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Expression and characterization of recombinant interferon gamma (IFN-γ) from the nine-banded armadillo (*Dasypus novemcinctus*) and its effect on *Mycobacterium leprae*-infected macrophages

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ABSTRACT

Armadillos (*Dasypus novemcinctus*) manifest the full histopathological spectrum of leprosy, and are hosts of choice for *in vivo* propagation of *Mycobacterium leprae*. Though potentially useful as a model of leprosy pathogenesis, few armadillo-specific reagents exist. We have identified a region of high homology to the interferon gamma (IFN-γ) of other mammals within the recently published armadillo whole genomic sequence. cDNA was made from ConA-stimulated armadillo peripheral blood mononuclear cells (PBMC), amplified, and cloned into a pET expression vector for transformation and over-expression in *Escherichia coli*. The recombinant protein (r*Dn*IFN-γ) was characterized by western blot and its biological function confirmed with bioassays including intracellular killing of *Toxoplasma gondii* and induction of indoleamine 2, 3-dioxygenase activity. In using rIFN-γ to activate macrophages from mice, humans or armadillos, similar to humans, rIFN-γ-activated armadillo MΦ did not produce nitrite and or inhibit the viability of *M. leprae in vitro*. Conversely, murine rIFN-γ-activated mouse MΦ produced high levels of nitrite and killed intracellular *M. leprae in vitro*. These data indicate that the response of armadillo MΦ to r*Dn*IFN-γ is similar to that which occurs in humans, and demonstrates a potentially important value of the armadillo as a model in leprosy research.

1. Introduction

Leprosy continues to be an important public health problem for the developing world and an estimated 2–3 million people currently live with deformity brought by their disease [1]. Though significant progress has been made in reducing new case presentations [2], improved diagnostic tests and therapeutic regimen are still needed. Two major obstacles impeding the progress of research in these areas have been our inability to cultivate *Mycobacterium leprae* (the etiological agent for leprosy) on artificial media in the laboratory, and the lack of a robust animal model for studying this infection [3].

Nine-banded armadillos (*Dasypus novemcinctus*) are the only immunologically intact animal species that exhibits high susceptibility to *M. leprae*. Like man, armadillos manifest leprosy over a broad clinical and histopathological spectrum that is classifiable from lepromatous to tuberculoid [4], and they have been developed as the hosts of choice for *in vivo* propagation of leprosy bacilli [5] [6]. Because of their unique natural susceptibility to infection with *M. leprae*, armadillos could be valuable models of leprosy pathogenesis and help advance development of new diagnostic tests, immunotherapies or vaccines [6]. Unfortunately, because of their exotic nature and scant commercial value, relatively few armadillo-specific immunological reagents have been generated, and consequently few translational benefits actually have been realized with this model to date.

Resistance to *M. leprae* is mediated through cellular immune processes and involves a complex interplay of cytokines and chemokines. Prominent among these is interferon gamma (IFN-γ), which stimulates macrophages (MΦ) to up-regulate anti-microbial, anti-tumour, and antigen processing and presentation pathways [7]. In rodent immune systems, activation of MΦ by IFN-γ results in effective growth restriction and clearance of mycobacteria with production of reactive nitrogen intermediates (RNI) as effector molecules [8,9]. However, this potent anti-microbial mechanism varies from species to species. Human IFN-γ-activated peripheral blood MΦ demonstrate little or no production of nitric oxide (NO) [10,11] and are unable to kill several different mycobacterial species.

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The IFN-γ genes of many other mammals have been cloned and over-expressed in *E. coli* [12,13]. Commercially available recombinant IFN-γ proteins and antibodies also are available for a variety of species, but they rarely exhibit functional cross reactivity between species and it has not been possible to monitor the production of IFN-γ among armadillos over the course of infection by *M. leprae*. However, because of the armadillo's evolutionary and medical significance, a low (2×) coverage of the *D. novemcinctus* genome sequence (http://www.ncbi.nlm.nih.gov/BLAST) was recently published, and more extensive 6× sequence coverage also is underway. Genomic sequence data is an invaluable resource for the identification and generation of specific immunological reagents [14] and exploitation of the armadillo sequence data can significantly benefit efforts to advance these animals as models for leprosy. We probed the available sequence data for an armadillo homolog to human IFN-γ, and report here the sequence, cloning, expression, biological activity and development of associated specific reagents of recombinant *D. novemcinctus* IFN-γ (r*Dn*IFN-γ). In addition, we also used these reagents to examine the functional character of armadillo IFN-γ-activated armadillo MΦ to live *M. leprae* and compared their function to both human and mouse activated macrophages.

2. Materials and methods

2.1. Identification of DnIFN-γ

Bioinformatic tools were used to identify the putative coding sequence of *Dn*IFN-γ. The amino acid sequence of *H. sapiens* IFN-γ (GI: 56786138) and tBLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) were used to search for homologous translated sequences in the *D. novemcinctus* whole genome sequence (WGS) [15]. The putative coding region of *Dn*IFN-γ (GI: DQ094083) was found in two *D. novemcinctus* genomic contigs (GI: 64640499 and GI: 64640497). The partial genomic sequence was used to derive a putative cDNA and a corresponding translation for the putative amino acid sequence was identified using ExPASy Translate tool (http://us.expasy.org/tools/dna.html). The cDNA and the amino acid sequence were submitted to BLAST to compare homology to other IFN-γ molecules [15].

2.2. Generation of D. novemcinctus cDNA

Armadillo peripheral blood mononuclear cells (PBMC) were purified from 8 mL peripheral blood collected in BD Vacutainer® CPT Mononuclear Cell Preparation Tubes (BD Biosciences, San Jose, CA) and mononuclear cells were isolated after centrifugation (1600*g* for 45 min, 25 °C). The mononuclear cell layer was removed, washed 3× with cold PBS, resuspended in culture medium (RPMI 1640 medium containing 2 mM glutamine and HEPES) supplemented with 20% fetal bovine serum (FBS), and plated at 2×10^6 cells/mL in a T_{75} tissue culture flask. The cells were stimulated with ConA (Sigma–Aldrich, St. Louis, MO) at a final concentration of 5 μg/mL for 4 h at 37 °C. Aliquots of the ConA-stimulated cells were washed 3× in cold PBS, resuspended in 500 μL cold PBS, snap frozen in liquid nitrogen, and stored at −70 °C for RNA purification. Total RNA was purified from these cells using the FASTRNA™ kit and the FastPrep® FP120 Instrument and manufacturer's recommendations (Q–Biogene, Carlsbad, CA). The cDNA was generated from 1 μg total RNA using the Advantage RT-for-PCR kit with random hexamers (BD Biosciences Clontech, Palo Alto, CA) in a final volume of 50 μL according to the manufacturer's recommendations.

2.3. DnIFN-γ amplification and recombinant plasmid construction

Primers (DnIFN-γ-F 5′-AGAAAAGATCAGCCAAGTCC-3′ and *Dn*IFN-γ-R 5′-TTCAAATATTACAGGGAGGATG-3′) (BIOMEDD, Baton Rouge, LA) and armadillo cDNA from Con A-stimulated PBMCs were used with high fidelity polymerase, (*Pfu,* Strategene, La Jolla, CA), and PCR to generate a fragment encoding the entire *Dn*IFN-γ cDNA. This product was purified using QIAquick columns (QIAgen, Valencia, CA) and verified by automated DNA sequencing using an ABI prism 3310 DNA sequencer (Applied Biosystems, Foster City, CA) (BIOMMED). DNA encoding the mature peptide (the protein without the signal peptide) was amplified from the cDNA using primers containing the "topo" sequence (CACC) on the 5′ terminus (*Dn*IFN-γ TOPOF: 5′-CACCTGCTACTGCCAGGCCAC-3′ and *Dn*IFN-γ TOPO-R: 5′-CAAATATTACAGGGAGGATGACCA-3′) and ligated into pET 200/DTOPO® vector (Invitrogen, Carlsbad, CA) using standard procedures. The recombinant plasmid was transformed into *E. coli* BL21star competent cells (Invitrogen) according to manufacturer's recommendations. Clones were identified by antibiotic selection and further characterized using *Dn*IFN-γ PCR/direct sequencing.

2.4. DnIFN-γ protein expression and characterization

r*Dn*IFN-γ protein was produced from a positive clone using the following protocol. Recombinant bacteria were grown to mid-log phase ($OD_{600} = 0.6$) and induced with a final concentration of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich). The expressed protein was purified using a Ni-NTA Purification System (Invitrogen) and dialyzed using the recommended, denaturing regimen (Invitrogen). The r*Dn*IFN-γ was refolded as described by Jeevan et al. [16] and concentrated using a Vivaspin 15 mL concentrator with a 10 kDa molecular weight cut-off (Vivascience, Hanover, Germany). The recombinant protein was separated by SDS–PAGE using a 4–20% gradient Novex TBE-urea polyacrylamide gel (Invitrogen), stained with Coomassie® Brilliant Blue (Bio-Rad, Hercules, CA), and compared to Kaleidoscope prestained polypeptide standards (Bio-Rad). Western blot analysis using rabbit polyclonal antibody prepared with synthetic *Dn*IFN-γ peptide epitopes (below) was used to verify the size and presence of the recombinant product.

2.5. Generation of anti-rDnIFN-γ antibodies

Polyclonal antibodies (Abgent, Inc, San Diego, CA) were prepared in rabbits against selected synthetic *Dn*IFN-γ peptide epitopes without Freund's complete adjuvant. Based on the deduced amino acid sequence, two epitopes Anti-*Dn*IFN-γ #1 (LKNWKEESDKKIIQS) and Anti-*Dn*IFN-γ #2 (PKSNLRKRKRSQSTF) were selected using *in silico* predictions of antigenicity. The complexity, hydropathy (Hopp–Woods method) [17], ß-turns (Chou–Fasman method) [18], flexibility, and accessibility of epitopes in the deduced *Dn*IFN-γ amino acid sequence were assessed to generate the regions of the protein most likely to produce antibodies.

Monoclonal antibodies (Mabs) to *Dn*IFN-γ were produced by immunizing Balb/c mice (Harlan, Indianapolis, IN) intra-peritoneally (IP) with 50 μg of the purified recombinant protein in 1:1 TiterMax Gold (Titermax, Inc., Norcross, Ga.) twice in 3-week intervals followed by a final IP injection of 20 μg protein in PBS. Hybridomas were made by fusing primed mouse spleen cells and the myeloma B-cell line SP2/0 and cultured in hypoxanthine, aminopterin and thymidine (HAT) selection medium using a protocol previously described [19]. Hybridoma culture supernatants were screened in an ELISA with either r*Dn*IFN-γ or synthetic peptides. HRP-Rabbit anti-mouse IgG conjugate or HRP-Goat anti-Rabbit IgG (Zymed laboratories, San Francisco, CA) was used to detect the positive clones. Specific antibody reactive sites were determined by ELISA using synthetic 15mer peptides overlapping by 5 amino acids each (15 × 5) and extending over the entire length of the *Dn*IFN-γ (Mimotopes: PharmAus, Ltd., Nedlands, Aus.).