

Development of a mouse-feline chimeric antibody against feline tumor necrosis factor-alpha

Tomoyoshi DOKI¹⁾, Tomomi TAKANO¹⁾ and Tsutomu HOHDATSU^{1)*}

¹⁾Laboratory of Veterinary Infectious Disease, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan

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ABSTRACT. Feline infectious peritonitis (FIP) is a fatal inflammatory disease caused by FIP virus infection. Feline tumor necrosis factor (fTNF)-alpha is closely involved in the aggravation of FIP pathology. We previously described the preparation of neutralizing mouse anti-fTNF-alpha monoclonal antibody (mAb 2-4) and clarified its role in the clinical condition of cats with FIP using *in vitro* systems. However, administration of mouse mAb 2-4 to cat may lead to a production of feline anti-mouse antibodies. In the present study, we prepared a mouse-feline chimeric mAb (chimeric mAb 2-4) by fusing the variable region of mouse mAb 2-4 to the constant region of feline antibody. The chimeric mAb 2-4 was confirmed to have fTNF-alpha neutralization activity. Purified mouse mAb 2-4 and chimeric mAb 2-4 were repeatedly administered to cats, and the changes in the ability to induce feline anti-mouse antibody response were investigated. In the serum of cats treated with mouse mAb 2-4, feline anti-mouse antibody production was induced, and the fTNF-alpha neutralization effect of mouse mAb 2-4 was reduced. In contrast, in cats treated with chimeric mAb 2-4, the feline anti-mouse antibody response was decreased compared to that of mouse mAb 2-4-treated cats.

KEY WORDS: cat, chimeric antibody, monoclonal antibody, tumor necrosis factor-alpha

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Feline infectious peritonitis virus (FIP virus: FIPV), a feline coronavirus (FCoV) of the family *Coronaviridae*, causes a fatal disease called FIP in wild and domestic cat species. Several organs, including the liver, lungs, spleen and central nervous system, are affected in cats that develop FIP, and the formation of lesions in these organs is accompanied by necrosis and pyogenic granulomatous inflammation [13]. Pleural effusion and ascitic fluid were reported to accumulate in some cats. Macrophages/monocytes play an important role in the pathogenesis of FIP. For example, differences in the proliferation of macrophages/monocytes were shown to be related to differences in pathogenicity between feline enteric coronavirus (FECV) and FIPV [2, 16]. FECV and FIPV cannot be serologically or genetically distinguished from each other; however, FECV infection is normally asymptomatic in cats.

We previously reported that tumor necrosis factor (TNF)-alpha is involved in the aggravation of FIP [17–19]. TNF-alpha binds to cell surface TNF receptors and induces various physiological activities [15, 23]. TNF-alpha plays a critical role in many aspects of immunity. However, the excessive production of TNF-alpha can lead to acute inflammation and immune system abnormalities in human and other animals. The involvement of TNF-alpha in aggravating the symptoms of rheumatoid arthritis, psoriasis and inflammatory bowel

disease has been reported [1, 9, 24]. Moreover, previous studies have described aggravation of the pathologies of viral infections (such as human immunodeficiency virus, influenza A virus, herpes simplex virus (HSV) and dengue virus infections) due to increased TNF-alpha production [5, 12, 14, 21, 25]. TNF-alpha was produced excessively by FIPV-infected macrophages. TNF-alpha was involved in lymphopenia and increase in the level of the cellular receptor of serotype II FIPV, aminopeptidase N (APN) [18, 19]. It is also reported that neutrophil apoptosis in cats with FIP was inhibited by TNF-alpha. This finding suggests neutrophilia in cats with FIP due to TNF-alpha-induced neutrophil survival [17].

Anti-TNF-alpha agents are expected to improve the symptoms of FIP caused by viral infection. We previously described the preparation of a feline TNF-alpha (fTNF-alpha)-neutralizing mouse monoclonal antibody (anti-fTNF-alpha mAb, mAb 2-4). This mAb 2-4 exhibited high neutralizing activity against recombinant and natural TNF-alpha, and was confirmed to inhibit the following fTNF-alpha-induced conditions *in vitro*: i) an increase in the survival rate of neutrophils from cats with FIP, ii) APN mRNA expression in macrophages and iii) apoptosis of a feline T-lymphocyte cell line [4]. These findings strongly suggested that mAb 2-4 is applicable as a therapeutic drug against FIP. We are also investigating that mouse mAb 2-4 administration to SPF cats experimentally infected with FIPV alleviated the clinical condition of cat with FIP [3]. However, because this is a mouse-derived antibody and is a xenogeneic protein for cats, induction of feline anti-mouse antibodies may reduce the reactivity of mouse mAb 2-4 against fTNF-alpha.

In humans, infliximab is used as a therapeutic drug for rheumatoid arthritis (RA). In addition to neutralizing human TNF-alpha, which is a factor aggravating the pathology of

*CORRESPONDENCE TO: HOHDATSU, T., Laboratory of Veterinary Infectious Disease, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan.

e-mail: hohdatsu@vmas.kitasato-u.ac.jp

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Table 1. Sequence of PCR primers for variable region of mAb 2-4 and constant region of feline IgG

	Orientation	Sequence
HV	Forward	5'-AAGCTTGCCGCCACCATGGCCTGGCTGTGGAAGCTT-3'
	Reverse	5'-GGATCCACTCACCTGAGGAGACGGTGACCGT-3'
LV	Forward	5'-AAGCTTGCCGCCACCATGGCCCCGTCTATTCAGTT-3'
	Reverse	5'-GGATCCACTTATTGATTTCAGCTT-3'
CH	Forward	5'-GGTGAGTGATCCAGACCACGGCCCCATCG-3'
	Reverse	5'-TGCTCATTACCCGGAGA-3'
CL	Forward	5'-AAGTGGATCCAGAGTGATGCTCAGCCATCT-3'
	Reverse	5'-CTACTCTCTGACACTCGCTCT-3'

RA, infliximab directly injures TNF-alpha-producing cells expressing TNF-alpha on the cell surface through antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Infliximab exhibits its treatment effect against RA through these actions. Infliximab is repeatedly administered at 4- or 8-week intervals until RA remission is observed. Accordingly, to reduce antigenicity for humans, infliximab is expressed in mammalian cells as a mouse-human chimeric antibody prepared by fusing the variable region of mouse mAb and the constant region of human antibody [20]. Human anti-mouse antibody response to the mouse-human chimeric mAb was reduced compared to that of the mouse mAb response, and the adverse reactions after administration were also reduced. Based on these findings, it was hypothesized that the feline anti-mouse antibody response and the development of adverse reactions after administration may be reduced by substituting the amino acid sequence of the feline antibody constant region for that of the mAb 2-4 constant region.

In the present study, we prepared mouse-feline chimeric mAb (chimeric mAb 2-4), in which the variable region of the previously reported anti-fTNF-alpha mAb 2-4 was fused to the feline antibody constant region, and confirmed its fTNF-alpha neutralization activity. In addition, purified mouse mAb 2-4 and chimeric mAb 2-4 were repeatedly administered to cats to investigate the changes in ability to induce a feline anti-mouse antibody response.

MATERIALS AND METHODS

Cell cultures: FO mouse myeloma cells (ATCC CRL-1646) and WEHI-164 murine sarcoma cells (ATCC CRL1751) were obtained from the American Type Culture Collection. The hybridoma mAb 2-4 cell was developed in our laboratory [4]. FO cells and hybridoma mAb 2-4 cell producing the antibody to feline TNF-alpha were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% FCS and antibiotics. WEHI-164 cells were maintained in RPMI 1640 growth medium supplemented with 10% FCS, antibiotics, 50 μ M 2-mercaptoethanol and 2 μ g/ml of polybrene.

Cloning of variable regions of heavy chain and light chain of mAb 2-4 and constant regions of heavy chain and light chain of feline immunoglobulin: RNA isolation from

cells and cDNA preparation were performed employing the method of [18]. The variable region genes of the mAb 2-4 (V_H and V_L) were amplified by PCR from cDNA of hybridoma mAb 2-4 mRNA. The constant region gene of feline immunoglobulin heavy chain (C_H) was amplified from cDNA of feline peripheral blood mononuclear cell mRNA. The constant region gene of feline immunoglobulin light chain (C_L) was artificially synthesized by Life Technologies (Carlsbad, CA, U.S.A.) based on a published nucleotide sequence (Genbank AF198257.1) and inserted into the pMA-T plasmid. The primer sequences used for PCR are shown in Table 1. V_H , V_L , C_H and C_L were individually cloned in pCR-blunt II-TOPO vectors using the Zero Blunt TOPO PCR cloning kit (Life Technologies).

Construction and expression of chimeric mAb 2-4: The C_H and C_L genes and V_H and V_L fragments inserted in the pCR-bluntII-TOPO vectors were connected using a Bam HI linker to prepare chimeric H and L chains (Fig. S1), respectively. The chimeric H chain fragment was inserted into the EcoRI site of the pCDNA3.1 (+)/Neo expression vector. The chimeric L chain fragment was inserted into the HindIII/EcoRV site of the pCDNA3.1 (+)/Hygro expression vector. FO cells were co-transfected with the H- and L-chain expression vectors with Lipofectamine 2000 (Life Technologies). The transfected FO cells were cultured in medium containing G418 (Roche Diagnostics, Basel, Switzerland) and hygromycin B (Roche Diagnostics), to acquire a stably expressing cell line (FOCM24). FOCM24 cells were cloned twice employing the limiting dilution method.

Purification of mouse mAb 2-4 and chimeric mAb 2-4: Mouse mAb 2-4 was purified from the hybridoma mAb 2-4 culture supernatant with Protein G Sepharose (GE Healthcare, Chicago, IL, U.S.A.) according to the product manual. Chimeric mAb 2-4 was purified from the FOCM24 culture supernatant with Protein A Sepharose (GE Healthcare). After purification, the buffer of mAbs was exchanged to PBS (pH 7.4) by Amicon Ultra-15 centrifugal filter devices (NMWL 30,000; Millipore, Billerica, MA, U.S.A.). The concentrations of purified mAbs were assayed by the Bradford method.

Western immunoblotting assay: Purified mouse mAb 2-4 and chimeric mAb 2-4 were incubated without 2-mercaptoethanol at RT for 5 min (non-reducing condition). mAbs were incubated with 2-mercaptoethanol at 100°C for 10 min (reducing condition). mAbs were run using 15% sodium

dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a transferred nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk powder in TBST (20 mM Tris-HCl, pH 8.0, 0.88% NaCl and 0.05% Tween-20) for 1 hr at 37°C. Following washing, the membrane was incubated with horseradish peroxidase conjugated goat anti-mouse IgG (H+L chain specific) (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A.) or horseradish peroxidase conjugated goat anti-feline IgG (whole molecular) (MP Biomedicals, Santa Ana, CA, U.S.A.) for 1 hr at 37°C and then visualized in the substrate for 10 min.

Neutralization test of mouse mAb 2-4 and chimeric mAb 2-4 against feline TNF-alpha using WEHI-164 cells: Neutralization test was performed as described previously [4]. Briefly, WEHI-164 cells were suspended at 1×10^6 cells/ml in the dilution medium containing 1 μ g/ml of Actinomycin D (Sigma Aldrich, St. Louis, MO, U.S.A.) and pre-incubated at 37°C for 3 hr. Serially diluted mouse mAb 2-4, chimeric mAb 2-4 or anti feline APN mAb (mAb R-G-4, as a control for mAb 2-4) was mixed with 40 ng/ml recombinant fTNF-alpha (R&D systems, Minneapolis, MN, U.S.A., 75% cytotoxic activity against WEHI-164 cells) or ascites of cats with FIP that were used as natural feline TNF-alpha samples (final concentration of 1:8, 80% cytotoxic activity against WEHI-164 cells). The mixture was incubated at 37°C for 1 hr. Pre-incubated cells were seeded in a volume of 50 μ l in the wells of a 96-well plate. Fifty microliters of the mixture was then added into each well. After incubation at 37°C for 24 hr, 10 μ l of WST-8 solution (WST-8 cell proliferation assay kit; Kishida Chemical Co., Ltd., Osaka, Japan) was added, and the cells were returned to the incubator for 1 hr. The absorbance of formazan produced was measured at 450 nm with a 96-well spectrophotometric plate reader, as described by the manufacturer. The percent neutralization was calculated by the following formula: Neutralization (%)=(O.D. of wells containing mAb and samples -O.D. of wells containing samples alone)/O.D. of wells without mAb and samples \times 100.

Repeated-dose test in cats: The mAb repeated-dose test was performed referring to the method reported by Umehashi *et al.* [22]. Purified mouse mAb 2-4, chimeric mAb 2-4 or PBS as a control was administered to 5 specific pathogen free (SPF) cats aged 2 months. After sedation with Medetomidine (Domitor, Orion Corporation, Espoo, Finland), the SPF cats received low- (1 mg/kg) or high-dose (5 mg/kg) mAb injection into the cervical vein 5 times at 2- or 4-week intervals. Serum was collected immediately before administration. Blood pressure and pulse were measured at the forearm or root of the tail before mAb administration and 10 min after administration, using a fully automatic electronic sphygmomanometer (Pettrust, Aster Electric Co., Yokohama, Japan). The measurements were performed in triplicate. This animal experiment was performed in accordance with the Guidelines for Animal Experiments of Kitasato University (the number of approval is 14-045). SPF cats were maintained in a temperature-controlled isolated facility.

Changes in anti-mouse antibody in mouse mAb 2-4- or chimeric mAb 2-4-injected cat serum: Immulon 4HBX

ELISA plates (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) were coated overnight at 4°C with purified mouse mAb 2-4 (500 ng/100 μ l/well) diluted with carbonate buffer (0.05 M, pH 9.6). After washing with phosphate buffered saline (PBS) containing 0.02% Tween-20, the plates were blocked with a blocking buffer containing 0.5% skim milk in PBS at 37°C for 60 min. Each well of the plates then received 100 μ l of 200-fold diluted serum collected from mAb treated cats. After 60 min incubation at 37°C, the plates were washed, and horseradish peroxidase conjugated goat anti-feline IgG (whole molecular) was diluted to the optimal concentrations, and then, 100 μ l of the dilution was added to each well of the plates. After incubation at 37°C for 30 min, the plates were washed, and each well received 100 μ l of substrate solution and was incubated at 25°C for 10 min in the dark. The substrate solution was prepared by dissolving o-phenylenediamine dihydrochloride at a concentration of 0.4 mg/ml in 0.1 M citric acid and 0.2 M Na₂HPO₄ buffer (pH 4.8) and adding 0.2 μ l/ml of 30% H₂O₂. The reaction was stopped with 3 N H₂SO₄ solution, and the optical density (OD) at 492 nm was determined.

Changes in neutralization activity of mouse mAb 2-4 and chimeric mAb 2-4 reacted with mouse mAb 2-4- or chimeric mAb 2-4-injected cat serum: Sera collected from the mAb-treated cats were diluted 10-fold with medium. The diluted sera were reacted for one hour with mouse mAb 2-4 or chimeric mAb 2-4 at the minimum concentration necessary for 80% or higher neutralization of 10 ng/ml recombinant fTNF-alpha. The reactant was then combined with recombinant fTNF-alpha (final concentration: 10 ng/ml). One hour later, the reactants were administered to WEHI-164 cells. The level of TNF-alpha-induced cytotoxicity was measured after 24 hr.

RESULTS

Cloning of the variable regions of the heavy and light chains of mAb 2-4 and the constant regions of heavy and light chains of feline immunoglobulin: The V_H (414 bp) and V_L (378 bp) genes were amplified from cDNA derived from hybridoma mAb 2-4 (Fig. S2), and the C_H gene (1005 bp) was amplified from cDNA derived from feline PBMCs. The C_L gene (330 bp) was amplified using a plasmid containing a known C_L gene as a template. Each PCR product was cloned into the pCR-blunt II-TOPO vector and sequenced. The V_H and C_H genes were ligated, inserted into the pCDNA 3.1 (+)/Neo expression vector and sequenced (Fig. S3). The amino acid sequence deduced from the base sequence was confirmed to show the characteristics of the variable region of mAb 2-4 and the constant region of the feline immunoglobulin heavy chain. Similarly, the amino acid sequences of the V_L and C_L genes were deduced from the base sequences, and it was confirmed that the recombinant protein had the characteristics of the variable region of mAb 2-4 and the constant region of the feline immunoglobulin kappa light chain (Fig. S4).

Western immunoblotting assay of mouse mAb 2-4 and chimeric mAb 2-4: FO cells harboring the expression vector

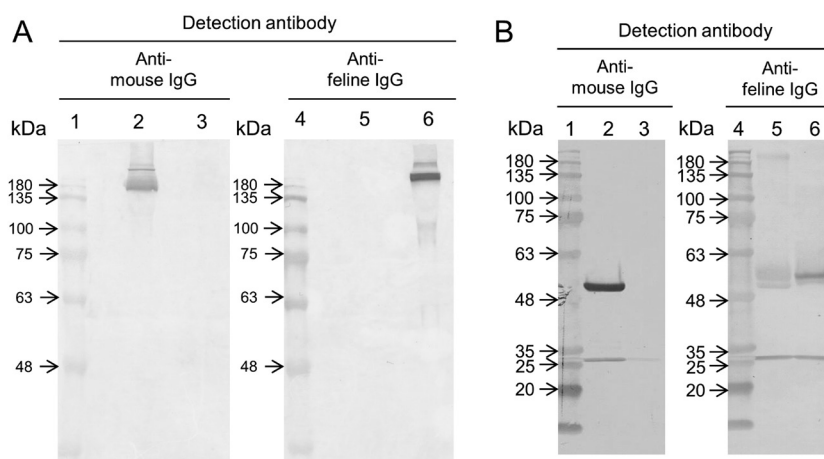


Fig. 1. Western blot analysis of purified mAb 2-4 and chimeric mAb 2-4. Mouse mAb 2-4 and chimeric mAb 2-4 were electrophoresed on 12.5% polyacrylamide gel under reducing and non-reducing conditions. Mouse mAb 2-4 and chimeric mAb 2-4 were detected by western blotting with anti-mouse IgG antibody and anti-feline IgG antibody. Lanes 1 and 4; marker, Lanes 2 and 5; mouse mAb 2-4, Lanes 3 and 6; chimeric mAb 2-4. (A) Non-reducing condition. (B) Reducing condition.

were cultured, and chimeric mAb 2-4 in the culture supernatant was collected. Chimeric mAb 2-4 and mouse mAb 2-4 were individually purified using a protein A or protein G column. The purified mAbs were subjected to SDS-PAGE, and their purities were confirmed by CBB staining. No protein band other than those of mAbs was detected by SDS-PAGE. The purified chimeric mAb 2-4 was western blotted with mouse mAb 2-4. When non-reduced mouse mAb 2-4 and chimeric mAb 2-4 were electrophoresed, smear-like bands were detected at 135-kDa and higher (Fig. 1A). When the blot was reacted with anti-mouse IgG antibody, bands were detected in the lane applied with mouse mAb 2-4, but no band was detected in the lane applied with chimeric mAb 2-4. When the blot was reacted with anti-feline IgG antibody, bands were detected in the lane applied with chimeric mAb 2-4, but not in the lane with mouse mAb 2-4.

When reduced mouse mAb 2-4 and chimeric mAb 2-4 were electrophoresed, approximately 25-kDa and 50-kDa bands were visible (Fig. 1B). When the blot was reacted with anti-mouse IgG antibody, 50- and 25-kDa bands were detected in the lane applied with mouse mAb 2-4, but no band was observed in the lane with chimeric mAb 2-4. When reacted with anti-feline IgG antibody, a band approximately 25-kDa was detected in the lane with mouse mAb 2-4, and 50- and 25-kDa bands were detected in the lane with chimeric mAb 2-4.

Neutralizing activity of mAbs against feline TNF- α : Mouse mAb 2-4 and chimeric mAb 2-4 were tested for their neutralization activity against recombinant fTNF- α using the WEHI-164 cytotoxicity assay system. Mouse mAb 2-4 and chimeric mAb 2-4 neutralized the cytotoxic activity of recombinant fTNF- α on WEHI-164 cells in a concentration-dependent manner (Fig. 2A). Similarly, the neutralization activity of mouse mAb 2-4 and chimeric mAb 2-4

against endogenous fTNF- α contained in ascites of cats with FIP was confirmed. Mouse mAb 2-4 and chimeric mAb 2-4 neutralized the cytotoxic activity of ascites from cats with FIP on WEHI-164 cells in a concentration-dependent manner (Fig. 2B). mAb R-G-4 (as a control for mAb 2-4) did not neutralize the cytotoxic activity of both recombinant fTNF- α and ascites of cats with FIP (Fig. 2A and 2B).

Repeated-dose test in cats: Purified mouse mAb 2-4 and chimeric mAb 2-4 were repeatedly administered to SPF cats at 2- or 4-week intervals, and the blood pressure and pulse were measured before and after administration (Table 2). Body temperature tended to decrease after administration of PBS, mouse mAb 2-4 and chimeric mAb 2-4 throughout the period with 5 administrations, but no significant difference was observed among the 3 groups. Neither were there any differences in blood pressure or pulse between the groups or due to dosing frequency.

Changes in anti-mouse antibody in mouse mAb 2-4- or chimeric mAb 2-4-injected cat serum: Serum was collected at various time points from cats treated with PBS, mouse mAb 2-4 and chimeric mAb 2-4. The feline anti-mouse antibodies in mAb-injected cat serum were detected using ELISA against mouse mAb 2-4 (Fig. 3). Feline anti-mouse antibodies were detected in mAb-injected cat serum.

To investigate whether there were neutralizing antibodies against mouse and chimeric mAb 2-4 in mAb treated cat serum, the collected sera were reacted with mouse mAb 2-4 or chimeric mAb 2-4, and changes in the fTNF- α neutralization activities of these antibodies were measured. When mouse mAb 2-4 was reacted with sera from cats treated with 1 or 5 mg/kg mouse mAb 2-4, its neutralizing activity was decreased (Fig. 4A), but when it was reacted with serum from PBS-treated cats, no decrease in the neutralizing activity was observed.

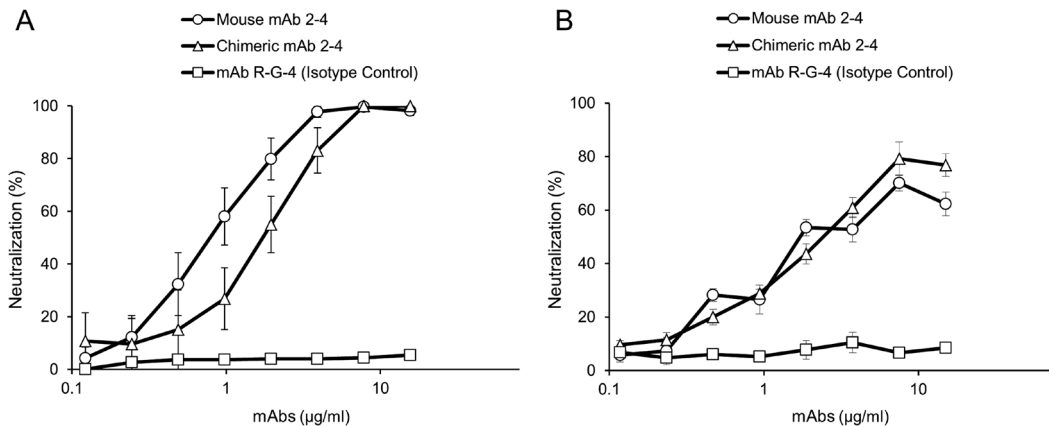


Fig. 2. Neutralization dose-response curve of mAb 2-4 and chimeric mAb 2-4 against fTNF-alpha. The neutralizing activities of mAbs against fTNF-alpha-induced cytotoxicity on WEHI-164 cells were measured. WEHI-164 cells were treated with mixtures of serial dilutions of mAbs and fTNF-alpha, and the level of TNF-alpha-induced cytotoxicity was measured after 24 hr. Experiments were performed in triplicate, and the figure shows mean \pm standard errors. (A) The neutralizing activities of mAbs against recombinant fTNF-alpha. (B) The neutralizing activities of mAbs against natural fTNF-alpha.

When chimeric mAb 2-4 was reacted with serum from cats treated with 1 mg/kg chimeric mAb 2-4, no decrease in the neutralizing activity of chimeric mAb 2-4 was observed, similarly to that observed when reacted with serum of PBS-treated cats (Fig. 4B). Regarding serum from cats treated with 5 mg/kg of chimeric mAb 2-4, the neutralizing activity of chimeric mAb 2-4 decreased when it was reacted with sera collected after the 3rd administration and thereafter (28 days after the initial administration) (Fig. 4B).

DISCUSSION

FIP is a fatal inflammatory disease caused by FIPV. Treatment of FIP-induced systemic inflammation with anti-inflammatory drugs, such as steroids, has been investigated, but existing anti-inflammatory drugs only transiently improves FIP symptoms, and the survival time and quality of life remain unable to be improved [6]. We previously reported that progression to the FIP was prevented by inhibition of the physiological activity of an inflammatory cytokine, TNF-alpha. When anti-fTNF-alpha mouse mAb was administered to cats with FIP, their survival time and quality of life were improved [3]. However, administration of mouse mAb to xenogeneic animals may cause adverse reactions. We prepared mouse-feline chimeric mAb (chimeric mAb 2-4) by modifying the mouse anti-fTNF-alpha mAb 2-4 and investigated its fTNF-alpha neutralization activity. In addition, changes in the feline anti-mouse antibody response-inducing ability induced by repeated administration of purified mouse mAb 2-4 and chimeric mAb 2-4 in cats were investigated.

Chimeric mAb 2-4 was prepared by fusion of the variable region of anti-fTNF-alpha mouse mAb 2-4 and the feline antibody constant region and expressed in FO cells. Mouse mAb 2-4 and chimeric mAb 2-4 were purified and analyzed using western blotting. Anti-mouse IgG antibody reacted

with mouse mAb 2-4 but did not react with chimeric mAb 2-4, whereas anti-feline IgG antibody did not react with mouse mAb 2-4 but reacted with chimeric mAb 2-4, confirming that the antigenicity of chimeric mAb 2-4 is similar to that of feline IgG. When neutralization of fTNF-alpha by purified chimeric mAb 2-4 was investigated, chimeric mAb 2-4 neutralized recombinant and natural fTNF-alpha, similarly to mouse mAb 2-4. Based on these findings, chimeric mAb 2-4 possesses fTNF-alpha neutralization activity similar to that of mouse mAb 2-4 while maintaining the characteristics of feline IgG.

Purified mouse mAb 2-4 and chimeric mAb 2-4 were administered to cats. Five administrations of the mAb did not cause any anaphylactic reaction in either group. Umehashi *et al.* reported the relationship between repeated administration of mouse mAb and mouse-feline chimeric mAb and the induction of anaphylactic reactions, in which several administrations of 10 mg/kg mouse mAb or chimeric mAb did not cause any anaphylactic reaction in cats, whereas 50 mg/kg mouse mAb did [22]. Based on this finding, the antibody dose and dosing frequency adopted in our study were within the range shown not to induce anaphylactic reaction. Because a human-mouse chimeric mAb, infliximab, is repeatedly administered to humans at doses of 3 or 5 mg/kg, and mouse mAb 2-4 exhibited a therapeutic effect at 3 mg/kg in FIP cats, the doses used in the present study were set based on these findings [8, 10, 11]. Experiments to confirm whether or not an anaphylactic reaction is induced by high-dose repeated administration are warranted.

Single administration of mouse mAb 2-4 improved the survival time and quality of life of cats with FIP [4]; however, single administration did not exhibit a therapeutic effect in some cats. It is known that single administration of an anti-fTNF-alpha drug is unlikely to exhibit a therapeutic effect on cats with FIP; thus, several administrations of mAb 2-4 are

Table 2. Blood pressure, body temperature and heart rate of mAb injected cats

	Dose of mAb (mg/ml)	Parameter	First injection (Day 0)	Second injection (Day 14)	Third injection (Day 28)	Fourth injection (Day 42)	Fifth injection (Day 72)
PBS (control)	0	Blood pressure (systolic blood pressure/ diastolic blood pressure, mmHg)	182.0/162.7	162.3/129.7	169.3/140.7	179.7/152.0	163.7/140.0
		Body temperature (°C)	164.0/146.3	156.0/142.0	156.3/139.0	158.3/131.7	209.7/139.7
	Heart rate (BPM)	Pre injection ^{a)}	39.2	38.6	38.9	38.8	39.0
		Post injection ^{b)}	38.2	38.0	38.0	38.3	37.4
		Pre injection ^{a)}	138.0	130.0	151.0	115.3	121.0
		Post injection ^{b)}	136.3	140.7	140.7	113.7	114.7
Mouse mAb 2-4	1	Blood pressure (systolic blood pressure/ diastolic blood pressure, mmHg)	192.3/162.7	156.0/130.7	151.3/117.0	179.0/165.0	169.3/113.0
		Body temperature (°C)	156.0/126.7	168.0/116.7	143.0/121.0	208.7/168.7	156.7/114.0
	Heart rate (BPM)	Pre injection ^{a)}	39.3	38.7	38.2	38.8	39.0
		Post injection ^{b)}	38.0	36.9	36.1	37.5	38.9
		Pre injection ^{a)}	128.7	135.3	125.0	114.0	136.3
		Post injection ^{b)}	129.0	115.7	127.0	114.3	128.0
Chimeric mAb 2-4	5	Blood pressure (systolic blood pressure/ diastolic blood pressure, mmHg)	160.7/134.0	161.3/116.3	159.7/135.0	189.7/156.0	153.3/95.0
		Body temperature (°C)	148.7/128.7	170.7/152.0	145.3/93.3	126.3/98.3	165.0/93.3
	Heart rate (BPM)	Pre injection ^{a)}	38.6	37.7	37.3	38.6	38.8
		Post injection ^{b)}	38.0	37.7	36.9	38.3	38.2
		Pre injection ^{a)}	141.0	142.0	127.0	146.0	145.0
		Post injection ^{b)}	144.7	131.3	131.7	140.7	138.7
Chimeric mAb 2-4	1	Blood pressure (systolic blood pressure/ diastolic blood pressure, mmHg)	160.7/132.3	186.7/161.7	196.7/146.0	188.0/157.3	168.7/136.0
		Body temperature (°C)	161.3/113.0	149.3/128.3	140.7/120.3	161.7/127.3	142.7/120.3
	Heart rate (BPM)	Pre injection ^{a)}	38.6	38.9	38.3	38.5	38.6
		Post injection ^{b)}	37.5	37.9	36.8	37.9	37.9
		Pre injection ^{a)}	105.0	141.3	141.7	108.0	115.0
		Post injection ^{b)}	86.7	138.0	132.0	120.3	113.0
Chimeric mAb 2-4	5	Blood pressure (systolic blood pressure/ diastolic blood pressure, mmHg)	172.7/136.0	175.0/138.7	164.7/140.3	160.7/152.0	178.3/140.3
		Body temperature (°C)	149.3/103.3	163.3/143.3	149.0/113.3	175.3/147.0	148.3/99.3
	Heart rate (BPM)	Pre injection ^{a)}	39.8	38.7	39.1	39.4	39.2
		Post injection ^{b)}	38.1	38.4	38.2	38.2	38.7
		Pre injection ^{a)}	146.3	153.0	139.0	116.3	131.3
		Post injection ^{b)}	145.0	148.0	134.7	115.7	135.7

a) Immediately before administration of PBS or mAb. b) 10 min after administration of PBS or mAb.

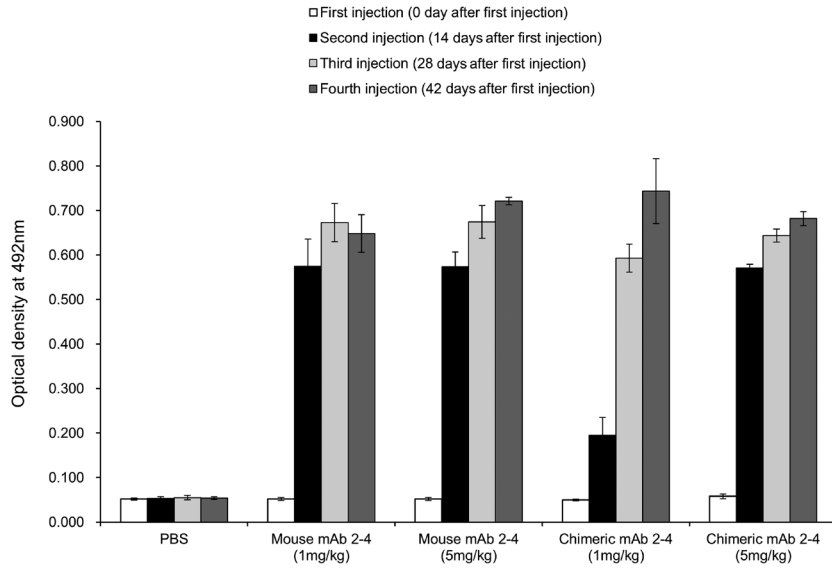


Fig. 3. Changes in anti-mouse antibody in mouse mAb 2-4- or chimeric mAb 2-4-injected cat serum. The ELISA plates were coated with purified mouse mAb 2-4. Each well of the plates received serum collected from mAb treated cats. After incubation, the feline anti-mouse antibody was detected with anti-feline IgG antibody.

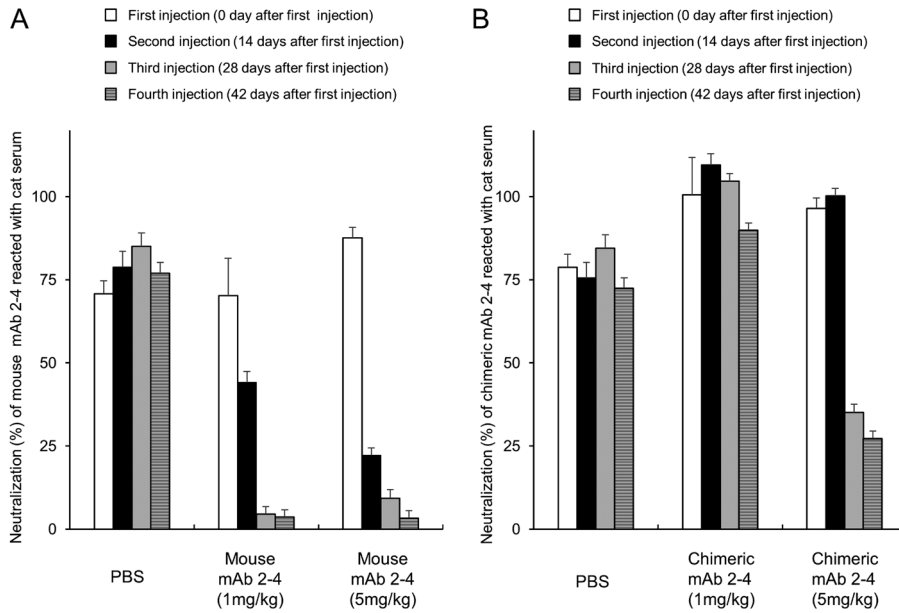


Fig. 4. The changes of neutralization activity of mouse mAb 2-4 and chimeric mAb 2-4 reacted with mouse mAb 2-4 or chimeric mAb 2-4 injected cat serum. Sera were collected from mAb-treated cats at various time points and reacted for one hour with mouse mAb 2-4 or chimeric mAb 2-4 at the minimum concentration neutralizing 80% or more of 10 ng/ml recombinant fTNF- α . Each reactant was then combined with recombinant fTNF- α (final concentration: 10 ng/ml). One hour later, the reactant was administered to WEHI-164 cells. The level of TNF- α -induced cytotoxicity was measured after 24 hr. Experiments were performed in triplicate, and the figure shows mean \pm standard errors. (A) The rates of recombinant fTNF- α neutralization by sera from cats treated with PBS or mAb 2-4 following reaction with mAb 2-4. (B) The rates of recombinant fTNF- α neutralization by sera from cats treated with PBS or chimeric mAb 2-4 following reaction with chimeric mAb 2-4.

necessary for treatment. However, several administrations of mouse mAb may induce anaphylactic reactions, as described above. Moreover, mouse mAb is a xenogeneic protein for cats, suggesting that a feline anti-mouse antibody response is induced in cats treated with mouse mAb 2–4, decreasing the reactivity against fTNF-alpha and thus reducing the therapeutic effect. The possibility of feline anti-chimeric antibody production similar to the production of anti-mouse mAb 2–4 antibody cannot be ruled out due to the variable region of chimeric mAb 2–4 being derived from mouse protein. Thus, the induction of feline anti-mouse antibodies in cats treated with mouse and chimeric mAb was investigated. As the feline anti-mouse antibody was increased in serum, the fTNF-alpha neutralization effect of mAb 2–4 was reduced in cats treated with mouse mAb 2–4 and 5 mg/kg chimeric mAb 2–4. That is, no neutralizing antibody against chimeric mAb 2–4 induction was observed in cats treated with 1 mg/kg chimeric mAb 2–4, suggesting that feline anti-mouse antibody induction was prevented by modifying mouse mAb 2–4 to chimeric mAb 2–4. Accordingly, repeated administration of chimeric mAb 2–4 may not decrease the therapeutic effect in cats. However, in cats receiving 5 mg/kg chimeric mAb 2–4 administration, the neutralizing antibody against chimeric mAb 2–4 was induced after the 2nd administration, strongly suggesting that chimeric mAb 2–4 also induces production of neutralizing antibody against chimeric mAb 2–4 when administered at a high dose. A decrease in the therapeutic effect of infliximab due to production of neutralizing antibody against chimeric mAb has similarly been reported [7]. However, neutralizing antibody against chimeric mAb production can be inhibited by concomitant administration of an anti-inflammatory drug, methotrexate (MTX). MTX suppresses the humoral immune response and is thought to reduce production of antibody against chimeric mAb after infliximab administration [10, 11]. Concomitant MTX administration with chimeric mAb 2–4 may also inhibit production of neutralizing antibody against chimeric mAb. Future studies are necessary to investigate the applicability of concomitant MTX administration with chimeric mAb 2–4 in cats with FIP.

In conclusion, we prepared mouse-feline chimeric mAb 2–4 by fusing the variable region of anti-fTNF-alpha mAb 2–4 to the feline antibody constant region and confirmed that it maintained fTNF-alpha neutralization activity. When the chimeric mAb 2–4 was administered to cats, the induction of feline anti-mouse antibody response was decreased compared to that after mouse mAb 2–4 administration. These results warrant further investigation on the dose and dosing frequency of chimeric mAb 2–4 appropriate for effective treatment of cats with FIP.

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