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Effect of anticoagulants and heat on the detection of tumor necrosis factor in murine blood

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Assays for tumor necrosis factor (TNF) may be inhibited by nonspecific factors present in body fluids. We found that the ability to quantitate TNF is greatly affected by blood processing methods. Mice were anesthetized by inhalation of methoxyflurane before obtaining blood by cardiac puncture. The blood from a group of mice was allowed to clot before recovery of serum. Plasmas were obtained from three other groups of mice after collection of blood into tubes containing EDTA, sodium citrate, or preservative-free heparin. The pooled serum or plasmas were spiked with rhTNF to a final concentration of 1 μg/ml and aliquoted for frozen storage. The serum and plasmas were divided into heated (56°C for 30 min) and unheated portions prior to a standard L929 cytotoxicity assay. Comparison of absorbances at 595 nm after crystal violet staining of cells revealed differences in detection of TNF in plasmas compared to serum and in heated compared to nonheated samples. Citrated plasma clotted in the assay at dilutions at or below 1 : 25. EDTA plasma consistently produced unexplained lysis of L929 cells in both heated and unheated unspiked samples. We conclude that TNF levels should be determined only in heat treated serum samples, and that comparisons be made against both a TNF standard and a TNF standard prepared in normal homologous serum that can be heated and assayed in parallel with the test samples.

Key words: Tumor necrosis factor; Bioassay; Plasma; Serum; (Murine)

Introduction

Tumor necrosis factor (TNF) has both beneficial and adverse effects on the animal host (Beutler et al., 1986). It is the mediator of the toxic effects of endotoxin (Hesse et al., 1988). An increase in circulating TNF is generally considered to be deleterious (Waage et al., 1987). Circulating TNF has been detected in some nonbacterial disease states (Kern et al., 1989), but not in others (Moldawer et al., 1987).

Current methods to assay for TNF in body fluids or culture supernatants depend on immunologic and biologic endpoints (Meager et al., 1989). The presence of serum in culture medium may interfere with the sensitivity of bioassays (Kramer et al., 1986). This does not reconcile the inherent variability present among individual serum or plasma samples to be tested. The manner in which a blood sample is handled greatly alters the levels of detectable TNF; thus, endotoxin-contaminated tubes cause rapid increases in TNF released from blood monocytes (Leroux-Roels et al., 1988).

Our research is focussed on the effects of immunomodulators administered following exposure to radiation in murine models. The results of our

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initial TNF assays using EDTA plasma were difficult to interpret because we noted the almost total lysis of the indicator cells at the lowest dilution and the apparent absence of a dilution-response relationship. When serum from parallel sets of mice were studied, this phenomenon did not occur. The experiments described here were performed to determine whether anticoagulants affect the detection of recombinant human TNF (rhTNF) added to plasmas. We also examined heat treatment of serum and plasma samples to determine whether TNF detection could be enhanced. We found that detection of TNF is optimal from mouse serum that is heat treated prior to assay.

Materials and methods

JAX: B6D2F1 female mice, 12–15 weeks of age (20–25 g) were quarantined on arrival and screened for evidence of disease before being released for experimental use. They were maintained in an AAALAC-accredited facility in plastic Micro-Isolator cages (Lab Products, Maywood, NJ) containing autoclaved hardwood chip contact bedding. Mice were provided commercial rodent chow and acidified tap water (pH 2.5 with concentrated HCl) ad libitum. Animal holding rooms were maintained at 21°C ± 1°C with 50% ± 10% relative humidity using at least ten air changes/h of 100% conditioned fresh air. The mice were on a 12 h light/dark full-spectrum lighting cycle with no twilight. All research was conducted in accordance with NIH and our Institutional Animal Care and Use Committee guidelines for the care and use of laboratory animals.

Mice were anesthetized by inhalation of methoxyflurane immediately before 1 ml of blood was obtained by cardiac puncture before being killed by cervical dislocation. The serum or plasma obtained from eight mice was pooled. Serum was obtained after allowing blood to clot in sterile microcentrifuge tubes for 1 h at ambient temperature before centrifugation at 4000 rpm for 4 min. Pooled sera were spun again. Citrated plasma was obtained by placing blood from individual mice into a microfuge tube containing 10 μl of 32% sodium citrate. EDTA plasma was obtained by placing blood from individual mice into 2 ml tubes (Starstedt, Princeton, NJ) containing 3 mg EDTA. Heparinized plasma was obtained by depositing blood from individual animals into sterile microfuge tubes containing 100 U preservative-free heparin (Fisher Scientific, Pittsburgh, PA). All plasmas were rocked on a blood mixer (Coulter, Hialeah, FL) prior to centrifugation at 4000 rpm for 4 min at 4°C. The plasma groups were pooled and spun again to remove any debris and aliquoted into sterile microfuge tubes.

The rhTNF-α produced in *Escherichia coli* was purchased from Sigma Chemical Co., St. Louis, MO, and has a biologic activity of 2.2×10^3 U/μg. Dilutions of rhTNF were added to serum and plasma aliquots to yield a final concentration of 1 μg/ml TNF. These spiked aliquots and untreated control samples were stored at −20°C until assayed. Some aliquots were heated to 56°C for 30 min immediately before assay.

TNF activity in serum or plasma was assayed using a modification of the method of Hogan et al. (1988). Briefly, 1.25×10^4 L929 cells were deposited in each well of 96-well tissue culture plates in plate medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 250 U/ml penicillin and 125 μg/ml streptomycin) and allowed to grow overnight at 37°C in a humidified 5% CO_2 incubator.

Samples for assay and the TNF standard were diluted in separate 96-well plates in assay medium (RPMI 1640 supplemented with 5% FBS, 2 mM glutamine, 250 U/ml penicillin, 125 μg/ml streptomycin, 30 mM Hepes, and 3 mg/ml sodium bicarbonate). In some assays, an additional TNF standard was prepared in homologous normal mouse serum and heated to 56°C for 30 min before further dilution in assay medium. Culture medium was removed from the seeded plates, and 100 μl of diluted sample or standard was added. Actinomycin D (Sigma Chemical Co., St. Louis, MO) was diluted to 10 μg/ml in assay medium, and 100 μl was added to the wells. Plates were returned to the incubator for 18 h.

Culture medium was aspirated and wells were washed once with 200 μl saline and aspirated again. Cells were stained with 50 μl of 0.05% crystal violet in 20% ethanol for 20 min; plates

were washed with cool tap water and allowed to dry in air. Absorbances at 595 nm were recorded using a Titertek spectrophotometer (Flow Laboratories, McLean, VA) after the addition of 50 μl methanol/well. Absorbance readings for dilutions of standard TNF were plotted to compare the amount of cytolytic activity present in samples. Student's t tests were performed to determine levels of significance.

TNF-dependent cytolytic activity in spiked samples was confirmed by neutralization of this activity by using a modification of the TNF assay described earlier (Meager et al., 1989). Briefly, 50 μl of dilutions of the rhTNF standard were mixed with 50 μl of dilutions of polyclonal rabbit-anti-mouse TNF antibody (Genzyme, Boston, MA) in microtiter wells and incubated for 2 h at 37°C. 100 μl of actinomycin-D treated L929 cells were then added (2×10^4/well) and incubated for an additional 18 h before staining with crystal violet as previously described.

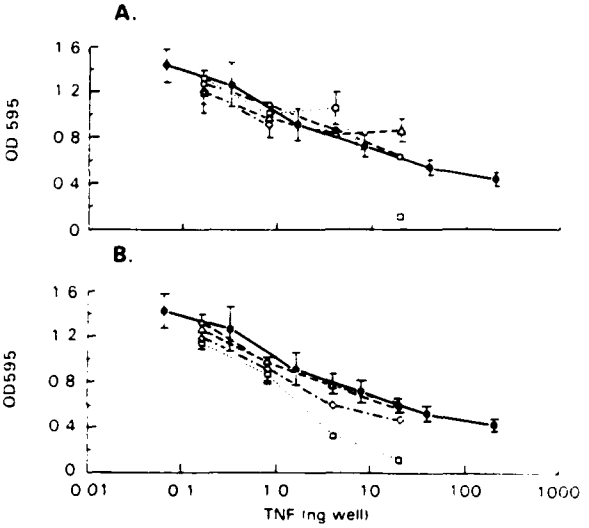

Fig. 1. Absorbances of serum or plasma pools spiked with 1 μg/ml of rhTNF. Five-fold dilutions of mouse serum (○------○), EDTA plasma (□·····□), citrated plasma (◇·–·–·◇) and heparinized plasma (△-----△) were compared to a rhTNF standard curve (●———●). Curves represent means of absorbances ± standard error. *A*: Spikes serum and plasmas tested for cytolytic activity. *B*: Absorbances following heat treatment of serum and plasma samples. The standard TNF contains approximately 2.2 biological activity U/ng.

Results

The detection of TNF in plasma and serum samples varied according to the method of collection. The results of a typical experiment using spiked serum and plasma samples are presented in Fig. 1*A*. In unheated samples to which TNF had been added, only serum showed a consistent dilutional effect for the entire series tested. The absorbances of spiked serum were similar to the standard curve, while there was greater variability among the spiked plasma samples.

EDTA plasma samples consistently produced extensive lysis in dilutions at or below 1/10 in both spiked and unspiked samples. This non-specific lysis was removed by dialysis against Hanks' balanced salt solution containing Mg^{2+} and Ca^{2+} (data not shown). Citrated plasma consistently clotted in the assay at dilutions at or below 1/25. The absorbances from wells containing clots ranged from that of the control (no lysis) to those of a normal standard TNF curve. Heparinized plasma responded similarly to serum, but was more variable at the higher TNF concentrations; at lower concentrations the absorbances were similar to those of the standard curve.

Heat treatment of spiked samples resulted in improved TNF detection (Fig. 1*B*). This treatment eliminated the problem of clotting found with citrated plasma, although the lytic activity detected in the assay was still above that of the standard curve. Heat treatment of spiked EDTA

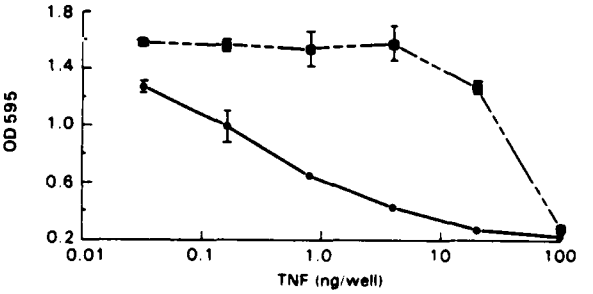

Fig. 2. Absorbances of rhTNF in L929 bioassay with (■) or without (●) the addition of rabbit antibody against mouse TNF. Curves are mean ± standard error. All values except the highest tested were significant at $P < 0.02$.

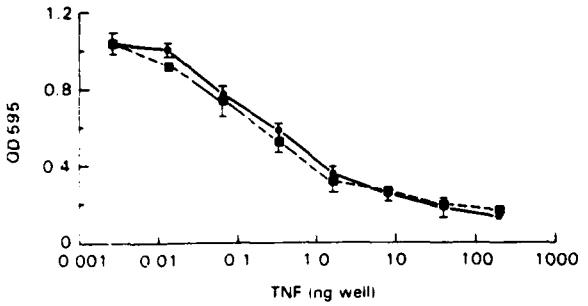

Fig. 3. Absorbances of rhTNF diluted in medium (●) or normal mouse serum (■) heated to 56°C for 30 min before bioassay. Curves are mean ± standard error. There were no significant differences between values ($P > 0.05$).

plasma did not alter the almost total lysis of L929 cells. Both serum and heparinized plasma that were heat treated before assay demonstrated excellent detection of spiked TNF.

The lytic activity in our assay was neutralized by antibody against TNF (Fig. 2). The anti-mouse TNF was able to neutralize rhTNF in all but the highest concentration of rhTNF tested. The anti-mouse TNF antibody was used in these experiments so that our results could be attributable to TNF alone. In addition, we plan to test this anti-mouse TNF antibody in future in vivo experiments.

We also examined whether normal mouse serum might nonspecifically bind spiked TNF. Normal homologous mouse serum heated to 56°C for 30 min before assay was used as the diluent for the first well of a five-fold dilution sequence. Fig. 3 shows that the activity of TNF diluted in pure mouse serum was indistinguishable from the activity of TNF diluted in medium alone, and that heating did not compromise TNF quantitation.

Discussion

We have demonstrated that reagents used in the processing of blood affect the subsequent bioassay for TNF. EDTA plasma was found to be unsuitable for use in a TNF bioassay because of nonspecific lysis of indicator cells. This effect could not be reversed by heat treatment of samples prior to assay. The effects of dilution on EDTA plasma were found to be inconsistent and, therefore, unsuitable for calculating TNF activity.

Citrated plasma clotted in our assay at the lower dilutions, but after heat treatment, cell lysis was found to be more than what was anticipated from the standard curve, and was attributed to a nonspecific mechanism. Citrated plasma, therefore, is not recommended if TNF activity is to be quantitated in a sample.

Heparinized plasma was found to be more suitable for TNF quantitation. Heat treatment of heparinized plasma was shown to optimize the detection of TNF from intentionally spiked samples. However, it has been reported that many commercial blood collection tubes are contaminated with endotoxin and that this can result in TNF release by cells within a blood sample destined for TNF quantitation (Leroux-Roels et al., 1988). In addition, many commercially available heparin preparations contain preservatives that interfere with any bioassay.

TNF was demonstrated from spiked serum samples in a quantitative manner that was consistent within a dilution series. Heat treatment of serum samples resulted in absorbances that were slightly, but insignificantly, lower than those of the unheated serum samples. The detection of TNF that was intentionally added to mouse serum was unaffected by heat treatment, and this simple step has been incorporated as routine in our laboratory. It has been reported (Ruff et al., 1981) that TNF from rabbit and mouse are stable at 56°C. Others have adopted heat treatment in their TNF assays (Kawasaki et al., 1989; Van de Wiel et al., 1989), but have not presented quantitative evidence as shown here.

We, therefore, recommend that circulating TNF should be quantitated from serum collected in sterile pyrogen-free tubes, and heated to 56°C for 30 min prior to assay. In addition, a standard curve prepared in normal homologous serum should be heated and assayed in parallel to assure that nonspecific factors are not present because they could affect the quantitation of TNF in test samples.

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