Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 118 (2007) 75–83
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Molecular characterization and expression of Interferon-γ of Asian elephant (*Elephas maximus*)

E. Sreekumar [a,*], M.B.V. Janki [a], D.S. Arathy [a], R. Hariharan [a], C. Avinash Premraj [a], T.J. Rasool [b]

[a] *Rajiv Gandhi Centre for Biotechnology (RGCB), Thycaud P.O., Thiruvananthapuram 695014, Kerala, India*
[b] *Indian Veterinary Research Institute (IVRI), Mukteswar, Nainital District, Uttaranchal 263138, India*

Received 3 February 2007; received in revised form 13 March 2007; accepted 26 April 2007

Abstract

Tuberculosis (TB) caused by Mycobacterial organisms has emerged as one of the major diseases in captive elephants. *In vitro* Interferon-gamma (IFN-γ) assay is being used as an ancillary test for early detection of TB in domestic and captive wild animals. In the present study, basic sequence information and immunological cross-reactivity of this major cytokine of Asian elephants were explored. At predicted amino acid level, IFN-γ of Asian elephant showed maximum identity to that of horse (73%). Other IFN-γ amino acid sequences that showed high level identity were that of giant panda (72%), dog (71%), nine-banded armadillo (69%), cattle (63%) and human (62%). IFN-γ promoter sequences of Asian elephant, human, cattle and mouse showed high level conservation of the putative transcription factor binding sites, TATA box and transcriptional start site. The functionally important human IFN-γ promoter elements, such as AP-2IRE-BE, YY1-γIFN-BED, ATFCS and AP-1γINF binding sites, were absolutely conserved in the corresponding elephant sequence. There was only a single nucleotide variation in the other two important elements, NFAT-γINF and IFN-γPE, indicating the highly conserved regulation of IFN-γ expression across different species. Phylogenetic analysis based on IFN-γ protein sequences revealed a closer relation of Asian elephants and nine-banded armadillo. This shows a closer evolution of these members of Afrotheria and Xenarthra, respectively; and supports the previous reports based on mitochondrial DNA studies. In Western blot analysis, IFN-γ of Asian elephant expressed in *Escherichia coli* was detected using an anti-bovine IFN-γ monoclonal antibody, indicating immunological cross-reactivity.

Keywords: Interferon-gamma; Elephant; Recombinant protein; Promoter; Phylogeny

1. Introduction

Large-scale conservation efforts are going on worldwide for the protection of the endangered Asian elephants (*Elephas maximus*). Western and Eastern Ghat mountain ranges in South India are considered to be the strongholds of these animals and are indicated as core area for their long-term conservation (Vidya et al., 2005; AERCC, 1998). *Ex situ* conservation of Asian elephants in captivity has resulted in infectious diseases, which are spread either from humans, e.g. Tuberculosis (TB) (Mikota et al., 2000, 2001; Montali et al., 2001), or from animals that are reared in proximity, e.g. endotheliotropic herpes virus infection (Ossent et al., 1990; Richman et al., 1999; Ehlers et al., 2006). An early diagnosis of TB is very essential for effective management of the infection and also to prevent the spread of infection to other elephants and its human handlers. In elephants, conventional diagnostic methods

* Corresponding author. Tel.: +91 471 2345899; fax: +91 471 2348096.
E-mail address: esreekumar@gmail.com (E. Sreekumar).

0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2007.04.012

such as intra-dermal tests based on skin response and serum enzyme-linked immunosorbent assays (ELISA) using mycobacterial antigens showed poor repeatability and low correlation with the infection (Mikota et al., 2001). Improved diagnostic technique based on multi-antigen print immunoassay (MAPIA) has been recently developed for diagnosis of TB in elephants (Lyashchenko et al., 2006) and is being used for early detection and management of the disease.

Interferon-γ (IFN-γ), a pleiotropic cytokine produced by activated Th cells and NK cells, is a major mediator involved in the immuno-pathogenesis of tuberculosis (Yang and Mitsuyama, 1997; Flynn et al., 1993) and herpes viral infections (Parr and Parr, 1999; Minami et al., 2002; Mikloska and Cunningham, 2001). It is a glycoprotein that plays a pivotal role in the modulation of Th1 mediated immune response and in the antigen presentation (Farrar and Schreiber, 1993). Cultured lymphocytes from mycobacterium infected animals show an augmented IFN-γ production on subsequent *in vitro* exposure to the mycobacterial antigens. Measurement of this enhanced IFN-γ level is being used in a sensitive, ELISA based assay for the early diagnosis of bovine TB (Wood and Jones, 2001). A recent study has attempted the development of a similar assay for the diagnosis of TB in African elephants (*Loxodonta africana*) (Morar, 2003). This diagnostic technique has so far not been developed for use in Asian elephants. A basic understanding of the Interferon-γ in this species is essential to facilitate such an effort.

In this study, we identified, cloned and analyzed the cDNA and the essential promoter sequences of IFN-γ of Asian elephant. In addition, we expressed the recombinant IFN-γ protein in *Escherichia coli* and Western blot was done with anti-bovine Interferon-γ antibodies to assess the immunological cross-reactivity.

2. Materials and methods

2.1. Culture and mitogen stimulation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood collected from a captive male Asian elephant was used in the experiment. Sixteen milliliters of the blood was transported to the laboratory on ice and processed within 8 h of collection. The samples were centrifuged at 200 × *g* for 10 min at room temperature and the buffy coat was separated. Three milliliters of the buffy coat collected was further diluted to 10 ml with phosphate buffered saline (PBS; pH 7.2) and was carefully layered over 5 ml Histopaque (Sigma, St. Louis, MI) and subjected to density gradient centrifugation at 400 × *g* for 30 min at room temperature. The mononuclear cells at the interface were collected and washed with PBS and subsequently with RPMI-1640 (Invitrogen, Carlsbad, CA). Viability was assessed by Trypan blue staining. The cells were re-suspended (8×10^6 viable cells per ml) in complete RPMI-1640 medium containing 20 mM HEPES, 2 mM L-Glutamine, 50 μM β-mercaptoethanol, 1× antibiotic-antimycotic solution and 10% foetal bovine calf serum (all from Sigma). Ten micrograms per milliliters Concanavalin A (Sigma) and 10 μg/ml Phytohaemagglutinin (Sigma) were added and cells were incubated at 37 °C in a 5% CO_2 incubator for 6 h.

2.2. RNA isolation and RT-PCR

Total RNA from mitogen-stimulated PBMCs was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's direction. Five micrograms of the RNA was reverse transcribed in a 20 μl reaction using oligodT primer and Avian Myeloblastosis Virus (AMV) Reverse Transcriptase as previously described (Premraj et al., 2006). Two microliters of the first strand reaction was subsequently used for PCR amplification. Bovine IFN-γ cDNA-specific primers were designed based on the Genbank Accession No. M29867 (Table 1) and used in the PCR reaction. Fifty microliters PCR reaction contained 1 × PCR buffer, 1 unit of Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 μM dNTPs (all from Promega, Madison, WI); 20 pmol of B-IFN-F and B-IFN-R primers. The cycling conditions were—denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min and extension at 72 °C for 2 min for 35 cycles, and a final extension at 72 °C for 15 min.

2.3. Amplification of the IFN-γ promoter sequence

Genomic DNA was isolated from 1 ml of the elephant blood buffy coat sample as per standard protocol (Sambrook et al., 1989). Genome Walker libraries were constructed using a Universal Genome Walker library kit (Clontech, Mountain View, CA). In order to amplify the 5′ region of the translational start site, nested PCR was done using the adapter-ligated PvuII and Eco*R*V GenomeWalker libraries as the template and adapter-specific forward primers (AP1 and AP2; provided in the kit) and Asian elephant IFN-γ sequence-specific (EIFNPMR1 and EIFNPMR2; Table 1) reverse primers. Construction of the libraries and PCR reactions were carried out as per manufacturer's protocol.