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Measurement of Tumor Necrosis Factor and Lymphotoxins

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Abstract

The tumor necrosis factor (TNF) superfamily of cytokines plays critical roles in all aspects of the immune response. TNF and the lymphotoxins (LT), LT α and LT β , are particularly important as major effector cytokines and mediators of lymphoid organ development. One of the classical methods for the measurement of TNF and LT α activity is by demonstrating their ability to lyse certain target cells. A detailed protocol for the measurement of this activity using a highly sensitive indicator cell line is presented. More recently, ELISA assays have been developed to measure the protein concentration of these cytokines in any type of biologic fluid.

Keywords

TNF; lymphotoxin; cell death

INTRODUCTION

The Tumor Necrosis Factor (TNF)/Lymphotoxin (LT) family is encoded by three genes linked within the Major Histocompatibility Complex—*TNFA*, *LT α* , and *LT β* . TNF is expressed as a membrane molecule, but can be enzymatically cleaved from the cell surface to form a homotrimer which binds to two distinct receptors, TNFR1 and TNFR2. LT α (formerly called TNF β) is secreted as a homotrimer and also binds to both TNF receptors, but in addition can bind to a second member of the TNF receptor family, herpes virus entry receptor mediator (HVEM). LT β anchors LT α to the cell surface by the formation of heterotrimers. The predominant form is an LT α 1/LT β 2 complex, but a complex of LT α 2/LT β 1 has also been reported. LT α 1/LT β 2 is the primary ligand for the LT- β R. TNF is a multifunctional cytokine produced by many different cell types, including macrophages after stimulation via Toll-like receptors and T and B lymphocytes after triggering via their antigen receptors. LT α and LT β are produced by a more restricted number of cells, including T and B lymphocytes. Most importantly, lymphoid tissue inducer (LTi) cells are an important source of both LT α and LT β , which play critical roles in the development of secondary and tertiary lymphoid organs (Ruddle, 2014).

The following protocol employs TNF-sensitive, actinomycin D–treated murine L929 fibroblasts to quantify TNF or LT α activity in supernatants derived from cell cultures, serum samples, or cerebral spinal fluid. While the assay can measure picogram concentrations of human, rat, and murine TNF or LT α , it cannot distinguish between these two cytokines.

This unit presents a Basic Protocol for for measuring TNF and LT α . Many commercial suppliers provide species-specific ELISA kits for detection of TNF and LT α and LT β . These are summarized in Table 6.10.1.

BASIC PROTOCOL

CELL DEATH ASSAY FOR THE DETECTION OF TNF/LT- α

Some cells that bind TNF and LT α on their cell surface lyse as a consequence of the binding reaction.

Materials—TNF-sensitive L929 fibroblasts (Support Protocol)

Supplemented Eagles minimum essential medium (EMEM; *UNIT 6.9*; Vogel et al., 1999)

Actinomycin D solution

0.9% (w/v) saline (Abbott)

0.05% crystal violet (w/v) in 20% ethanol

100% methanol

96-well flat-bottom microtiter plate (Falcon, cat. no. 3072 or Costar, cat. no. 3596) 8-well aspirator (Drummond; e.g., Thomas Scientific cat. no. 7691-R32)

8-channel pipettor (fixed or adjustable to 50 μ l and 100 μ l; e.g., Costar Octapette #4850, #4800)

Microtiter plate reader with 595-nm filter (optional)

Grow the fibroblasts

1. Plate L929 fibroblasts by adding 100 μ l fibroblast suspension to each well of a 96-well flat-bottom microtiter plate. Allow one complete horizontal row for each sample to be tested. Incubate overnight in a 37°C, 6% CO₂ humidified incubator.

Using an inverted microscope, check the confluency of cells in the wells before proceeding. Each well must be equivalently confluent for the assay to be reproducible.

2. Aspirate all medium from each well of the microtiter plate with an 8-well aspirator.

Tilt the plate slightly to avoid damaging the monolayer with the aspirator tips.

3. Add 50 μ l supplemented EMEM to every well.
4. Add 50 μ l test sample to the second well of each row (column 2). Make 2-fold serial dilutions by gently mixing the contents of well 2 and transferring 50 μ l from well 2 into well 3. Mix the contents of well 3 gently, and transfer 50 μ l

from well 3 into well 4. Continue this procedure through well 12. Finally, gently mix the contents of all wells in column 12 and discard 50 µl from each well. At this point all wells should contain 50 µl.

Serial dilutions done in this manner are greatly facilitated by the use of an 8-channel pipettor set for 50 µl.

5. Add 50 µl actinomycin D solution to each well. Incubate the plates 18 hr in a 37°C, 6% CO₂ humidified incubator.

Harvest the plates

6. Aspirate all supernatant in each well and wash L929 cells once with 200 µl of 0.9% saline in each well.
7. Aspirate the saline and add 50 µl of 0.05% crystal violet in 20% ethanol to each well. Stain wells 10 min at room temperature.

After staining cells, the control wells containing medium only (column 1) should be confluent, intact, and stained a very dark violet (Fig. 6.10.1).

8. Rinse the crystal violet thoroughly out of the wells using cold tap water. With a sharp flick of the wrist, shake each plate once to rid the wells of excess water.

Do this action in the air. Do not bang the plates against a hard surface (e.g., the benchtop) in an effort to remove excess water as this will dislodge the remaining adherent cells.

9. Discard lids, lay plates face down on absorbent paper, and allow to dry overnight.

Score the plates for TNF or LTα activity

6. Add 100 µl of 100% methanol to each stained well to elute stain from cells.
7. Read each well immediately with a microtiter plate reader at an absorbance of 595 nm. Use wells that exhibit maximum lysis as blanks and medium control wells (column 1) as a measure of maximal uptake of crystal violet.
8. Wells that exhibit an A_{595} closest to 50% of the arithmetic mean of the maximal control are considered to represent 50% lysis of the L929 cells. To calculate TNF or LTα U/ml of a sample, multiply the reciprocal of the highest dilution resulting in 50% lysis by 20 (20 is the dilution factor of the sample added to each well).

L929 fibroblasts lines vary widely in TNF sensitivity from laboratory to laboratory. Therefore, it is essential to establish that the fibroblasts acquired for use in this assay are TNF-sensitive before assaying test samples and include a positive control (e.g., recombinant TNF of an established bioactivity measured in U/ml) in the assay to assess the sensitivity of the assay and to establish “lab units.”

ALTERNATE PROTOCOL

MEASUREMENT OF TNF AND LYMPHOTOXINS BY ELISA ASSAYS

A number of kits are available from commercial sources for the specific detection of TNF, LT α , and LT β in serum, culture supernatants, or other sources. A list of several of these sources for measurement of human and mouse TNF and the lymphotoxins is given in Table 6.10.1. Kits for measurement of TNF and lymphotoxins from other species are also available.

SUPPORT PROTOCOL

PROPAGATION AND PREPARATION OF L929 FIBROBLASTS

Additional Materials (also see Basic Protocol)—L929 fibroblasts (see critical parameters)

Trypsin solution (*UNIT 6.9*; Vogel et al., 1999)

150-cm² tissue culture flask

50-ml conical centrifuge tube

Additional reagents and equipment for cell counting and evaluating cell viability by trypan blue exclusion (*APPENDIX 3B*; Strober, 2015)

1. Inoculate 50 ml supplemented EMEM with 4×10^5 L929 fibroblasts in a 150-cm² tissue culture flask.
2. Incubate flask for 5 to 7 days (or until the cells have reached confluency) in a 37°C, 6% CO₂ incubator. On day 4 or 5, aspirate the medium, leaving the adherent cells, and replace with 50 ml fresh supplemented EMEM.

As these are adherent cells, it is essential that the flasks incubate horizontally, with the cells resting on the treated side of the flask.
3. When cells have reached confluency, aspirate medium from flask and add 4 ml trypsin solution to the cell monolayer. Incubate ~ 1 to 2 min at 37°C.
4. Strike flask vigorously against the palm of the hand to dislodge cells. Quickly add ~ 15 ml supplemented EMEM to stop the enzymatic action of the trypsin.
5. Transfer cell suspension into a sterile 50-ml conical centrifuge tube and centrifuge 10 min in a tabletop centrifuge at $\sim 650 \times g$, room temperature. Decant supernatant from cell pellet.
6. Add 10 ml supplemented EMEM to the pellet and gently resuspend by repeated pipetting. Count cells and evaluate viability by trypan blue exclusion (*APPENDIX 3B*; Strober, 2015). Resuspend in supplemented EMEM to a final concentration of 4×10^5 cells/ml.

The cells are now ready to be used in the TNF- $\alpha\beta$ assay. To continue propagation of the cells for subsequent assays, repeat step 1.

REAGENTS AND SOLUTIONS

Actinomycin D solution

Stock solution, 500 µg/ml: Add 2 ml sterile H₂O to a vial containing 1 mg actinomycin D-mannitol (water soluble). Store at –20°C. Stock solution can be frozen and thawed repeatedly.

Working solution, 8 µg/ml: Dilute stock solution 1:62.5 just before use with cold supplemented EMEM (*UNIT 6.9*). Keep on ice until used.

COMMENTARY

Background Information

TNF, a multifunctional cytokine elaborated primarily by monocytes and macrophages (Rosenblum and Donato, 1989), was first observed when a serum factor directly caused the hemorrhagic necrosis of transplantable tumors in vivo (Carswell et al., 1975). Subsequently, it was discovered that the T cell product, lymphotoxin-α (TNF-β), was structurally related to TNF and exhibited an overlapping spectrum of bioactivities. Many cells bear receptors on their surfaces which bind these two distinct cytokines (Le and Vilcek, 1987). For some of these cells, the consequence of binding TNF or LTα to their receptors is cell lysis. This fact facilitates the detection and quantification of TNF/LTα in bioassays. The assay described here employs TNF/LTα sensitive L929 fibroblasts (Wang et al., 1985; Hogan and Vogel, 1988). It is sensitive, inexpensive, does not require radioactivity, and accommodates many samples; however, the assay cannot distinguish between TNF and LTα.

An alternative assay described by Chen et al. (1985) uses a ⁵¹Cr-labelled WEHI 164 cell line. The natural killer cell-resistant murine fibrosarcoma cell line, WEHI 164, when treated with actinomycin D, has been shown to be a sensitive target for the lytic action of human monocyte-derived TNF/LTα (Chen et al., 1985). This cell line is weakly adherent and is employed as a target in short-term, radiolabeled, chromium release assays. As such, WEHI 164 can be cocultured with monocytes (effector cells) or exposed to cell-free supernatants. This assay is faster than the fibroblast procedure (6 hr vs. 48 hr) but requires the use of radioactive material, and is ~10 times less sensitive for TNF than L929 fibroblasts (Espevik and Nissen-Meyer, 1986).

A clone of this parent cell line, WEHI 164 clone 13, has been reported to be ~1000 times more sensitive than WEHI 164 and 100 times more sensitive than L929 fibroblasts for human monocyte-derived TNF. This hypersensitive clone was obtained via limiting dilution of the parent cell line and screening of subsequent progeny, a procedure that is highly labor intensive. WEHI 164 clone 13 cells do not require actinomycin D treatment nor will actinomycin D treatment enhance their sensitivity. This cell line is used as a target for both cell-free supernatant and monocyte coculture assays. This assay is desirable for samples containing low levels of TNF.

TNF and lymphotoxins can also be identified and quantitated in supernatants derived from activated macrophages using an immunoconcentration/immunoblotting technique (Beutler et

al., 1986). This technique involves concentrating supernatants from activated macrophages and analyzing them on immunoblots, using an immunoreactive antibody to TNF. The sensitivity of the immunoblot technique appears to be highly dependent upon the anti-TNF antibody used in the assay, although a direct comparison of its sensitivity with that of the other assays has not been made. Finally, while the immunoblot assay will detect TNF protein, it does not provide information as to the functional activity of the sample. One other approach that can be used to determine if an observed biologic activity in vitro or in vivo is mediated by TNF or the lymphotoxins is to attempt to neutralize that activity with a neutralizing monoclonal antibody or a soluble receptor. A truncated version of the TNFRII fused to the Fc of immunoglobulin is available as an Etanercept (EMBREL; Amgen, Pfizer) and will inhibit the biologic activities of TNF and LT α . A soluble form of the LTBR has been generated which is capable of neutralizing the activity of LT α 1b2 (Browning, 2008).

Critical Parameters

The reproducibility of this assay is critical and is dependent on many parameters, including technical skill in performing the assay and precise propagation of the cell lines. For every laboratory, week-to-week variability of the assay must be established. In addition, laboratory units of TNF should be established relative to a standard. At this time, there is no international reference reagent for the standardization of TNF units as exists for the interferons; therefore, we have used human recombinant TNF (Cetus) as a positive control to standardize our assays.

Not all L929 fibroblasts are TNF-sensitive and those that are, vary in their degree of sensitivity. To establish a sensitive L929 fibroblast cell line, and eliminate the tedium of screening many cell lines, it is recommended that TNF-sensitive L929 fibroblasts be obtained from a laboratory that routinely carries out this assay. Once an optimal assay system has been established, aliquots of this stock cell line should be frozen for future use. Long-term storage in liquid nitrogen is recommended. As thawing and reestablishment of the stock cell line becomes necessary, fresh cells should be frozen to ensure an adequate future supply. Finally, it is important to establish a schedule of cell feeding and splitting and to adhere to that schedule, as variations in growth times can have an influence on the stability of the cell line. If possible, keep long-term cultures in a separate incubator to minimize disturbances.

Troubleshooting

Irregularities or holes in the cell controls and in the experimental wells after staining with crystal violet may have a variety of causes. (1) Check the confluency of the L929 fibroblasts monolayers 24 hr after the initial plating in 96-well plates. They may not be confluent, necessitating seeding at a slightly higher density. Alternatively, they may be overgrown, necessitating seeding at a lower density. (2) Cells may have dislodged during “flicking” after staining. Don’t be too vigorous or strike the plate against any hard surface. (3) Check the position of the tips on the pipettor when adding samples as well as the position of the tips of the aspirator. Cells will be damaged by jabbing into the monolayer. (4) Check for contamination. If cell culturing techniques are practiced carefully, contamination may not be immediately apparent. Mycoplasma contamination is more difficult to discern than fungal or

bacterial contamination. Cells in mycoplasma-contaminated cultures may have erratic growth kinetics, may become nonadherent, or appear “peppered” intracellularly. If contamination is suspected, discard the contaminated culture and repropagate with frozen stock aliquots.

TNF activity can be lost by acquired resistance or contamination. Test L929 fibroblasts with a TNF preparation (e.g., rTNF) of previously established activity. If there is a loss of activity, thaw an aliquot of frozen stock L929 cells from the original culture and repropagate. Discard the TNF-resistant L929 cells and check for contamination (as above).

Anticipated Results

A representative plate from a TNF- $\alpha\beta$ assay is shown in Figure 6.10.1. Column 1 is the untreated cell control. These are L929 fibroblasts that were exposed to medium only. They are undisrupted and darkly stained. The wells in column 2 (rows A-H) are the highest concentration of the experimental samples tested (1:4). Column 3 is a two-fold dilution of column 2 (1:8), column 4 is a two-fold dilution of column 3, and so forth. For any sample, a titration of the TNF can be observed, as evidenced by the gradual reappearance of staining. This increased staining corresponds to increased L929 fibroblast viability as the lytic effect of TNF becomes diluted out. Ideally, a total lysis of L929 cells is seen with the higher concentrations of experimental sample (evidenced by clear, unstained wells), gradually moving toward darkly stained, confluent cells in the wells where no TNF activity is detected. This pattern indicates that the sample TNF concentration is within the range of the assay (<80 to 81, 920 U/ml). If the sample does not titer within this range (i.e., if all wells are clear), dilute the sample prior to adding it to column 2. If, on the other hand, the sample has no apparent lytic activity (all wells are darkly stained), it can be presumed that there is no detectable TNF activity as measured by the protocol. The detection limit of the assay is 50 to 100 pg of TNF or LT α .

Time Considerations

The TNF/LT α bioassay using actinomycin D–treated L929 fibroblasts can be performed in 3 days. The fibroblasts are split in the afternoon of the first day for propagation and for plating the TNF assay. In the hands of an experienced technician, splitting and cell counting takes ~30 min and plating takes ~1 min per plate. The plates are incubated for ~24 hr, at which time they are checked for confluence. If the cells are sufficiently confluent, experimental samples are added, serially diluted, and actinomycin D–treated. This takes ~3 to 5 min per plate. These plates incubate ~18 hr, at which time the wells are aspirated, washed, and stained. The plates are left to dry overnight and scored the next day or at anytime thereafter.

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Key References

- Hogan and Vogel, 1988. See above.
- Wang et al., 1985. See above.

Figure 6.10.1.
Representative microtiter of a plate for the TNF- α bioassay using actinomycin D-treated L929 fibroblasts.

Table 6.10.1

Commercially Available Kits for the Specific Detection of TNF, LT- α , and LT- β in Serum, Culture Supernatants, and Other Sources

KIT	SOURCE
Human TNF	R&D Systems; ebioscience; ThermoFisher Scientific
Mouse TNF	abcam; ebioscience; R&D Systems
Human LT α	ebioscience; R&D Systems; ThermoFisherScientific
Mouse LT α	Abbexa Ltd; antibodies-online.com ; My Biosource
Human LT β	LifeSpan Biosciences; Ray Biotech; Biomatk
Mouse LT β	LifeSpan Biosciences; My BioSource

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.