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FIV-infected cats respond to short-term rHuG-CSF treatment which results in anti-G-CSF neutralizing antibody production that inactivates drug activity

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Abstract

The hematological and virological effects of recombinant human granulocyte colony-stimulating factor (rHuG-CSF) were evaluated in feline immunodeficiency virus (FIV)-infected cats. Six age-matched, FIV-infected cats used in this cross-over study were injected subcutaneously with 5 μg/kg of rHuG-CSF daily for 3 weeks, while six control cats received a placebo. Five of six rHuG-CSF-treated cats had significant increases in neutrophil counts that peaked on days 11–21 of treatment. All rHuG-CSF-treated cats exhibited an increase in myeloid:erythroid ratios of the bone marrow cells without significant changes in lymphocyte, CD4 counts, CD4/CD8 ratios, RBC counts, FIV antibody titers, and FIV loads in peripheral blood, and without clinical and hematological toxicities. Five of six rHuG-CSF-treated cats developed antibodies to rHuG-CSF by 14–21 days of treatment, which correlated with decreasing neutrophil counts and increasing neutralizing antibodies to rHuG-CSF. Three cats re-treated with rHuG-CSF rapidly developed neutralizing antibodies to rHuG-CSF, while one cat also developed neutralizing antibodies to recombinant feline G-CSF (rFeG-CSF). Overall, rHuG-CSF treatment increased neutrophil counts in FIV-infected cats without affecting the infection status of cats. However, long-term use of rHuG-CSF is not recommended in cats because of the neutralizing antibody production to rHuG-CSF that affects the drug activity. In addition, a preliminary finding suggests that repeated treatment cycle can also induce cross-neutralizing antibodies to rFeG-CSF, which may potentially affect the homeostasis of endogenous FeG-CSF.

Keywords: G-CSF; neutralizing antibodies; FIV

Abbreviations: BM, bone marrow; CID_{50}, cat median infectious dose; CI, confidence interval; ED_{50}, efficacy dose 50%; FIV, feline immunodeficiency virus; FIP, feline infectious peritonitis; M:E, myeloid:erythroid; rHuEPO, recombinant human erythropoietin; rHuG-CSF, recombinant human granulocyte-CSF; rHuGM-CSF, recombinant human granulocyte-macrophage-CSF; rFeG-CSF, recombinant feline G-CSF; rCaG-CSF, recombinant canine G-CSF; RT, reverse transcriptase; $TCID_{50}$, tissue culture median infectious dose

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1. Introduction

Hematopoietic growth factors, which regulate the growth, development, and function of hematopoietic lineages, have been used in immune reconstitution therapies in humans and animals. Most clinically relevant hematopoietic growth factors in veterinary medicine are erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Ogilvie, 1995). Since species-specific growth factors are commercially unavailable for veterinary medicine, recombinant human growth factors that are FDA-approved for human use are being used in animals (Ogilvie, 1995). Recombinant human EPO (rHuEPO), which enhances red blood cell production, can be used in veterinary medicine as a therapy for anemia resulting from chronic renal failure, cancer, chemotherapy, and to reduce the need for transfusion during surgery (Ogilvie, 1995). Similar to their use in humans, recombinant human G-CSF (rHuG-CSF) and GM-CSF (rHuGM-CSF) can be used to increase the production and functional activity of neutrophils and monocytes in animals that have infectious disease or chemotherapy-induced myelosuppression (Ogilvie, 1995). However, these drugs are not veterinary-labeled products and the potential risks must be considered in relation to the therapeutic benefits for veterinary use. Foremost, the potential for adverse reactions caused by the development of antibodies to these human products may be a limiting factor for the long-term use.

Feline immunodeficiency virus (FIV) is a lentivirus that causes immunodeficiency syndrome in domestic cats (Pedersen et al., 1987; Yamamoto et al., 1988a). Hematological abnormalities, such as anemia, neutropenia, and lymphopenia are typical manifestations of FIV infection in cats. Hence, the use of hematopoietic growth factor(s) can greatly benefit the FIV-infected cats by improving the quality of life. Findings from short-term studies have already been reported on the use of rHuEPO and rHuGM-CSF as supportive therapy in FIV-infected cats (Arai et al., 2000). These studies demonstrate that rHuEPO could be used safely in FIV-infected cats to increase RBC count without changing FIV status or developing anti-rHuEPO antibodies. When compared to rHuEPO, the efficacy of rHuGM-CSF was remarkably low, resulting in 50% increase of neutrophils and monocytes in treated cats with the production of anti-rHuGM-CSF antibodies and an increase in FIV load (Arai et al., 2000). As a result, the use of rHuGM-CSF is not recommended in FIV-infected cats, requiring an alternative drug be identified for treating neutropenia in these animals.

Like FIV-infected cats, neutropenia is a well-known clinical outcome of HIV infection in humans (Yamamoto et al., 1988a; Coyle, 1997) and a common side effect of some anti-FIV and anti-HIV therapies (Coyle, 1997; Arai et al., 2002, 2002). To help counter the loss of neutrophils, HIV-infected individuals have been treated successfully with rHuG-CSF (Coyle, 1997; Aladdin et al., 2000). However, no studies have been reported to date on the efficacy of rHuG-CSF treatment in FIV-infected cats. Unlike GM-CSF that regulates the development of earlier uncommitted progenitor cells, such as stem cells and multipotent progenitors, G-CSF stimulates predominantly the proliferation, differentiation, and activation of committed neutrophil–granulocyte progenitors into functionally mature neutrophils (Hollingshead and Goa, 1991). In this paper, we report the hematological and virological effects of rHuG-CSF treatment in FIV-infected cats.

2. Materials and methods

2.1. Effect of rHuG-CSF on proliferation of peripheral blood mononuclear cells (PBMC) and bone marrow (BM) cells

In vitro ^{3}H thymidine incorporation assays were performed to assess the effect of rHuG-CSF on the proliferation of PBMC and BM cells. Primary PBMC and BM cells from specific pathogen-free (SPF) cats were purified by Cellgro Lymphocyte Separation Medium (Mediatech Inc. Herndon, VA) (Yamamoto et al., 1988a) and cultured in triplicate at 2×10^5 PBMC/100 μl per well and 5×10^4 BM cells/100 μl per well for 3 days in assay media supplemented with varying concentrations (0–100 ng/ml) of rHuG-CSF (Neupogen® Filgrastim; Amgen Inc., Thousand Oaks, CA). The assay media consisted of RPMI 1640 media containing 5% heat-inactivated FCS, 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, and 50 μg/ml gentamycin. The ^{3}H thymidine was added 18 h prior to cell

harvest on day 3. The level of ^{3}H thymidine incorporation was determined by liquid scintillation analysis and presented as cpm per culture.

2.2. Effect of rHuG-CSF on FIV replication in T-cell-enriched PBMC, primary PBMC, and BM cells

The effect of rHuG-CSF on FIV replication was determined in T-cell-enriched PBMC, primary PBMC, and BM cells. The T-cell-enriched PBMC were derived by stimulating PBMC with T-cell mitogen, ConA, for 3 days and culturing the cells for an additional 2 weeks in culture media. The culture media was the same as the assay media for proliferation with the exception of 10% heat-inactivated FCS and the addition of 100 U/ml recombinant human IL-2. The T-cell-enriched PBMC were inoculated with FIV at 25–100 tissue culture median infectious dose ($TCID_{50}$) and cultured for 3 weeks in culture media that were supplemented with set concentrations of rHuG-CSF (0–100 ng/ml). The culture supernatants were collected every 3 days and the cells were resuspended in fresh culture media containing appropriate concentrations of rHuG-CSF. The supernatants were tested for FIV titers by reverse transcriptase (RT) assay as previously described (Rey et al., 1984). In other studies, primary PBMC or BM cells from FIV-infected cats were cocultured with uninfected T-cell-enriched PBMC for 3 weeks in culture medium that was supplemented with set concentrations of rHuG-CSF. T-cell-enriched PBMC were >98% T-cells based on phenotypic analysis with Pan-T antibody (Yamamoto et al., 1998). The primary PBMC had neutrophil contamination of 15–25%, whereas T-cell-enriched PBMC had no detectable neutrophils based on microscopy with Wright-stain. The supernatants were collected for RT assay as described above.

2.3. Animal studies

Cats were bred at the Laboratory of Comparative Immunology and Retrovirology (LCIR) and housed in Animal Care Facilities at the University of Florida. Six SPF cats, 15 weeks of age (unrelated to the cats in the in vitro studies in Sections 2.1 and 2.2) were inoculated with 200 cats median infectious dose (CID_{50}) of FIV_{UK8} and were chronically infected for 3.5 months prior to initiation of this study. Upon confirmation of FIV infection, these cats were used in a cross-over study consisting of two 3-week treatments separated by a 5-week rest period. Initially (study part 1), three experimental cats (#9QE, #9QG, and #9QJ) received 5 mg/kg s.c. injection of rHuG-CSF (Neupogen® Filgrastim) daily for 3 weeks, while three control cats (#9QF, #9QH, and #9QI) received an equivalent volume of s.c. saline as a placebo. Following a 5-week rest period, the cat groups were reversed (study part 2) and the study repeated using the same established treatment regimen. Approximately, 3–5 months after completion of the cross-over study, three cats (#9QG, #9QH, and #9QI) were re-treated s.c. once a day for 1 week with 5 μg/kg of rHuG-CSF.

All cats were monitored daily for clinical signs including loss of appetite, ocular/nasal discharges, and rectal temperature. Body weights were taken several times over the course of the study. Blood was drawn twice before treatment, every 3–4 days during treatment, and three times after treatment for complete blood counts (CBC), blood chemistry, CD4, and CD8 phenotyping (Yamamoto et al., 1998), FIV load analysis of PBMC (Yamamoto et al., 1998), anti-FIV antibody titration (Yamamoto et al., 1988b), and anti-G-CSF antibody analyses. Cats in the re-treatment study were monitored only for CBC and anti-G-CSF antibodies during the re-treatment period.

Bone marrow cells from all cats were evaluated for myeloid:erythroid (M:E) ratios once before the start of treatment, once during the treatment, and once following the end of the treatment. Bone marrow aspirate (1 ml) collected from a femur was prepared in 0.5 ml of 5% EDTA solution for BM smear slides as previously described (Freeman, 2000) and submitted to the Clinical Pathology Laboratory, College of Veterinary Medicine at the University of Florida for analysis.

2.4. Determination of anti-rHuG-CSF antibodies and anti-rFeG-CSF antibodies

Recombinant FeG-CSF was produced using pET-Blue-1 vector in *Escherichia coli* Origami™ (DE3) pLacI strain as recommended by the manufacturer (Novagen®, EMD Biosciences Inc., San Diego, CA). The expressed product was purified using Macro-prep High-S and Bio-Gel HTP Hydroxyapatite Chromatographies (Bio-Rad Inc., Hercules, CA). The purity of

rFeG-CSF was over 97% based on silver stain analysis.

Anti-rHuG-CSF and anti-rFeG-CSF antibody levels were determined by ELISA. Recombinant HuG-CSF or rFeG-CSF was coated on 96-well plates at 100 ng/well. After blocking and washing, plates were incubated with serial dilutions of serum in triplicate for 1 h. The remaining procedure for the ELISA was the same as previously described (Yamamoto et al., 1991), except for the use of 300 μg/ml of 2,2′-azino-di-(3-ethybenzthiozoline sulfonic acid) with 0.03% H_2O_2 as ELISA substrate solution.

2.5. Detection of neutralizing antibodies against rHuG-CSF and rFeG-CSF

Sera from rHuG-CSF-treated cats were tested for neutralizing antibodies to rHuG-CSF and rFeG-CSF using a neutralization assay based on proliferation of G-CSF-sensitive NFS-60 cells (Weinstein et al., 1986; Shirafuji et al., 1989); 50 μl of serially diluted sera were mixed with 50 μl of eight efficacy dose 50% (ED_{50}) of either rHuG-CSF or rFeG-CSF and incubated at 37 °C for 1 h. The ED_{50} of rHuG-CSF and rFeG-CSF was 0.0473 and 0.141 ng/ml, respectively. After the initial incubation, 50 μl of each suspension samples were added in duplicate to wells containing 50 μl of 2.5×10^4 NFS-60 cells and subsequently incubated for an additional 48 h at 37 °C. Cell proliferation was measured with CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corp. Madison, WI). The representative result from three separate assays is shown as the dilution titer that provided over 30% inhibition as compared to the value of NFS-60 cell-proliferation from pre-treatment sera.

2.6. Statistical analyses

Results from in vitro studies were analyzed for statistical significance using the Student's *T*-test. Cats were divided into treatment versus placebo groups for *T*-test analysis of M:E ratios, FIV load, and anti-FIV antibody titers. Confidence interval analysis was used to evaluate the hematological results from in vivo studies. Confidence intervals were used to compare the following groups: rHuG-CSF-treated cat samples versus control cat samples, pre-treatment versus treatment samples, pre-treatment versus post-treatment samples, and treatment versus post-treatment samples. The groups, which were considered statistically different based on 95% confidence interval (CI), were further analyzed by *T*-test for final statistical comparisons. The *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of rHuG-CSF on in vitro cell-proliferation and FIV replication in PBMC and BM cells

In vitro studies were performed to evaluate the direct effects of rHuG-CSF on the proliferation of feline PBMC and BM cells and to determine the effects of rHuG-CSF on FIV replication in feline PBMC and BM cells. The primary PBMC from two of four SPF cats had slight irregular non-dose-dependent increases in proliferation response to varying concentrations of rHuG-CSF (Fig. 1, part A1). In contrast, slight but more consistent dose-dependent increases in proliferation response were observed with BM cells from all four SPF cats (Fig. 1, part A2). However, these increases in BM proliferation were not statistically significant. Consequently, PBMC and BM cells from FIV-infected cats were not tested for proliferation response to rHuG-CSF but they were used to determine the in vitro effects of rHuG-CSF treatment on FIV replication. None of the PBMC and BM cell cultures (in vitroly infected T-cell-enriched PBMC, and PBMC and BM cells from FIV-infected cats) had an increase in FIV replication due to rHuG-CSF treatment (Fig. 1, parts B1–B3). Hence, these in vitro results demonstrate that rHuG-CSF has no direct effect on the FIV replication in PBMC and BM cells even though slight proliferation of uninfected BM cells occurred in response to rHuG-CSF stimulation.

3.2. Clinical observations of FIV-infected cats

Six SPF cats from a single litter were inoculated with a highly virulent FIV-UK8 (FIV_{UK8}) strain. Five of six cats developed fever prior to the study starting 4–8 weeks post-inoculation and were extensively treated with antibiotics and fluid therapy until their

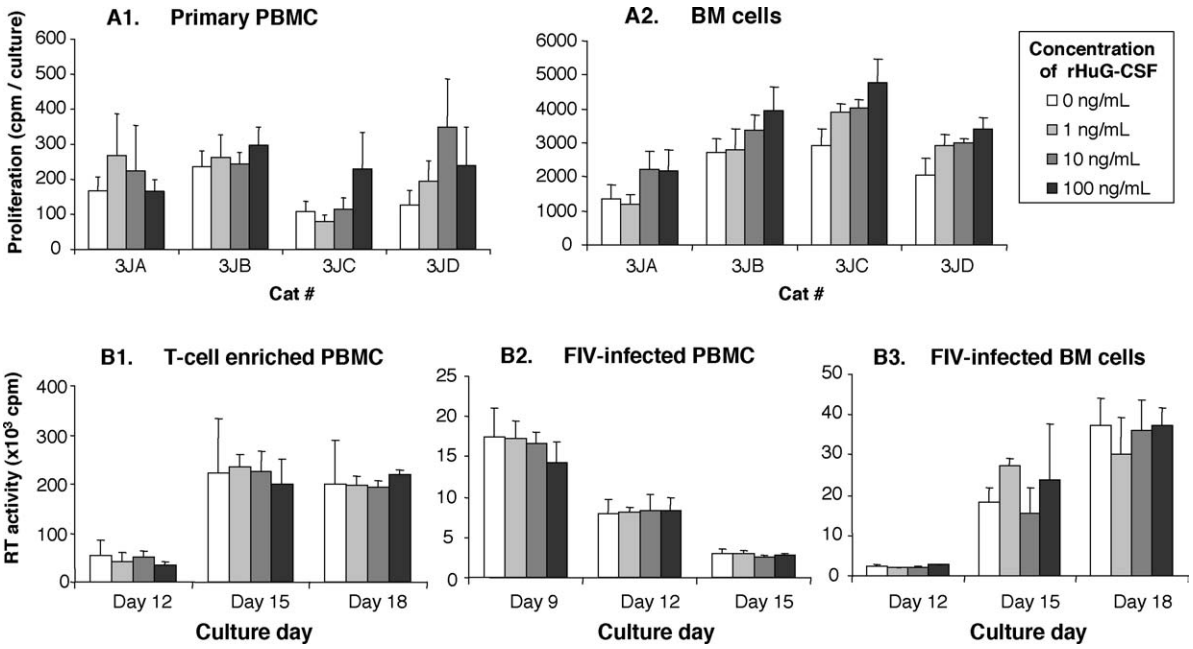

Fig. 1. Effects of rHuG-CSF on the in vitro cell-proliferation and FIV replication in PBMC and BM cells. Primary feline PBMC (A1) and BM cells (A2) from SPF cats were tested for its proliferation responses to 10-fold dilutions (1–100 ng/ml) of rHuG-CSF. In the next set of experiments, the ability of rHuG-CSF in 10-fold dilutions to enhance the in vitro FIV infection was tested. T-cell-enriched PBMC were inoculated with FIV at the time of first rHuG-CSF treatment and subsequently monitored for FIV replication by measuring RT activity (B1). In other experiments, PBMC (B2) and BM cells (B3) from FIV-infected cats were co-cultured with uninfected T-cell-enriched PBMC in the presence of rHuG-CSF and monitored for FIV replication. All assays were performed in triplicate and typical results from three to five separate experiments are shown.

temperature returned to normal at 4–8 weeks post-supportive therapy. Except for cat #9QI, all of the remaining cats were clinically healthy, afebrile, and not receiving any drug therapy at the start of the study. Cat #9QI was treated with prednisolone (2.5 mg, orally once as day) before and throughout both parts of the study to control the recurrent fever caused potentially by FIV infection, with the exception on days 1 and 4–6 of placebo treatment in study part 1 and throughout re-treatment study starting day 42 of study part 2.

Two cats developed mild reactions to rHuG-CSF treatments. During study part 1, cat #9QG developed a fever of 103.5 °F with redness at the injection site and a limp on its right fore-foot starting day 2 of rHuG-CSF treatment. All clinical signs resolved by treatment day 5. In study part 2, cat #9QF developed what appeared to be sensitivity to the injection, which may be instead generalized resistance to injection as there was no redness or swelling at the site of injection. During both parts of the study, the hematological findings were within normal limits in all cats, suggesting no rHuG-CSF-induced drug toxicity occurred. Although blood chemistry values from several of the cats were outside the normal range during the treatment period, the baseline values were outside the normal range in these cats prior to the treatment, suggesting that the abnormal values were most likely due to FIV infection.

3.3. Hematological results

Except for cat #9QJ, five of six rHuG-CSF-treated cats had a significant increase in the average WBC counts during treatment compared to the baseline (Fig. 2A). These cats exhibited elevated WBC counts of at least 10,000 neutrophils/μl increase over baseline values that peaked on treatment days 4, 11, 14, and 18. Moreover, the WBC counts in the six rHuG-CSF-treated cats were statistically higher than those in the

placebo group on treatment days 4, 11, 14, and 18 (Fig. 2A and B). In addition, the rHuG-CSF-treated group had significantly higher average WBC counts on treatment days 11–18 than the average baseline (day 0) WBC counts.

The neutrophil counts of the rHuG-CSF-treated group showed a similar trend as that observed with the WBC counts over the course of study (Figs. 2C and 3). The five rHuG-CSF-treated cats, which had statistically significant increase in WBC counts,

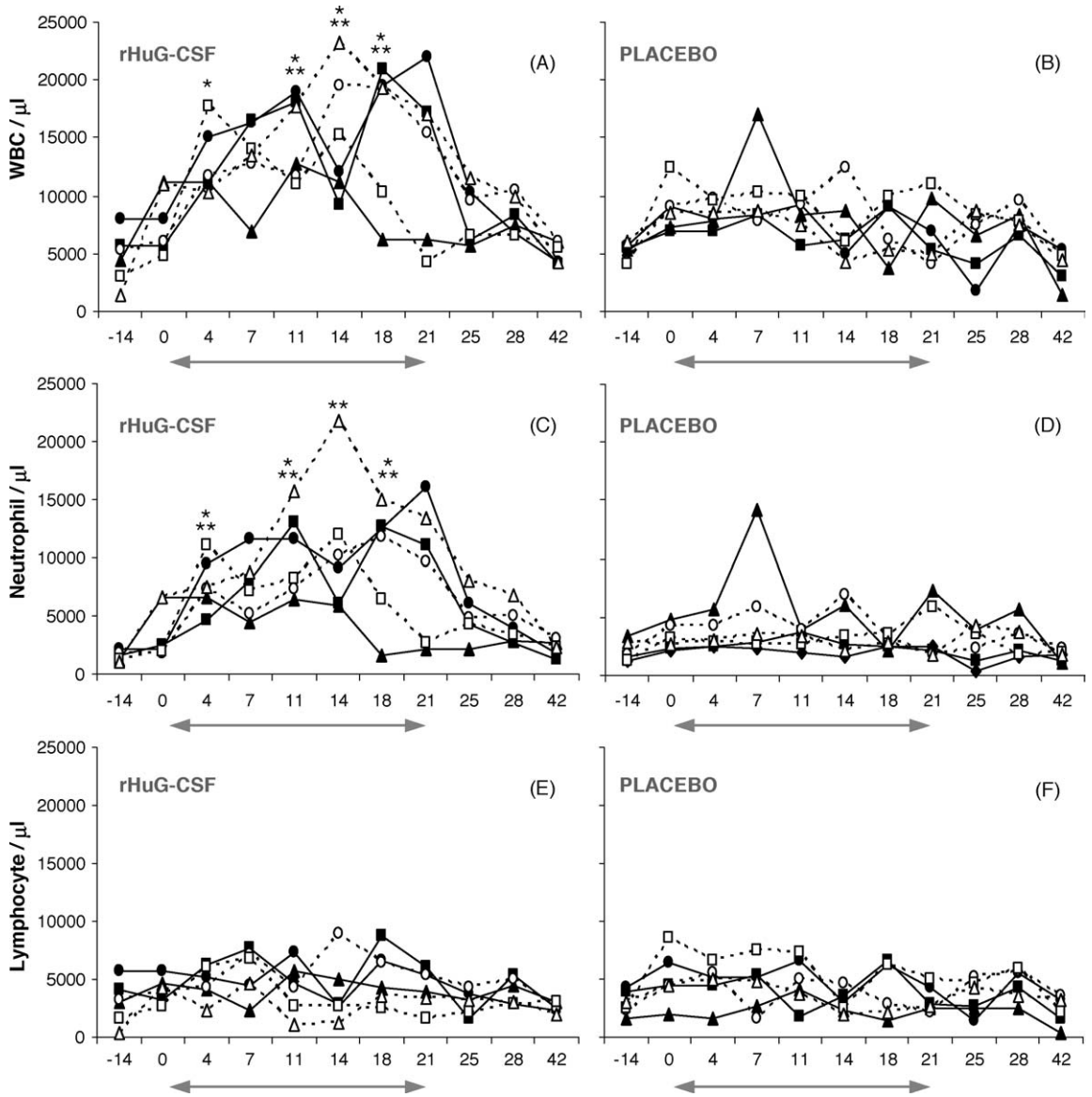

Fig. 2. WBC, neutrophil, lymphocyte, eosinophil, and monocyte counts of FIV-infected cats undergoing rHuG-CSF treatment. The arrow bar represents time of treatment in days. Statistically significant differences demonstrated between rHuG-CSF treatment group and placebo group from the same time point are shown as (*) and between post-treatment and pre-treatment values of the rHuG-CSF treatment group are shown as (**). Statistically significant differences established by both comparisons (treated group vs. placebo group; post-treatment vs. pre-treatment) are shown as (*,**).

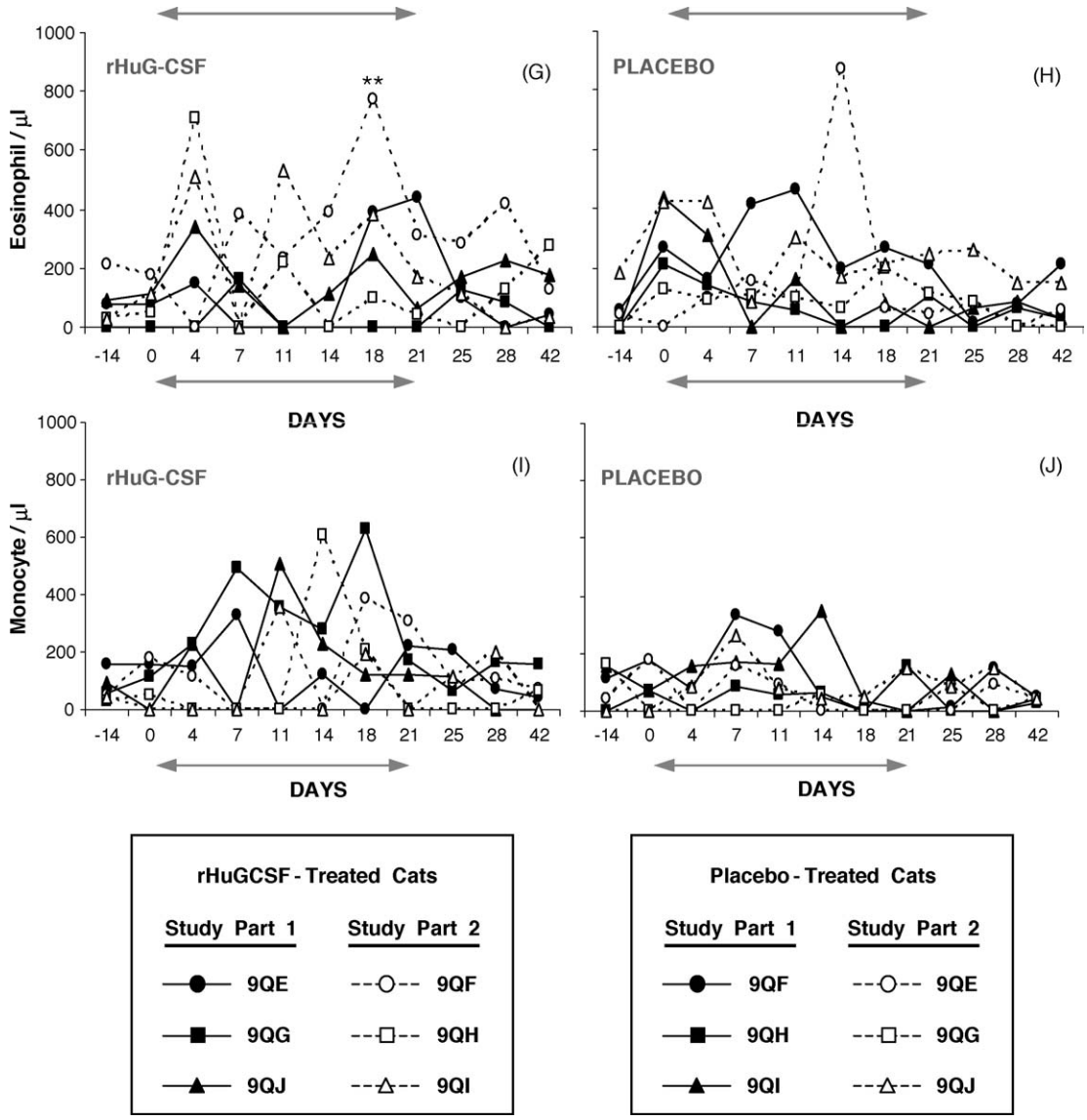

Fig. 2. (*Continued*).

also had significant increases in neutrophil counts (i.e., at least 10,000 neutrophils/µl increase). Statistically significant increases in the average neutrophil counts of the rHuG-CSF-treated versus placebo groups were observed on treatment days 4, 11, and 18 (Fig. 2C and D). Similar to the WBC counts, the average neutrophil counts were significantly higher in the rHuG-CSF-treated group on treatment days 4, 11, 14, and 18 when compared to the average baseline (day 0) counts. In contrast, WBC and neutrophil counts remained relatively stable in the placebo group, with the exception of cat #9QI in part 1 on treatment day 7 (Fig. 2B and D). As described before, cat #9QI received prednisolone throughout part 1, except for placebo treatment days 1, and 4–6, which coincides with the increases in both WBC and neutrophil counts on treatment day 7. However, in part 2, the increases in WBC counts

in this cat during the rHuG-CSF treatment were not affected by the prednisolone given to the cat throughout this study.

No statistically significant increases in the lymphocyte counts were observed in rHuG-CSF-treated cats when compared to the placebo controls and when compared to the baseline pre-treatment values on day 0 (Fig. 2E and F). Eosinophil counts were highly variable in both treatment groups, although not significantly different when compared to either their baseline pre-treatment values on day 0 or to the placebo groups, except on treatment day 18 (Fig. 2G and H). Although monocyte counts were highly variable between cats, there appeared to be more pronounced elevation of monocyte counts in the rHuG-CSF-treated cats compared to placebo controls (Fig. 2I and J). However, no statistically significant increases in monocyte counts were observed in the rHuG-CSF-treated group when compared to either the placebo controls or their baseline values on day 0. Furthermore, the monocyte counts of the placebo-treated group had no significant increases throughout the study. Other hematological parameters, such as RBC, hemoglobin, platelet, and hematocrit were not affected by rHuG-CSF treatment (data not shown).

3.4. Effect of rHuG-CSF on M:E ratios

All FIV-infected cats had low M:E ratios before rHuG-CSF administration (Table 1). When compared to pre-treatment baseline (day −14) values, statistically significant increases in the M:E ratios of the rHuG-CSF-treated cats were observed at 2 weeks of treatment (day 14, Table 1) and at 3 weeks after the cessation of treatment (day 42). The increases in average M:E ratios were more consistent and pronounced in the rHuG-CSF-treated group, resulting in statistically significant M:E ratio compared to the placebo group on treatment day 14 in addition to the baseline value on treatment day 14 and post-treatment day 42. Although slight transient M:E ratio increases were observed in two placebo-treated cats, no placebo-treated cat individually or as a group exhibited a statistically significant increase in M:E ratios (Table 1). Hence, significant M:E ratios were only observed in the rHuG-CSF-treated group.

3.5. Effect of rHuG-CSF on the FIV status

Fluctuations in FIV load were observed in the PBMC from both rHuG-CSF-treated and placebo-treated cats (Table 2), but there was no statistical significance in the results. Moreover, all cats maintained relatively constant anti-FIV antibody titers (Table 2), CD4 counts (average, 453 cells/μl; range, 41–1714 cells), and CD4/CD8 ratios (average, 0.42; range, 0.09–1.0) throughout the study. These results taken together demonstrate that rHuG-CSF treatment had no adverse effect on FIV load or disease of the treated animals.

3.6. Detection of antibodies to rHuG-CSF and rFeG-CSF by ELISA

Except for one cat (#9QE), five of six cats developed antibodies to rHuG-CSF by 14 or 21 days post-treatment (Table 3). The peak anti-rHuG-CSF antibody titers were detected on treatment day 21, ranging in titer from 1:200 to 1:3200. Four of five cats maintained detectable anti-rHuG-CSF antibody titers as long as 3 weeks after the withdrawal of the treatment (day 42, Table 3). The anti-rHuG-CSF antibodies from two cats (#9QJ and #9QH), which had the highest titer of antibodies, also cross-reacted to the rFeG-CSF by ELISA (Table 3).

3.7. Detection of anti-G-CSF neutralizing antibodies

Four of six cats had both ELISA and neutralizing antibody titer to rHuG-CSF (Table 3, Fig. 3). In addition, two of these cats (#9QJ and #9QH) had ELISA antibody titer to rFeG-CSF but no detectable neutralizing antibodies to rFeG-CSF during treatment and at post-treatment day 42. Neutralizing antibodies to either rHuG-CSF or rFeG-CSF were not detected in the pre-treatment sera.

Approximately 3–5 months after the withdrawal of rHuG-CSF treatment, three cats (#9QG, #9QH, and #9QI) were re-treated for 1 week with the same daily rHuG-CSF dose as previously described. The ability of their sera to neutralize rHuG-CSF and rFeG-CSF was tested. All three cats developed significant levels of neutralizing antibodies to rHuG-CSF by 1 week of re-treatment (Fig. 3C, E and F). In addition, cat #9QH

Table 1
Myeloid:erythroid (M:E) ratios of FIV-infected cats from study parts 1 and 2

Treatment (study part #)	Cat ID#	Pre-treatment (day −14 PT[a])	During treatment (day 14 PT[a])	Post-treatment (day 42 PT[a])
rHuG-CSF				
Part 1	9QE	0.22	1.40	0.50
	9QG	0.27	0.74	0.40
	9QJ	0.23	0.68	0.30
Part 2	9QF	0.13	1.20	0.65
	9QH	0.32	1.90	0.74
	9QI	0.27	3.10	0.64
Mean ± S.D.		0.24 ± 0.06	1.50 ± 0.90[**b]	0.54 ± 0.17[*b]
Placebo				
Part 1	9QF	0.30	0.25	0.13
	9QH	0.33	0.38	0.32
	9QI	0.24	0.56	0.27
Part 2	9QE	0.50	0.40	0.29
	9QG	0.40	0.30	0.36
	9QJ	0.30	0.80	0.67
Mean ± S.D.		0.34 ± 0.09	0.45 ± 0.20	0.34 ± 0.18

[a] Post-first treatment (PT).
[b] Statistically significant differences exist between pre- and post-treatment values of the rHuG-CSF treatment group denoted by * and also between rHuG-CSF treatment group and placebo groupdenoted by **.

Table 2
Effect of rHuG-CSF treatment on FIV Load in PBMC and anti-FIV antibody titers

Treatment (study part #)	Cat #	FIV load in PBMC (number of cells in $\log^{-x}$)					Anti-FIV antibody titers (dilution, X^{-1})		
		Pre-treatment		During treatment		Post-treatment	Pre-treatment	During Treatment	Post-treatment
		Day –48[a]	Day –14[a]	Day 14[a]	Day 21[a]	Day 42[a]	Day –14[a]	Day 21[a]	Day 42[a]
rHuG-CSF									
Part 1	9QE	4	3	4	5	4	7	7	7
	9QG	6	3	5	5	4	7	7	7
	9QJ	5	5	5	4	4	8	8	8
Part 2	9QF	ND	4	6	5	5	7	7	7
	9QH	ND	5	5	5	4	7	7	7
	9QI	ND	5	6	6	4	6	6	6
Mean + S.D.[b]		5.0 ± 1.0	4.2 ± 1.0	5.2 ± 0.7	5.0 ± 0.6	4.2 ± 0.4	7.0 ± 0.6	7.0 ± 0.6	7.0 ± 0.6
Placebo									
Part 1	9QF	5	3	4	5	4	7	7	7
	9QH	5	5	4	4	5	7	7	7
	9QI	5	4	5	5	5	6	6	6
Part 2	9QE	ND	4	5	4	4	7	7	7
	9QG	ND	4	5	5	5	7	7	7
	9QJ	ND	4	5	5	4	8	8	8
Mean + S.D.[b]		5.0 ± 0.0	4.0 ± 0.6	4.7 ± 0.5	4.7 ± 0.5	4.5 ± 0.5	7.0 ± 0.6	7.0 ± 0.6	7.0 ± 0.6

[a] Number of days post-first treatment.
[b] Statistically significant differences were not observed between pre-treatment and post-treatment and between treatment and placebo groups.

Table 3
Production of anti-rHuG-CSF and anti-rFeG-CSF antibodies in rHuG-CSF-treated cats

Study (part and cat #)[a]	ELISA Anti-G-CSF antibody titers[b]					Neutralizing antibody titers[b]		
	Pre-treatment	During treatment			Post-treatment	During treatment		Post-treatment
	Day –14	Day 7	Day 14	Day 21	Day 42	D 14	D 21	D 42
Part 1								
9QE	U	U	U	U	U	U	U	U
9QG	U	U	U	200 (u)	U	U	45 (u)	15 (u)
9QJ	U	U	400 (u)	3200 (200)	1600 (100)	135 (u)	135 (u)	U
Part 2								
9QF	U	U	200 (u)	800 (u)	400 (u)	U	U	U
9QH	U	U	800 (200)	3200 (400)	800 (100)	15 (u)	405 (u)	405 (u)
9QI	U	U	100 (u)	800 (u)	400 (u)	U	135 (u)	135 (u)

All placebo-treated cats had no anti-G-CSF antibodies except for cats #9QJ and #9QG. Cat #9QJ had ELISA antibodies to rHuG-CSF and rFeG-CSF remaining from study part 1 at the time of placebo treatment in study part 2. Cat #9QG had residual anti-rHuG-CSF antibodies at the time of placebo treatment in study part 2.

[a] Only the results from rHuG-CSF-treated cats are shown.

[b] Antibody titer to rHuG-CSF is shown followed by titer to rFeG-CSF in parenthesis. The symbol U represents undetectable to both anti-rHuG-CSF and anti-rFeG-CSF antibodies at the lowest serum dilution of 1:50 for ELISA and 1:15 for neutralizing antibody assay. The symbol (u) represents undetectable for only anti-rFeG-CSF antibodies.

developed a low neutralizing antibody titer (1:15) to rFeG-CSF after 1 week of re-treatment (Fig. 3E). Since this observation is the first reported case of cross-neutralizing antibodies to FeG-CSF, this result was confirmed by performing three additional assays with all results confirming the same finding. Overall, these studies suggest that repeated cycles of rHuG-CSF treatment can lead to the induction of neutralizing antibodies to the HuG-CSF and potentially to the FeG-CSF.

4. Discussion

FIV-infected cats are known to have multilineage hematopoietic defects, such as lymphopenia, neutropenia, and anemia (Shelton et al., 1990; Fleming et al., 1991; Sparkes et al., 1993). In our previous study, we treated FIV-infected cats with rHuGM-CSF instead of rHuG-CSF because multilineage activity of GM-CSF could be more useful for immune reconstitution of FIV-infected cats (Arai et al., 2000). However, only 50% of rHuGM-CSF-treated cats responded to the treatment by having an increase in neutrophil, eosinophil, lymphocyte, monocyte, RBC, or their combination. In addition to increasing multiple populations of hematocytes in FIV-infected cats, concomitant 1–2 log increases in FIV load were observed during the rHuGM-CSF treatment. Unlike the rHuGM-CSF studies, current rHuG-CSF studies clearly demonstrate that FIV load is not increased by rHuG-CSF treatment of cats, suggesting that this drug is more selective for neutrophils than for monocytes. In fact, pronounced statistically significant increases in neutrophil counts were observed with rHuG-CSF treatment (Fig. 2C and D) when compared to slight irregular increases in monocyte counts with no statistical significance (Fig. 2I and J). Moreover, rHuGM-CSF had significant effect on FIV load in peripheral blood whereas rHuG-CSF did not. Since FIV productively infects monocytes/macrophages but not neutrophils (Kubes et al., 2003), this observation demonstrates the importance of neutrophil count increases compared to the slight irregular monocyte increases. Unlike rHuGM-CSF, rHuG-CSF did not increase the in vitro FIV replication of monocyte-containing PBMC and BM cells from FIV-infected cats (Fig. 1, parts B2 and 1B3).

G-CSF functions as a growth factor to neutrophil progenitors by inducing proliferation and differentiation of myeloid cells into neutrophils (Frampton et al., 1994). However, mature neutrophils are affected only at differentiation and function, but not at proliferation (Frampton et al., 1994). Consequently, the current study evaluated the effect of rHuG-CSF treatment on myeloid cells by measuring the M:E ratios which

monitor progenitor neutrophil proliferation. Recombinant HuG-CSF treatment in FIV-infected cats significantly increased M:E ratios along with concomitant increases in neutrophil counts in blood. This observation is important because FIV, especially, FIV_{UK8}, has been reported to infect BM cells and cause hematological abnormality in the marrow by 24 weeks post-FIV inoculation (Tanabe and Yamamoto,

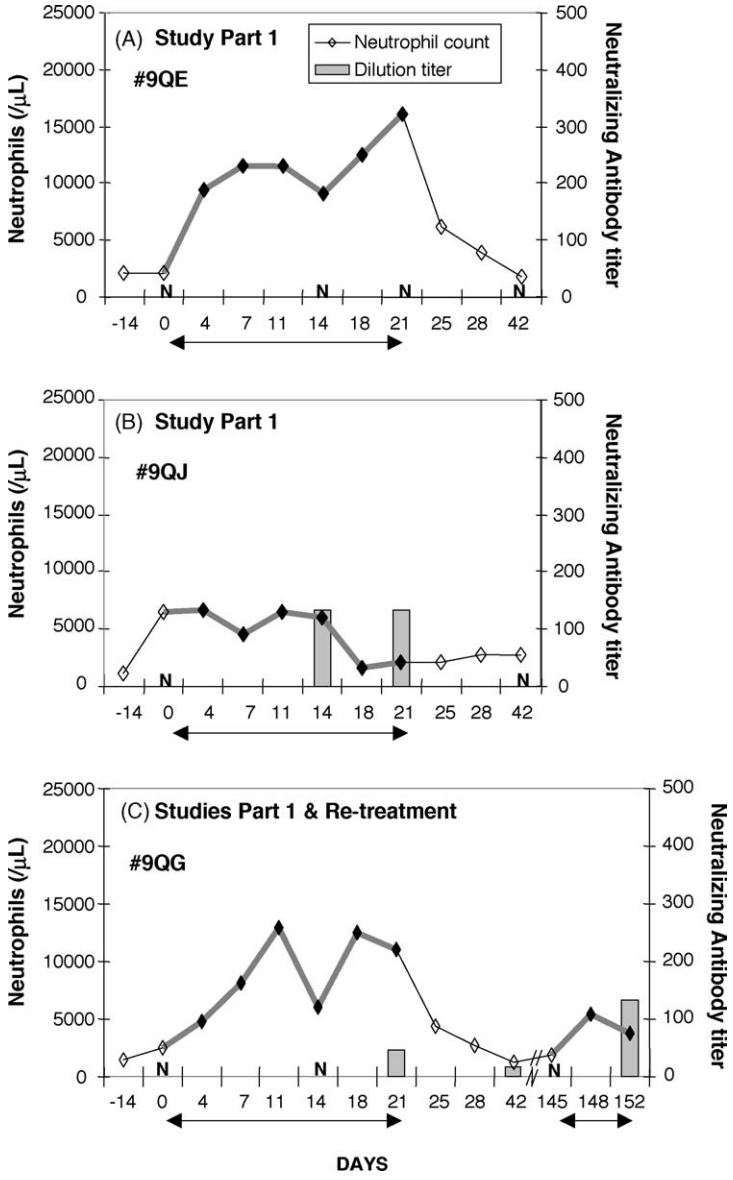

Fig. 3. Neutrophil production kinetics and neutralizing antibody titers of individual cats treated with rHuG-CSF. Sera from pre-treatment (day 0), during treatment (days 14 and 21), and post-treatment (day 42) were tested for neutralizing antibody titers. Those sera with undetected neutralizing antibody titer are shown as (N). Cats #9QG (panel C), #9QH (panel E), and #9QI (panel F) were re-treated with rHuG-CSF for 7 days, and their sera tested for neutralizing antibody titers to rHuG-CSF (▭) and rFeG-CSF (■) on days 0 and 7 of re-treatment. A thick grey line with solid symbol (◆) represents neutrophil counts during rHuG-CSF treatment. All three cats developed significant neutralizing antibody titers to rHuG-CSF but only cat #9QH had neutralizing antibody to rFeG-CSF on 7 days of re-treatment.

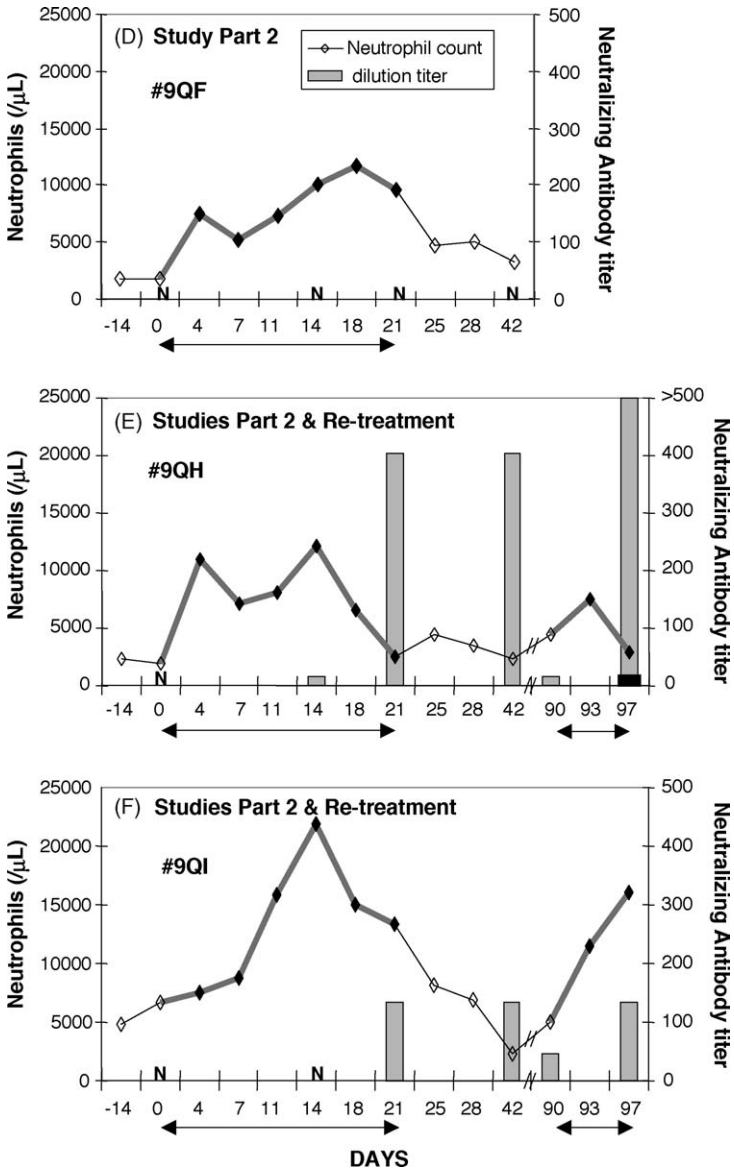

Fig. 3. (*Continued*).

2001). Compared to the reported increases in M:E ratios and neutrophil counts of FIV-free cats undergoing G-CSF treatment (Phillips, 2002), current results indicate that the FIV_{UK8} infection status did not significantly affect the ability of BM cells to respond to the rHuG-CSF treatment.

The neutrophil counts from the majority of rHuG-CSF-treated, FIV-infected cats peaked at treatment days 11–21 and steadily declined thereafter. Since these cats were continuously treated daily for 3 weeks including day 21, the moderate to rapid decline in neutrophil counts during the treatment suggested the potential development of neutralizing antibodies to rHuG-CSF. Two cats (#9QJ and #9QH) exhibited declines in neutrophil counts to baseline levels by treatment days 18–21, while developing the highest titers of ELISA anti-rHuG-CSF antibodies along with anti-rHuG-CSF neutralizing antibody production by

14–21 days of treatment (Fig. 3B and E; Table 3). Observed neutrophil production kinetics and the corresponding neutralizing antibody production in these cats were similar to neutrophil production kinetics and anti-rHuG-CSF antibody titers reported in dogs (Lothrop et al., 1988). These results represent the first reported anti-rHuG-CSF ELISA and neutralizing antibody productions in FIV-infected cats undergoing rHuG-CSF treatment.

Three cats, which developed anti-rHuG-CSF neutralizing antibody titers in the first 3 weeks of treatment, were re-treated with rHuG-CSF for 7 days to determine whether neutralizing antibody development would reoccur after 3–5 months without supplemental treatment (Fig. 3C, E and F). All three cats had minimal ($\leq$1:50) or no detectable anti-rHuG-CSF neutralizing antibody titers at the beginning of the re-treatment period. The titers from two cats significantly increased to 1:135 and >1:500 by re-treatment day 7 (Fig. 3C and E). Corresponding to this antibody increase, there was a slight increase in neutrophil counts by re-treatment day 4 followed by a sharp decrease in neutrophil numbers by re-treatment day 7 returning to or near to baseline titers. In contrast, cat #9QI in absence of prednisolone therapy responded to rHuG-CSF re-treatment with a sharp neutrophil increase on re-treatment day 4 that continued through day 7, despite the developing anti-rHuG-CSF neutralizing antibodies (Fig. 3F). Three weeks post-re-treatment study, this cat developed high fever (105.7 °F), underwent high-dose prednisolone therapy, and was euthanized shortly thereafter. At the time of euthanasia, this cat had proteinuria (8.9 g/dl), feline infectious peritonitis (FIP)/coronavirus titer of 1:400, and a histopathology revealing uncharacteristic FIP-related intestinal lesions (Harvey et al., 1996). Thus, the neutrophilic increase of cat #9QI during the re-treatment study may be caused by FIP-associated neutrophilia (Hoskins, 1997). Whereas, the other two cats (without prednisolone) responded to the re-treatment with a decrease in neutrophil counts upon development of high anti-rHuG-CSF neutralizing antibody titers, even though retrospective study of their re-treatment sera demonstrated FIP/coronavirus titers of 1:50 in cat #9QG and 1:200 in cat #9QH.

In retrospect, the sudden increase in neutrophil count on day 7 of placebo treatment (study part 1) of cat #9QI may have resulted from the FIP-induced neutrophilia, since it coincided with prednisolone withdrawal and a concomitant transient increase in FIP/coronavirus titers (from <1:50 to 1:200) (Fig. 2D). Even under a prednisolone treatment, this cat was able to respond to rHuG-CSF treatment (study part 2) with a neutrophil increase and with only a transient two-fold increase in FIP/coronavirus antibody titer (from 1:50 to 1:100) and no signs of FIP disease. This result suggests that rHuG-CSF treatment in combination with prednisolone therapy safely elevated the neutrophil counts and M:E ratio in the FIV and FIP co-infected cat without causing clinical illness (Fig. 2C and Table 1). Moreover, no other cats housed in the same quarter, including cats #9QG and #9QH, had detectable titers to FIP/coronavirus or exhibited clinical FIP signs during Parts 1 and 2 of the study. Hence, their rHuG-CSF treatment results during this period were not affected by ongoing FIP infection in cat #9QI.

Recombinant HuG-CSF differs from FeG-CSF by 28.8% in amino acid (aa) identity (Yamamoto, unpublished data). Consequently, at least 28.8% of the rHuG-CSF is foreign to the feline immune system and such foreign epitopes can potentially stimulate the feline lymphocytes that may enhance the replication of FIV. In addition, rHuGM-CSF, which differs from FeGM-CSF by 31.2% in aa identity, has been reported to increase FIV replication of primary feline PBMC and T-cell-enriched PBMC cultures (Arai et al., 2000). Unlike rHuGM-CSF, our studies demonstrate that rHuG-CSF can be used for FIV-infected cats to increase neutrophil counts without enhancing FIV infection. However, rHuG-CSF neutralizing antibodies developed as early as day 14 of treatment since 28.8% of the rHuG-CSF aa sequence is foreign to the feline immune system. Moreover, the long-term use or repeated cycle of treatment may result in the production of neutralizing antibodies to HuG-CSF, including cross-neutralizing antibodies to endogenous FeG-CSF. Similar studies on the effect of recombinant canine G-CSF (rCaG-CSF) treatment in cats show the lack of neutrophil decline beyond 21 days of treatment with rCaG-CSF, suggesting no production of neutralizing antibodies to rCaG-CSF (Colgan et al., 1992; Obradovich et al., 1993). Since G-CSF of cats and dogs differ by 18.5% in aa identity, the potential of developing neutralizing antibodies to rCaG-CSF in cats is a concern and the use of rFeG-CSF is preferred.

A recent short-term rFeG-CSF study suggests that treatment of cats with rFeG-CSF is a better treatment option than with either rHuG-CSF or rCaG-CSF (Yamamoto et al., unpublished observation). The 11-day treatment study with high daily dose (50 μg/dose) of rFeG-CSF demonstrates a rapid increase in neutrophil counts with no signs of anti-rFeG-CSF antibody production based solely on the neutrophil kinetics at the time of drug withdrawal (Yamamoto et al., unpublished observation). However, our studies suggest that 11 days of treatment may be too short to observe the antibodies to rFeG-CSF, since this product is homologous in aa sequence to endogenous FeG-CSF. According to published reports of antibody production to homologous growth factor in humans, about 3% of subjects treated with rHuG-CSF only developed ELISA-based antibodies without developing neutralizing antibodies to rHuG-CSF (Amgen Inc., 2004). Like rHuG-CSF treatment of HIV-infected individuals, long-term and recurrent treatments with rFeG-CSF may be important for FIV-infected cats that are more susceptible to recurrent bacterial infection due to their immunodeficient state (Pedersen et al., 1987; Yamamoto et al., 1988a; Shelton et al., 1990; Fleming et al., 1991; Sparkes et al., 1993). The commercial development of rFeG-CSF is in its infancy and cost prohibitive due to the difficulty of large-scale production of rFeG-CSF because of the low yield in more conventional expression systems, such as *E. coli* (Yamamoto et al., 2002). In addition, rHuG-CSF is still considered cost prohibitive for long-term use in feline medicine. Hence, future effort should be placed on the cost effective, commercial development of rFeG-CSF, which can be used effectively and safely in cats without the production of neutralizing antibodies that block drug activity.

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