Quantitative Determination of TNFá, a Multipotent Modulator of Immune Response

1. Introduction

Tumor Necrosis Factor á (TNFá), also known as cachectin, is a polypeptide cytokine produced by monocytes and macrophages. It functions as a multipotent modulator of immune response and further acts as a potent pyrogen (1, 2). TNFá circulates throughout the body responding to stimuli (infectious agents or tissue injury), activating neutrophils, altering the properties of vascular endothelial cells, regulating metabolic activities of other tissues, as well as exhibiting tumoricidal activity by inducing localized blood clotting. TNFá also inhibits lipoprotein lipase activity resulting in cachexia, a physical wasting condition (1, 2). Activation of B-cells by the Epstein Barr virus can be inhibited by TNFá (3). Due to its varied actions throughout the immune system, TNFá may play a role in the pathogenesis of many disease states.

TNFá production is mediated by the action of lymphokines and endotoxins on the macrophage. Purified monocytes produce TNFá within four hours of stimulation by recombinant IL-2 (4) and there is some *in vitro* evidence to suggest that TNFá is expressed at high levels and with prolonged kinetics in T cells stimulated by both CD2 and CD28 (5). Secretion of TNFá is enhanced by gamma interferon. TNF then induces or enhances the specific production of Class I MHC antigen, GM-CSF, and IL-1. Recent evidence has suggested an intracellular role for this peptide (6).

TNFá may play a significant role in the pathogenesis of inflammatory disease of the joints and other tissues. Chin et al. (7) found that TNFá, along with gamma interferon and IL-1â increased cell surface expression of ICAM-1 on synovial fibroblasts. Alvaro-Garcia et al. (8) report that TNFá stimulates synovial proliferation.

Waage et al. (9) found that increased levels of TNFá in patients with septicemia and meningococcal disease correlated with fatal outcome. Scuderi et al. (10) suggest that increased levels of this cytokine may play a role in the host defense mechanism against parasitic infections. Girardin et al. (11) reported that increased serum TNFá levels correlated with the number of risk factors involved in children with gram-negative sepsis and *purpura fulminians.* Elevated levels of TNFá were also found in individuals suffering from myocarditis (12).

Recently, a growing body of information has pointed to a role for TNFá in the pathogenesis of AIDS.

Alveolar macrophages (AM) from HIV positive individuals with opportunistic lung infections have been shown to spontaneously produce higher levels of TNFá *in vitro* than those HIV positive individuals without infection and HIV negative controls (13, 14). Krishnan et al. (14) report that higher TNFá production by AM was associated with lower counts of *pneumocystis carinii* in broncheoalveolar lavage fluid, indicating that TNFá may play a role in the control of this infection in AIDS. Israel-Biet et al. (13) also reported in *in vitro* studies, that AM that express HIV($p24^+$) released significantly higher levels of TNFá than p24- alveolar macrophages and controls. Reddy et al. (15) found persistently elevated levels of circulating TNFá in HIV seropositive individuals and suggest a possible involvement of this cytokine in the development of AIDS.

Measurement of TNFá levels has also been shown to be useful in transplant research, where Maury et al. (16) and McLaughlin et al. (17). both reported TNFá to be markedly elevated in renal allograft rejection episodes. Recent evidence has been presented on increased TNFá levels in Bone Marrow Transplant (BMT) (19, 18). BMT patients with major transplant related complications such as interstitial pneumonitis and severe acute graft-versus-host disease had TNFá levels significantly increase over controls (19).

2. Materials

- human TNFá Instant ELISA (Bender Medsystems)
- Anthos Zenyth 340 Microplate Reader with Evaluation Software
- Adjustable single- and multichannel micropipettes
- Beakers, flasks, cylinders necessary for preparation of reagents

3. Specimen Collection

Cell culture supernatants, human serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8°C and separated rapidly before storing at -20°C to avoid loss of bioactive TNFá. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

4. Method

Natural human serum samples were applied to **Bender MedSystems Instant ELISA^{TM}** microplates. For incubation times and wash cycles refer to the corresponding instruction manual. Samples were measured with an **Anthos Zenyth 340** reader at 450nm, reference measurement was taken at 620nm.

5. Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.

- Standard curve is automatically calculated by Zenyth 340 microplate reader. In addition the instrument features 4 different modes of curve fitting:

  - point to point
  - linear regression
  -cubic spline
  - 4 parameter fit

- To determine the concentration of circulating TNFá for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding TNFá concentration.

- ***Samples have been diluted 1:2, thus the concentration read from the standard curve must be multiplied by the dilution factor (x2).**

- It is suggested that each testing facility establishes a control sample of known TNFá concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- A representative standard curve is shown in Figure 2. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

- N.B: There is a common dilution factor for standards and samples due to the conjugate. However only the dilution factor given in this manual has to be considered for the calculation of sample concentrations.

Standard	TNFαx Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	500	1.893	1.844	3.8
	500	1.794		
2	250	1.048	1.061	1.7
	250	1.074		
3	125	0.578	0.543	9.1
	125	0.503		
4	62.5	0.310	0.301	4.2
	62.5	0.292		
5	31.3	0.172	0.168	3.8
	31.3	0.163		
6	15.6	0.138	0.136	2.6
	15.6	0.133		
7	7.8	0.075	0.073	3.9
	7.8	0.071		
Blank	0	0.041	0.038	
	0	0.034		

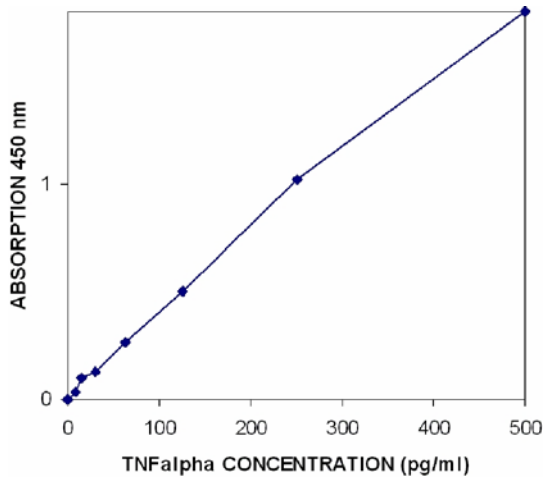

Fig. 1: Shows a representative standard curve, calculated by Zenyth 340 S onboard software

6. Results/Summary:

The ideal platform for absorbance measurement for the **Bender MedSystems Instant ELISA**TM turned out to be the **Anthos Zenyth 340 microplate reader.** This absorbance detector allows through its very flexible software a rapid and convenient read out and data processing. **Bender MedSystems' Instant ELISA** technology and **Anthos reader Zenyth 340** provide a complete solution for busy customers in Biotech and High Throughput laboratories. Both products ensure significant boost of production.

7. Literature

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