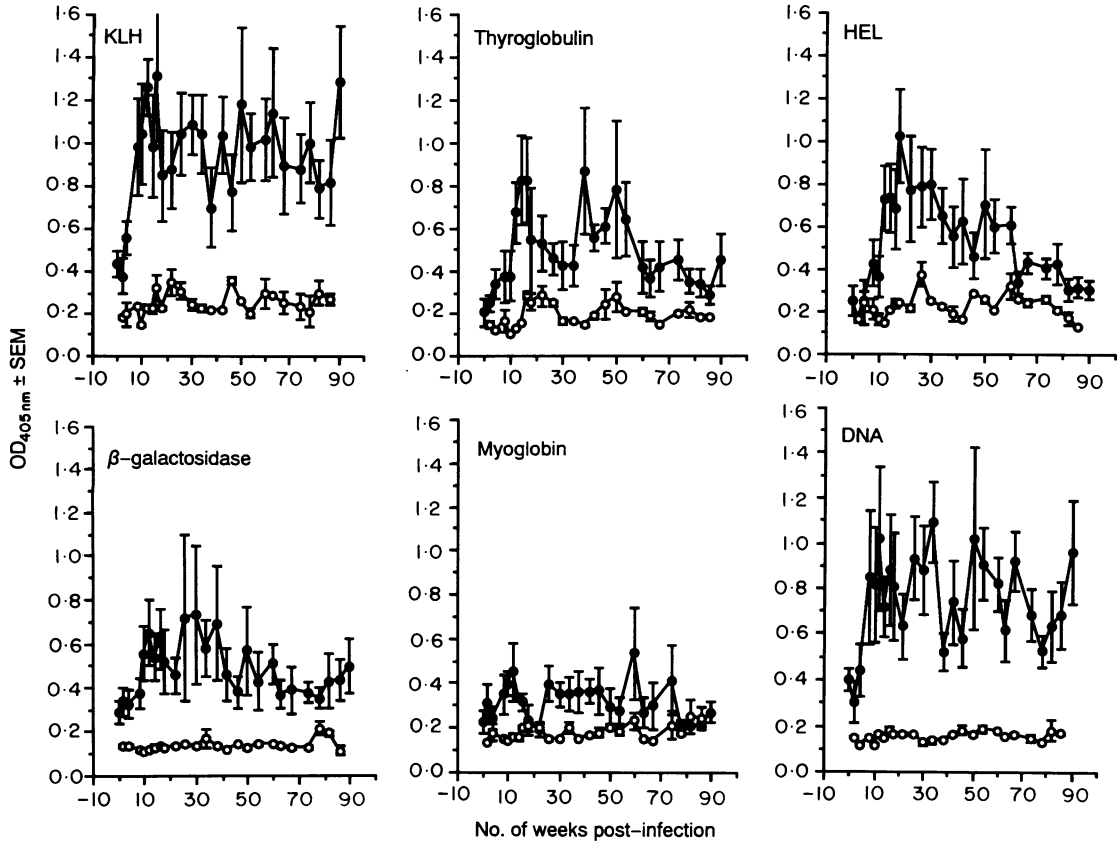

Figure 1. Heterophile antibody responses in experimentally FIV-infected cats. Plasma collected from cats experimentally infected with FIV-GL 8 (●) was examined by ELISA for the presence of antibodies to KLH, thyroglobulin, HEL, β-galactosidase, myoglobin and DNA throughout the first 90 weeks following infection. Non-infected cat served as controls (○). The results shown represent the mean absorbance value at OD_{405nm} for duplicate samples from each cat (less the background absorbance obtained with alkaline phosphatase-conjugated goat anti-cat IgG alone) ± SEM.

galactosidase (one cat), calf thymus DNA (one cat) and horse heart myoglobin (two cats).

The association between FIV infection and production of heterophile antibodies was tested with the general association CMH statistic, which gave a highly significant value of 441·8 ($P<0·001$) with six degrees of freedom. Differences for the individual antigens recognized were significant for TNP ($P<0·0001$), KLH ($P<0·0001$) and β-galactosidase ($P<0·0001$), calf thymus DNA ($P<0·05$), of borderline significance for HEL ($0·05<P<0·5$) and horse heart myoglobin ($0·05<P<0·10$), but not significant for ovalbumin ($0·2<P<0·5$).

Competition binding studies

Plasma from FIV-infected cats known to react with β-galactosidase, TNP or KLH was selected because these antigens were the most commonly recognized non-viral antigens, and preincubated with recombinant FIV p24 or p17 before repeating the assays to detect antibody reactivity to β-galactosidase, TNP or KLH. The results shown in Fig. 1a clearly demonstrate that the antibody recognition of β-galactosidase, TNP or KLH was not inhibited by preincubation with either FIV p24 or p17. This result was confirmed by the reciprocal assay in which preincubation with β-galactosidase, TNP or KLH failed to competitively inhibit the recognition of FIV p24 antigen (Fig. 1b). To test the integrity of the blocking protocol, the assay to detect anti-FIV p24 antibodies was performed following preincubation of the plasma with FIV p24. This procedure completely abrogated any anti-FIV p24 antibody activity in the sample.

Development of heterophile antibodies in experimentally FIV-infected cats

To study the onset and duration of the heterophile antibody response, samples were collected from a group of cats experimentally infected with the $Glasgow_8$ strain of FIV (FIV-GL8). The samples were examined by ELISA for antibodies to KLH, β-galactosidase, horse heart myoglobin, thyroglobulin, DNA and HEL throughout the first 90 weeks following infection. Reactivity to these antigens was first observed between 6 and 8 weeks post-infection (Fig. 2) and, although all the antigens were recognized by infected cats, the OD_{405nm} values recorded for KLH, DNA, HEL and thyrogobulin were higher than those for myoglobin and β-galactosidase. There appeared to be two peaks of heterophile antibody activity; the first occurred between 10 and 20 weeks post-infection, the second occurred later, in the range of 40–60 weeks post-infection. This biphasic response was most noticeable with KLH and thyroglobulin. By the end of the 90-week study the antibody responses to thyroglobulin, HEL

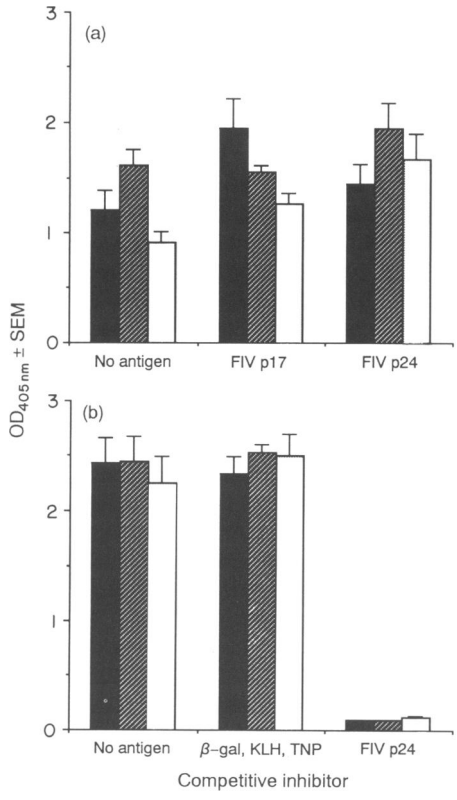

Figure 2. Heterophile antibody is not associated with the generation of antibodies to cross-reactive epitopes on FIV p17 or p24. (a) Plasma from naturally infected FIV seropositive cats was examined by ELISA for the presence of antibodies to β-galactosidase ($n = 10$) (■), KLH ($n = 14$) (▨) and TNP ($n = 15$) (□) without preincubation of the plasma with another antigen. The heterophile antibody activity was then competitively inhibited by either FIV p17 or by FIV p24, by preincubation of the samples for 1 hr at 37° with a 1 mg/ml solution of either of these proteins. (b) Plasma from cats with known antibody activity to β-galactosidase (■), KLH (▨) and TNP (□) was examined for anti-FIV p24 antibody activity without preincubation of the plasma with another antigen. In a reciprocal assay to that performed in (a), the anti-FIV p24 antibody activity was competitively inhibited by preincubation of the plasma samples with β-galactosidase, KLH or TNP, respectively. To test the integrity of the blocking protocol, the anti-FIV p24 antibody activity was inhibited by preincubation of the samples with FIV p24. The results shown represent the mean absorbance value at $OD_{405nm} \pm SEM$.

and myoglobin were approaching those observed in control cats, whereas the responses to the other antigens were still elevated. Indeed, the responses to KLH, β-galactosidase and DNA are starting to increase at 90 weeks post-infection, possibly indicating a further wave of heterophile antibody production and release. The responses of the uninfected control cats fluctuated around the values recorded prior to infection throughout the study period.

DISCUSSION

Due to the remarkable similarities between the human and feline lentiviruses and the diseases associated with infection, FIV infection of domestic cats has become an important animal model system in which to study the mechanisms of immunopathology and protective immunity involved in these two diseases. Studies to assess modulation of the immunocompetence of FIV-infected cats have revealed a progressive depression of immune function, with deficits in the lymphocyte proliferative response to mitogens such as Con A, PHA and PWM.[3-5] Furthermore, the release of IL-2 from mitogen-activated lymphocytes from naturally infected symptomatic cats was also significantly reduced.[3] However, the effect of infection on B-cell function has been less comprehensively examined. There appers to be no deficit in the ability of cats to mount an antibody response to T-independent antigens throughout infection, and a diminished ability to mount an *in vivo* antibody response to a T-dependent immunogen correlated with depressed responses to Con A.[4] Indeed, infection results in an expansion of the B-cell areas[6] and hypergammaglobulinaemia, although the specificity of this response is unclear.

Accordingly, in this study we have examined the production of heterophile antibodies in domestic cats naturally or experimentally infected with FIV. The results presented in Table 1 demonstrate that the B-cell activation, differentiation and consequent antibody production following infection are not entirely FIV-specific and antibodies are produced against a variety of antigens, in particular TNP, KLH and β-galactosidase. The production of antibodies against the hapten TNP is frequently acknowledged as an indicator of polyclonal B-cell activation.[15] When these studies were extended to include experimentally infected SPF cats a similar phenomenon was observed, thus confirming our observations in naturally infected cats and demonstrating that the deveopment of a heterophile antibody response was not associated with intercurrent disease in these field cases of FIV infection. Furthermore, competitive inhibition studies revealed that the reactivity to the heterologous antigens was not associated with the generation of antibodies to cross-reacting epitopes on viral proteins. These preliminary studies provide strong evidence that infection with FIV induces a polyclonal activation of B cells similar to that described in HIV-infected patients.[9,10] We were unable to find any evidence to support the generation of polyreactive antibodies in the FIV-infected cats examined in this study. Such antibodies are usually low-affinity IgM antibodies which react with a broad range of antigens, whereas the antibodies demonstrated in the present study were IgG and their recognition of either viral or heterologous antigens was not blocked in the competitive inhibition studies.

The results of the longitudinal study demonstrated the presence of heterophile antibodies as early as 6–8 weeks post-infection. At this stage of infection defects in T-cell function are already present.[5] Two peaks of heterophile antibody activity were apparent, the firt occurred between 10 and 20 weeks post-infection, and the second between 40 and 60 weeks post-infection. These peaks of antibody release did not correlate with elevated plasma tumour necrosis factor (TNF)-α levels or IL-6 production by lipolysaccharide (LPS)-stimulated PBMC from experimentally infected cats (C. E. Lawrence, personal communication).

The mechanisms underlying the polyclonal B-cell activation are not known at present. In HIV infection, it has been proposed that virus particles in immune complexes trapped in the lymph nodes by follicular dendritic cells are capable of stimulating adjacent T and B cells directly.[16,17] Whereas activated T cells become infected, the activated B cells are induced to proliferate and differentiate. Although the peaks of heterophile antibody

activity observed in the experimentally infected cats did not correlate with TNF-α levels in the plasma, membrane TNF-α on HIV-infected human T cells has recently been shown to be involved in the induction of polyclonal B-cell activation.[18] IL-6 levels are also elevated in FIV-infected cats and in HIV-infected humans,[19] and given the role that this cytokine plays in B-cell development and differentiation it is likely that over-production will be associated with B-cell dysfunction.

The role of polyclonal B-cell activation in disease pathogenesis is still open to speculation, but such B-cell hyperactivity may be associated with the development of B-cell neoplasia.[20–22] Also, the hypergammaglobulinaemia may contribute to the renal lesions observed in FIV-infected cats either directly or indirectly as immune complexes (A. Poli, personal communication). The polyclonal activation may ultimately result in B-cell exhaustion, which will contribute to the generalized immunosuppression associated with FIV infection. Polyclonal B-cell activation also appears to result in the induction of autoantibody production, as evidenced by the development of antibodies to DNA in both naturally and experimentally infected cats and antibodies to thyroglobulin in experimentally infected animals. Anti-nuclear antibodies have also been described in HIV-infected patients,[11] although their significance is unclear.

In summary, natural and experimental infection of domestic cats with FIV results in a sustained polyclonal activation of B cells with the production of antibodies to a variety of non-viral antigens including self-antigens. Given the similarities between FIV and HIV, the feline sytem represents a valuable model for the development of a vaccine against HIV. The majority of vaccination regimes aim to stimulate B cells in an appropriate way to induce maximal levels of neutralizing antibodies, which may be important in protective immunity, whilst minimalizing deleterious B-cell activity such as non-specific polyclonal activation of B cells. Thus a comprehensive understanding of the aberrations of B-cell function associated with infection is an important prerequisite to the rational design of an effective vaccine.

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