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Detecting Wildlife Orthologues for Tumor Necrosis Factor (TNF) and Interleukin-6 (IL-6)

Abstract

Detection and quantification of cytokines is important to better understand disease processes in populations. This study assessed the feasibility of quantifying wildlife orthologues for tumor necrosis factor (TNF) and interleukin-6 (IL-6) using two bioassays combined with an *in vitro* model for immune stimulation. For all species, heparinized blood (1 ml) was exposed to lipopolysaccharide (LPS; 10 pg to 50 μg) for 6 hr (37°C) after which plasma was collected. Neutralizing antibodies were used to demonstrate that grizzly bear (*Ursus arctos*) TNF can be quantified with a WEHI-164 bioassay, and expression of TNF in the LPS stimulated model is dose-dependent. A B9 bioassay demonstrated LPS-dose-dependent production of IL-6 for grizzly bears, although neutralizing antibodies were not available to confirm that IL-6 was responsible for these results. The B9 assay may have detected IL-6 orthologues for bighorn sheep (*Ovis canadensis canadensis*), elk (*Cervus elaphus*) and bison (*Bison bison*). These assays combined with the LPS stimulated blood model could provide a rapid means for assessing immunological effects of pathogens and toxicants.

Introduction

Cytokines are low molecular weight proteins that play a significant role in human and mouse immunopathology (Remick and Friedland 1997). The ability to quantify orthologous cytokines in wildlife will be an important step to developing a better mechanistic understanding of disease processes in these species. In this paper we assess the feasibility of quantifying two pro-inflammatory cytokines in several wildlife species. Tumor necrosis factor (TNF) plays a significant role in initiating an inflammatory response in human and mouse models of disease. Interleukin-6 (IL-6) is also associated with inflammatory events and has been used as an indicator of severity of septicemia in humans (Damas et al. 1992). In this paper we studied the production of cytokines in whole blood; this could serve as a nondestructive *in vitro* system for assessing effects of antigens and toxicants on immune systems of wildlife species.

Quantifying cytokine concentrations usually involves either an enzyme-linked immunoassay (ELISA) or a bioassay (Remick and Friedland 1997). ELISAs provide information about the presence of proteins of interest, but provide no information about the biological activity of these proteins. In addition, ELISAs typically employ antigen specific antibodies that are rarely suitable for interspecific assays. Alternatively, bioassays provide information about the biological activity of the protein of interest and they tend to be more suitable for interspecific assays. For instance, the WEHI cytotoxicity assay described below has been used to quantify TNF in humans (Call and Remick 1998), mice (Ebong et al. 1999), and several species of domestic livestock (Su et al. 1992, Ellis et al. 1993, Egan et al. 1994, Boury et al. 1997). In this paper we used two bioassays in conjunction with a whole blood model (Strieter et al. 1990) to quantify TNF and IL-6 in several wildlife species.

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Methods

Up to 25 ml of blood was collected into heparinized tubes from captive grizzly bears (*Ursus arctos*; n = 4), bighorn sheep (*Ovis canadensis canadensis*; n = 1), elk (*Cervus elaphus*; n = 1) and bison (*Bison bison*; n = 1). The choice of these species was based on their ready availability at the time of this study. All animals appeared to be in good health. Blood was also collected from normal human volunteers (n = 4) into heparinized syringes. Grizzly bears were housed by the Washington State University Bear Program, Pullman, Washington. Remaining wildlife were maintained at the Idaho Fish and Game Wildlife Health Laboratory, Caldwell, Idaho. All animal husbandry practices and protocols for blood collection were approved by the host Institutional Animal Care and Use Committees. Protocols for collection of human blood were approved by the Institutional Review Board, University of Michigan, Ann Arbor, Michigan.

Within 1 hr of collection, 1 ml heparinized blood was aliquoted into 1.5 ml microcentrifuge tubes and stimulated with between 10 pg and 50 µg lipopolysacharride (LPS, Sigma, St. Louis, Missouri). LPS is a component of Gram negative cell membranes and it typically induces leukocytes to rapidly upregulate expression of pro-inflammatory cytokines. The LPS (50 mg/ml) was stored frozen in RPMI-1640 media (RPMI; BioWhittaker, Walkersville, Maryland) and the same lot was used for all experiments. RPMI alone was used as a negative control. Blood was then held for 6 hr at 37°C with gentle agitation every 30 min. After incubation, samples were centrifuged (2000 × *g*, 5 min) and plasma was collected for analysis.

TNF bioactivity was assessed using the WEHI 164 subclone-13 bioassay (Espevick and Nissen-Meyer 1986). The WEHI cells (mouse macrophages) undergo lysis when membrane-bound TNF receptors are activated. Consequently cell death can be used as an indicator of the presence of biologically active TNF. Two replicates of each plasma sample were serially diluted (1:10 initial dilution and 1:2 subsequent dilutions) across 96-well cell culture plates (Costar, Cambridge, MA) in 100 µl of dilution media (RPMI, 1% fetal calf serum (FCS), 1 mM L-glutamine (BioWhittaker)). Trypsinized WEHI cells (5×10^5 cells/ml) were resuspended in suspension media (RPMI, 10% FCS, 1 mM L-glutamine, 3 µg/ml gentamicin (Sigma)). Actinomycin D (0.5 µg/ml; Calbiochem, La Jolla, California) was also included in the suspension media to halt cell proliferation during subsequent incubations. The cell suspension was added to the sample dilutions (1:1) and held overnight (37°C, 5% CO_2, humidified chamber). At approximately 18 h, cell viability was assessed by adding tetrazolium salt WST-1 (WST; 1:63 final dilution; Roche Diagnostics Corporation, Indianapolis, Indiana) followed by an additional 6 h incubation. Absorbance was quantified at 465 nm (630 nm background subtraction) using a BioKinetics plate reader (Bio-Tek Instruments, Winooski, Vermont) where higher absorbance correlated with greater cell viability and reduced levels of TNF. A standard curve was fitted to absorbance from serial dilutions (1:2) of recombinant human TNF (Cetus Corp, Emeryville, California) and used to quantify sample TNF. This assay was suitable for quantifying TNF between 1-2 pg/ml to 150 pg/ml.

Neutralizing antibodies were used to determine if cytolytic activity of bear plasma was due to TNF. The assay was conducted as described above except wells were prepared with anti-mouse-TNF (amTNF; 0.5% final concentration) serum, or control serum. Individual wells included either a 1:40 dilution of plasma from bear blood treated with 10 ng LPS, or a 1:40 dilution of plasma from bear blood treated with RPMI, or 375 pg recombinant human TNF.

IL-6 bioactivity was assessed using the B9 cell bioassay (Aarden et al. 1987). When IL-6 receptors are activated, these cells undergo rapid proliferation. Two replicates of each plasma sample were serially diluted (1:10, 1:2) across 96-well cell culture plates in 100 µl dilution media (Iscove's modified Dulbecco medium (BioWhittaker), 5% FCS, 1 mM L-glutamine, 100 U/ml each of penicillin and streptomycin (Gibco BRL)). Stock cultures were resuspended (5×10^5 cells/ml) in the above media plus 100 µM 2-mercaptoethanol (Sigma). The cell suspension was added to the sample dilutions (1:1) and incubated three days at 37°C (5% CO_2, humidified incubator). WST was added (1:63 final dilution) and after 6 h absorbance was quantified as described above. We used serial dilutions (1:2) of human recombinant IL-6 (PeproTech, Rocky Hill, NJ) to construct a standard curve with a minimum sensitivity of 1-2 pg/ml).

Results and Discussion

Both human and grizzly bear leukocytes had similar patterns of TNF production in response to increasing doses of LPS (Figure 1). The absolute activity of grizzly bear TNF appeared to be 50-fold lower than human TNF levels when blood was treated with 10 ng LPS. We expected that inherent differences in orthologous proteins would elicit different levels of cytolytic response from the WEHI cell line. Alternatively, the antigenic activity of the LPS used in this study may be weak for bear leukocytes. Consequently, while relative comparisons are meaningful, estimates of absolute TNF concentrations in different wildlife species would require species specific standard curves in the assay.

It is possible that residual LPS or some other protein in grizzly plasma produced a similar cytolytic effect unrelated to TNF concentration. To test this latter hypothesis, bear plasma was incubated in the bioassay with a polyclonal antibody (rabbit) that neutralizes the biological activity of both human and mouse TNF (Remick et al. 1990). Normal rabbit antisera was used for control treatments. As expected, human TNF treated with control antibody was not neutralized (low absorbance) while neutralizing antibodies blocked the cytolytic activity of the human TNF (Figure 2; high absorbance). A similar pattern was observed for LPS treated bear plasma and these results were replicated using a second neutralizing antibody (rabbit anti-human TNF; data not shown). Plasma from bear blood treated with RPMI showed no effect from either antibody treatment. These results demonstrate that the WEHI bioassay was responsive to TNF rather than experiencing cell death due to exposure to residual LPS or other unidentified proteins. These results also suggest that our antibodies would also be useful for immunohistochemistry with bear tissue. We were unable to detect any TNF in bighorn sheep, elk or bison plasma with the WEHI assay (data not shown).

Both human and bear leukocytes produced similar levels of IL-6 in response to increasing doses of LPS (Figure 3). These results might be explained by mitogenic effects of residual LPS contained in the plasma, except the B9 bioassay does not experience increased cell proliferation in the presence of LPS (Ulich et al. 1994). We did not have access to neutralizing antibodies against IL-6, but the dose-dependent response was consistent with

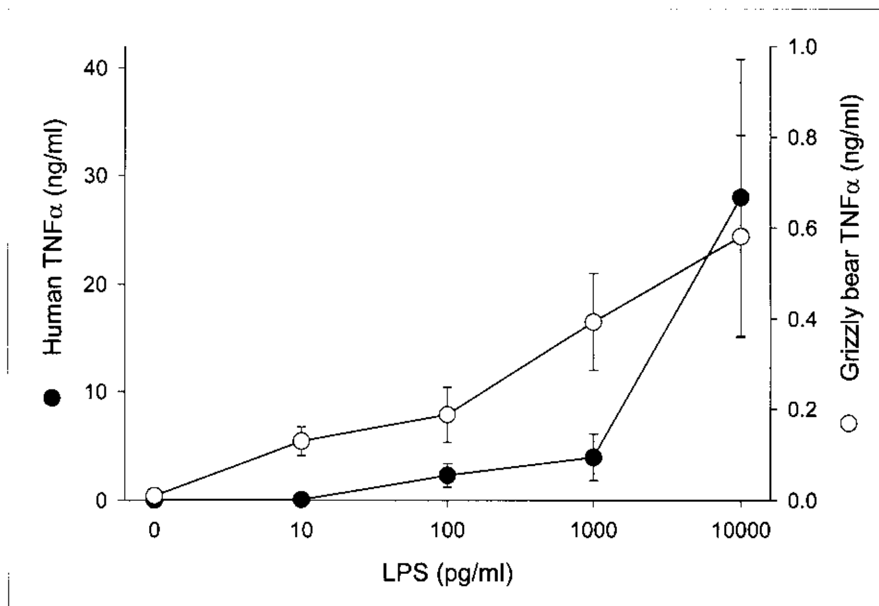

Figure 1. Absolute quantity of human (black circle, left axis) TNF and relative quantity of grizzly bear TNF (open circle, right axis) based on the WEHI bioassay. Data (avg ± SEM) were collected from an LPS-stimulated whole blood model involving four replicate donors for both human and bear experiments. Quantity of TNF was based on serial dilutions of human recombinant TNF and may not reflect absolute concentrations of bear TNF.

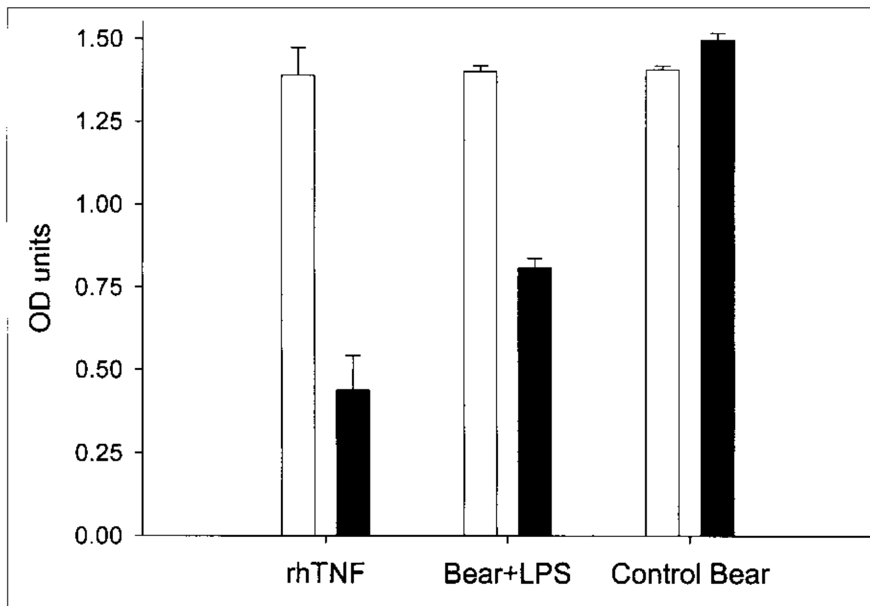

Figure 2. Effect of neutralizing antibodies on cytolytic activity of human and bear TNF. Either recombinant human TNF (n = 2; rhTNF) or bear plasma from 10 ng LPS treatment (n = 3; Bear + LPS) or bear plasma from RPMI treatment (n = 3; Control Bear) was combined with control antibody (black bar) or polyclonal antibody against mouse TNF (open bar). Control antibody had no effect on cytolytic activity of either human or bear TNF (low absorbance) whereas neutralizing antibody blocked TNF (high absorbance). Bars represent average absorbance ± SEM.

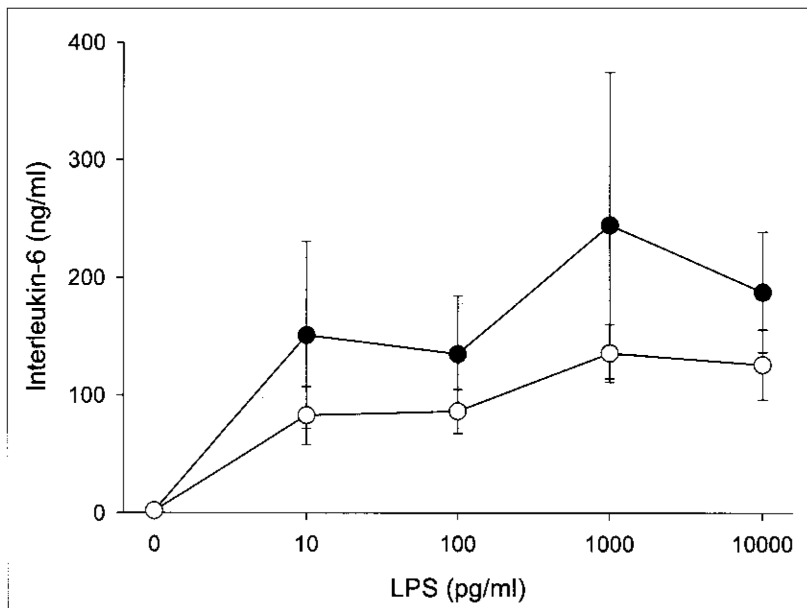

Figure 3. Absolute quantity of human IL-6 (black circle) and relative quantity of grizzly bear (open circle) IL-6 based on the B9 bioassay. Data (avg ± SEM) were collected from an LPS-stimulated whole blood experiments involving four replicate donors for both human and bear experiments. Quantity of IL-6 was based on serial dilutions of human recombinant IL-6 and may not reflect absolute concentrations of bear IL-6.

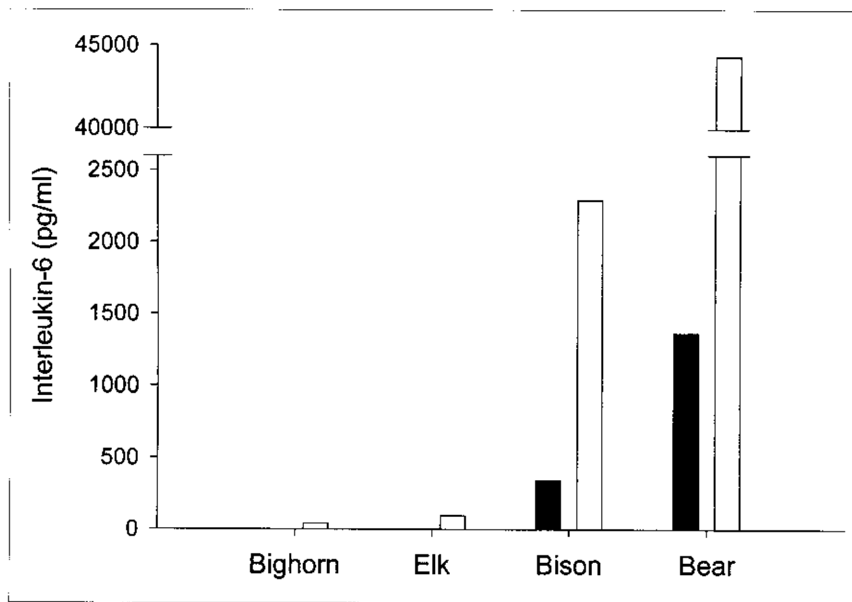

Figure 4. Demonstration of the feasibility of quantifying IL-6 for several wildlife species. Black bars represent control treatments (RPMI). LPS treatment (open bars) represent measurements from whole blood exposed to 10 mg LPS (bighorn sheep) or 50 mg LPS (all others) for six hours (n = 1 per species). IL-6 concentrations were estimated from serial dilutions of recombinant human IL-6.

IL-6 production. Some level of IL-6 was evident in all species of wildlife that we tested (Figure 4). As with the TNF assay, absolute quantification would require species specific standard curves. Despite this limitation, relative quantification in the context of experimental models could provide a rapid and simple means to quantify immunological responses to pathogens and toxins. *In vitro* blood experiments such as the ones described here could also be used to assess immunocompetence in free-ranging wildlife (Lochmiller et al. 1994).

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