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ORIGINAL ARTICLE

Therapeutic effect of an anti-human-TNF-alpha antibody and itraconazole on feline infectious peritonitis

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Abstract
Feline infectious peritonitis (FIP) is a fatal disease in wild and domestic cat species. Although several drugs are expected to be useful as treatments for FIP, no drugs are available in clinical practice. In this study, we evaluated the therapeutic effect of combined use of adalimumab (an anti-human-TNF-alpha monoclonal antibody, ADA) and itraconazole (ICZ), which are presently available to veterinarians. The neutralizing activity of ADA against fTNF-alpha-induced cytotoxicity was measured in WEHI-164 cells. Ten specific pathogen-free (SPF) cats were inoculated intraperitoneally with type I FIPV KU-2. To the cats that developed FIP, ADA (10 mg/animal) was administered twice between day 0 and day 4 after the start of treatment. ICZ (50 mg/head, SID) was orally administered daily from day 0 after the start of treatment. ADA demonstrated dose-dependent neutralizing activity against rfTNF-alpha. In an animal experiment, 2 of 3 cats showed improvements in FIP clinical symptoms and blood chemistry test results, an increase in the peripheral blood lymphocyte count, and a decrease in the plasma alpha 1-AGP level were observed after the beginning of treatment. One of the cats failed to respond to treatment and was euthanized, although the viral gene level in ascites temporarily decreased after the start of treatment. ADA was found to have neutralizing activity against rfTNF-alpha. The combined use of ADA and ICZ showed a therapeutic effect for experimentally induced FIP. We consider these drugs to be a treatment option until effective anti-FIPV drugs become available.

Introduction

Feline infectious peritonitis virus (FIPV), a feline coronavirus (FCoV) of the family *Coronaviridae*, causes a fatal disease called feline infectious peritonitis (FIP) in wild and domestic cat species. Several organs, including the liver, lungs, spleen, serosae, kidneys, eyes, 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. Pleural effusion and ascitic fluid have been reported to accumulate in some cats [1].

The FCoV virion is mainly composed of nucleocapsid (N), envelope (E), membrane (M), and peplomer spike (S) proteins. FCoVs are classified into two serotypes, type I and II FCoV, based on differences in the amino acid sequence of S protein [2, 3]. Type II FCoV was generated by genomic recombination between type I FCoV and type II **??**canine coronavirus**??** (CCoV) [4–6]. Several serological and genetic surveys have shown that type I FCoV is dominant and that most cases of FIP are caused by type I FIPV infection [7–9].

We previously reported that tumor necrosis factor (TNF)-alpha plays a critical role in the progression of FIP. TNF-alpha is produced excessively by FIPV-infected macrophages. TNF-alpha is involved in lymphopenia and increased levels of the cellular receptor of serotype II FIPV, aminopeptidase N (APN) [10, 11]. It has also been reported that neutrophil apoptosis in cats with FIP is inhibited by TNF-alpha [12]. This finding suggests that neutrophilia in

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cats with FIP is due to TNF-alpha-induced neutrophil survival. We previously reported that the monoclonal antibody (mAb) 2-4, with high neutralizing activity against feline TNF-alpha (fTNF-alpha), is applicable as a treatment for FIP [13, 14]. However, anti-fTNF-alpha mAb has not been marketed and cannot be used clinically in animal hospitals. In human medicine, an anti-human-TNF-alpha mAb is used to treat inflammatory diseases such as rheumatoid arthritis and Crohn's disease [15]. It is commercially available and can be used by veterinarians in their practice. However, whether this human antibody is effective against FIP, a cat disease, is unknown.

Several antiviral agents have been reported for FIPV [16–20]. These drugs have been confirmed to reduce the proliferation of FIPV, but they are not commercially distributed as veterinary drugs. We previously found that itraconazole (ICZ), which is used in veterinary medicine, reduces the proliferation of type I FIPV [21–23]. However, the therapeutic effects of ICZ in cats with FIP remain unclear.

In the present study, we evaluated whether adalimumab (ADA), an anti-human-TNF-alpha mAb, has neutralizing activity against fTNF-alpha. In addition, we evaluated the therapeutic effects of ADA and ICZ on FIP by administering them to cats with experimentally induced FIP.

Materials and methods

Cell cultures and virus

WEHI-164 murine sarcoma cells were maintained in RPMI 1640 growth medium supplemented with 10% FCS, 600 U of benzylpenicillin potassium per mL, 240 µg of streptomycin sulfate per mL, and 50 µM 2-mercaptoethanol. WEHI-164 murine sarcoma cells were obtained from the American Type Culture Collection (ATCC CRL1751).

Type I FIPV KU-2 was grown in *Felis catus* whole fetus (fcwf)-4 cells at 37 °C. The type I FIPV KU-2 was isolated in our laboratory.

Monoclonal antibody

Anti-fTNF-alpha mAb 2-4 has been described previously [13] and shown to have neutralizing activity for recombinant fTNF-alpha (rfTNF-alpha) and natural fTNF-alpha. mAb 2-4 were purified from a hybridoma culture supernatant using protein G Sepharose (GE Healthcare Life Sciences, Marlborough, MA, USA). Anti-human-TNF-alpha mAb, adalimumab (ADA, Humira®), was purchased from Eisai Co., Ltd. (Tokyo, Japan) as a 20 mg/0.2 mL solution. ADA was diluted to a concentration of 2 mg/mL in physiological saline.

Itraconazole

Itraconazole (ICZ) was purchased from NICHI-IKO (Toyama, Japan) and provided as a tablet (100 mg). Half of a tablet, pulverized and mixed with food, was administered to cats.

Neutralization of feline TNF-alpha by ADA in WEHI-164 cells

Neutralization of TNF-alpha in WEHI-164 cells was tested as described by Doki et al. [13]. Briefly, WEHI-164 cells were suspended at a density of 1×10^6 cells/mL in a dilution medium containing 1 µg of actinomycin D (Sigma-Aldrich, St. Louis, MO, USA) per mL and pre-incubated at 37 °C for 3 h. Serially diluted mAbs were mixed with 40 ng of rfTNF-alpha per mL. The mixture was incubated at 37 °C for 1 h. Pre-incubated cells were seeded in a volume of 50 µL in the wells of a 96-well plate. Fifty microliters of the mixture was then added to each well. After incubation at 37 °C for 24 h, 10 µL of WST-8 solution (WST-8 Cell Proliferation Assay Kit; Kishida Chemical Co., Ltd., Osaka, Japan) was added and the absorbance of formazan produced was measured at 450 nm. The percent neutralization was calculated using the following formula: Neutralization (%) = (OD of wells containing mAbs and rfTNF-alpha − OD of wells containing rfTNF-alpha without mAbs)/OD of wells without mAbs and rfTNF-alpha × 100.

Animal experiment

All applicable national and institutional guidelines for the care and use of animals were followed. The animal experimentation protocol was approved by the President of Kitasato University through the judgment of the Institutional Animal Care and Use Committee of Kitasato University (approval no. 18-152). Specific-pathogen-free (SPF) cats were bred in our own laboratory and maintained in a temperature-controlled isolated facility.

Experimental schedule

Following the experimental schedule shown in Fig. 1, 10 SPF cats (9- or 10-month-old domestic shorthair cats) were inoculated intraperitoneally with type I FIPV KU-2 ($10^{3.6}$ $TCID_{50}$/mL/animal). The cats were then examined daily for clinical signs, and their body temperature and weight were measured. To the cats that developed FIP, ADA (10 mg/animal) was administered twice intravenously between day 0 and day 4 after the start of treatment. ICZ (50 mg/animal, SID) was orally administered daily from day 0 after the start

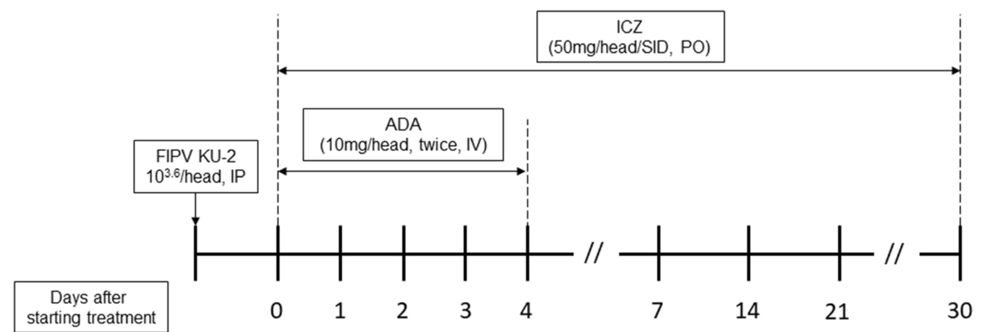

Fig. 1 Experimental schedule of treatment and FIPV inoculation of cats

of treatment. Blood was collected from the cats at 1-week intervals after viral inoculation and at the time of administration of ADA. Heparinized blood was used to measure complete and differential cell counts and to isolate plasma. The plasma samples were stored at −30 °C until the day of analysis. The cats were euthanized when reaching the humane endpoint or 60 days after challenge. FIP diagnoses were confirmed by postmortem examination, revealing peritoneal and pleural effusions, and pyogranuloma in the major organs.

Measurement of plasma alpha1-glycoprotein (AGP)

Plasma concentrations of AGP were determined using a feline alpha1 AG plate (The Institute for Metabolic Ecosystem Lab., Osaki, Japan) according to the manufacturer's protocol.

Measurement of plasma vascular endothelial growth factor (VEGF) concentration

Plasma concentrations of VEGF were determined using a human VEGF ELISA Kit (R & D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. The ELISA kit primarily detects the feline VEGF isoform 164 [24].

Quantitative real-time RT-PCR (qRT-PCR)

To quantify viral RNA in the supernatant and cells from ascites, qRT-PCR was performed using a primer targeting the 3'-UTR [17]. One milliliter of ascites was collected from cat no. 6, and after centrifugation, the supernatant and cells were stored separately. Total RNA was isolated from supernatant and cells from ascites using a High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany), following the manufacturer's instructions. RNA was reverse transcribed and amplified using RNA-direct Realtime PCR Master Mix (TOYOBO, Osaka, Japan) with the specific primers 3'-UTR-F (5'-GGAGGTACAAGCAACCCTATT-3') and 3'-UTR-R (5'-GATCCAGACGTTAGCTCTTCC-3') and a probe (FAM-5'-AGATCCGCTATGACGAGCCAACAA-3'-BHQ1). The reaction was carried out in a total volume of 20 µL/well in 48-well PCR plates using a StepOne Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) at 90 °C for 30 s, 60 °C for 20 min, and 95 °C for 1 min, followed by 45 cycles of 90 °C for 15 s and 60 °C for 1 min. To obtain control RNA for quantification, cDNA fragments amplified with primers 3'-UTR-F and 3'-UTR-R were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). The linearized and purified plasmid was transcribed using a RiboMAX Large Scale RNA Production System-T7 (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration of transcribed RNA was measured using a spectrophotometer. Tenfold dilutions of the RNA transcript, ranging from 1×10^1 to 1×10^9 copies/µL, were prepared with 10 ng of MS2 RNA per mL. The RNA copy number was calculated following the procedure described by Fronhoffs et al. [25]. The stock solutions of the *in vitro*-transcribed RNA were stored at −80 °C, and the diluted working solutions were stored at −30 °C.

Protein sequence

The deduced amino acid sequences of the feline and human TNF-alpha were obtained from RefSeq (accession numbers NP_001009835.1 and NP_000585.2, respectively). Comparison of the deduced amino acid sequences of feline and human TNF-alpha was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Results

Neutralizing activity of ADA against rfTNF-alpha

A comparison of the deduced amino acid sequences of feline and human TNF-alpha is shown in Fig. 2. The sequences were found to be 89.7% identical.

The neutralizing activity of ADA against rfTNF-alpha was measured using a WEHI-164 cytotoxicity assay. ADA neutralized rfTNF-alpha activity in a dose-dependent

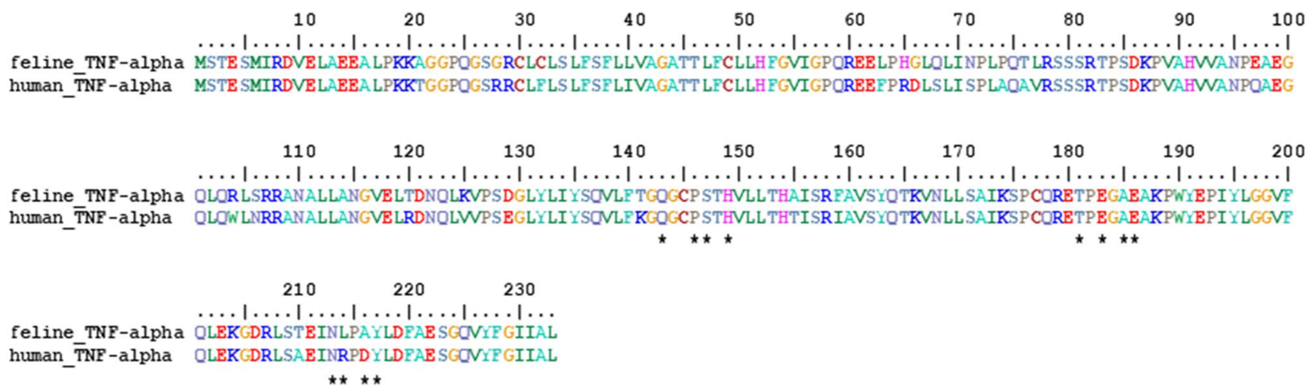

Fig. 2 Comparison of amino acid sequence of feline and human TNF-alpha. *: residues that interact with adalimumab

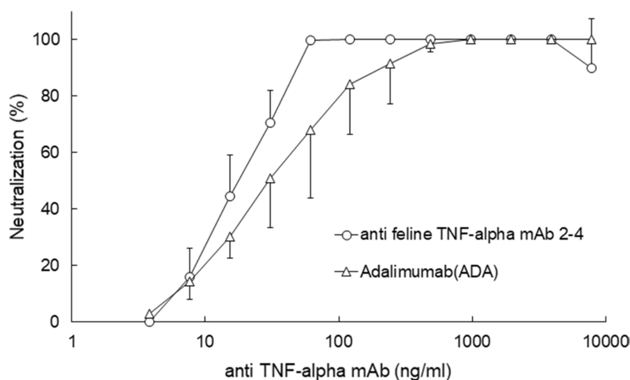

Fig. 3 Neutralization dose-response curve against recombinant fTNF-alpha. The neutralizing activity of ADA against fTNF-alpha-induced cytotoxicity was measured in WEHI-164 cells. WEHI-164 cells were treated with mixtures of serial dilutions of ADA and fTNF-alpha, and the level of TNF-alpha-induced cytotoxicity was measured after 24 h

manner, similar to anti-fTNF-alpha mAb 2-4 (Fig. 3). The dose of mAb2-4 and ADA required to inhibit cell death by 50% (IC_{50}) was 18.1 ± 4.9 ng/mL and 36.9 ± 23.4 ng/mL, respectively.

Changes in body temperature and body weight

Ten SPF cats were inoculated intraperitoneally with $10^{3.6}$ $TCID_{50}$ of type I FIPV KU-2. Three cats (2, 3, and 6) exhibited fever, anorexia, lethargy, and jaundice. Abdominal distension caused by ascites was observed in cat no. 6. These 3 cats were used in the subsequent experiments as FIP cats. Treatment of cats 2, 3 and 6 was started on day 34, 32 and 21, respectively, after virus inoculation.

The changes in body temperature and body weight in the three cats are shown in Fig. 4. They all developed fever of ≥ 39°C a few days before the start of treatment (Fig. 4A). In cats 2 and 3, the body temperature decreased to the range before the onset of FIP on day 7 after the start of treatment. Thereafter, the body temperature of cat no. 3 increased to 38.7°C on day 31 after the start of treatment. The body temperature of cat no. 6 remained at ≥ 39.0°C even after starting treatment.

In cats 2 and 3, no body weight loss was observed compared with the body weight on day 0 of treatment (Fig. 4B). However, the body weight of cat no. 6 continued to decrease from day 0 to day 15, resulting in an 8% loss compared with day 0.

Changes in WBC and lymphocyte counts

The total white blood cell count and lymphocyte count were measured in cats challenged with FIPV. In cats 2 and 3, little change was observed in the total white blood cell count throughout the experiment (Fig. 5A). In cat no. 6, the total white blood cell count increased after the start of treatment and remained at ≥ 20,000 cells/μL after day 6.

In cats 2 and 3, the lymphocyte count increased slightly after the start of treatment (Fig. 5B). In cat no. 6, the lymphocyte count remained low throughout the experiment. It decreased to approximately 800 cells/μL on day 6 after the start of treatment but recovered nearly to the level before the development of FIP on day 13 and exhibited no marked change until the humane endpoint.

Changes in the plasma alpha1-acid glycoprotein (AGP) concentration

The plasma alpha 1-AGP level was measured sequentially in three cats. In all three cats, it increased to a high level of ≥ 750 μg/mL at the beginning of treatment (Fig. 6). In cats 2 and 3, the plasma alpha 1-AGP level decreased nearly to the level before the development of FIP on days 14 and 16 after the start of treatment. However, in cat no. 6, the increase in the plasma alpha 1-AGP level was suppressed until day 6 after the start of treatment, but it increased to approximately 1,900 μg/mL on day 13.

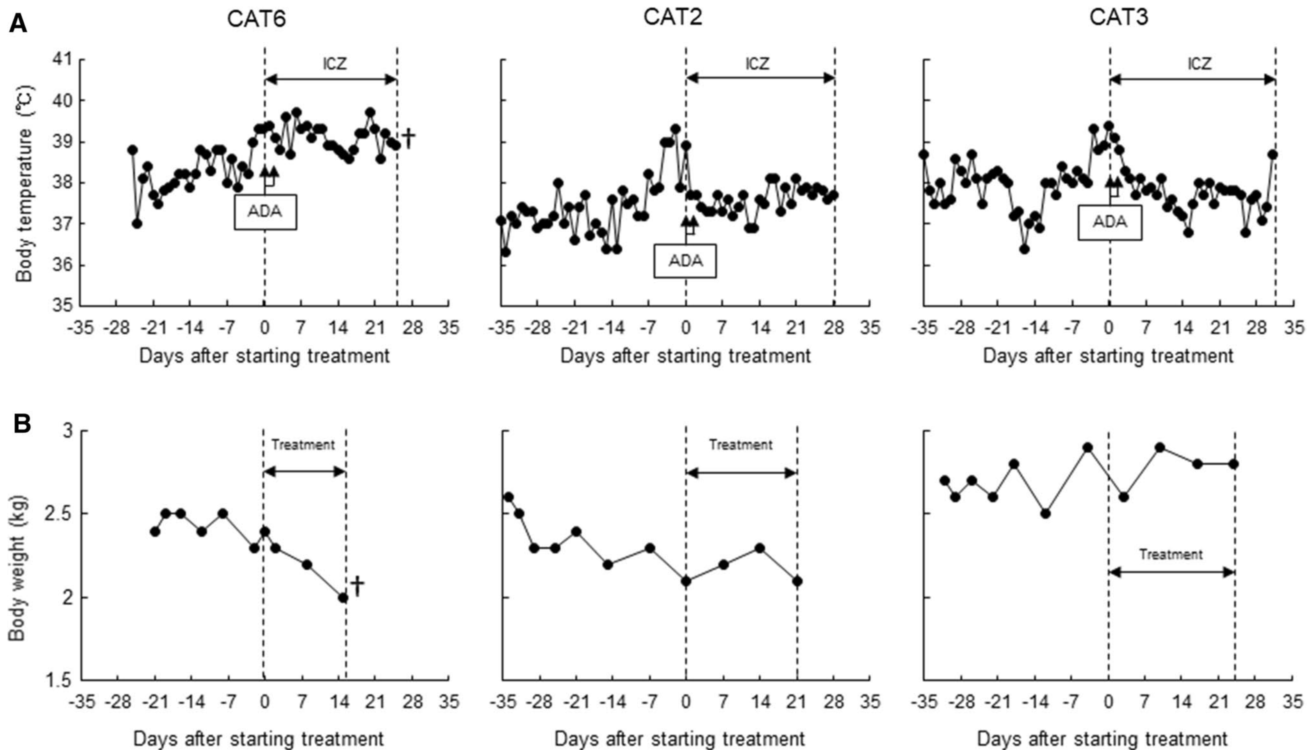

Fig. 4 Changes in body temperature and weight of cats. †: The cat was euthanized because its clinical condition had reached the humane endpoint

Changes in the plasma vascular endothelial growth factor (VEGF) concentration

The plasma VEGF level was sequentially measured in three cats. In cat no. 2, it remained low (Fig. 7). In cat no. 3, it temporarily increased to approximately 200 pg/mL on day 5 before the start of treatment but decreased nearly to the level before the development of FIP on day 0 of treatment and exhibited no increase thereafter. In cat no. 6, the plasma VEGF level increased after FIPV inoculation to approximately 170 pg/mL on day 0 of treatment. Thereafter, it decreased starting on day 6 of treatment and was similar to the level before the development of FIP on day 13.

Viral RNA levels in the supernatant and cells from ascites in cat no. 6

Viral RNA levels in the supernatant and cells from ascites collected from cat no. 6 were quantified by qRT-PCR. The viral RNA level temporarily decreased on day 4 after the start of treatment in both the supernatant and cells from ascites (Fig. 8). On day 20 after the start of treatment, however, the viral RNA levels in the supernatant and cells from ascites increased to twice those on day 0 of treatment.

Blood chemistry panel

TP and the A/G ratio, which are diagnostic indices of FIP, and AST, LDH, and TB, which are related to liver function, were measured after treatment (Fig. 9). TP increased slightly in cat no. 3 but remained in the normal range in all three cats. The A/G ratio, on the other hand, decreased after FIPV challenge in all three cats and was ≤0.8 on day 0 of treatment. In cat no. 2, the A/G ratio remained similar to that after the start of treatment until the end of the experiment. In cat no. 3, it decreased until day 16 of treatment but exhibited no further decrease thereafter. In cat no. 6, it continued to decrease even after the start of treatment.

AST, LDH, and TB were higher than the normal range on day 0 or 3 of treatment in all three cats. In cats 2 and 3, AST, LDH, and TB decreased nearly to the levels before the onset of FIP on day 7 or 9 after the start of treatment. In cat no. 6, AST, LDH, and TB were higher than the normal range on day 0 or 4 after the start of treatment and continued to increase until the end of the experiment.

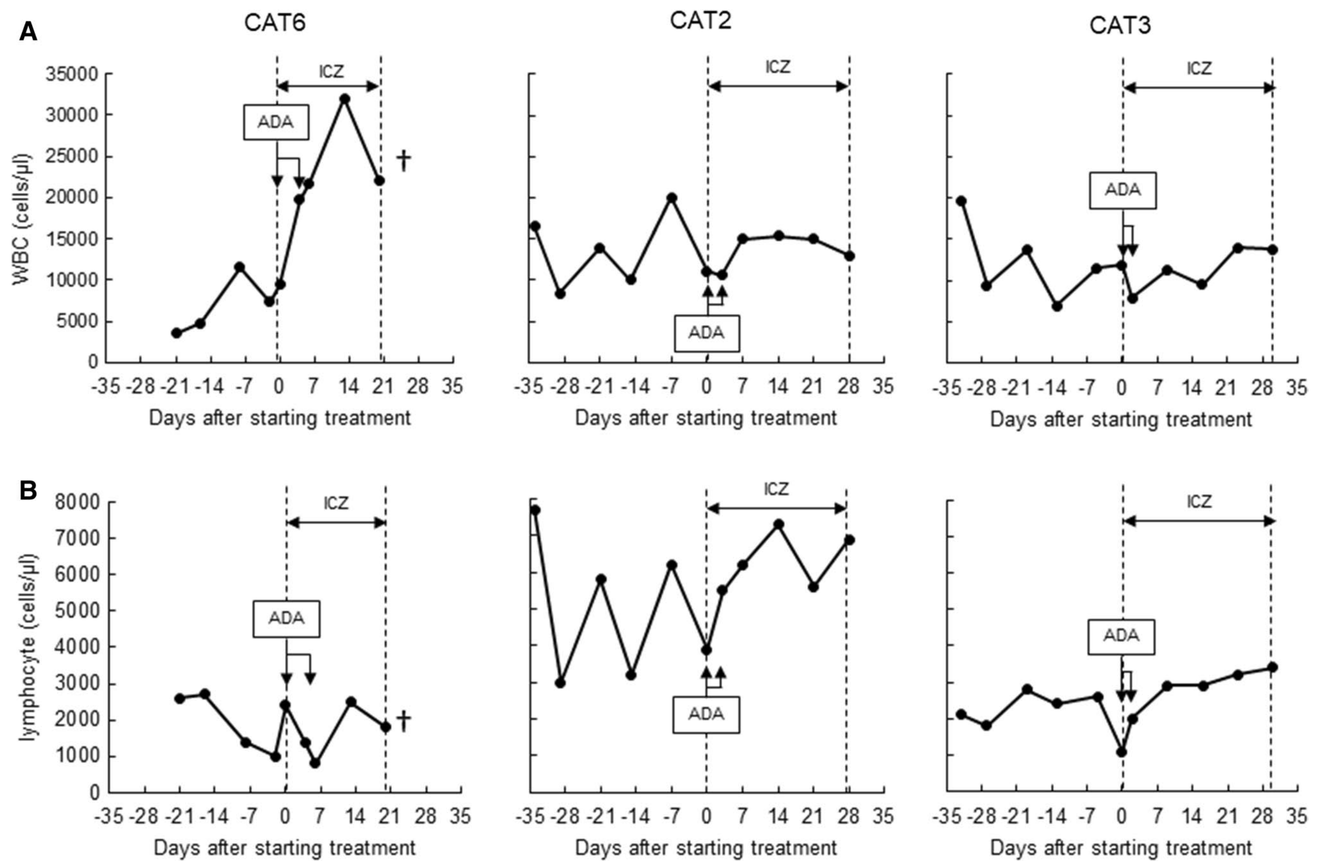

Fig. 5 Changes in WBC and lymphocyte counts in cats. †: The cat was euthanized because its clinical condition had reached the humane endpoint

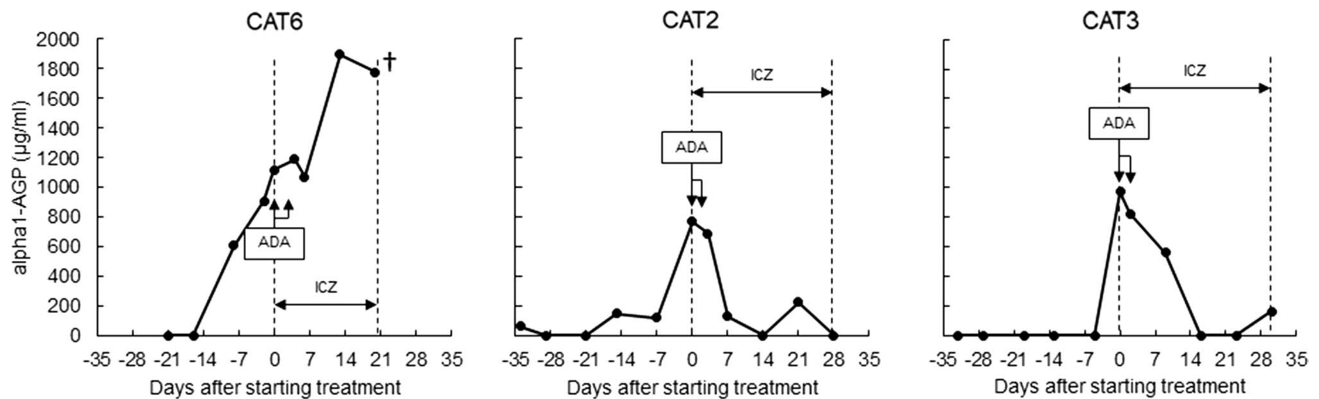

Fig. 6 Changes in plasma alpha1-acid glycoprotein (AGP) concentration in cats. †: The cat was euthanized because its clinical condition had reached the humane endpoint

Discussion

A strong therapeutic effect may be obtained if anti-fTNF-alpha mAb, which suppresses the activity of TNF-alpha, an exacerbator of FIP, is administered in combination with ICZ, which reduces the proliferation of FIPV, to cats with FIP. However, it is costly and time-consuming for anti-fTNF-alpha mAb to be approved as a veterinary drug in each country. We therefore focused on an anti-human TNF-alpha mAb that is already being used to treat human inflammatory diseases.

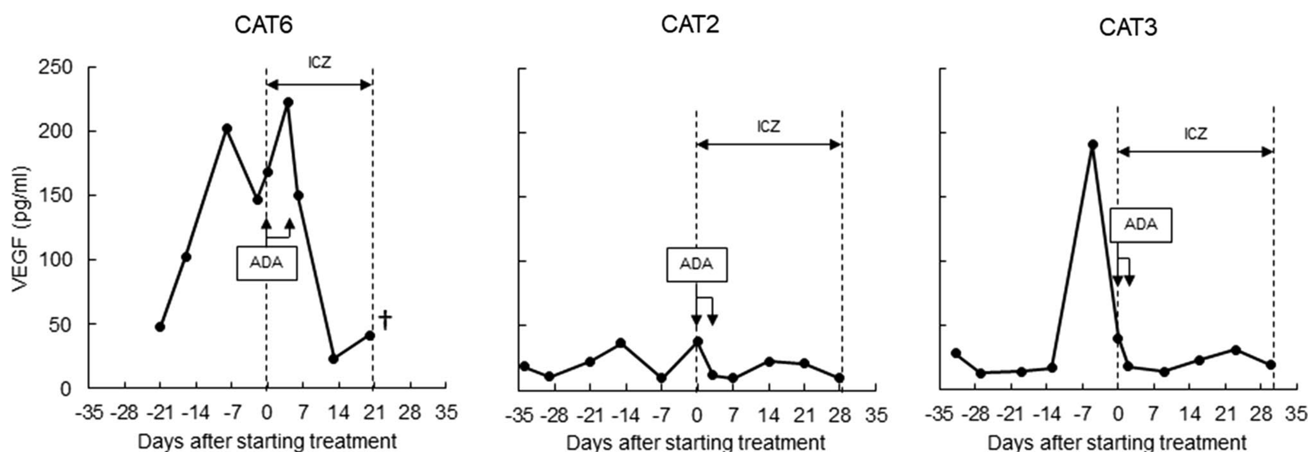

Fig. 7 Changes in plasma vascular endothelial growth factor (VEGF) concentration in cats. †: The cat was euthanized because its clinical condition had reached the humane endpoint

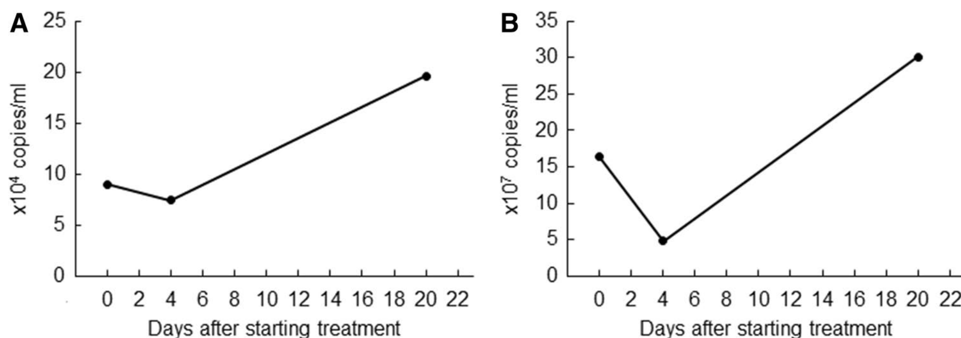

Fig. 8 Changes in viral RNA levels in ascites and cells from ascites collected from CAT No. 6. The viral RNA levels in the supernatant and cells from ascites collected from cat no. 6 were quantified using qRT-PCR. (A) supernatant of the ascites. (B) cells from the ascites

Before conducting animal experiments, we examined whether the anti-human-TNF-alpha mAb can neutralize fTNF-alpha. ADA, which is an anti-human-TNF-alpha mAb, demonstrated dose-dependent neutralizing activity against rfTNF-alpha, similar to anti-fTNF-alpha mAb 2-4. The nine anti-fTNF-alpha mAbs that we reported previously had an IC_{50} ranging from 5 to 700 ng/mL against rfTNF-alpha at the same concentration [13]. The IC_{50} of ADA (36.9 ± 23.4 ng/mL) suggests that it has high neutralizing activity against fTNF-alpha compared with these anti-fTNF-alpha mAbs. Generally, mAbs against human cytokines show no cross-reactive neutralizing activity against animal cytokines. However, Aguirre et al. reported that human and feline TNF-alpha have similar neutralizing epitopes [26]. Furthermore, the deduced amino acid sequence of human TNF-alpha is 89.7% identical to that of feline TNF-alpha, and 10 of 12 residues that interact with ADA are present in both [27]. This appears to be the reason that adalimumab has potent neutralizing activity against feline TNF-alpha.

We administered ADA and ICZ to cats with experimentally induced FIP, and examined their therapeutic effect. Of the 10 SPF cats that received intraperitoneally administered type I FIPV KU-2, three developed FIP. Of these three cats, cat no. 6 had obvious ascites. These three cats were judged to have developed FIP for the following reasons: i) all three cats had been infected with FIPV, ii) clinical symptoms related to FIP were observed, iii) plasma levels of alpha-1 AGP and VEGF increased, and iv) data from our past animal experiments were used as references. ADA (10 mg/animal) was administered intravenously to the three cats on day 0 and within 4 days after the start of treatment. In addition, ICZ (50 mg/animal) was orally administered daily starting on day 0 of treatment. In cats 2 and 3, improvements in clinical symptoms and blood chemistry test results, increases in the peripheral blood lymphocyte count, and decreases in the plasma alpha 1-AGP level were observed after the beginning of treatment. Symptomatic improvements were observed between 1 and 2 weeks after the start of treatment. Furthermore, in cats 2 and 3, no ascites or pyogenic granulomas were observed pathologically at the end of the experiment, and no changes suggestive of the recurrence of FIP were observed until the end of the treatment period. Cat no. 6 failed to respond to treatment and was euthanized, although the viral gene level in ascites temporarily decreased after the start of the treatment. These

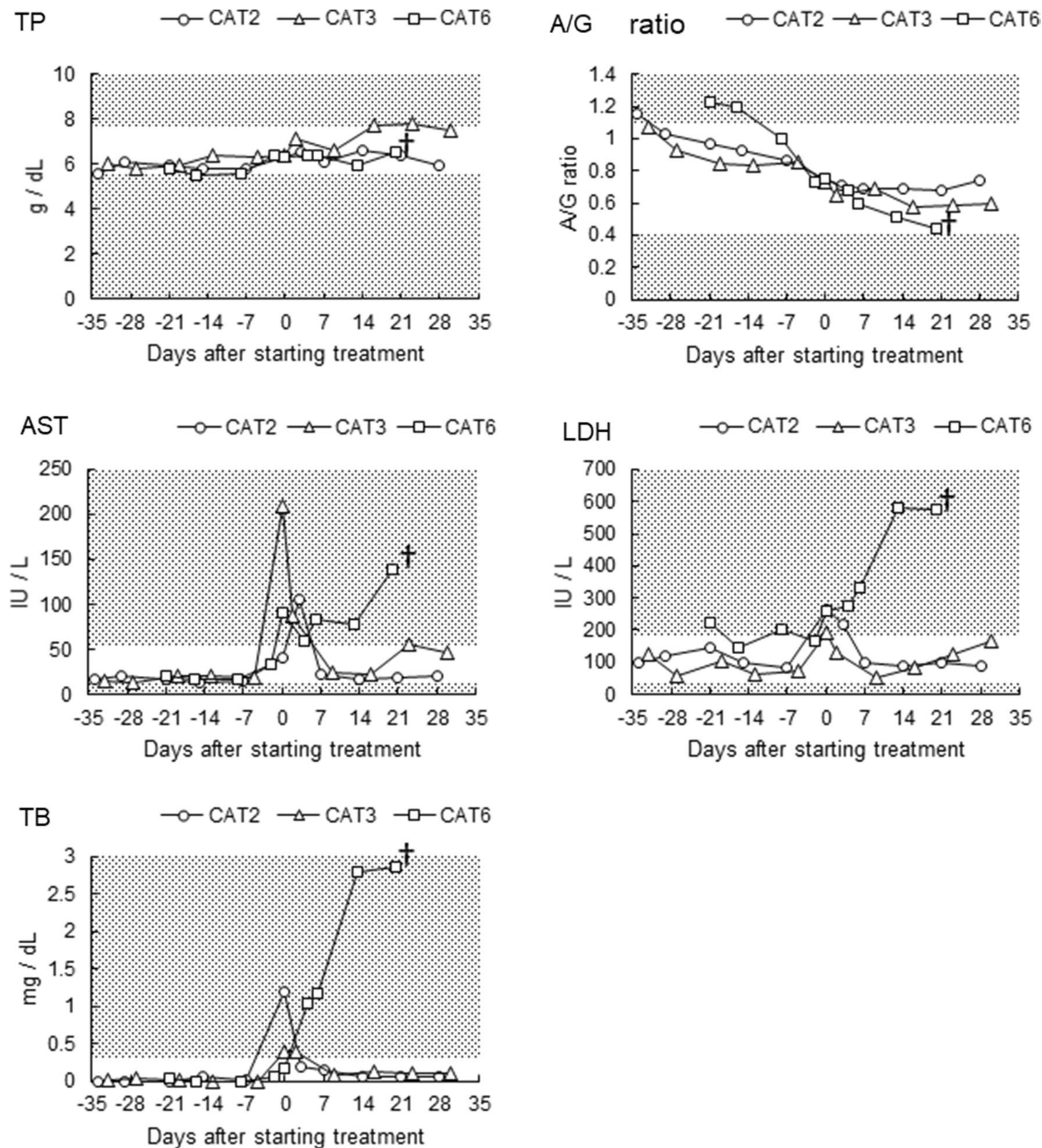

Fig. 9 Blood chemistry panel. The white areas of the graphs represent the normal ranges. †: The cat was euthanized because its clinical condition had reached the humane endpoint

results strongly suggest that anti-human TNF-alpha mAb and ICZ are effective as treatments for FIP.

VEGF is produced by FIPV-infected macrophages and improves the permeability of feline endothelial cells [28]. In cats with FIP, the plasma VEGF level is correlated with the amount of ascites. Cat no. 6, in which ascites was evident by gross examination and palpation (160 mL of ascites was collected at euthanasia), had a high plasma VEGF level of ≥ 100 pg/mL for 3 weeks. However, in cats 2 and 3, in which no signs of ascites were noted, the plasma VEGF level remained low or only increased temporarily. Thus, the plasma VEGF level was confirmed to be correlated with the amount of ascites in cats with FIP.

Azole antifungal agents, including ICZ, are known to have hepatotoxicity as a side effect and possibly to induce cholestasis and jaundice [29–32]. As increases in liver enzymes are observed in cats with FIP, the development of adverse effects after ICZ administration was of concern. However, in cats 2 and 3, liver function marker levels, which increased during treatment progression, returned to the normal range, and plasma jaundice disappeared. Therefore, no adverse effects of ICZ were observed. Thus, from the

viewpoint of adverse effects, the administration of ICZ is considered to be safe for FIP cats.

Recently, GC-376, a 3C-like protease inhibitor, and GS-441524, a nucleoside analog, have been developed as drugs to inhibit the proliferation of FIPV [16–20]. GC-376 and GS-441524 are efficacious drugs that produce therapeutic effects in 30-80% of FIP cats. Although these drugs are expected to be useful as treatments for FIP, they have not been approved. Therefore, no drugs are available in clinical practice for the treatment of FIP. In this study, we demonstrated that the combined use of ADA and ICZ, which are presently available to veterinarians, is effective for the treatment of FIP. We consider these drugs to be a treatment option until antiviral drugs such as GC-376 and GS-441524 become available. In addition, these antiviral drugs exert therapeutic effects on FIP by mechanisms different from those of ADA and ICZ [16, 18, 21, 23]. In the future, the evaluation of the therapeutic effects of their concomitant use may aid in the development of more-effective treatments for FIP.

Conclusions

The therapeutic effects of adalimumab, an anti-human TNF-alpha mAb, and itraconazole on FIP were examined. Adalimumab was found to have neutralizing activity against rfTNF-alpha, similar to an anti-fTNF-alpha mAb. FIP was successfully treated in 2 of 3 cats with experimentally induced FIP by administering adalimumab and itraconazole.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval All applicable national and institutional guidelines for the care and use of animals were followed. The animal experimentation protocol was approved by the President of Kitasato University through the judgment of the Institutional Animal Care and Use Committee of Kitasato University (approval no. 18-152). The SPF cats were bred in our own laboratory and maintained in a temperature-controlled isolated facility. Sample sizes were determined based on our experience with FIPV infection models, and the minimum number of cats was used.

Informed consent Informed consent was obtained from the legal custodian of all experimental animals described in this work for the procedures undertaken. No animals or humans are identifiable within this publication, and therefore additional informed consent for publication was not required.

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