CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, July 1997, p. 487–490 Vol. 4, No. 4
1071-412X/97/$04.00+0
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Production of Polyclonal Antibodies to Feline Tumor Necrosis Factor

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Received 23 January 1995/Returned for modification 20 March 1995/Accepted 11 April 1997

Two 13-amino-acid peptides were synthesized based on the putative feline tumor necrosis factor (FeTNF) sequence. The synthesized peptides were conjugated to keyhole limpet hemocyanin, emulsified in complete Freund's adjuvant, and injected into rabbits. The gene for FeTNF was cloned into the FLAG (International Biotechnologies Inc. [IBI], Kodak, New Haven, Conn.) fusion protein expression vector. The expressed fusion protein was purified by using the M-1 anti-FLAG octapeptide monoclonal antibody (IBI, Kodak). The fusion protein was emulsified in complete Freund's adjuvant and injected into chickens. The immune sera generated to the synthetic peptides and the fusion protein recognized the recombinant FeTNF fusion protein on Western or dot blot assay. The preimmune and immune sera were incubated with naturally occurring FeTNF (supernatants from lipopolysaccharide-stimulated cultured feline peritoneal exudate or peripheral mononuclear cells). The antibody raised to the recombinant FeTNF fusion protein and N-terminal synthetic peptide neutralized bioactivity of native FeTNF and recombinant human TNF. Preimmune sera did not have any neutralizing activity. The polyclonal antibodies were not specific for FeTNF, since both porcine and human recombinant TNF were neutralized by the fusion protein antibodies. The synthetic peptide antibodies recognized recombinant feline and equine TNF on a Western blot.

Tumor necrosis factor (TNF) is a 17-kDa protein released from macrophages following stimulation by lipopolysaccharide, parasites, or other invasive stimuli (4, 32). TNF was initially identified as a mediator of tumor necrosis and parasite-induced cachexia. TNF has also been referred to as cachectin (1). With the availability of recombinant human and murine TNF and antibodies to TNF, it has been recognized that the effects of TNF are not limited to neoplastic cells and adipocytes. TNF is also an immune modulator. It stimulates neutrophil accumulation and activation, and it promotes thrombosis. TNF is involved in tissue and bone remodeling. When released in large amounts, TNF produces the signs of septic shock (12).

Antibodies to TNF have been generated in several species (3, 27, 29). These antibodies have been useful in the development of immunologic assays (6, 8) and in blocking the effects of TNF in vivo and in vitro. It has been shown that TNF-neutralizing antibodies protect baboons and mice from septic shock (2, 25, 33) and inhibit cachexia associated with cancer in mice (24).

It has been recognized that there is a species specificity for TNF. Human TNF can cause a response in mouse cells but only binds one of the two cellular receptors (34), and it exerts a greater cytotoxic effect on human cells (4, 30). The receptor binding site is thought to lie in a highly conserved hydrophobic area that is protected from immune surveillance. It is thought that neutralizing antibodies bind the highly variable hydrophilic regions and sterically interfere with receptor binding. The interspecies variation of the hydrophilic regions of the TNF molecule confers an immunologic specificity necessitating the development of species-specific antibodies (13).

The bioassays currently available produce variable cytolytic results when measuring the effects of TNF on mouse L929 fibroblast (16) or WEHI 164 (7) cells. This variability may be related to changes in cell line sensitivity following culture or serial passage (6). In addition to providing an improved method to measure TNF, species-specific antibodies can be used in immunocytochemistry, immunoaffinity purification, and neutralization (in vivo and in vitro) of TNF.

We generated polyclonal antibodies to the feline TNF protein. This was done prior to expression of the feline TNF gene by using synthetic peptides derived from the putative feline TNF protein sequence. Following expression of the feline TNF gene as a fusion protein, this recombinant protein was used to generate antibodies.

Materials and methods. (i) Synthetic peptides. The sequences for generation of synthetic peptides were identified by selecting regions of the feline TNF sequence (17) that fulfilled the criteria for antigenicity (23). The peptides chosen for synthesis represented predominantly hydrophilic sequences. The peptides were synthesized by J. Wunderlich at the University of Georgia, Molecular Genetics Core Facility.

The first peptide, NTERFTNF, consisted of the 12 N-terminal amino acids plus a cysteine: Leu Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Cys. This sequence is identical to the N-terminal sequences of human and horse TNF. The second peptide, CYSFPTNF, was initiated with the cysteine occurring at amino acid 101 and incorporated the next 12 amino acids: Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro. It is located in a predicted variable-loop region connecting beta-pleated sheets and is similar to the porcine and human sequences.

Five milligrams of synthetic peptide was coupled to 10 mg of keyhole limpet hemocyanin (Sigma Chemical Co., St. Louis, Mo.) according to the method of Sambrook et al. (23). The conjugated peptide was emulsified in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) (both from Sigma). For each synthetic peptide, three rabbits were immunized with 300 μg of protein in CFA (total volume, 0.3 ml) injected subcutaneously into three sites. Rabbits were boosted

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with 200 μg of protein in IFA every 2 weeks for 6 weeks. Blood samples were collected by ear vein venipuncture before immunization and at 6, 8, and 10 weeks after initial immunization. Rabbits were housed and treated according to University of Georgia animal care guidelines.

(ii) Recombinant feline TNF. Preparation of the feline TNF gene for expression followed the methods of Su and coworkers (28). Briefly, PCR overlap extension was used to delete the introns (11) from the gene cloned by Otto and coworkers (18). After the fused PCR product was ligated into the FLAG fusion protein expression vector system (International Biotechnologies Inc. [IBI], Kodak, New Haven, Conn.), the N-terminal sequence and reading frame were corrected by oligonucleotide-mediated site-directed mutagenesis. The vector containing the modified insert was used to transform *Escherichia coli*. Protein induction and purification were performed according to the manufacturer's recommendations. The presence of the protein in inclusion bodies prevented protein purification by standard methods. Therefore, the denaturation-renaturation procedure described by Lin and Cheng (15) was used.

The FLAG technology results in production of a fusion protein consisting of the cloned gene and an octapeptide "flag" at the N terminus (10). The commercially available M-1 (IBI) antibody recognizes this octapeptide and was used for Western blot analysis and affinity purification of the fusion protein.

The fusion protein was collected for assay of activity and generation of polyclonal antibodies. Three 6- to 8-week-old white bantam chickens were each inoculated with 1 ml of an emulsion containing IFA and 36 μg of the fusion protein. Subsequent injections contained 30 to 40 μg of protein. Routes of injection were intradermal for bird 1, subcutaneous for bird 2, and intramuscular for bird 3. Birds were boosted at 14 and 28 days. Serum was collected from the wing vein at 21 days and then every 7 days.

(iii) Western and dot blots. One to five microliters of bacterial culture, culture supernatant, or bacteria resuspended in phosphate-buffered saline was loaded into a 96-well blotting manifold (GIBCO-BRL, Life Technologies, Inc., Gaithersburg, Md.) for dot blot analysis according to the method recommended by IBI in its FLAG manual. Western blots were prepared by electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels (14) to nitrocellulose membranes (31).

To confirm the presence of the fusion protein, membranes were incubated with 10 μg of anti-FLAG monoclonal antibody (M-1; IBI, Kodak)/ml. Biotinylated anti-mouse immunoglobulin G (Bio-Rad) (1:100,000 in Tris-buffered saline) was used as the secondary antibody. Following incubation with strepavidin-alkaline phosphatase (1:2,500; Sigma), nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolyl phosphate) (both from Bio-Rad) (each diluted 1:10 in deionized water) was added to develop the color.

Rabbit polyclonal antibodies to the synthetic peptides were used to identify the TNF protein. Membranes were incubated with 1:100 dilutions of rabbit serum containing anti-NTERFTNF or anti-CYSFPTNF antibodies. The secondary antibody was goat anti-rabbit immunoglobulin G horseradish peroxidase (1:500; Sigma). Diaminobenzadine (10 mg; Sigma) in 20 ml of phosphate-buffered saline and 3 μl of 3% H_2O_2 were added to develop the color.

(iv) WEHI assay. A previously described cytolytic bioassay using mouse WEHI 164 fibrosarcoma cells was employed to measure TNF activity (7). The cells were grown in 96-well flat-bottomed trays (Costar, Cambridge, Mass.) at 30,000 cells (in 0.1-ml volumes) per well in the presence of 1 μg of actinomycin D/ml and were incubated with serially diluted test samples or a recombinant human TNF standard (Genzyme, Cambridge, Mass.) in a humidified atmosphere at 37°C with 5% CO_2. Alamar blue (Alamar Biosciences, Inc., Sacramento, Calif.) provided a colorimetric index of cell viability as determined by a Bio-Rad microtiter autoreader set for absorbance at 570 nm. The absorbance from cells exposed to culture medium alone represented 0% lysis, and the absorbance from cells exposed to 0.5% Triton X-100 (Sigma) solution provided an end point for 100% lysis. One unit of TNF was defined as the amount required for 50% cell lysis.

TABLE 1. Recognition of feline, equine, and porcine recombinant TNF fusion proteins by antibodies or the corresponding preimmune sera on a Western or dot immunoblot assay

Antibody or serum	Recognition of fusion protein[b]		
	Feline	Equine	Porcine
Anti-rFeTNF[a]	+	ND	+
Preimmune serum	−	ND	ND
Anti-NTERFTNF	+	+	±
Preimmune serum	−	−	−
Anti-CYSFPTNF	+	+	±
Preimmune serum	−	−	−

[a] rFeTNF, recombinant feline TNF.
[b] ND, not done. The anti-synthetic peptide antibodies, anti-NTERFTNF and anti-CYSFPTNF, did not recognize the 17-kDa recombinant porcine fusion protein but recognized a band in the 60-kDa region that may have been the trimeric form of porcine TNF.

(v) Neutralization of TNF activity. Samples with known TNF activity (obtained from lipopolysaccharide-stimulated feline peritoneal macrophages, cultured feline mononuclear cells, or recombinant human TNF) were mixed with different dilutions of the sera containing the antibodies prior to the WEHI cell bioassay. The sera were also tested for cytolytic or stimulatory activity on the WEHI assay.

Results. (i) Expression of recombinant feline TNF. Expression of the recombinant feline TNF was toxic to *E. coli* and limited the ability to obtain recombinant fusion protein (19). Early harvesting of the induced cultures (at 2 h following IPTG [isopropyl-β-D-thiogalactopyranoside] treatment) provided an opportunity to obtain a small amount of the recombinant feline TNF fusion protein.

(ii) Fusion protein extraction. The routine extraction protocol failed to produce a purified protein. Electrophoretic protein gel and dot blot assays identified fusion protein in the bacterial cultures and cell pellet but not in the supernatants. The failure to produce a soluble fusion protein was not corrected by manipulation of the culture temperature or IPTG concentration. It was concluded that the protein was associated with inclusion bodies and necessitated the denaturation-renaturation extraction procedure. The product of this extraction was applied to the M-1 immunoaffinity column and resulted in a purified fusion protein for the generation of antibodies. Unfortunately, this protein lacked bioactivity on the WEHI assay.

(iii) Antibodies. Antibodies to the synthetic peptides, NTERFTNF and CYSFPTNF, and the recombinant feline TNF fusion protein were successfully generated in rabbits and chickens, respectively. The ability of these antibodies to recognize the TNF protein is shown in Table 1. Identification of the fusion protein was confirmed by anti-M-1 antibody.

The anti-synthetic peptide antibodies did not recognize the 17-kDa recombinant porcine fusion protein but recognized a band in the 60-kDa region that may have been the trimeric

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TABLE 2. Neutralization of TNF activity, as measured by a WEHI bioassay, by antibodies generated to recombinant feline TNF and synthetic peptides

TNF and serum dilution	% Neutralization by:		
	Anti-rFeTNF[a]	Anti-NTERFTNF	Anti-CYSFPTNF
Natural feline			
Preimmune	0	0	0
1:10	59	76	59
Recombinant human			
Preimmune	0	0	0
1:5	88	89	0
1:50	100	100	0
1:100	100	100	0

[a] rFeTNF, recombinant feline TNF.

form of porcine TNF. Trimeric forms of TNF can be seen despite the presence of reducing conditions associated with the protein gel. There was weak recognition of a similar-sized band with the recombinant equine fusion protein. A dot blot of the porcine fusion protein was not recognized by NTERFTNF (which represents a different sequence from that found in the N-terminal sequence of porcine TNF).

The antibodies were tested for the ability to block the cytolytic activity of TNF in the WEHI bioassay (Table 2). The antibody to the N-terminal TNF synthetic peptide (NTERFTNF) was able to neutralize both human and feline TNF. The antibody to the synthetic peptide unique to the feline sequence (CYSFPTNF) demonstrated partial neutralization of the natural feline TNF but not neutralization of recombinant human TNF. The antibodies to recombinant feline TNF fusion protein partially neutralized naturally occurring feline TNF and completely neutralized recombinant human TNF.

Discussion. The use of synthetic peptides for the generation of antibodies is a procedure that can aid in identification, characterization, and purification of proteins of interest (22, 35). Synthetic peptides can be created to represent antigenic epitopes and have been shown to be able to induce a neutralizing antibody response to the parent protein/pathogen (22, 26). Regions of the protein that possess potential antigenicity can be predicted by using computer programs or can be identified as regions of high sequence variability (9). While three-dimensional structure is important, T cells can recognize the linear amino acid sequence (22, 35).

Synthetic peptides are useful in the identification of active sites. A synthetic peptide to the first 15 amino acids of the human TNF sequence neutralizes TNF activity (26). Deletion of the first 8 amino acids of human TNF does not affect its activity (13). This is compatible with our results. The ability of the 13-amino-acid synthetic peptide to block activity suggests that amino acids 14 and 15 are not critical for the epitope binding. The inability of the second synthetic peptide to neutralize human TNF suggests that the minor differences between species in this region are sufficient to prevent neutralization activity. The limited neutralization of the feline TNF is not surprising, considering the three-dimensional structure and putative active sites (13).

While antibodies to synthetic peptides can be used for affinity purification (35), the two generated to feline TNF were not. These antibodies were used to confirm the presence of feline TNF on Western blot and dot blot. The failure of the CYSFPTNF antibody to recognize porcine TNF cannot be explained. This antibody recognized the feline recombinant protein, which shares this sequence with the porcine protein. The equine sequence is different from those of both the feline and the porcine proteins but was recognized by this antibody. It is possible that the expressed porcine TNF contains a mutation in this region that alters the epitope, or that the folding of the porcine protein prevents adequate exposure of this site. The ability of this synthetic peptide to recognize the equine TNF suggests that the epitope recognized involves the last 5 amino acids of the synthetic peptide, which are identical in the cat and the horse.

Expression of feline TNF in *E. coli* was toxic to the *E. coli* (20). This, along with the localization of the protein in inclusion bodies, complicated collection of the fusion protein. When the fusion protein was obtained, only small quantities were available. The extraction procedure to separate the fusion protein from the bacteria required denaturation of the protein, followed by renaturation. This process produced a fusion protein of the expected size (17 kDa). This protein was recognized by the monoclonal antibody to the FLAG octapeptide and by the polyclonal antibodies to the two synthetic peptides. The protein lacked bioactivity on the WEHI assay. This could have resulted from the denaturation or from the nature of the recombinant. It has been shown that the activity of TNF depends on the presence of TNF in trimers (5). The tertiary and quaternary structure of the recombinant feline TNF might have been altered by the introduction of a mutation during amplification of the gene. Successful expression of recombinant feline TNF has been accomplished with a cDNA possessing two significant amino acid differences (21). These amino acid differences could result in alterations of protein folding (13) and may explain our difficulty in expression of an active recombinant.

Despite the difficulties in obtaining the recombinant feline TNF, antibodies to the expressed protein neutralize feline and human TNF and have been used in preliminary studies for affinity purification of naturally occurring feline TNF. The ability of these polyclonal antibodies to neutralize TNF is most likely the result of binding of multiple epitopes and steric hindrance. The lack of complete neutralization for feline TNF compared to human TNF may be a result of the altered folding of the recombinant feline TNF used to generate the antibody or the presence of TNF-β in the supernatants from feline mononuclear cell cultures. The polyclonal nature of this antibody and the cross-reactivity limit its use as a therapeutic agent. The ability of the fusion protein to generate neutralizing antibodies, despite its own lack of bioactivity, suggests that it may provide a suitable antigen for production of monoclonal antibodies. Alternatively, the polyclonal antibody may provide an efficient means for immunopurification of naturally occurring feline TNF. The purified TNF could then be used to generate monoclonal antibodies for development of an enzyme-linked immunoassay and neutralization of feline TNF. Antibodies to feline TNF provide reagents that may help further characterize the role of TNF in feline diseases and experimental models that utilize the cat.

We thank Donald Dawe, Denise Pesti, and Pat Lindl for their technical advice and assistance.

This research was supported by a grant from the University of Georgia Veterinary Medical Experiment Station and the Clara B. West Research Foundation. C. M. Otto was an American Heart Association, Georgia Affiliate research fellow.

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