Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Cloning, expression and characterization of biologically active feline tumour necrosis factor-\(\alpha\)

Espen Rimstad*, Gerhard H. Reubel, Gregg A. Dean, Joanne Higgins, Niels C. Pedersen

Department of Medicine, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

Accepted 10 May 1994

Abstract

We report the cloning, expression and characterization of biologically active feline tumour necrosis factor-\(\alpha\) (fTNF-\(\alpha\)). Messenger RNA was extracted from feline peritoneal macrophage cultures and used to synthesize cDNA for polymerase chain reaction (PCR) amplification. The PCR products were cloned into the plasmid vector pCRII and sequenced, showing 99.3% homology with a published fTNF-\(\alpha\) gene sequence. Subcloning into the vector pGEX-2T and subsequent expression resulted in a 43 kDa fusion protein of fTNF-\(\alpha\) and glutathione S-transferase (GST). Thrombin cleavage of the fusion protein yielded a 17 kDa protein. This protein cross-reacted with a monoclonal anti-human TNF-\(\alpha\) antibody in Western blotting, but not with a polyclonal anti-murine TNF-\(\alpha\) serum. Recombinant fTNF-\(\alpha\) (rfTNF-\(\alpha\)) and rfTNF-\(\alpha\)-GST had a \(CD_{50}\) of 15 ng ml\(^{-1}\) and 230 ng ml\(^{-1}\), respectively, in the L929 cytotoxicity assay. Cats given rfTNF-\(\alpha\)-GST intravenously manifested the typical biological effects of TNF-\(\alpha\), including fever, depression, and piloerection. The rfTNF-\(\alpha\)-GST upregulated IL-2 receptor and MHC-II antigen expression on peripheral blood mononuclear cells stimulated in vitro, but had no effect on TNF-\(\alpha\) receptor and MHC-I antigen expression.

1. Introduction

Tumour necrosis factor-alpha (TNF-\(\alpha\)) is a cytokine with multifunctional activity. Although its original activity was recognised against tumour cells (Carswell et al., 1975), it is now known to play an important role in immune and

* Corresponding author at: Department of Pharmacology, Microbiology and Food Hygiene, Norwegian College of Veterinary Medicine, Oslo, Norway. Tel. +47-22-964821; Fax. +47-22 964818.

0165-2427/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved

SSDI 0165-2427(94)05345-S
inflammatory responses as well as in the pathogenesis of many human and animal diseases (reviewed by Jäätelä, 1991). TNF-α may also play a crucial role in the pathogenesis of human AIDS (Matsuyama et al., 1991). TNF-α stimulates human immunodeficiency virus (HIV) replication in both established lymphoid and primary T cell cultures (Suzuki et al., 1989). This enhanced replication is mediated through TNF-α inducible nuclear factors like NFκB and the κB enhancer elements of the HIV LTR (Osborn et al., 1989). Induction of HIV gene expression is regulated by interactions of DNA binding proteins with specific gene sequences (Folks et al., 1989; Osborn et al., 1989; Poli et al., 1990). The levels of TNF-α are increased in patients with AIDS and may upregulate virus replication in an autocrine fashion (Poli et al., 1990). In addition TNF-α may play an important role in some clinical manifestations of HIV infection; dramatic improvement in aphthous stomatitis and esophagitis is seen in AIDS patients treated with a TNF-α inhibitor (thalidomide) (Nicolau and West, 1990). TNF-α is an important reagent; therefore, for studies of HIV pathogenesis.

The nucleotide sequences for human, mouse, sheep, pig, rabbit and cat TNF-α has previously been reported (Pennica et al., 1985; Shirai et al., 1985; Ito et al., 1986; Drews et al., 1990; McGraw et al., 1990; Green and Sargan, 1991). Both human and murine recombinant TNF-α proteins have been expressed in different systems, and used in several studies (Pennica et al., 1985; Shirai et al., 1985). However, recombinant feline TNF-α (rfTNF-α) protein has not been expressed.

The aim of this study was to clone the cDNA of feline TNF-α and to express it in *Escherichia coli* in a biologically active form. Our goal is to use rfTNF-α to study immunodeficiency virus pathogenesis using the feline immunodeficiency virus (FIV) infection model. FIV infection has been shown to be a valid animal model for HIV studies because similar changes in TNF-α expression have also been observed in FIV infected cats (Lawrence et al., 1992; Lehmann et al., 1992; Pedersen, 1993).

**2. Materials and methods**

**2.1. Experimental animals**

Adult specific pathogen free (SPF) cats were obtained from the breeding colony of the Feline Retrovirus Research Laboratory, University of California, Davis. Animals were housed in quarters provided by the Animal Research Service, University of California, Davis.

**2.2. Macrophage cultures**

Peritoneal macrophages were obtained by peritoneal saline lavage from two specific pathogen free cats as previously described (Stoddart and Scott, 1988; Brunner and Pedersen, 1989). Cats were inoculated intraperitoneally with 0.75 ml of human diphtheria/pertussis/tetanus vaccine, and the peritoneal cavities
lavaged 4 days later. Cells were pelleted by centrifugation and resuspended in RPMI medium with 10% fetal bovine serum (FBS) and cultured for 8 h. Adherent cells were determined to be virtually 100% macrophages by non-specific alpha-naphthyl esterase staining (Stoddart and Scott, 1988). The macrophage cultures were then stimulated with 100 ng ml⁻¹ of *E. coli* lipopolysaccharide (LPS) (Sigma, St. Louis, MO) for 2 h, and frozen at −70°C until further use. The 2 h duration of LPS stimulation was based upon the kinetics of TNF-α production after LPS stimulation in macrophages from other animal species (Green and Sargan, 1991).

2.3. Extraction of RNA and mRNA-polymerase chain reaction

Messenger RNA was extracted both from 4×10⁶ unstimulated and 4×10⁶ LPS stimulated macrophages using an mRNA extraction kit (Micro-Fast Trac, Invitrogen, San Diego, CA); 0.08 µg mRNA from each source was used for cDNA synthesis. Synthesis of cDNA was performed at 42°C for 2×60 min using oligo-dT primers (cDNA-cycle kit, Invitrogen). The cDNA was then used in a polymerase chain reaction (PCR) with a 100 µl reaction mixture consisting of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatine, 200 µM of each dNTP, 30 pmol of each primer, and 2.5 U of Pfu DNA polymerase.

Three primers, making up two pairs P1/P3 and P2/P3, were constructed from a previously published sequence of feline TNF-α (McGraw et al., 1990): P1, GGGATCCATGAGCACTGAAAGCATGATCCG; P2, GGGGATCCCAGAACACTCAGATCATCTTCTC; P3, GGCTGCAGAATTCACAGGGCAATGATCCCAAAGTA. The primers had restriction sites for *BamHI*, *PstI* and *EcoRI* in the 5'-ends to make directionally cloning of the PCR products possible. The forward primer P1 was located at the start of the fTNF-α gene and the forward primer P2 at the assumed start of the coding sequences for the mature TNF-α protein, while the backward primer P3 was located at the 3'-end of the coding sequences of the gene. The mixtures were overlaid with 30 µl mineral oil and heated at 94°C for 5 min and then cycled 35 times at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min with a final extension at 72°C for 7 min. The PCR products were subjected to electrophoresis on a 1.7% agarose gel using 3 V cm⁻¹ for 2 h in 0.5X TBE-buffer (1XTBE = 0.09 M Tris–borate, 2 mM EDTA) and then stained with ethidium bromide. To assure that stimulated macrophage mRNA was the actual source for cDNA, cDNA derived by reverse transcription of mRNA from unstimulated macrophage cultures, as well as feline genomic DNA, were extracted and used as targets in separate and parallel PCRs.

2.4. Expression and purification of rfTNF-α

The amplified fragments generated by both the P1/P3 and P2/P3 primers were separately cloned into the plasmid vector pCRII (TA-cloning kit, Invitrogen), and the nucleotide sequences were determined by conventional dideoxy sequencing of both strands. The cloned fragment from the amplification with P2/P3 was
digested out with \textit{BamHI/EcoRI}, and subcloned directly into the expression vector pGEX-2T (Pharmacia, Uppsala, Sweden). The pGEX-2T vector has been used previously to express FIV-p17 and -p24 proteins (Reid et al., 1991). This expression vector contains an open reading frame encoding glutathione S-transferase (GST), followed by unique restriction endonuclease sites for \textit{BamHI}, \textit{Smal} and \textit{EcoRI}, followed in turn by termination codons in all three frames. A thrombin cleavage site is constructed into the vector between GST and the protein to be expressed (Chang, 1985). The resulting plasmid, designated pGEX-ftNF, was used to transform the \textit{E. coli} strain XL1-blue. A 100 ml overnight terrific-broth (T-broth) culture of \textit{E. coli} containing pGEX-ftNF was diluted 1:10 in T-broth and incubated for 1 h at 37°C. All bacterial cultures contained 50 µg ml\(^{-1}\) ampicillin and 12.5 µg ml\(^{-1}\) tetracycline. Expression of the recombinant protein was induced by adding isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) to a concentration of 0.3 mM. The culture was further incubated at 37°C for 5 h and then centrifuged for 10 min at 5000 \(\times\) g. The supernatant was discarded. The bacterial pellet was resuspended in 10 ml ice-cold phosphate-buffered saline (PBS), and sonicated twice for 30 s; samples were kept on ice. Triton-x-100 was added to a concentration of 1%, the solution was centrifuged for 5 min at 10 000 \(\times\) g, and the supernatant collected. One milliliter of a 50% slurry of glutathione–agarose beads was added to the supernatant and gently mixed for 3 min at room temperature, followed by three times washing with PBS. The fusion protein was eluted by adding 1 ml of 50 mM Tris-Cl (pH 8.0) with 10 mM reduced glutathione. The elution was repeated three times.

The purity of the rfTNF-\(\alpha\), the efficiency of the elutions, and its relative molecular size were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The part of the rfTNF-\(\alpha\)-GST that bound to the glutathione–agarose beads, was washed with thrombin cleavage buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 2.5 mM CaCl\(_2\)) and incubated with 1% thrombin for 1 h at room temperature (Chang, 1985). The cleaved protein products were analyzed on SDS-PAGE. The concentrations of the purified recombinant proteins were analysed as described earlier (Bradford, 1976).

2.5. \textit{TNF-\(\alpha\) stimulation studies}

Peripheral blood mononuclear cells (PMBCs) from four different normal donor cats were purified on ficoll-hypaque density gradients, and resuspended in growth medium (RPMI, 10% FBS, 1 µg ml\(^{-1}\) Concanavalin A (ConA)) to a concentration of \(10^6\) cells ml\(^{-1}\). Tenfold increasing concentrations (0, 1, 10, 100 and 1000 ng ml\(^{-1}\)) of rfTNF-\(\alpha\)-GST or rfTNF-\(\alpha\) were added to quintuplicate wells and the cells maintained in culture for 48 h before being analyzed for cell surface receptor expression. Control wells contained growth medium without ConA and no TNF-\(\alpha\).

PMBCs from each culture were pelleted by low speed centrifugation, washed twice with PBS containing 2% FBS and 0.1% sodium azide (PBS/FBS/Na\(_3\)). The pelleted cells were then resuspended in one of the following reagents and
incubated for 15 min: (1) 15 μl of tissue culture supernatant from mouse hybridoma cell line 9F23 containing antibodies against the alpha subunit of the feline interleukin-2 receptor (IL-2R) (Kindly provided by Dr. K. Ohno, Tokyo, Japan) (Ohno et al., 1992); (2) 10 μl of tissue culture supernatant from mouse hybridoma cell line 42.3H2, which contains antibodies to feline MHC class II antigen (Rideout et al., 1992); (3) 15 μl of tissue culture supernatant from mouse hybridoma W6/32 which contains antibodies against feline MHC class I antigen (Pollack et al., 1988); (4) 10 μg of rtTNF-α. Control cultures were left untreated. Following incubation, cells were pelleted, and washed twice with PBS/FBS/NaN₃. The TNF-α treated cells were incubated for an additional 15 min with 25 μl of mouse monoclonal antibody to human TNF-α receptor (Biosource International, Camarillo, CA), washed twice with PBS/FBS/NaN₃, and pelleted. Cell pellets were then resuspended in 25 μl of a 1:25 dilution of goat F(ab′)2 anti-mouse IgG-FITC (Caltag Laboratories, San Francisco, CA) and 10 μl propidium iodide (100 μg ml⁻¹) were added to each tube and the samples incubated at 37°C for 15 min. Samples were then washed twice with PBS/FBS/NaN₃ buffer and 10 000 cells were analyzed immediately by flow immunocytometry using a 488 nm argon laser, standard filter configuration for two color analysis and Consort 30 software (FACScan, Becton-Dickinson, San Jose, CA). Data were analyzed with LYSYS software. Events were gated on forward and log side scatter light characteristics and dead cells were eliminated from analysis based on propidium iodide staining. Live cells were evaluated using log green fluorescence and analysis regions were set such that less than 2% of control cells were in the positive analysis region.

2.6. Immunoblotting

Western blot analysis of rtTNF-α and rtTNF-α-GST were performed as described earlier (Lutz et al., 1980). Polyclonal rabbit anti-murine TNF-α (Genzyme, Cambridge, MA) and monoclonal anti-human TNF-α (Biosource International, Camarillo, CA) were used.

2.7. Rabbit anti-fTNF-α serum

Two adult New Zealand white rabbits were immunised subcutaneously with 100 μg of rtTNF-α-GST at weeks 0 and 2, and with the same amount of rtTNF-α on weeks 4, 6, 14, and 20. The first dose of antigen was in Freund’s complete adjuvant, while subsequent doses were in Freund’s incomplete adjuvant. Major antibody activity was demonstrated against both GST and TNF-α component by Western blotting.

2.8. L929 cytotoxicity assay

Serial dilutions of recombinant proteins were tested for cytotoxic activity using the murine fibroblast cell line L929 as described (Flick and Gifford, 1984).
Briefly, L929 cells were seeded into flat bottomed 96-well microtiter plates and incubated overnight in Eagle's minimum essential medium (MEM) supplemented with 5% FBS. Medium was then replaced with MEM containing 5 μg ml⁻¹ actinomycin D (Sigma, St. Louis, MO), samples were tested in quadruplicates of 100 μl and incubated for 16 h at 37°C and 5% CO₂. The cell supernatant was removed thereafter and the monolayer stained with crystal violet for 10 min. The absorbance of washed stained cell monolayers was measured at a wavelength of 595 nm using an automatic plate reader (BioRad, Hercules, CA). Medium and recombinant feline immunodeficiency virus p24-GST (FIV-rp24-GST) were used as a negative control and recombinant mouse TNF-α (rmTNF-α, Genzyme) as a positive control. The concentration of fTNF-α resulting in 50% of the absorbance of the controls was considered the 50% cytotoxic dose (CD₅₀).

2.9. Inhibition of cytotoxicity by anti-rfTNF-α polyclonal serum

Recombinant fTNF-α (500 ng ml⁻¹) was incubated with different dilutions of both preimmune and immune rabbit anti-rfTNF-α serum for 30 min at room temperature. The fTNF-α antiserum mixtures were then tested with L929 cells as described above.

2.10. In vivo studies

The first study involved three groups of adult SPF cats, each consisting of two animals. Each group was injected i.v. with 25 or 50 μg kg⁻¹ of rfTNF-α-GST or 50 μg kg⁻¹ of GST dissolved in PBS. The cats were observed for clinical symptoms for a period of 48 h and rectal temperature was measured every 20 min the first 3 h.

In a second study, two adult cats were each inoculated intravenously with 50 μg kg⁻¹ of rfTNF-α-GST dissolved in PBS. Clinical symptoms and rectal temperatures were measured 0, 2, 6, 12, 24 and 48 h following treatment.

3. Results

3.1. PCR, cloning and sequencing of fTNF-α cDNA

A 700 bp DNA fragment was amplified using primer pair P1/P3 and cDNA from LPS stimulated macrophages as target (Fig. 1, Lane 1). No PCR product was amplified from cDNA produced from mRNA of unstimulated macrophages (Fig. 1, Lane 2). Three fragments of 1.7 kb, 400 bp and 250 bp were amplified from genomic DNA (Fig. 1, Lane 3).

Primer pair P2/P3 amplified a 500 bp DNA fragment from cDNA of stimulated macrophages (Fig. 1, Lane 4). No PCR products were amplified from cDNA derived from the mRNA of unstimulated macrophages (Fig. 1, Lane 5), while an 850 bp fragment was amplified from genomic feline DNA (Fig. 1, Lane 6). The
Fig. 1. Electrophoresis in a 1.5% agarose gel of the PCR products of feline TNF-α gene amplification. M₁, φX174-HaeIII DNA fragments; M₂, λ-BstEII DNA fragments. Lanes 1, 2 and 3: RT-PCR using primers P₁/P₃ of LPS-stimulated, unstimulated feline macrophages, and genomic DNA as targets, respectively. Lanes 4, 5 and 6: RT-PCR using primers P₂/P₃ of LPS-stimulated, unstimulated feline macrophages, and genomic DNA as targets, respectively.

sizes of the 1.7 kb and 850 bp amplified fragments from genomic feline DNA corresponded to the distance between the P₁–P₃ and P₂–P₃ primers in the genomic fTNF-α nucleotide sequence, respectively. Similarly the 700 bp and 500 bp bands amplified from cDNA from LPS-stimulated macrophages corresponded to the estimated respective sizes of mRNA for the pre- and mature-proteins of TNF-α.

Sequence analysis of the P₁/P₃ amplified product from cDNA from stimulated macrophages showed a 99.3% homology to previously reported genomic DNA sequence of fTNF-α gene, and 98.7% homology on the amino acid level. There were 91% and 81% sequence homologies between the mRNA of fTNF-α and the respective human and murine TNF-α genes. The homologies between the deduced amino acid sequence of the mature part of fTNF-α, i.e. between primer pair P₂/P₃, to those of human and murine TNF-α were 92% and 78%, respectively.

3.2. Expression and immunoblotting

A single band with a molecular size of 43 kDa was observed in SDS-PAGE gels of the purified fusion protein rfTNF-GST expressed by pGEX-fTNF (Fig. 2, Lane 1). Thrombin cleaved the fusion protein into two fragments one with a size of 17 kDa, which corresponds to the size of TNF-α in other species (Marmenout et al., 1985; Pennica, et al., 1985) (Fig. 2, Lane 2), the other was retained on the glutathione agarose beads after thrombin cleavage had thus a size of 26 kDa, which corresponded to the size of GST (Smith et al., 1986). The amount of purified rfTNF-GST produced was about 4 mg 1⁻¹ bacterial culture medium. However, the amount of rfTNF-α after thrombin cleavage was only about one-tenth of this.

No cross-reactivity was observed between polyclonal rabbit anti-murine TNF-α antibodies and rfTNF-α in Western blot. As predicted from sequence homologies and previous studies (Lehmann, et al., 1992) monoclonal anti-human TNF-α antibodies reacted specifically to both rfTNF-GST and rfTNF-α (Fig. 3).
Fig. 2. Relative migration of purified rat TNF-GST fusion protein (Lane 1), and rat TNF-α after thrombin cleavage (Lane 2). Lanes M₁ and M₂ are molecular size markers.

Fig. 3. Western blot using monoclonal anti-human TNF-α serum. Lane 1, rat TNF-α-GST fusion protein; Lane 2, rat TNF-α after thrombin cleavage; Lane M, biotinylated molecular size marker.

3.3. Titration of rat TNF-α in L929 cytotoxicity assay

L929 mouse fibroblast cells were susceptible to the toxic effects of both rat TNF-α-GST and rat TNF-α (Fig. 4). At concentration below 125 ng ml⁻¹ the rat TNF-α was significantly more toxic than a similar concentration of rat TNF-α-GST. The CD₅₀ for the L929 cells was estimated to be 230 ng ml⁻¹ and 15 ng ml⁻¹ for rat TNF-α-GST and the rat TNF-α, respectively.

3.4. Inhibition of rat TNF-α activity by polyclonal antiserum

The cytotoxic effect of purified rat TNF-α on L929 cells was completely neutralised by a 1:10 dilution, and partially neutralised by a 1:100 dilution, of rabbit anti rat TNF-α serum. Preimmune serum had no inhibitory effect on the cytotoxicity.

3.5. Clinical observations

Cats given rat TNF-α-GST, became clinically ill between 15 min and 10 h after treatment. A fever peaked at 4–5 h after treatment and disappeared after 10 h and was the most prominent syndrome (Fig. 5). Other clinical signs included
Fig. 4. Titration of rTNF-α and rTNF-α-GST cytotoxicity on mouse L929 cells. Toxicity is inversely proportional to uptake of crystal violet stain by the monolayer, i.e. absorbance at 595 nm. The positive control was rmTNF-α while the negative control was normal cell culture medium and non-related recombinant purified FIV-p24 (made by the same expression and purification system). The cytotoxic activity of the rTNF-α-GST paralleled that of the purified rTNF-α at concentrations of 125 ng ml\(^{-1}\) and greater.

Fig. 5. The rectal temperatures of four cats given 50 μg kg\(^{-1}\) of either rTNF-α-GST or GST intravenously. An increase in the rectal temperature was apparent within 2 h in cats treated with rTNF-α-GST but not with GST alone.

depression, immobility caused by malaise, protrusion of the nictitating membranes, piloerection (especially along the dorsum of neck and back and on the tail) and hemoconcentration. Clinical signs peaked at 4 h following treatment and had largely disappeared by 10 h. The clinical signs and their severity were similar in cats given either 25 and 50 μg kg\(^{-1}\) of the fusion protein, except for one cat that was treated with 50 μg kg\(^{-1}\) of rTNF-α-GST and developed moderately severe hypovolemic shock and signs of cerebellar hypoxemia (disorientation, loss of balance). These signs appeared 4 h following treatment and lasted for about 1
h before spontaneously resolving. No clinical signs of illness were seen in two control cats that were given only the GST portion of the fusion protein (Fig. 5).

3.6. TNF-α mediated regulation of receptor expression

Although some variation was evident between the four individual donor cats, rFTNF-α induced a dose related increase of IL-2R and MHC class II antigen expression on the cell surface of in vitro stimulated feline PMBCs (Fig. 6). At the highest rFTNF-α concentration, this increase was 13–23% for IL-2R and 7–30% for MHC class II antigen expression. Recombinant FTNF-α had no stimulatory effect in vitro on MHC class I or TNF-α receptor expression.

Fig. 6. (a) The upregulation of IL-2 receptors, as measured by flow cytometry, on feline PMBCs exposed in vitro to rFTNF-α-GST. (b) The upregulation of MHC class II antigen, as measured by flow cytometry, on the surface of feline PMBCs exposed in vitro to rFTNF-α-GST.
4. Discussion

Biologically active recombinant feline TNF-α was expressed in *E. coli*. The entire fTNF-α gene was cloned from cDNA by PCR since the feline genomic TNF-α gene is interrupted by three introns, making it difficult to clone a functional TNF-α gene directly from genomic DNA. Although genomic DNA was not used in the experiment the PCR primers were constructed from a TNF-α sequence that was itself derived from genomic DNA (McGraw et al., 1990). The cDNA encoding the functional TNF-α gene was derived from mRNA by reverse transcription. The mRNA was extracted from peritoneal macrophages that were induced to produce high levels of TNF-α (and specific mRNA) by *E. coli* LPS stimulation. This procedure allowed for the ultimate construction of a plasmid containing only the relevant portions of the TNF-α gene in a continuous linear configuration.

The protein expression system used the pGEX-2T vector. The GST fusion protein encoded by the plasmid pGEX-fTNF had a molecular size of 43 kDa of which rffTNF was making up 17 kDa. This is in about the same size range as human and murine TNF-α (Marmenout et al., 1985), and correlates well with the estimated molecular size (17.9 kDa) of the 157 amino acid long mature feline TNF-α. The reduction in the yield of rffTNF-α after the fusion protein had been cleaved with thrombin could have been caused by the lower solubility of the cleaved protein compared with the fusion protein. A possible obstructive effect of the agarose beads on the efficacy of thrombin cleavage could not be ruled out.

Analysis of the nucleotide sequence of the cloned mRNA encoding for the preprotein of feline TNF-α showed a homology of 99.3% with the coding sequences of a previously sequenced feline TNF-α gene (McGraw et al., 1990), and 98.7% homology at the amino acid level (98.8% homology for the mature part). The small nucleotide sequence divergence (0.7%) between these two fTNF-α genes reflects either limited genetic variations between individual cats and/or small errors in reverse transcription/PCR. If the differences were due to errors, however, the errors were not sufficient to alter the biological activity of the protein.

The fusion protein and rffTNF-α both appeared to be biologically active. Both proteins killed TNF-α-sensitive L929 mouse cells; rffTNF-α-GST had a toxic effect on these cells comparable to rffTNF-α at concentrations above 125 ng ml⁻¹, but at levels below 125 ng ml⁻¹ rffTNF-α was significantly more toxic than rffTNF-α-GST. This indicated some interference by the GST moiety on the rffTNF-α portion of the molecule. Polyclonal rabbit anti-human TNF-α prevented the toxic effect of both rffTNF-α-GST and rffTNF-α on L929 cells, again demonstrating that both the cleaved and fused fTNF-α proteins were biologically and antigenically intact. The rffTNF-α-GST was also biologically active when injected intravenously into cats. The onset and the character of clinical signs resembled those observed for TNF-α in other species (Creagan et al., 1988). Since i.v. administration of recombinant GST did not induce any clinical signs, it can be deduced that the in vivo effects were caused by the rffTNF-α portion of the fusion protein and not by the GST. As expected, it was shown that rffTNF-α-GST upregulated
the expression of IL-2R and MHC class II antigens in normal cultures of feline PMBCs. The induction of IL-2R and MHC class II antigen mRNA by TNF-α involves the activation of transcriptional factors (Maniatis et al., 1987). TNF-α activates NFkB proteins that can induce expression of genes possessing kB-like enhancer elements in their regulatory regions (Lowenthal et al., 1989b). Included in this group are the genes encoding IL-2R (Lowenthal et al., 1989a), MHC class II antigen (Pessara and Koch, 1990), and human, feline and simian immunodeficiency viruses (Folks et al., 1989; Osborn et al., 1989; Dewhurst et al., 1990; Poli et al., 1990; Sparger et al., 1992).

Feline TNF-α possessed considerable antigenic cross-reactivity with human, but not with murine TNF-α in Western blotting. The degree of antigenic cross-reactivity corresponded with the deduced amino acid sequence of the TNF-α coding regions of the three species; rTFN-α showed a genetic homology of 92% and 78% with human and murine TNF-α, respectively.

In conclusion, our results demonstrate that rTFN-α could prove to be a useful reagent for the study and treatment of feline disease. Studies of the cytokine-virus interactions in the FIV infection of cats could be useful for human AIDS research.

Acknowledgments

This work was supported by Public Health Service grants AI-25802-03 and CA-50179-01.

References

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilising the principle of protein dye binding. Anal. Biochem., 72: 248-254.
Brunner, D. and Pedersen, N.C., 1989. Infection of peritoneal macrophages in vitro and in vivo with feline immunodeficiency virus. J. Virol., 63: 5483-5488.
Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Wiliamson, B., 1975. An endotoxin-induced serum factor that causes necrosis of tumours. Proc. Natl. Acad. Sci. USA, 72: 3666-3670.
Chang, J.-Y., 1985. Thrombin specificity. Requirement for a polar amino acid adjacent to the thrombin cleavage site of a polypeptide substrate. Eur. J. Biochem., 151: 217-224.
Creagan, E.T., Kovach, J.S., Moertel, C.G., Frytak, S. and Kvol, L.K., 1988. A phase I clinical trial of recombinant human tumour necrosis factor. Cancer, 62: 2467-2471.
Dewhurst, S., Embretson, J.E., Anderson, D.C., Mullins, J.I. and Fultz, P.N., 1990. sequence analysis and acute pathogenicity of molecularly cloned SIVSM.RB14-. Nature, 345: 636-640.
Drews, R.T., Coffee, B.W., Prestwood, A.K. and McGraw, R.A., 1990. Gene sequence of porcine tumour necrosis factor alpha. Nucleic Acids Res., 18: 5564.
Flick, D.A. and Gifford, G.E., 1984. Comparison of in vitro cell cytotoxic assays for tumour necrosis factor. J. Immunol. Meth., 68: 167-175.
Folks, T.M., Clouse, K.A., Justement, J., Rabson, A., Duh, E., Kehrl, J.H. and Fauci, A.D., 1989. Tumor necrosis factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc. Natl. Acad. Sci. USA, 86: 2365.
Green, I.R. and Sargan, D.R., 1991. Sequence of the cDNA encoding ovine tumour necrosis factor-alpha: problems with cloning by inverse PCR. Gene, 109: 203–210.

Ito, H., Shirai, T., Yamamoto, S., Akira, M., Kawahara, S., Todd, S. and Wallace, R.B., 1986. Molecular cloning of the gene encoding rabbit tumour necrosis factor. DNA, 5: 157–165.

Jäättelä, M., 1991. Biologic activities and mechanisms of action of tumour necrosis factor-α/cachectin. Lab. Invest., 64: 724–742.

Lawrence, C.E., Callanen, J.J. and Jarrett, O., 1992. Decreased mitogen responsiveness and elevated tumour necrosis factor production in cats shortly after feline immunodeficiency virus infection. In: S. Dandekar and N. Pedersen (Editors), Feline Immunodeficiency Virus. Vet. Immunol. Immunopathol., 35: 51–59.

Lehmann, R., Joller, H., Haagmans, B.L. and Lutz, H., 1992. Tumor necrosis factor α levels in cats experimentally infected with feline immunodeficiency virus: effects of immunization and feline leukemia virus infection. In: S. Dandekar and N. Pedersen (Editors), Feline Immunodeficiency Virus. Vet. Immunol. Immunopathol., 35: 61–69.

Lowenthal, J.W., Ballard, D.W., Bogerd, H., Böhlein, E. and Greene, W.C., 1989a. Tumor necrosis factor α activation of the IL-2 receptor-alpha gene involves the induction of kappa B-specific DNA binding proteins. J. Immunol., 142: 3121–3128.

Lowenthal, J.W., Ballard, D.W., Böhlein, E. and Greene, W.C., 1989b. Tumor necrosis factor α induces proteins that bind specifically to kB-like enhancer elements and regulate interleukin 2 receptor a-chain gene expression in primary human T-lymphocytes. Proc. Natl. Acad. Sci. USA, 86: 2331.

Lutz, H., Pedersen, N.C., Higgins, J., Hübscher, U., Troy, F.A. and Theilen, G.H., 1980. Humoral immune reactivity to feline leukemia virus and associated antigens in cats naturally infected with feline leukemia virus. Cancer Res., 40: 3642–3651.

Maniatis, T., Goodbourn, S. and Fisher, J.A., 1987. Regulation of inducible and tissue-specific gene expression. Science, 236: 1237–1245.

Marmenout, A., Fransen, L., Tavernier, J., van der Hayden, J., Tijard, R., Kawashima, E., Shaw, A., Johnson, M.J., Semon, D., Muller, R., Ruysschaert, M.R., van Vliet, A. and Fiers, W., 1985. Molecular cloning and expression of human tumour necrosis factor and comparison with mouse tumour necrosis factor. Eur. J. Biochem., 152: 512–522.

Matsuyama, T., Kobayashi, N. and Yamamoto, N., 1991. Cytokines and HIV infection: is AIDS a tumour necrosis factor disease? AIDS, 5: 1405–1417.

McGraw, R.A., Coffee, B.W., Otto, C.M., Drews, R.T. and Rawlings, C.A., 1990. Gene sequence of feline tumour necrosis factor alpha. Nucleic Acids Res., 18: 5563.

Nicola, D.P. and West, T.E., 1990. Thalidomide: treatment of severe recurrent aphthous stomatitis in patients with AIDS. J. Clin. Pathol., 43: 1054–1056.

Ohno, K., Goitsuaka, R., Kitamura, K., Hasegawa, A., Tokunaga, T. and Honda, M., 1992. Production of a monoclonal antibody that defines the alpha subunit of the feline IL-2 receptor. Hybridoma, 11: 595–605.

Osborn, L., Kunkel, S. and Nabel, G.J., 1989. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancers by activation of the nuclear factor KB. Proc. Natl. Acad. Sci. USA, 86: 2336–2340.

Pedersen, N.C., 1993. The feline immunodeficiency virus. In: J. Levy (Editor), The Retroviridae. Vol. 2. Plenum Press, New York, pp. 181–228.

Pennica, D., Hayflick, J.S., Bringham, T.S., Palladino, M.A. and Goeddel, D.V., 1985. Cloning and expression in E. coli of the cDNA for murine tumour necrosis factor. Proc. Natl. Acad. Sci., 82: 6060–6064.

Pessara, U. and Koch, N., 1990. Tumor necrosis factor α regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-κB-like factor to a pro-motor element. Mol. Cell. Biol., 10: 4146.

Poli, G., Kinter, A., Justement, J.S., Kehrl, J.H., Bressler, P., Stanley, S. and Fauci, A.D., 1990. Tumor necrosis factor α functions in an autocrine manner in the induction of human immunodeficient virus expression. Proc. Natl. Acad. Sci. USA, 87: 782.

Pollack, M.S., Hayes, A., Mooney, S., Pedersen, N.C. and Cook, R.G., 1988. The detection of conven-
tional class I and class II I-E homologue major histocompatibility complex molecules on feline cells. Vet. Immunol. Immunopathol., 19: 79–91.

Reid, G., Rigby, M.A., McDonald, M., Hosie, M.J., Neil, J.C. and Jarrett, O., 1991. Immunodiagnosis of feline immunodeficiency virus infection using recombinant viral p17 and p24. AIDS, 5: 1477–1483.

Rideout, B.A., Moore, P.F. and Pedresen, N.C., 1992. Persistent upregulation of MHC class II antigen expression on T lymphocytes from cats experimentally infected with feline immunodeficiency virus. In: S. Dandekar and N. Pedersen (Editors), Feline Immunodeficiency Virus. Vet. Immunol. Immunopathol., 35: 71–82.

Shirai, T., Yamaguchi, H., Ito, H., Todd, C.W. and Wallace, R.B., 1985. Cloning and expression in Escherichia coli of the gene for human tumour necrosis factor. Nature, 313: 803–806.

Smith, D.B., Davern, K.M., Board, P.G., Tiu, W.U., Garcia, E.G. and Mitchell, G.F., 1986. M, 26000 antigen of Schistosoma japonicum recognized by resistant WEHI 129/J mice is a parasite glutathione S-transferase. Proc. Natl. Acad. Sci. USA, 83: 8703–8707.

Sparger, E.E., Shacklett, B.L., Renshaw, G.L., Barry, P.A., Pedersen, N.C., Elder, J.H. and Luciw, P.A., 1992. Regulation of gene expression directed by the long terminal repeat of the feline immunodeficiency virus. Virology, 187: 165–177.

Stoddart, C.A. and Scott, F.W., 1988. Isolation and identification of feline peritoneal macrophages for in vitro studies of coronavirus–macrophage interactions. J. Leuc. Biol., 44: 319–328.

Suzuki, M., Yamamoto, N. and Shinozaki, F., 1989. Augmentation of in vitro HIV replication in peripheral blood mononuclear cells of AIDS and ARC patients by tumour necrosis factor. Lancet, i: 1206.