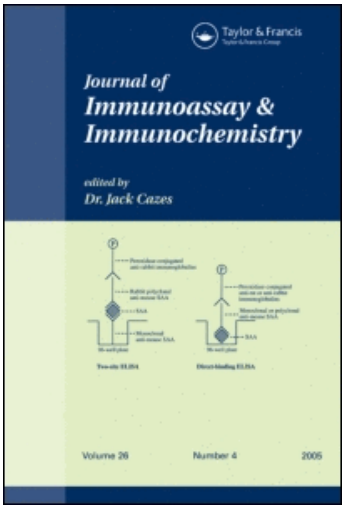

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MONOCLONAL ANTIBODIES TO PORCINE TUMOR NECROSIS FACTOR ALPHA: DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

Five hybridomas (4F4, 14H1, 9B4, 6E10, and 8G7) secreting antibodies to porcine tumor necrosis factor alpha (PTNF-α) were obtained from one fusion. Four of the 5 monoclonal antibodies (Mab) recognized recombinant PTNF-α (rPTNF-α) on western blot and were able to neutralize both rPTNF-α and native (released by porcine macrophages) PTNF (nPTNF-α, only 4F4, 14H1, and 9B4 tested for the neutralization of nPTNF-α) in vitro. A sandwich enzyme-linked immunosorbent assay (ELISA) for PTNF-α was developed using Mab 4F4 and purified rabbit polyclonal antibodies against PTNF-α. The test detected PTNF-α concentrations as low as 400 pg/ml and did not cross react with native porcine TNF-ß, recombinant human TNF-α, recombinant mouse TNF-ß or native mouse TNF-α. The Mabs and the ELISA should be useful for assessing PTNF-α levels in swine serum during disease processes and possibly for alleviation of toxic effects of TNF-α. (Key Words: Porcine Tumor Necrosis Factor alpha, Monoclonal Antibodies, Enzyme-linked Immunosorbent Assay).

INTRODUCTION

Tumor necrosis factor alpha (TNF-α) is a cytokine released predominantly by activated macrophages and to a lesser extent by monocytes. TNF-α usually is found as a 17 Kd soluble secreted molecule, although other forms have been reported (1, 2, and 3). TNF-α has diverse immunomodulatory, antitumor, antiviral, antiparasitic, and toxic effects (4, 5, 6, 7, 8, 9, 10, and 11).

Recently, TNF-α has been applied clinically for treatment of certain cancers and various parasitic infections (12). TNF-neutralizing antibodies have been shown to alleviate symptoms of toxic shock (8, 13 and 14). A sensitive and specific assay is required to monitor concentrations of TNF-α in tissue and serum. Cytotoxic assays using TNF-α sensitive cell lines (WEHI 164 clone 13 or L929) often are used for this purpose, but the assays are time-consuming and can be influenced by the presence of other cytokines, such as interleukin 1 and gamma interferon (15). Furthermore, these bioassays cannot distinguish TNF-ß from TNF-α. Enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays have been developed to detect human, mouse, and bovine TNF-α, and test kits for mouse and

human TNF-α are available commercially (16, 17, and 18). These tests, however, cannot be used to determine the level of TNF-α in other species due to species specificity of the antibodies used in the tests.

Previously, we described the molecular cloning and DNA sequence analysis of the gene encoding porcine TNF-α (PTNF-α) (20). More recently, we reconstructed the gene for production of the recombinant protein (rPTNF-α) in Escherichia coli (unpublished). In this report, we describe the production of murine monoclonal antibodies (Mabs) and rabbit polyclonal antibodies against rPTNF-α, and the development of a sensitive and specific sandwich ELISA for detection of native PTNF-α. This assay will facilitate studies on the role of TNF-α in porcine disease processes such as parasitic infections.

MATERIALS AND METHODS

TNF-α and TNF-β

The production and isolation of rPTNF-α using the FLAG™ system (IBI) will be described in detail elsewhere. rPTNF-α isolated by M1 Mab (anti-FLAG, IBI) chromatography was used to immunize mice and rabbits.

rPTNF-α to be used as the standard in an ELISA was further purified by a column packed with Mab against rPTNF-α as described below. Recombinant human TNF-α (rHTNF-α), native mouse TNF-α (nMTNF-α), and recombinant mouse TNF-ß (rMTNF-ß) were gifts from Dr. Rick Tarleton, The University of Georgia.

Native PTNF-α (nPTNF-α) released by porcine macrophages was obtained from swine peritoneal exudate cells. A 20 Kg pig was injected intraperitoneally with 13 mg of Propionibacterium acnes (Immunoregulin, Immunovet INC., Tampa, FL) suspended in 50 ml Minimal Essential Medium (MEM). The pig was anesthetized with Azaperone (0.5 mg/Kg) and Ketamine-HCl (1.75 mg/Kg) 7 days later and 500 ml of MEM was lavaged into the peritoneal cavity via a dialysis catheter (Peritoneal Dialysis Catheter, Travenol). The medium was withdrawn and cells were centrifuged, washed 3X with MEM, and incubated for 3 hr at 37 C in 5% CO_2. Adherent cells were stimulated with lipopolysaccaride (1-5 ug/ml) for 20 hr. The medium was harvested and tested for WEHI cell cytotoxicity. Samples with cytotoxic activity were pooled and concentrated with an ultrafiltration cell (Centricell, Polysciences).

Native porcine TNF-ß (nPTNF-ß) was obtained by stimulating porcine peripheral blood lymphocytes with 4-ß-phorbol-12-ß-myristate-13-α-acetate (PMA) and phytohemagglutinin (PHA). Peripheral blood in sodium-EDTA was obtained from swine and mixed (1:1) with RPMI-1640 supplemented with 10% newborn calf serum. Ten ml of this suspension was underlayered with 5 ml Histopaque 1077 (Sigma) and centrifuged for 45 min at 800 g. The mononuclear cell layer was removed and washed 3X with MEM. Remaining red blood cells were removed by hypotonic shock. Adherent cells were removed by incubating cells at 4 X 10^6 cells/ml in 175 cm^2 flasks for 1.5 hr at 37 C, 5% CO_2. Nonadherent cells were subjected to a second round of panning under the same conditions. Nonadherent cells from the second round of panning were washed 2X with Dulbecco's Minimal Essential Medium (DMEM), adjusted to 5 X 10^6 cells/ml, and cultured in RPMI-1640 supplemented with gentamycin (100 ug/ml), 10 ng/ml of PMA, and 5 ug/ml of PHA for 24 hr. Culture supernatants were tested by WEHI cell cytotoxicity assays and the ELISA described below.

Generation of Mabs Against PTNF-α

Three mice were immunized with rPTNF-α with 200 ul/each (100 ug) in Freund's complete adjuvant (FCA),

intraperitoneally and intradermally, in 50 ul aliquots, with 3 injections intradermally and 1 intraperitoneally. Two boosters were given with 100 ug/each in Freund's incomplete adjuvant (FIA), intraperitoneally. Procedures for fusion and cloning were performed by the Monoclonal Antibody Facility, The University of Georgia. Tissue cultures were screened by a dot-ELISA described below and by western blotting. Isotypes of the Mabs were determined by Ouchterlony assays using a mouse Mab typing kit (Sigma).

Dot-ELISA

rPTNF-α purified by M1 Mab column chromatography was applied to nitrocellulose (NC) membrane with 50 ng/dot of rPTNF-α in phosphate buffered saline (PBS, 0.01 M phosphate and 0.15 M NaCl, pH 7.3). The NC membrane was allowed to dry at room temperature (R.T.) for 30 min and was blocked with PBS/Tween20/bovine serum albumin (PTB, pH 7.3, 0.05% Tween20, and 0.2% BSA) for 40 min at R.T.. The NC membrane was incubated with 100 ul culture supernatants for 2 hr at R.T. with gentle shaking. Goat anti-mouse multivalent (IgG, A, M) peroxidase conjugate (1:500 dilution in PTB, Sigma) was added to

the NC membrane after washing 5X with dH_2O. The membrane was washed 5X with PBS, pH 7.3, after incubation with conjugate for 1 hr at R.T.. Subsequently, it was developed in substrate solution (3,3'-diaminobenzidine, Sigma). To exclude any Mabs recognizing the short FLAG fusion peptide, rPTNF treated with enterokinase (to remove the FLAG peptide) was also used in the dot-ELISA as a secondary test. Because free FLAG peptide does not bind to NC membrane, culture supernatants positive by this test were expected to contain Mabs against rPTNF-α itself. Supernatants positive by both dot-ELISAs also were tested for their ability to recognize rPTNF-α in western blot.

Western Blot

The western blot procedure has been described (21). Briefly, bacterial lysates containing rPTNF-α were resolved on 12% polyacrylamide gels and transblotted to NC membranes. Detection procedures were similar to those of the dot-ELISA.

Rabbit Polyclonal Antibodies

Two NZW rabbits were immunized with 200-400 ug rPTNF-α in FCA (1:1, v/v, in 1 ml), s.c., at several

sites. Four boosters were given with the same amount of rPTNF-α in PBS or FIA (1:1, v/v) at 2 wk intervals. Sera were evaluated by the dot-ELISA (above).

Affinity Chromatography

Mab (4F4) produced in mouse ascitic fluid was precipitated with 40% ammonium sulfate and dialysed in 4 liters of PBS, pH 7.2, at 4 C overnight, with 3 changes of buffer. Purified mouse Mab (4F4) was coupled to CNBr-activated Sepharose 4B according to manufacturer's instructions (Pharmacia). rPTNF-α isolated by the M1 Mab column was then passed through the 4F4 column. Bound rPTNF-α was eluted with 0.1 M glycine buffer, pH 3.0. The purity of eluted product was checked by SDS-PAGE with silver staining (21).

The rPTNF-α purified by both Mab columns was again coupled to CNBr-activated Sepharose 4B. About 10 ml (in PBS) of rabbit polyclonal antibody solution precipitated and dialysed as above from 15 ml sera was passed through the rPNFT-α column, washed with 5 X 2 ml of wash buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl), and eluted with 5 X 1 ml of 0.1 M glycine buffer (pH 3.0). Protein concentrations were determined by optical density measurements (260 nm and 280 nm).

ELISA

Ninety-six well plates (Immulon4, Dynatech) were coated with purified rabbit anti-PTNF-α antibodies (1.5 ug/ml in 0.1 M bicarbonate buffer, pH 9.6, 100 ul/well) at 4 C overnight. After washing 2 X with PBS/Tween20 (PBS, pH 7.2, 0.05% Tween20), the plates were blocked with 150 ul/well PTB at R.T. for 40 min. One hundred microliters of samples or rPTNF-α diluted in PTB was added to each well after washing 2X. The plates were incubated at R.T. for 2 hr, washed 2X, and then incubated with 100 ul of PTB containing Mab (4F4, 1:100) and goat anti-mouse IgG peroxidase conjugate (1:1000, Sigma) at R.T. for 1 hr. The plates were washed 6X, incubated with 100 ul/well substrate (ABTS, Kirkegaard and Perry Laboratories) at R.T. for 40 min, and read in an ELISA reader (Bio-Rad) at 405 nm.

Bio-assay for TNF Activity

The biological activity of rPTNF-α was determined by the in vitro cytotoxicity assay using WEHI 164 clone 13 murine fibrosarcoma cells as described by Morris et al. (22). For the antibody neutralizing assay, samples containing PTNF-α were incubated with different dilutions of antibodies at 4 C for 3 to 11 hrs before introduction to wells.

TABLE 1

Characteristics and Isotypes of Mouse Mabs Against PTNF-α

Mabs	Isotype	Reactivity on W-blot	Neutralizing Activity	Titer* Super.	Binding to Other TNF#
4F4	IgG_1	++++**	+	1:640	None
14H1	IgG_{2b}	++++	+	1:1280	None
9B4	IGg_1	++++	+	1:2560	None
6E10	IgG_1	++	+	1:2560	rHTNF-α
8G7	IgG_1	-	-	1:16	Not Done

* Titers of Mabs were determined by ELISA. Plates were coated with 5 ug/ml rPTNF-α. The dilution that had an O.D. higher than 2X O.D. of negative control was considered to be the titer.

TNF tested included rHTNF-α, rMTNF-ß, nMTNF-α, and nPTNF-ß.

** Reactivities were assigned visually according to color intensity on western blot.

RESULTS

Monoclonal Antibodies

Five stable hybridomas secreting antibodies against rPTNF-α (4F4, 14H1, 9B4, 6E10, and 8G7) were identified by dot-ELISA and western blotting (Table 1 and Fig. 1). Mab from 14H1 was determined to be in the IgG_{2b} subclass by Ouchterlony Assay while the remainder were IgG_1. Four of the 5 Mabs (except 8G7)

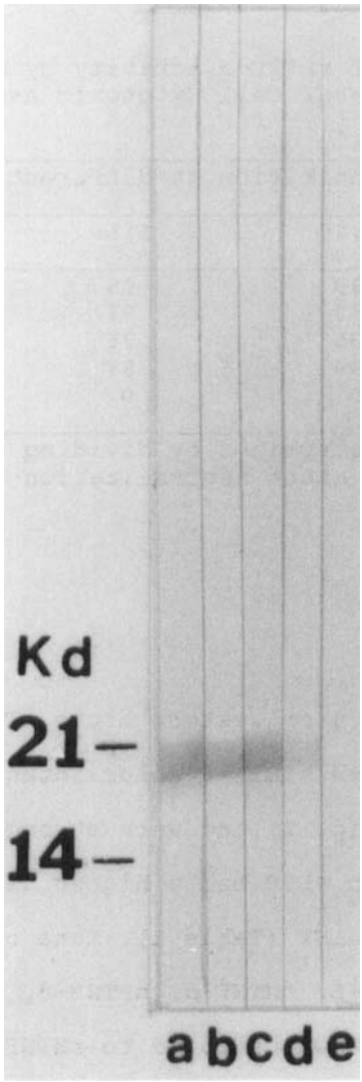

FIGURE 1. Western blot analysis of Mabs against PTNF-α. Bacterial lysate containing induced recombinant PTNF-α was resolved in 12% SDS-PAGE, transblotted to NC membrane, and probed with murine Mabs against PTNF-α. Lane a, Mab 4F4; lane b, 14H1; lane c, 9B4; lane d, 6E10, and lane e, 8G7. Four of the 5 mabs (except 8G7) recognized rPTNF-α on western blot.

TABLE 2

Neutralization of rPTNF-α Activity by Monoclonal Antibodies in a WEHI Cell Cytotoxic Assay

Mabs	% inhibition at different dilutions*		
	1:10	1:50	1:200
4F4	99	98	48
14H1	99	97	58
9B4	95	76	38
6E10	99	87	46
8G7	0	0	N/D**

* % inhibition determined by dividing the amount of rTNF-α remaining after neutralization by the amount of rPTNF-α added.

** not done

recognized rPTNF-α on western blots. The Mabs 4F4, 14H1, and 9B4 gave similar color intensities on western blots (Fig. 1) and were stronger than that of 6E10, even though 6E10 had a higher titer than 4F4 and 14H1 in an ELISA (Table 1). None of the Mabs recognized rMTNF-ß, nMTNF-α, nPTNF-ß, or rHTNF-α with exception of 6E10 which bound to rHTNF-α in the sandwich assay.

Four of the 5 Mabs neutralized rPTNF-α in the WEHI cell cytotoxicity assay (Table 2). The 4F4 and 14H1 neutralized about 60 ng/ml (16 Units/ml) rPTNF-α completely at a 1:50 dilution of culture supernatants

(titers see Table 1), while 9B4 and 6E10 neutralized the same amount of rPTNF-α at a 1:10 dilution in this assay. The Mabs (4F4, 14H1, and 9B4; 6E10 not done) also neutralized nPTNF-α (1.4 U/ml) completely at 1:10 dilution. Mab 8G7 did not show neutralizing activity in these assays.

Sandwich ELISA

A sandwich ELISA was developed for PTNF-α using purified rabbit polyclonal antibodies and murine Mab 4F4. The standard curve of the sandwich ELISA is presented in Figure 2. The lowest detection limit was 400 pg/ml, which was established by determining the mean absorbance +2SD of repetitive (PTB only) controls. Linear regression analysis showed a correlation coefficient of >0.99 for samples (triplicates) ranging from 400 - 25,000 pg/ml. The percent coefficient of variation (%CV) for samples ranging in concentration from 400 to 25,000 pg/ml were generally <10%. A test of a sample of rPTNF-α (0.06 mg/ml) by WEHI assay gave 16,000 Units/ml or 2.7×10^5 Units/mg. This indicates that the ELISA can detect as little as 0.1 units as determined by the WEHI assay, or in other words, the ELISA is about 10 times more sensitive than the WEHI assay for PTNF-α.

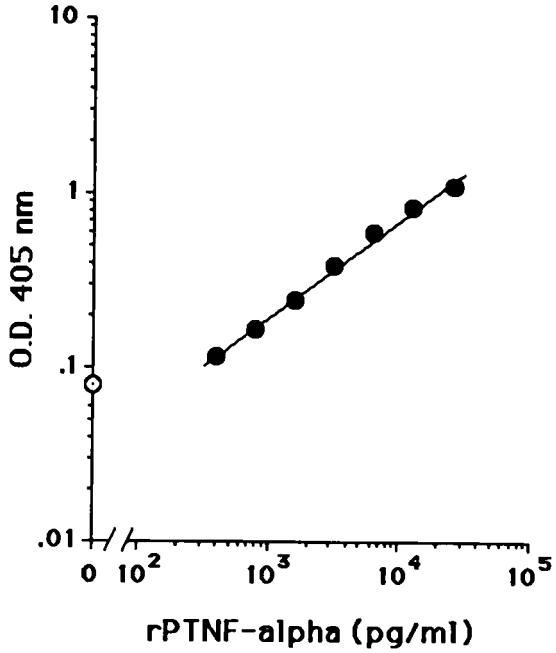

FIGURE 2. Typical standard curve for the sandwich ELISA assay. The open circle indicates the optical density of a TNF-α negative control.

TABLE 3

Specificity of the Sandwich ELISA in Testing Human And Mouse TNF-α and Other Closely Related Factors.

Samples	Concentration	O.D.405 nm*
rPTNF-α	0.4 ng/ml	0.108±0.006
Neg. (BSA)	0 ng/ml	0.074±0.004
rHTNF-α	490 ng/ml	0.067±0.009
nMTNF-α	16 U/ml#	0.075±0.007
rMTNF-ß	104 ng/ml	0.056±0.009
nPTNF-α	1.4 U/ml#	0.415±0.008
nPTNF-ß	2.3 U/ml#	0.061±0.004

* O.D.s are means of triplicates.

\# Culture media with TNF activities were determined by WEHI cell cytotoxic assays.

In order to examine specificity of the ELISA, we also tested human and mouse TNF-α and porcine and mouse TNF-ß by the sandwich ELISA. No cross reactivities were detected for nPTNF-ß, rHTNF-α, nMTNF-α, or rMTNF-ß (Table 3).

DISCUSSION

Monoclonal antibodies and polyclonal antibodies against human, mouse, and bovine TNF-α have been reported (8, 13, 16, 18, and 24). Some of these antibodies neutralize TNF-α activity and prevent the lethal effect of endotoxin. Highly sensitive and specific ELISAs for human and mouse TNF-α have been developed by using Mabs and/or rabbit polyclonal antibodies against human and mouse TNF-α. In this report, we describe the production of Mabs to PTNF-α and the development of a sensitive and specific sandwich ELISA for PTNF-α. Five stable Mab secreting hybridomas were obtained. Four of the 5 Mabs (except 8G7) neutralized rPTNF-α activities in vitro. Three of these were also shown to neutralize nPTNF-α (6E10 not tested). The 8G7 Mab gave a good precipitation band (comparable to 4F4 and 14H1) in the Ouchterlony assay (data not shown) but was very low in titer

(1:16), indicating a low affinity of this Mab for PTNF-α.

It was interesting to note that even though supernatants of Mabs 9B4 and 6E10 had higher titers (Table 1) than those of 4F4 and 14H1, they had lower neutralizing abilities than 4F4 and 14H1. Mab 6E10 bound weakly to rPTNF-α on western blot even though it showed a high titer in ELISA. This Mab was the only one which bound to rHTNF-α and partially neutralized feline TNF-α activity in WEHI assay (data not shown).

To determine PTNF-α in tissue and serum, we developed a sandwich ELISA using purified rabbit polyclonal antibodies and one of the Mabs (4F4). This ELISA detected both rPTNF-α and nPTNF-α and was more sensitive and consistent than the WEHI cell assay. This ELISA did not cross react with human or murine TNF-α, murine TNF-ß, or porcine TNF-ß.

Since TNF-α acts as a mediator of toxic shock and some inflammatory diseases (8, and 14), the Mabs and ELISA described herein should be useful for the evaluation of the functions of PTNF-α in porcine systems.

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