Improvement of a cytokine (TNF- α) bioassay by serum-free target cell (WEHI 164) cultivation

Yngve Figenschau^{1*}, Baldur Sveinbjörnsson² & Kjell Bertheussen¹

¹ Department of Obstetrics and Gynaecology, University Hospital of Tromsø, Tromsø, N-9038, Norway

² Department of Experimental Pathology, University of Tromsø, Tromsø, N-9037, Norway

Received 18 March 1998; accepted 21 September 1998

Key words: bioassay, cytotoxicity, serum-free, tetrazolium, tumour necrosis factor

Abstract

The elaboration of a sensitive bioassay for assessment of tumour necrosis factor alpha (TNF- α) in a defined medium is described. The assay is based on the cytotoxic effect of TNF- α on a target cell line, the murine fibrosarcoma WEHI 164 clone 13. Cytotoxicity was assessed by detecting the rate of tetrazolium salt reduction employing a spectrophotometer (ELISA-reader). A similar bioassay was used previously to assess TNF- α , though this was dependent on cell growth in a medium containing serum. By employing a synthetic serum replacement, the WEHI cells were adapted to growth in a defined medium which allowed both the propagation of the cell line and the assay to be performed under completely defined conditions. Thus, factors in serum that may influence the TNF- α assessment, such as growth factors, cytokines, soluble cytokine-receptors and macroglobulin, were avoided. The only protein required in this bioassay was insulin, while albumin was added as a carrier protein and to protect the cytokine against loss of biological activity during multiple freeze and thaw cycles. The present assay was optimised to achieve a high sensitivity and, by testing endogenous TNF- α originating from the macrophage-like cell line RAW in both the serum-free and serum-based assay, we found the highest sensitivity in the assay based on defined medium. The LC₅₀ of recombinant mouse and human TNF- α were in the serum-free and serum-based assays considered to be 25 and 50 pg mL⁻¹, respectively. The demonstration of a culture condition that enables long-term cultivation of target cells and a bioassay in a completely defined medium is in our opinion a substantial contribution to more reliable cytokine assessment.

Introduction

There are two main classes of assays for assessment of tumour necrosis factor alpha (TNF- α): bioassays and immunochemical assays. The bioassays are usually based on cytotoxicity towards different target cells, e.g. L-929 cells, L-M cells, EMT-6 and WEHI 164 cells. The objective measurement of cytotoxicity is carried out by a variety of techniques including uptake of vital dye, incorporation or release of radioactive labelled materials and reduction of a tetrazolium salt (Meager et al., 1989). The last method was demonstrated by Mosmann (1983) to be a rapid and simple technique for detection of cell proliferation and cyto-

toxicity, and was later utilized in a bioassay for TNF- α with the target cell WEHI 164 clone 13, a murine fibrosarcoma cell line cultivated in serum containing medium (Espevik and Nissen-Meyer, 1986). Bioassays have the advantage of detecting the amount of cytokines that are biologically active, in contrast to immunoassays, that are based on antibodies recognizing an epitope on a TNF- α molecule which may not be biologically active due to aggregation, fragmentation and denaturation (Meager et al., 1989). Thus, immunoassays often numerically exceed bioassays in the amount of TNF- α detected. Bioassays are claimed to be less specific than immunoassays and also associated with high inter-assay variation, which has been suggested caused by the serum supplement in the cell

* Author for all correspondence.

cultivation media (Kramer and Carver, 1986; Meager et al., 1989).

Recently, the traditional use of serum has been questioned and efforts made to elaborate culture conditions that support long-term cell growth and bioassays in a defined medium (Bertheussen, 1993; Figenschau et al., 1997a; Figenschau et al., 1997b). Under normal physiologic condition, only blood cells are surrounded by plasma and the use of serum when cultivating other cell types may thus be controversial. Although TNF- α is considered to be a pro-inflammatory cytokine and is produced by cells within the white blood-cell family, these cells are not necessarily surrounded by serum at the site of action. There is also a large body of evidence for the existence of other TNF- α producing cells which are not blood-cells and hence are foreign to the serum that cells usually are exposed to *in vitro*. The undefined nature of serum includes a multitude of factors that in an unknown way may influence cell function or the parameters investigated. The amount of polypeptides, hormones, growth factors and cytokines in serum is unknown. Such factors may act synergistically or antagonistically with TNF- α , e.g. the synergistic action of Interferon gamma (IFN- γ) or the antagonistic action of Interleukin 1 (IL-1) and Transforming Growth Factor beta (TGF- β) (Aggarwal et al., 1985; Holtmann and Wallach, 1987; Sugarman et al., 1987). Also, the assessment of cytokines may be complicated by serum factors that bind specifically to cytokines, like secreted forms of soluble membrane receptors (Van Zee et al., 1992; Olsson et al., 1993), or by interference with α -2-macroglobulin (Fomsgaard et al., 1989; James, 1990). Two soluble receptors for TNF- α have been identified: sTNF-R55 and sTNF-R75 (Lewis et al., 1991). These have been detected in serum from healthy individuals at levels of ng mL⁻¹, typically exceeding the circulating pg mL⁻¹ levels of TNF- α (Aderka et al., 1992; Olsson et al., 1993).

The use of slaughterhouse-derived sera is an additional source of microbial contamination which may require termination of the cell culture, or complicate interpretation of the results if contamination is obscure. This has been demonstrated by the findings that pathogenic organisms like viruses may subvert the cytokine network by encoding secreted cytokine binding proteins, e.g. Shope fibroma virus encodes a secreted binding protein for TNF (Smith et al., 1991).

It has also been observed that WEHI 164 clone 13 cells may lose their TNF- α susceptibility during long-term propagation (Espevik and Nissen-Meyer, 1986;

Fomsgaard et al., 1988; Meager et al., 1989). This may be attributed to batch to batch variation of the serum sources (Bertheussen et al., 1989; Meager et al., 1989) causing selection of less susceptible subclones which explains some of the problems with standardization of TNF- α measurement (Elborn et al., 1995).

Finally, the proteins in serum may cause an additional source of error by precipitating in the acidic solvent that is usually employed in the bioassays. This causes additional absorbance when the tests are read in a spectrophotometer and complicates interpretation of the results (Denizot and Lang, 1986).

Yet, there are to our knowledge no reports describing a bioassay that allows assessment of TNF- α under defined conditions. Kramer and Carver (1986) managed to exclude serum from a TNF- α bioassay by cultivating the murine L-M cells in a medium supplemented with Bacto-PeptoneTM. However, their culture conditions were undefined due to the nature of Bacto-PeptoneTM which consists of materials originating from enzymatic digestion of animal tissue. A medium supplemented with Bacto-PeptoneTM has been demonstrated to contain a large amount of the hormone estrone (Feldman and Krishnan, 1995), clearly illustrating the undefined nature of that substance.

In the present investigation we used the medium DME/F-12 supplemented with 0.1% RenCyte3TM, to propagate the target cell line WEHI 164 clone 13 until decided termination, after ca. 6 months. The only proteins present were 0.5 μ g mL⁻¹ recombinant insulin (Bertheussen, 1993) and albumin (0.1%). Albumin was added to conserve the biological activity of TNF- α (Eskandari et al., 1990) when samples were subjected to freezing and thawing cycles and as a carrier protein according to the TNF producer's suggestion. Full control of all metals in solution (iron, trace elements and heavy metals) was achieved by a novel metal buffer. All metals necessary for cell growth, including Fe, Zn, Cu, Mn, Ni, Al, Cr, Co and Se are presented as stabilised chelated compounds without transferrin or other binding proteins (Bertheussen, 1993).

Since intracellular formazan production is linked to the energy generating process in the cells, i.e. glycolysis (Berridge et al., 1996), and the energy source in a serum-free medium is different from that in serum (Bertheussen, 1993), we investigated several critical parameters that specifically influence both production and measurement of formazan as recommended in a previous report (Vistica et al., 1991). We aimed

to elaborate an optimized and sensitive bioassay for TNF- α based on a stable cell line propagated in a defined medium, thereby enabling cytokine assessment exclusive of any adverse effects emanating from serum.

Materials and Methods

The anchorage dependant murine fibrosarcoma cell line WEHI 164 (American Type Culture Collection, ATCC; CRL-1751) clone 13 (Espevik and Nissen-Meyer, 1986) was a kind gift from Dr. A. Waage (Institute of Cancer Research, University of Trondheim, Trondheim, Norway). The cell line had been kept in our laboratory for >1 yr by propagation in tissue culture flasks (Cat. No. 3813; Falcon®, Oxnard, CA, U.S.A.), employing the culture medium RPMI 1640 (Cat. No. R-6504; Sigma®, St. Louis, MO, U.S.A.) supplemented with 10% FCS (Fetal Calf Serum, Hyclone®, Lund, Sweden). The adaptation of the WEHI cells to serum-free growth was carried out by transferring the cells to the medium DME/F12 (Dulbecco's Modified Eagle's medium/Ham's F-12 medium, Cat. No. 32-500-035; Gibco®, Paisley, UK), supplemented to 10% with heat-inactivated FCS. The incubation was carried out at 37 °C in humidified air containing 5% CO₂ in tissue culture vessels coated with Primaria™ (Cat. No. 3846; Falcon®). The portion of serum in the medium was successively reduced in consecutive passages to 1.25% FCS, i.e. 10–7.5–5–2.5–1.25%. Then the subsequent cultivation was carried out with 0.1% of the synthetic serum replacement RenCyte3™ (Cat. No. 22363010; Medi-Cult®, Copenhagen, Denmark).

The growth rate of the adapted WEHI cells was tested in tissue culture flasks with different coatings: on gelatine-extracted fibronectin (from serum); on gelatine that had not been exposed to serum; on Primaria™ coated vessels; and on uncoated plastic vessels (Cat. No. 3013E; Falcon®).

Fibronectin was extracted from serum as briefly described here: a stock solution of gelatine (Cat. No. 1038; Medi-Cult®) was diluted 1:20 (1 mg mL⁻¹, final concentration) in MEM (Minimal Essential Medium Eagle, Cat. No. M-0268; Sigma®) and added to vessels in volumes that equalled half the volume of cultivation medium later used. After a further incubation of one hour (h) in room temperature, the gelatine solution was removed and the vessels were washed twice with PBS (Dulbecco's Phosphate

Buffered Saline, Cat. No. D-8662; Sigma®). FCS was added to the vessels in 1/4 the volume of medium later used and incubated overnight in room temperature. Thereafter, FCS was removed and the vessels were washed three times with PBS before use.

The passing of the cell cultures was done by discharging the old medium, adding fresh warm medium and subsequently detaching the cells with a cell scraper (Cat. No. 3010, Costar Europe Ltd.®, Badhoevedorp, the Netherlands). The suspension was further centrifuged for five minutes (min) at 200 times gravity (200G) and, when the supernatant had been removed, the pellet was dispersed in 5 mL fresh medium before the centrifugation procedure was repeated. The viability was assessed in a Bürker chamber by Trypan-blue exclusion and the cells were seeded in Primaria™ coated 96-well plates/microtiter plates (Cat. No. 3872, Falcon®) in a total volume of 100 μ L medium. Each plate contained a serial dilution of triplicates of laboratory standards with known TNF- α activity calculated according to the International Units (NIBSC international standard for TNF- α , code 87/650).

To confirm that the observed cytotoxicity was due to TNF- α activity, neutralising antibodies were used in an additional serial dilution of the test materials. Neutralisation was achieved by a 4 h preincubation of the test material in 45 μ L medium plus 5 μ L antibody before the cells were added in an appropriate number in 50 μ L.

After 20 h of incubation, each well except those that served as blanks received the tetrazolium salt [3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide], MTT (Cat. No. M-2128; Sigma®) at a final concentration of 0.8 mg mL⁻¹. The MTT solution was prepared in PBS (phosphate buffered saline, Cat. No. D-8662, Sigma®) by sterile filtration (0.2 μ m). After an additional 3 h incubation interval which allowed the cells to produce formazan, the old medium was removed and 200 μ L of a solvent was added to each well. Subsequently, the wells were subjected to stirring with a multichannel pipette and the absorbance was assessed at 570 nm with reference wavelength 690 nm in a spectrophotometer/ELISA reader (Reader 510; Organon Teknica®, Boxtel, the Netherlands).

The cytokines used were recombinant mouse (rmTNF- α ; Genzyme®, Cambridge, MA, U.S.A.) and recombinant human TNF- α (rh TNF- α , Cat. No. T-0157; Sigma®). These were neutralised by polyclonal rabbit anti-mouse TNF- α (Cat. No. IP-400, Genzyme®) and polyclonal rabbit anti-human TNF- α (Cat. No. T-8300, Sigma®), respectively. As

suggested by the producer, a dose response assay was performed to determine the optimal antibody concentration, which was considered to be 10% of the purchased stock solution. The dilution was done in PBS plus 0.1% BSA (Bovine Serum Albumin; Cat. No. A-8022, Sigma®). Biologically produced (endogenous) TNF- α was prepared by challenging the murine macrophage-like cell line RAW 264.7 (ATCC; TIB-71; 1.0×10^5 cells mL⁻¹ medium) with 1 μ g mL⁻¹ (final) lipopolysaccharide (LPS, Cat. No. L-8274; Sigma®) for 24 h. The RAW cells were cultivated in tissue culture flasks (Cat. No. 3013E; Falcon®) in DME/F12 supplemented with 10% serum. TNF- α production by these cells was stimulated in a serum-free medium; the cells were detached with a cell scraper, centrifuged at 200G for 5 min and the pellet resuspended in DME/F12 with 0.1% Rencyte3™. After repeating this procedure twice, the cells were seeded in tissue culture flasks and further cultivated in DME/F12-RenCyt3™ for two weeks with medium exchange every second day until challenge with LPS.

The serum-based bioassay was performed in accordance with an established method (Espevik and Nissen Meyer, 1986). Briefly, 2.0×10^4 cells were seeded in microplates with 200 μ L RPMI 1640 supplemented with 10% heat inactivated FCS and incubated for 20 h, then MTT (1.0 mg mL⁻¹ final) was added and, after a further 4 h incubation, the formazan crystals were dissolved in 200 μ L isopropanol to which had been added 0.04 N HCl. The plates were read at 570 nm (nano meter) and all samples were tested as triplicates.

Three different solvents were examined: acidic alcohol (isopropanol with 0.3% hydrochloric acid); SDS/DMF (Sodium Dodecyl Sulphate, Cat. No. 822050; Merck®, Darmstadt, Germany and Dimethylformamide, Cat. No. 40250; Fluka Chemika®, Buchs, Germany), 1:1, pH 4.7, adjusted with sulphuric acid and acetic acid prepared as described by Hansen et al. (1989); and DMSO (Dimethylsulphoxide, Cat. No. D2650; Sigma®). Scanning of the optical densities in the solvents containing dissolved formazan was carried out in a spectrophotometer (U-2000; Hitachi®, Tokyo, Japan).

The cytotoxicity was calculated as % dead cell (Espevik and Nissen-Meyer 1986):

$$100\% - \left[\frac{\text{Absorbance in test* wells}}{\text{Absorbance in control** wells}} \times 100 \right]$$

* Test wells were the TNF containing wells. ** Con-

trol wells (sets $\pm$ antibody) were wells without TNF, i.e. 100% survival.

The equation used to calculate specific growth rate was that used to calculate generation time:

$$n = (\log N_t - \log N_0) / \log 2,$$

where n is number of generations, N₀ is cell number at zero time and N_t is cell number at finish. Generation time was found by dividing n with the number of hours of cell incubation.

Statistics

Data were imported from a PC-based spreadsheet to a standard statistical package (Statgraphics® Plus for Microsoft® Windows™, Manugistics, Inc.®, MD, U.S.A.). The samples were tested for normal distribution with Shapiro-Wilk's W statistic and plotted in normal probability plots. Non-parametric methods for testing significance were applied when sample distribution was unknown. The significance was tested at a 5% α -level and $p < 0.05$ accepted as statistically significant. The type of tests employed is noted. N is the number of experiments.

Results

Choice of vessel coating

The growth rate of WEHI cells adapted to the serum-free medium was investigated in tissue culture flasks with four different anchoring materials: gelatine, gelatine-extracted fibronectin, Primaria™ and uncoated plastic. These were compared with the growth of the stock cell suspension in gelatine+1% serum and 10% serum. The growth rate was estimated by counting successively in an inverted microscope during seven days and the specific growth was determined in the log-phase of the growth curve by calculating the generation time.

Table I demonstrates that the WEHI cells did not grow unless provided an anchorage material, and that the growth rates were approximately similar with the different coatings. It also shows that the defined medium supported cell growth at a rate similar to that in 10% serum. Although the other coatings supported cell growth better than Primaria™, the latter was considered sufficient and was selected for use as the standard to save labour and time and to avoid macromolecules in the cultures. However, when the

Table 1. Serum-free growth¹ of WEHI 164 cells in vessels with different coatings

Type of coating	Gelatine + fibronectin	Gelatine	Serum 10%	Gelatine + 1% serum	Primaria™	Uncoated
Generation time in hours	18	21	18	20	23	∞

¹ Growth in the log-phase: day 2-day 4.

culture became very dense, a growth arrest occurred in some tissue culture flasks by a sudden and complete detachment of the cells. This may be attributed to limitations within the coating and not to cell death as the nutrients in the medium supported cell growth well in differently coated vessels containing denser cultures. Within the time schedule and cell densities presented here, detachment was avoided.

Seeding densities

The influence of seeding densities on the growth of WEHI 164 cells during seven days of cultivation was investigated. The cells were cultivated at different densities in microplates supplemented with 100 μ L defined medium. Every 24 h octoplicates of cell-containing wells were trypsinized and the viability was determined in a Bürker chamber by Trypan-blue exclusion. In addition, the growth in tissue culture flasks (mother cell culture) was studied during a similar period by counting successively in an inverted microscope. In these, the WEHI cells were seeded at density (cell number/cm²) and volume (cell number mL⁻¹ culture medium) equalling those in the microplates.

The growth rate demonstrated in Figure 1 indicates that a seeding density $>5.0 \times 10^4$ cells/well (c/w) does not assure log-phase cell cultures beyond 24 h of incubation. In the log-phase, metabolism is not compromised by exhausted medium nutrients and the formazan production in each cell is expected to be optimal. A high cell number is advantageous to obtain a high amount of formazan. Thus, a seeding density of 4.0×10^4 was chosen.

The seeding density in the tissue culture flasks that could give a sufficient number of log-phase cells to perform an assay using all wells in a microplate was estimated to be 1.5×10^3 cells/cm², i.e. 7.5×10^5 cells/flask (results not shown). Further harvesting of the cells in the flasks was done within 3 days.

Maximal absorbance

The wavelength of maximal absorbance in formazan dissolved in three different solvents was examined by scanning in a spectrophotometer. WEHI 164 cells (1.0×10^5 c/w) were challenged with 1.0 mg mL⁻¹ MTT (final concentration) and cultivated in the defined medium for 3 h. The absorbance was subsequently investigated in acidic isopropanol, SDS/DMF and SDS (Figure 2). In addition, the absorbance in wells containing cells without MTT was determined (results not shown).

The maximal absorbance in all solvents was found at 570 nm and there was a preference for using acidic isopropanol due to the high absorbance. In wells containing cells unexposed to MTT, the absorbance at 570 nm was low (0.020–0.040) and approximately similar in the three solvents. This indicates low absorbance in wells containing denaturated cells. The reference wavelength was chosen whereby the absorbance in the wells containing dissolved formazan was very low (690 nm) and further reading was done by dual wavelength (570 nm/690 nm) to eliminate non-specific absorbance originating from moisture, dirt, scratches etc.

Optimal MTT concentration

The optimal concentration of MTT was investigated by preparing stock solutions that gave final concentrations (mg mL⁻¹) of 0.05–0.1–0.25–0.5–1.0 and 2.0. The cells were seeded in wells at a number of 4.0×10^4 c/w to which MTT was added after 20 h of cultivation. They were further incubated for 3 h until the absorbance was read.

A MTT concentration (final) of 0.8 mg mL⁻¹ was considered to be the optimum due to the high absorbance that was nonetheless considered within the linear phase of the production curve (Figure 3). Higher concentrations of MTT did not result in higher absorbance. Subsequent experiments were carried out using 0.8 mg mL⁻¹ of MTT.

Figure 1. The growth of WEHI 164 clone 13 cells during seven days of cultivation in a defined medium. The cells were seeded in different numbers of cells per well (c/w) and counted daily. N = 6 (triplicates), error bars not shown.

Optimal incubation time

4.0×10^4 c/w cells were seeded in eight separate plates and cultivated for 20 h and subsequently challenged with MTT. The formazan production was investigated by reading a new plate at 30 min intervals. There was an approximately linear relation until 3.5 h and a MTT incubation time of 3 h was considered preferable (Figure 4). Further incubation gave little additional absorbance and, by microscopy, we observed that crystals of formazan were present from 4 h onwards, indicating saturation of the solvent.

Optimal cell number

The correlation between cell number and formazan production was investigated by seeding cells in microplates at these numbers (c/w): 1.0×10^4 , 2.5×10^4 , 5.0×10^4 , 7.5×10^4 , 1.0×10^5 , 1.5×10^5 , 2.0×10^5 .

After incubation for 20 h, MTT was added and incubation was continued for a further 3 h, before acidic isopropanol was added and the plates read (results not shown). The correlation coefficient was $r = +0.99$ between absorbance and seeded cell number until 5.0×10^4 c/w. From this number onwards there was a weaker correlation which indicated that higher cell numbers, i.e. $>5.0 \times 10^4$ c/w, gave little additional absorbance. This was probably due to saturation of the solvent.

The cytotoxicity of TNF- α in assays $\pm$ defined medium

We compared the TNF assay based on the defined medium with that based on serum by testing the cytotoxicity of recombinant human and mouse TNF- α towards WEHI 164 cells. In these assays, a sigmoidal shape of the TNF- α cytotoxicity curves was found for

Figure 2. The absorbance (by scanning) in formazan dissolved in three different solvents: Isopropanol with 0.3% hydrochloric acid, acidic sodiumdodecylsulphate/dimethylformamide and dimethylsulfoxide. The numbers are mean of octoplicates.

both human and mouse r-TNF- α (Figures 5a and b). It was typical in these experiments that even the highest concentration of the cytokine did not kill 100% of the cells. At the lower concentrations (<1 pg mL $^{-1}$), there was 100% survival only in the defined medium. The approximately linear part of the cytotoxicity curves was by graphical estimation situated from 1.0×10^{-1} to 1.0×10^3 pg mL $^{-1}$ (Figures 5a, b), which suggests 0.1 pg mL $^{-1}$ to be the lowest detection limit in both assays. Correlations between the assays in the linear part of the slopes were $r = +0.99$ and $r = +0.98$ for human and mouse TNF- α , respectively Figures 5a and b.

Test of endogenous TNF- α

The two bioassays were estimated for capability of detecting endogenous TNF- α originating from the murine cell line RAW. The supernatants from the

LPS challenged RAW cells were tested for TNF- α activity in seven different concentration in the two assays by serial dilution, 1:1, 1:2 and 1:4, onwards. Recombinant mouse TNF- α was used as the laboratory standard in final concentrations (pg mL $^{-1}$) of: 1.0×10^3 , 0.5×10^3 and 1.0×10^2 , onwards. The detected concentration in pg mL $^{-1}$ was multiplied with the reciprocal number of the sample dilution to illustrate the effect on recovery of varying cytokine concentrations.

The serum-free assay detected TNF- α with a higher sensitivity than did that based on serum (600 pg mL $^{-1}$ versus 400 pg mL $^{-1}$), which was statistically significant (Mann-Whitney *U*-test) (Figure 6). Also, in both assays the undiluted sample exhibited a significantly higher cytokine concentration than did the diluted ones (Mann-Whitney *U*-test). This suggests that each sample should be tested as a dilution series to reveal a reliable value. The variance in detected

Figure 3. The absorbance in cell cultures (4.0×10^4 cells/well) grown in a defined medium for 20 h and subsequently challenged for 2.5 h with different concentrations of MTT. Two different solvents were used: isopropanol with 0.3% hydrochloric acid and sodiumdodecylsulphate/dimethylformamide. N = 4 (quadruplicates), error bars are SEM.

TNF- α levels between the parallels in one assay, expressed as intra-assay coefficients of variation (COV), was typically <10 for both assays. The highest inter-assay COV in the serum-free and serum-based assay for the data in Figure 6 were found in the undiluted samples: 50.8 and 126.6, respectively. Except for the most diluted samples, the COV for the latter numbers were approximately 25 in the serum-free assay and 50 in the serum-based assay. Thus, the assay in the defined medium reduced the inter-assay COV to approximately half of that in serum.

Discussion

Tetrazolium salts are used extensively in cell proliferation assays, cytotoxicity assays, enzyme assays, histochemistry and bacteriology. Despite a long his-

tory of employing these salts for such purposes, the mode of its intracellular bioreduction has been poorly understood. According to the early investigations of Slater et al. (1963) and Löw et al. (1964), MTT competes with O_2 as a hydrogen acceptor by linking up with different sites of the terminal electron transport in the oxidative phosphorylation in the mitochondria. The amount of formazan produced was believed to be a parameter of the cellular respiration when ATP is formed from the energy-rich molecules (NADH and $FADH_2$) originating in glycolysis, the citric acid circle or fatty acid oxidation. These findings have recently been challenged by investigators demonstrating that formazan production predominantly is carried out by microsomal enzymes that require the reduced pyridine nucleotides NADH and NADPH (Berridge and Tan, 1993; Berridge et al., 1996). Yet formazan production is a measure of intracellular glycolytic activity. These

Figure 4. The absorbance in cell cultures (4.0×10^4 cells/well) that were incubated for 20 h, subsequently challenged with MTT (0.8 mg mL^{-1} , final) for a period ranging from 0.5 h to 6 h. $N = 4$ (triplicates), error bars are SEM.

observations indicate that the amount of formazan generated in a cell culture is dependent on both the cell number and the metabolic state of the cells, which is in agreement with the demonstration of formazan production being affected by the glucose concentration in the medium (Vistica et al., 1991). These facts emphasize the importance of using cells that are not compromised by exhausted nutrients, i.e. using log-phase cells and fresh media. An undesirable failure of formazan production may occur in cells that are grown in exhausted medium, causing reduced spectrophotometrical absorbance in the wells that serve as controls, while the lower nutritional demand in the test wells may cause an unaffected formazan production in cells surviving TNF cytotoxicity. In this situation the result will be a loss of sensitivity.

Optimization of the production and extraction of formazan is crucial to obtain a wide range of ab-

sorbance in the spectrophotometer. According to Figure 3, the formazan production is associated with the concentration of MTT used. There is only a slight gain in absorbance when the concentration in the incubation medium exceeds 1.0 mg mL^{-1} . Considering the additional gain in absorbance that is achieved when the incubation time is extended using a concentration of 0.8 mg mL^{-1} (Figure 4), MTT probably inhibits the metabolic activity of the cells in the higher concentrations. Hence, $>1.0 \text{ mg mL}^{-1}$ (final) may be toxic.

Previous investigations have demonstrated that the solvent DMF/SDS is favourable due to the high capability of formazan extraction (Hansen et al., 1989). These authors employed serum in the medium which contains proteins that both aggregate with formazan (Tada et al., 1986) and precipitate at the low pH needed to eliminate additional absorbance from the

Figure 5a. A semilogarithmic plot of the cytotoxicity of recombinant human TNF- α towards WEHI 164 clone 13 cells detected as percent dead cells in two different bioassays: either based on cells grown in 10% serum or on cells adapted to a defined medium. N = 16 (triplicates), error bars are SEM.

phenol-red component of the medium (Denizot and Lang, 1986). When preparing the solvent as described (Hansen et al., 1989), we experienced precipitates in the stock solution and foam at the surface of the microwells. To avoid dispersion of the light in the ELISA-reader, which is an additional source of error, we had to use a hairdryer to eliminate the foam. We consider the preparation of acidic isopropanol to be less hazardous to personnel and less time consuming to prepare. Since isopropanol has been demonstrated to precipitate serum proteins which thereby impose additional error during reading in a spectrophotometer (Sandberg, 1977), the defined medium obviously serves well to limit that problem. Moreover, in our experiments acidic isopropanol was considered

favourable on account of the high extraction capability as demonstrated in Figures 1 and 2.

The optimal reading wavelength in our experiments is 570 nm (Figure 2); lower or higher wavelengths will cause less absorbance in the spectrophotometer. Previous investigators have used different reading wavelengths, e.g. 550, 620 and 570 nm (Espevik and Nissen-Meyer, 1986; Meager et al., 1989; Garbin et al., 1994). Also, different reference wavelengths have been used, e.g. 690 and 630 nm (Espevik and Nissen-Meyer, 1986; Garbin et al., 1994). According to Figure 2, there will be a high loss of information if the subtracted reference wavelength is too close to the reading wavelength. The latter should also be situated close to the optimum (570 nm). The consequence will be a narrowed absorbance scale and an apparent

Figure 5b. A semilogarithmic plot of the cytotoxicity of recombinant murine TNF- α towards WEHI 164 clone 13 cells detected as percent dead cells in two different bioassays: either based on cells grown in 10% serum or on cells adapted to a defined medium. N = 19 (triplicates), error bars are SEM.

loss of sensitivity in the assay, i.e. the cytotoxicity curve will flatten. Thus, a little change in absorbance will be read as a large difference in TNF- α concentration. Differences in the wavelengths employed can explain the variance in sensitivity observed between various laboratories (Meager et al., 1989; Elborn et al., 1995).

The titration curve of the laboratory standards (r-TNF- α) showed a sigmoidal shape (Figures 5a and b) for both human and mouse cytokine in the two assays. This is in accordance with previous data that suggest interpretation of the results by using only the linear scale of the titration curve as the standard curve (Meager et al., 1989). Yet it is necessary to do a graphical estimation in the absence of a microcomputer interfacing to the ELISA-reader. In these figures

we accepted a linear scale between 1.0×10^0 (at least) and 1.0×10^3 pg mL⁻¹. However, the plots of both human and mouse TNF- α in the serum-based assay were ambiguous. Cytotoxicity was detected even at the smallest concentrations, in contrast to the findings in the serum-free assay. This may be caused by a real TNF- α effect which is not detected in the serum-free assay, or to cytotoxicity originating from other sources. In the serum-free assay it was observed that the antibodies completely abolished the cytotoxicity, whereas in the serum-based assay there was an incomplete neutralisation of the cytotoxicity, typically between 5–10% (results not shown). Together, these findings suggest that cytotoxicity partly may come from other sources, possibly emanating from serum in the presence of small amounts of TNF- α . When inter-

Figure 6. The concentration in serial diluted samples of endogenous murine TNF- α assessed in two assays: either based on serum containing medium or defined medium. To demonstrate the recovery, the numbers (pg mL^{-1}) were multiplied with the reciprocal of the times the samples had been diluted. $N = 5$ (triplicates), error bars are SEM.

polating the results, non-specific cytotoxicity should be subtracted from the total cytotoxicity, consequently giving a less sensitive assay, i.e. higher TNF- α concentrations are needed to kill the same amount of cells. In the latter situation it can, by graphical estimation of Figures 5a and b, be concluded that the serum-free assay has the highest sensitivity.

The concentration of recombinant m-TNF- α that causes 50% cell death (LC_{50}) has in the serum-based WEHI 164 bioassay previously been found to be 750 pg mL^{-1} (Meager et al., 1989). Other investigators have claimed a sensitivity of 3 pg mL^{-1} (Waage et al., 1989) and 2 pg mL^{-1} (Espevik and Nissen-Meyer, 1986), while Kramer and Craver (1986) found a sensitivity of 88 pg mL^{-1} in their assay. However, these numbers are not comparable since the authors interpret the results differently. Kramer and Craver

(1986) considered sensitivity to be the lowest concentration of TNF- α that could be repeatedly measured, while Meager et al. (1989) used the more comprehensive LC_{50} . In the present serum-free assay the LC_{50} calculated in Figures 5a and b was 25 pg mL^{-1} for mouse TNF and 50 pg mL^{-1} for human TNF. Sensitivity calculated according to Kramer and Craver (1986) is considered to be $<1.0 \text{ pg mL}^{-1}$. This demonstrates that the present serum-free bioassay improves sensitivity considerably.

An additional problem concerned with serum-based assays is loss of susceptibility after serial passages of the cells (Meager et al., 1989). We believe that this observation may partly be attributed to selection of less susceptible subclones caused by differences in serum batches. This problem is reduced by serum-free cell cultivation.

We were not able to demonstrate enhanced susceptibility to TNF- α when $1 \mu\text{g mL}^{-1}$ of the transcriptase blocker actinomycin D (AMD) was added to the medium in both assays. This is in accordance with previous findings (Espevik and Nissen-Meyer, 1986). Furthermore, we confirmed that supplementing the test medium with 10 mM lithium chloride (LiCl) in the serum-based assay enhanced the sensitivity without affecting the viability (Beyaert et al., 1989). The equivalent concentration in the serum-free assay was found to be 1 mM LiCl. This enhanced the sensitivity approximately tenfold (results not shown). It is unclear how LiCl exerts an augmented cytotoxicity, but it has been suggested to comprise an increased intracellular accumulation of inositol phosphate (Beyaert et al., 1993). Yet it is not known whether such agents as AMD or LiCl also enhance the cytotoxicity of other substances that may be contained in the TNF- α preparations to be tested or in the serum source (Meager et al., 1989). They are therefore not recommended in bioassays. The commonly used TNF- α assays utilising the cell lines L-929 (ATCC; CCL-1) or L-M cell (ATCC; CCL-1.2) are both based on the use of AMD (Ostrove and Gifford, 1979; Kramer and Carver, 1986). Like the bioassay demonstrated here, an assay is preferable when it is based on a stable target cell that is independent of agents that augment cytotoxicity (Meager et al., 1989).

We observed a high inter-assay variation in the serum containing assay which was reduced when serum was excluded. There were no indications of reduced susceptibility during three months of repeated passages which would have explained the high inter-assay COV observed. The variation present in the serum-free assay is probably linked to unspecific factors usually connected with laboratory work. Among these a prominent source of error may arise from difference in the gas exchange between wells in the centre of the microplate and those on the periphery. This may cause an unequal milieu in the wells, causing differences in pH that may be decisive for the formazan production in the control wells where the buffer capacity of the medium is crucial regarding the high metabolic rate in these healthy cells. Therefore, control wells should be scattered in the plate (e.g. diagonally) and the traffic in the incubator should be reduced to a minimum during the assay period. The high inter-assay variations also indicate that if a large series of samples are to be tested, this should be carried out in one operation.

When endogenous TNF- α was tested, there was a significantly higher TNF- α concentration (Mann-Whitney *U*-test) detected by the serum-free assay, which was less evident when recombinant cytokine was tested. This is probably explained by the fact that endogenous TNF- α differs structurally and chemically from the recombinant source that may bind with higher affinity (specific or non-specific) to factors in serum. Also, it has previously been demonstrated that TNF- α has a species preference (Fransen et al., 1986), which in accordance with our observation (Figures 5a and b). Human TNF- α binds only one of the two murine TNF-receptors, i.e. TNF-RI (p55) (Lewis et al., 1991).

Conclusions

Several aspects of a TNF- α bioassay were investigated here and optimization was aimed at. We succeeded to improve the assay as judged by increased sensitivity and reproducibility by serum-free cell cultivation. Compared with an overview of both immunoassays and bioassays (Elborn et al., 1995), the present assay is the most sensitive. We also found evidence for serum components that interfered with cytokine assessment. This suggests that *in vitro* experiments similarly may be affected. In view of such facts we have succeeded to culture other cell types in defined media (Bertheussen, 1993; Figenschau et al., 1997a; Figenschau et al., 1997b) and accordingly, there should be a considerable potential for adapting other cell types to serum-free growth. This will enable both experiments and assays to be conducted in defined media which we believe will be required to increase the reliability of future research based on living cells, especially research concerned with cytokines.

References

- Aderka D, Engelmann H, Shemer Avni Y, Hornik V, Galil A, Sarov B and Wallach D (1992) Variation in serum levels of the soluble TNF receptors among healthy individuals. *Lymphokine Cytokine Res* 11: 157–159.
- Aggarwal BB, Eessalu TE and Hass PE (1985) Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon. *Nature* 318: 665–667.
- Berridge MV, Tan AS, McCoy KD and Wang R (1996) The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochem J* 4: 15–20.
- Berridge MV and Tan AS (1993) Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence,

- and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys* 303: 474–482.
- Bertheussen K, Holst N, Forsdahl F and Hoie KE (1989) A new cell culture assay for quality control in IVF. *Hum Reprod* 4: 531–535.
- Bertheussen K (1993) Growth of cells in a new defined protein-free medium. *Cytotechnology* 11: 219–231.
- Beyaert R, Vanhaesebroeck B, Suffys P, Van Roy F and Fiers W (1989) Lithium chloride potentiates tumor necrosis factor-mediated cytotoxicity in vitro and in vivo. *Proc Natl Acad Sci USA* 86: 9494–9498.
- Beyaert R, Heynink K, De Valck D, Boeykens F, Van Roy F and Fiers W (1993) Enhancement of tumor necrosis factor cytotoxicity by lithium chloride is associated with increased inositol phosphate accumulation. *J Immunol* 151: 291–300.
- Denizot F and Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 89: 271–277.
- Elborn JS, Delamare F, Bell SC and Shale DJ (1995) Can tumor necrosis factor- α (TNF- α) be reliably measured in blood? *Pediatr Pulmonol* 19: 226–230.
- Eskandari MK, Nguyen DT, Kunkel SL and Remick DG (1990) WEHI 164 subclone 13 assay for TNF: sensitivity, specificity, and reliability. *Immunol Invest* 19: 69–79.
- Espevik T and Nissen Meyer J (1986) A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Methods* 95: 99–105.
- Feldman D and Krishnan A (1995) Estrogens in unexpected places: possible implications for researchers and consumers. *Environ Health Perspect* 103 Suppl 7: 129–133.
- Figenschau Y, Sundsfjord JA, Yousef MI, Fuskevåg OM, Sveinbjörnsson B and Bertheussen K (1997a) A simplified serum-free method for preparation and cultivation of human granulosa-luteal cells. *Hum Reprod* 12: 523–531.
- Figenschau Y, Yousef MI, Sveinbjörnsson B and Bertheussen K (1997b) A sensitive serum-free colorimetric assay for the detection of cytotoxic effects of pesticides. *J Environ Sci Health B* 32: 177–194.
- Fomsgaard A, Worsaae H and Bendtzen K (1988) Detection of tumour necrosis factor from lipopolysaccharide-stimulated human mononuclear cells by enzyme-linked immunosorbent assay and cytotoxicity bioassay. *Scand J Immunol* 27: 143–147.
- Fomsgaard A, Svenson M and Bendtzen K (1989) Auto-antibodies to tumour necrosis factor alpha in healthy humans and patients with inflammatory diseases and gram-negative bacterial infections. *Scand J Immunol* 30: 219–223.
- Fransen L, Ruysschaert MR, Van der Heyden J and Fiers W (1986) Recombinant tumor necrosis factor: species specificity for a variety of human and murine transformed cell lines. *Cell Immunol* 100: 260–267.
- Garbin F, Eckert K and Maurer HR (1994) Evaluation of the MTT and SRB assays for testing LAK cell-mediated growth inhibition of various adherent and non-adherent tumor target cells [letter]. *J Immunol Methods* 170: 269–271.
- Hansen MB, Nielsen SE and Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 119: 203–210.
- Holtmann H and Wallach D (1987) Down regulation of the receptors for tumor necrosis factor by interleukin 1 and 4 beta-phorbol-12-myristate-13-acetate. *J Immunol* 139: 1161–1167.
- James K (1990) Interactions between cytokines and alpha 2-macroglobulin [see comments]. *Immunol Today* 11: 163–166.
- Kramer SM and Carver ME (1986) Serum-free in vitro bioassay for the detection of tumor necrosis factor. *J Immunol Methods* 93: 201–206.
- Lewis M, Tartaglia LA, Lee A, Bennett GL, Rice GC, Wong GH, Chen EY and Goeddel DV (1991) Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc Natl Acad Sci USA* 88: 2830–2834.
- Löw H, Alm B and Vallin I (1964) The use of phenazine methosulfate in the study of oxidative phosphorylation. *Biochem Biophys Res Commun* 14: 437–440.
- Meager A, Leung H and Woolley J (1989) Assays for tumour necrosis factor and related cytokines. *J Immunol Methods* 116: 1–17.
- Olsson I, Gatanaga T, Gullberg U, Lantz M and Granger GA (1993) Tumour necrosis factor (TNF) binding proteins (soluble TNF receptor forms) with possible roles in inflammation and malignancy. *Eur Cytokine Netw* 4: 169–180.
- Ostrove JM and Gifford GE (1979) Stimulation of RNA synthesis in L-929 cells by rabbit tumor necrosis factor. *Proc Soc Exp Biol Med* 160: 354–358.
- Sandberg HE (1977) Proceedings of the International Workshop on Technology for Protein Separation and Improvement of Blood Plasma Fractions. U.S. Dept. Health, Education, Welfare, U.S.A., Publ. no. (NIH) 78–1422.
- Slater TF, Sawyer B and Sträuli U (1963) Studies on succinate-tetrazolium reductase systems III. Points of coupling of four different tetrazolium salts. *Biochim Biophys Acta* 77: 383–387.
- Smith CA, Davis T, Wignall JM, Din WS, Farrah T, Upton C, McFadden G and Goodwin RG (1991) T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF receptor. *Biochem Biophys Res Commun* 176: 335–342.
- Sugarman BJ, Lewis GD, Eessalu TE, Aggarwal BB and Shepard HM (1987) Effects of growth factors on the antiproliferative activity of tumor necrosis factors. *Cancer Res* 47: 780–786.
- Tada H, Shiho O, Kuroshima K, Koyama M and Tsukamoto K (1986) An improved colorimetric assay for interleukin 2. *J Immunol Methods* 93: 157–165.
- Van Zee KJ, Kohno T, Fischer E, Rock CS, Moldawer LL and Lowry SF (1992) Tumor necrosis factor soluble receptors circulate during experimental and clinical inflammation and can protect against excessive tumor necrosis factor alpha in vitro and in vivo. *Proc Natl Acad Sci USA* 89: 4845–4849.
- Vistica DT, Skehan P, Scudiero D, Monks A, Pittman A and Boyd MR (1991) Tetrazolium-based assays for cellular viability: a critical examination of selected parameters affecting formazan production [published erratum appears in *Cancer Res* 1991 Aug. 51;51(16):4501]. *Cancer Res* 51: 2515–2520.
- Waage A, Brandtzaeg P, Halstensen A, Kierulf P and Espevik T (1989) The complex pattern of cytokines in serum from patients with meningococcal septic shock. Association between interleukin 6, interleukin 1, and fatal outcome. *J Exp Med* 169: 333–338.