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ScienceDirect
Veterinary Immunology and Immunopathology 115 (2007) 146–154
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Cloning, sequencing and expression of white rhinoceros (*Ceratotherium simum*) interferon-gamma (IFN-γ) and the production of rhinoceros IFN-γ specific antibodies

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Received 28 July 2006; received in revised form 16 October 2006; accepted 19 October 2006

Abstract

Bovine tuberculosis (BTB) is endemic in African buffalo (*Syncerus caffer*) in the Kruger National Park (KNP). In addition to buffalo, *Mycobacterium bovis* has been found in at least 14 other mammalian species in South Africa, including kudu (*Tragelaphus strepsiceros*), Chacma baboon (*Papio ursinus*) and lion (*Panthera leo*). This has raised concern about the spillover into other potentially susceptible species like rhinoceros, thus jeopardising breeding and relocation projects aiming at the conservation of biodiversity. Hence, procedures to screen for and diagnose BTB in black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) need to be in place. The Interferon-gamma (IFN-γ) assay is used as a routine diagnostic tool to determine infection of cattle and recently African buffalo, with *M. bovis* and other mycobacteria. The aim of the present work was to develop reagents to set up a rhinoceros IFN-γ (RhIFN-γ) assay. The white rhinoceros IFN-γ gene was cloned, sequenced and expressed as a mature protein. Amino acid (aa) sequence analysis revealed that RhIFN-γ shares a homology of 90% with equine IFN-γ. Monoclonal antibodies, as well as polyclonal chicken antibodies (Yolk Immunoglobulin-IgY) with specificity for recombinant RhIFN-γ were produced. Using the monoclonals as capture antibodies and the polyclonal IgY for detection, it was shown that recombinant as well as native white rhinoceros IFN-γ was recognised. This preliminary IFN-γ enzyme-linked immunosorbent assay (ELISA), has the potential to be developed into a diagnostic assay for *M. bovis* infection in rhinoceros.

Keywords: Interferon-gamma (IFN-γ); White rhinoceros (*Ceratotherium simum*); Bovine tuberculosis (BTB); *Mycobacterium bovis*; Kruger National Park (KNP)

1. Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB) and has mostly presented itself as a problem in cattle (O'Reilly and Daborn, 1995, pp. 1–46; Pollock et al., 2006, pp. 141–150) and related species including goats (Cousins et al., 1993, pp. 262–263; Liébana et al., 1998, pp. 50–53) worldwide. More recently *M. bovis* has also been found to infect wildlife species like lions (*Panthera leo*), kudu (*Tragelaphus strepsiceros*), African buffalo (*Syncerus caffer*) and Chacma baboon (*Papio ursinus*) in South Africa (Michel et al., 2006, pp. 91–100; Michel, 2002),

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doi:10.1016/j.vetimm.2006.10.016

European badger (*Meles meles*) (Cheeseman et al., 1989, pp. 113–125) in the United Kingdom and the Brushtail possum (*Trichosurus vulpecula*) in New Zealand (Buddle et al., 2000, pp. 1–16). In the KNP and elsewhere, potential spillover into other species, like rhinoceros, for which currently no validated *ante mortem* (indirect) diagnostic tools exist, may jeopardise breeding and relocation projects aiming towards the conservation of biodiversity. The development of practical and reliable procedures to diagnose BTB in black rhinoceros (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*) has therefore been identified as a priority area for research by conservation bodies. Although bovine tuberculosis has to date not been diagnosed in pachyderms in South Africa it is of utmost importance to be able to provide an additional guarantee on the Tb free status of these animals and to provide conservation bodies with an early warning system should bovine Tb enter the rhino population. While tests traditionally available for diagnosing bovine Tb include microscopic and bacterial culture techniques (Schaechter et al., 2006), as well as tuberculin skin tests (Wood et al., 1990, pp. 37–46; Monaghan et al., 1994, pp. 111–124), these are of little value for screening purposes in pachyderms. The culture techniques are most reliable and specific (Schaechter et al., 2006), but have the drawback that they require *post mortem* specimens and results are obtained only after 6–8 weeks. Specificity and sensitivity of Tb skin tests have been determined mostly for domesticated animals, especially cattle (Monaghan et al., 1994, pp. 111–124), while diagnosing *M. bovis* infection in wildlife is proving to be a challenge. In pachyderms, like rhinoceros and elephants, tuberculin skin tests are not practical both due to difficulties in defining suitable injection sites and the fact that these reactions have to be read after approximately 72 h, which necessitates the recapture of the animals. As an alternative to tuberculin skin tests, IFN-γ assays, for example the BOVIGAM™ test (Wood and Jones, 2001, pp. 147–155; Wood et al., 1990, pp. 37–49), have been used during the last decade to determine *M. bovis* specific immune responses in ruminants. These tests consist of antibody-based sandwich enzyme immunoassays that will detect IFN-γ produced by specific T cells after incubation of heparinized blood with *M. bovis* antigens. Infected animals are identified by their high IFN-γ responses, due to *M. bovis* antigen specific T cells induced by the mycobacterial infection.

In this paper we describe the first steps in developing an IFN-γ based ELISA for the detection of *M. bovis* infection in white rhinoceros and report the first evaluation of the specificity of the test in known Tb free rhinoceros. The Tb free status of these white rhinos was determined based mainly on the epidemiological evidence of the absence of *M. bovis* infection in rhinos in South Africa. Indeed to date, after more than 10 years of translocation programmes, no single case of Tb infected rhinoceros has been documented (Michel et al., 2006, pp. 91–100). The set up of the test includes the cloning, sequencing and the expression of the white rhinoceros IFN-γ gene and the production of RhIFN-γ specific monoclonal and polyclonal antibodies. Thus, the IFN-γ produced *in vitro* by antigen stimulation of sensitised T-lymphocytes can be measured to serve as a sensitive and specific indicator of *M. bovis* exposure. Development of this assay could ultimately yield a vital tool for detecting *M. bovis* infection in rhinoceros prior to the development of clinical signs.

2. Materials and methods

2.1. Cloning and sequencing white rhinoceros IFN-γ

Blood from an adult white rhinoceros was collected in EDTA Vacutainer tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (Amersham 17-1440-02). After 25 min of centrifugation at 2800 rpm, mononuclear cells were taken from the interphase and washed two times in RPMI-1640 medium supplemented with L-glutamine (Sigma, R8758) and 10% heat inactivated fetal calf serum (FCS). To induce IFN-γ production, purified mononuclear cells (1×10^6 cells/ml in wells of a 24-well plate) were stimulated with 5 μg/ml Concanavalin A (Con A) (Sigma, C2010-100 mg) for 18–24 h at 37 °C in a 5% CO_2 incubator. Total RNA was purified from stimulated lymphocytes using Trizol reagent (Gibco BRL, life technologies, 15596-018) and 1 μg of total RNA was subjected to first strand cDNA synthesis using reverse transcriptase Rnase H SuperscriptII™ (Gibco BRL, life technologies, 18064-014) and oligo-$(dT)_{12–18}$ as a primer for RT-PCR. The cDNA produced in this way was used as a template for a polymerase chain reaction (PCR) using *Pwo* DNA polymerase (Roche, 1644 947) according to the manufacturer's instructions. PCR primers (5′-end primer sequence: 5′-GCCGCGCGGGAGCCAGGCCGCGTTTTTTAAA-GAAATAG -3′ and 3′-end primer sequence: 5′-GCGGCGGCGGGAATTCAAATATTGCAGGC AG-G-3′) used were designed to amplify the part of the white rhinoceros IFN-γ gene that encodes the mature protein (without signal sequence). The sequence of the

primers was based on the IFN-γ gene of the horse (Genbank accession no. D28520), because this species has a close phylogenetic relationship with rhinoceros. A gradient PCR was performed for 35 cycles using a Bio-Rad Thermal iCycler. One cycle consisted of: DNA denaturation at 95 °C for 30 s, primer annealing at 56 °C, 58 °C, 61 °C or 64.7 °C (depending on the position of the PCR tube in the gradient) for 45 s and primer extension at 72 °C for 60 s. The underlined sequences in the primers above are not part of the IFN-γ sequence, but were included as annealing sites for a second PCR performed with the forward primer GW2-F (5′-GGGGACAAGTTT GTACAAAAAAGCAGGCT-TGGTGCCGCGCGGGAGC-3′) and the reverse primer GW-R (5′-GGGGACCACTTTGTACAAGAAAGCT-GGGTGCGGCGGCGGG-3′). This second PCR, introduced the *attB1* and *attB2* sites, which enabled subsequent Gateway® cloning (Invitrogen). The conditions for this second PCR were similar as described for the first PCR, apart from the annealing temperature that was set at 56 °C.

Following DNA electrophoresis, the PCR product was harvested from the low melting point agarose gel and inserted into the vector pDONR201 (Invitrogen) by the BP Gateway reaction (Invitrogen, Gateway™ BP Enzyme Mix, 11789-013) performed according to the manufacturers instructions. After transformation of *E. coli* strain DH5α, plasmid DNA was purified from selected colonies and sequenced to check the cloned fragment. Subsequently, the Interferon-gamma gene was subcloned into the expression vector pET15bGW by the LR Gateway reaction (Invitrogen, Life technologies, Gateway™ LR Clonase Mix, 11791-091). The resulting expression vector was designated pET15-RhIFN-γ. Vector pET15bGW is a derivative of pET15b (Novagen) that was adapted for Gateway cloning (Invitrogen) by ligation of the Gateway cassette containing *Xba*I–*Hin*dIII fragment of pDEST17 (Invitrogen) into the corresponding sites of pET15b. The resulting plasmid was purified from liquid culture from ampicillin and chloroamphenicol resistant colonies obtained after transformation of *E. coli* DB3.1 (Invitrogen).

To determine the 5′-end of the complete coding part of the RhIFN-γ-gene (including the sequence encoding the signal sequence), this end was cloned separately. First, it was PCR amplified using KOD hotstart polymerase (Novogen) according to the manufacturer's instructions. The white rhinoceros cDNA described above was used as a template. The forward primer F3 (3′-CCTGATCAGCT-TAGTACAGAAGTGA-5′) was based on the published equine IFN-γ sequence upstream of the start codon and the reverse primer R3 (5′-TCCTCTTTCCAGTTCTT-CAAGATATC-3′) based on the RhIFN-γ-gene sequence encoding the mature protein that had been cloned as described above. A gradient PCR, with annealing temperatures between 65 °C and 55 °C, resulted in a weak PCR band of the expected size for the reaction performed at 57 °C. To obtain enough material for cloning, additional PCR rounds were required. To avoid amplification of the smear of unspecific PCR bands present, a half-nested PCR was performed on the original PCR product to increase the specificity. For this half-nested PCR, the same forward primer (F3) was used, because the 5′-end sequence was not known, and a reverse primer (R7: 5′-TCATTCATCACTTTGATGAGTTCA-3′) which anneals to a sequence upstream of primer R3 (internal reverse primer). For the first 20 cycles, the annealing temperature was reduced with 0.5 °C each cycle (touch down PCR), starting at 65 °C. The following 20 cycles were performed at a constant annealing temperature of 55 °C. The resulting PCR product was run on a 1.5% low melting point agarose gel and a dominant PCR band of the expected size (approximately 450 bp) was cut out. After melting the agarose at 65 °C, this PCR band was used as a template for another half-nested PCR, using the same forward primer and as a reverse primer (R4: 5′-CCTCTTTCCAGTTCTTCAA-GATATC-3′) which anneals to a sequence upstream of primer R7. The PCR conditions were identical to those described for the previous half-nested PCR, except that only ten cycles were given, once the touch down PCR reached an annealing temperature of 55 °C. The obtained PCR product was cloned into the vector pCR4, using the "Zero-blunt-TOPO-PCR-cloning-kit" (Invitrogen) according to the manufacturer's instructions. After transformation of *E. coli* strain DH5α, plasmid DNA was purified from selected colonies and sequenced to verify the cloned fragment. The successive rounds of this half-nested PCR approach were performed to obtain enough material to allow efficient cloning and to increase the specificity.

2.2. Expression and purification of recombinant white rhinoceros IFN-γ

Vector pET15-RhIFN-γ was used to transform *E. coli* BL21-codon^{+}®(DE3)-RIL competent cells (Stratagene, 230245). A single ampicillin and chloroamphenicol resistant colony was spread on an LB agar plate containing ampicillin, chloroamphenicol and 1% (w/v) glucose. The glucose was added to repress expression of the recombinant protein. After overnight incubation at 37 °C, the bacteria were harvested from the plate with an inoculation loop and resuspended in 10 ml LB. After

resuspension the bacteria were transferred to 500 ml LB containing ampicillin and chloroamphenicol and incubated at 37 °C, with shaking, until the optical density at 600 nm (OD_{600}) reached 0.6–0.9. Gene expression was induced with 1 mM IPTG and incubation continued for 4 h, after which cells were harvested by centrifugation at 5000 × *g* for 15 min. Cell pellets were resuspended in 40 ml of Buffer B (20 mM Tris–HCl [pH 8], 500 mM NaCl) containing 0.1 mg/ml of lysozyme. The cell suspension was transferred to a 50 ml tube and was incubated at room temperature under rotation for 30 min. This was followed by addition of 5 ml buffer C (100 mM DTT, 50 mM EDTA, 10% Triton X-100). The contents of the tube were mixed by inverting the tubes several times and the lysate was prepared by repeating alternate freezing and thawing steps at −20 °C and room temperature, respectively. After the last freeze/thaw step, 1500 units of benzonase (Novagen) and 1.5 ml of a 0.5 M $MgCl_2$ solution was added and incubated at room temperature for 30 min to break down the DNA and reduce the viscosity. The total protein lysate was centrifuged at 5000 × *g* for 15 min. The pellet, containing the IFN-γ inclusion bodies, was washed with Buffer B containing 1% Triton X-100. After a final centrifugation step (5000 × *g* for 15 min) the pellet was solved in 10 M urea prepared freshly in buffer B containing 20 mM imidazole at room temperature. Any remaining insoluble material was removed by centrifugation at 5000 × *g* for 20 min at room temperature. The hexa-histidine tagged recombinant IFN-γ was purified by immobilized metal affinity chromatography (IMAC) on chelating sepharose fast flow (Amersham-Biosciences, 17-0575-02) charged with Ni^{2+} according to the manufacturer's instructions. After equilibration of the column with buffer B containing 20 mM imidazole and 8 M urea, the solved inclusion bodies were applied to the column. The bound protein was washed with 10 column volumes of buffer B containing 20 mM imidazole and 8 M urea. The protein was refolded on the column by fast replacement of wash buffer with two column volumes of refolding buffer (50 mM Tris–HCl [pH 8], 2 mM oxidized gluthathione, 0.22 mM reduced gluthathione, 1 M NDSB201, 0.5 M L-arginine) and incubated at 4 °C for 40 h. Refolding buffer was discarded and the column washed twice with one column volume PBS containing 1 M NDSB201. The refolded IFN-γ was eluted with a total of five column volumes of PBS containing 50 mM EDTA. The protein was dialysed against 1× PBS and subsequently centrifuged (5000 × *g* for 30 min) to remove any protein that had precipitated during the dialysis. The protein solution was mixed with an equal volume of glycerine then sterile filtered using a 0.2 μM filter and stored at −20 °C. Samples were taken during the whole purification process for analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. White rhinoceros IFN-γ specific poly- and monoclonal antibodies

Mice were immunised with recombinant IFN-γ according to routine procedures using Specol, an oil based adjuvant (Boersma et al., 1992, pp. 503–512; Bokhout et al., 1981, pp. 491–500; Hall et al., 1989, pp. 175–186) and boosted on three occasions. When desired serum antibody titres were achieved, as determined by indirect *r*RhIFN-γ ELISA, mice spleens cells were fused to SP2/0 cells to obtain hybridomas and plated in 96 well tissue culture plates (Kohler, 1975, pp. 495–497; Kohler, 1975, pp. 495–497). Supernatants from wells containing colonies of hybridoma cells were tested in the ELISA for the presence of *r*RhIFN-γ specific monoclonal antibodies. Positive colonies were subcloned by FACSVantage (Becton Dickson) single cell sorting, based on forward/sideward scatter characteristics, and tested again.

For the production of polyclonal antibodies, chickens were immunised intramuscularly using Specol as an adjuvant, and boosted on a regular basis to maintain antibody titres. Eggs were collected for up to 1 year following the first booster immunization and stored at 4 °C till further processing. Finally antibodies were purified from the egg yolk by the "water dilution method" followed by ammonium sulphate precipitation according to the procedure described by (Hansen et al., 1998, pp. 1–7). After extensive dialysis against PBS, poly-and monoclonal antibodies were sterile filtered using a 0.2 μM filter, aliquoted and stored at 4 °C until use.

The immunization protocols were approved by the Animal Ethics Committee of the Veterinary Faculty of the University of Utrecht.

2.4. Screening of hybridomas for antibody production by indirect ELISA

Fifty microlitres per well of the recombinant IFN-γ protein diluted to 1 μg/ml in carbonate/bicarbonate buffer (0.1 M, pH 9.6) was used to coat 96 well Costar high binding ELISA plates overnight at 4 °C. After removal of the coating, the plates were blocked with 100 μl/well of 2% non-fat powdered milk (Protifar) in PBS for 1 h at 37 °C. Plates were then washed five times using tap water with 0.1% Tween 20 using an ELISA plate washer. Hybridoma supernatants, 50 μl diluted 1:1 with 2% Protifar in PBS containing 0.1% Tween 20,

were added to the wells. After 1 h at 37 °C and five additional washings, goat anti-mouse IgG (1:2000) HRP conjugate (Boehringer Mannheim, 1047523) in Protifar + PBS containing 0.1% Tween 20 was added and plates were incubated for 1 h at 37 °C. After five additional washes, ABTS (5 mg/ml) (Roche, 1112597) was used as the substrate for the colour reaction. After 30 min at room temperature plates were read spectrophotometrically (Bio-Rad) at 492 nm.

2.5. Native IFN-γ

Blood was collected in EDTA Vacutainer tubes from three Tb free white rhinoceros. PBMC were isolated as described above. To induce IFN-γ production, purified mononuclear cells (1×10^6 cells/ml in wells of a 24-well plate) were stimulated with 10 μg/ml Con A (Sigma, C2010-100 mg) at 37 °C in a 5% CO_2 incubator. PBMC were also stimulated with Bovine and Avian purified protein derivatives (PPD). PPDs' are the antigens used in standard *in vivo* comparative skin tests and *in vitro* IFN-γ tests for the diagnosis of Tb in cattle. Bovine and Avian PPD are extracts produced from cultures of *M. bovis* strain AN5 (Hewinson et al., 2006, pp. 127–139) and *M. avium* strain D4ER which have been inactivated. A control sample was included consisting of PBMC cultured without mitogen. After 18–24 h incubation, cell cultures were collected and centrifuged at 3200 rpm for 10 min and the supernatant harvested. Production of IFN-γ was analysed in the capture ELISA described below.

2.6. Prototype capture ELISA for detection of native white rhinoceros IFN-γ

Microwell™ polysorb ELISA plates (Nunc, C96 446140) were coated with 50 μl of monoclonal antibody (mAb) 1H11 at 1 μg/ml and incubated overnight at 4 °C. Wells were blocked with 100 μl block buffer (2% fat free milk powder in 1× PBS) and incubated at 37 °C for 1 h. The plates were washed with wash buffer (H_2O/0.1% Tween 20) five times. As a positive control recombinant white rhinoceros IFN-γ was diluted in PBS to 1 μg/ml and tested in duplicate. Undiluted supernatants (50 μl) collected from overnight stimulated PBMC were added to the remainder of the wells. After the incubation the wells were washed five times with wash buffer and incubated with 50 μl polyclonal antibodies to white rhinoceros IFN-γ (chicken IgY (700 μg/ml), 1:100 dilution in block buffer) per well. After 1 h the plates were washed five times with wash buffer and rabbit polyclonal to chicken IgY H&L (HRP) (Abcam, ab6753) antibody was added (1:3000 dilution). The wash step with wash buffer was repeated and the addition of *o*-phenylenediamine (OPD) (Sigma, P3804) substrate followed. The reaction was stopped after 20 min with 50 μl of 2N H_2SO_4 and the OD was read 10 min later at 492 nm.

3. Results

3.1. Cloning and sequencing of the white rhinoceros (C. simum) IFN-γ gene

Initially the part of the IFN-γ gene encoding the mature protein was amplified by RT-PCR, using primers that were based on the horse IFN-γ sequence. A single PCR band was obtained (results not shown) that was cloned into the Gateway vector pDONR201. A BLAST search demonstrated strong homology of the cloned PCR fragment with the horse IFN-γ gene. This IFN-γ gene was subsequently cloned into an *E. coli* expression vector (pET15b-GW). To determine the total coding sequence of the RhIFN-γ gene, the missing 5′-end was cloned separately, using a forward primer that was based on the horse 5′-end IFN-γ sequence and reverse primers based on the cloned sequence encoding the mature part of white rhinoceros IFN-γ. The complete coding sequence was composed of this 5′-end sequence and the previously determined sequence encoding the mature IFN-γ. The nucleotide (nt) and predicted amino acid (aa) sequences of white rhinoceros IFN-γ are shown in Figs. 1 and 2, respectively.

The coding part of the white rhinoceros IFN-γ gene is 501 nucleotides long and encodes a protein with a predicted molecular weight (MW) of 19.4 kDa. According to the SignalP 3.0 prediction server (http://www.cbs.dtu.dk/services/SignalP/) the most likely signal peptidase cleavage site is located between aa 25 and 26, which would yield a mature protein of 141 aa and a signal peptide of 25 aa.

The predicted amino acid sequences of white rhinoceros and equine IFN-γ (Grünig et al., 1994, pp. 448–449) are aligned in Fig. 2.

Blast searches of the nucleotide sequences and the predicted aa sequences of RhIFN-γ demonstrated the highest homology with equine IFN-γ, that is 90% identity on the nucleotide as well as on the aa level.

3.2. Expression and purification of recombinant white rhinoceros IFN-γ (rRhIFN-γ)

The *r*RhIFN-γ protein with a hexa-histidine tag (his-6-tag) at its N-terminal end and the additional

Fig. 1. Nucleotide sequence of RhIFN-γ gene. Bold arrows under the sequence indicate annealing regions used by PCR primers used for cloning the sequence encoding the mature protein (bold arrows). Primers used for determining the 5′-end of the gene are indicated with thin arrows. The start codon (ATG) and the stop codon (TAA) are in bold and underlined (Genbank accession no. DQ305037).

aa's derived from the GW recombination sequence and thrombine cleavage site was expressed in *E. coli* by plasmid pET15-RhIFN-γ. Upon IPTG induction a strong protein band with the expected molecular weight for the tagged *r*RhIFN-γ was induced (Fig. 3, lane 3). The major part of the expressed *r*RhIFN-γ was present in the insoluble fraction as inclusion bodies (Fig. 3, lane 5). After solubilisation of the inclusion bodies in 8 M urea (Fig. 3, lane 6), the majority of the hexahistidine-tagged *r*RhIFN-γ bound to a column with immobilized Ni^{2+} (Fig. 3, lane 8) and a minor part failed to bind and showed up in the flow through fraction (Fig. 3, lane 7). After washing, the bound protein was refolded on the column. After

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Identities = 149/166 (90%), Positives = 157/166 (95%)

Rhino: 1   MNYTSFILAFQLCVILGSPSYYCQAVFFKEIENLKEYFNASNPDVADGGSLFLDILKNWK 60
           MNYTSFILAFQLC ILGS +YYCQA FFKEIENLKEYFNASNPDV DGG LFLDILKNWK
Horse: 1   MNYTSFILAFQLCAILGSSTYYCQAAFFKEIENLKEYFNASNPDVGDGGPLFLDILKNWK 60

Rhino: 61  EESDKKIIQSQIVSFYFKLFENLKDNQVIQKSMDIIKEDLFVKFFNSSTSKLDDFKKLIQ 120
           E+SDKKIIQSQIVSFYFKLFENLKDNQVIQKSMD IKEDLFVKFFNSSTSKL+DF+KLIQ
Horse: 61  EDSDKKIIQSQIVSFYFKLFENLKDNQVIQKSMDTIKEDLFVKFFNSSTSKLEDFQKLIQ 120

Rhino: 121 IPVDDLQVQRKAISELIKVMNDLSPRSNLRKRKRSQGQFRGRRALN 166
           IPV+DL+VQRKAISELIKVMNDLSP++NLRKRKRSQ  FRGRRAL
Horse: 121 IPVNDLKVQRKAISELIKVMNDLSPKANLRKRKRSQNPFRGRRALQ 166
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Fig. 2. Alignment of predicted protein sequences for white rhinoceros and equine IFN-γ. Amino acid identities are shown between the white rhinoceros and equine sequence; a plus sign denotes conserved substitution. Underlined is the predicted signal sequence of rhinoceros IFN-γ.

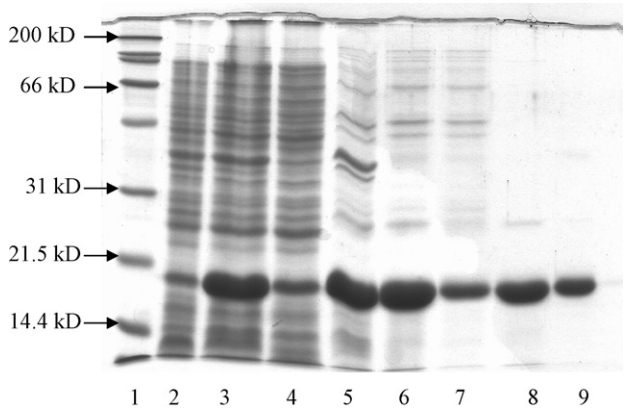

Fig. 3. SDS-PAGE gel showing the purification of recombinant IFN-γ. Lane 1: broad range Mw marker; lane 2: total bacterial lysate (uninduced); lane 3: total bacterial lysate (IPTG-induced); lane 4: soluble fraction; lane 5: insoluble fraction (inclusion bodies); lane 6: solved inclusion bodies (10 M urea); lane 7: flow-through Ni^{2+} column; lane 8: protein bound on column; lane 9: eluted protein.

elution and dialysis the refolded IFN-γ had a purity of at least 95% (Fig. 3, lane 9).

3.3. Prototype sandwich ELISA for the detection of white rhinoceros IFN-γ

The purified recombinant white rhinoceros IFN-γ was used to generate specific monoclonal mouse antibodies and polyclonal chicken antibodies (IgY). Initially, the supernatants of ten hybridomas showed strong binding to the recombinant RhIFN-γ in an indirect ELISA. Further analysis demonstrated that three hybridomas produced antibodies specific for the IFN-γ moiety of the fusion protein, while others were specific for the remainder of the recombinant molecule incorporated to allow affinity purification. Stable subclones could be obtained from two of the three IFN-γ specific hybridomas. The monoclonal antibodies produced by both hybridomas behaved similar when they were tested as capture antibodies in initial experiments. We chose to use the product of the best producing hybridoma, i.e. monoclonal antibody 1H11 (mAb 1H11, IgG1, kappa light chain). Recombinant white Rhinoceros IFN-γ could be detected using the preliminary sandwich ELISA, using mAb 1H11 as a capture antibody in combination with polyclonal anti-IFN-γ IgY as a detecting antibody. This sandwich ELISA was also able to detect native white rhinoceros IFN-γ (Fig. 4).

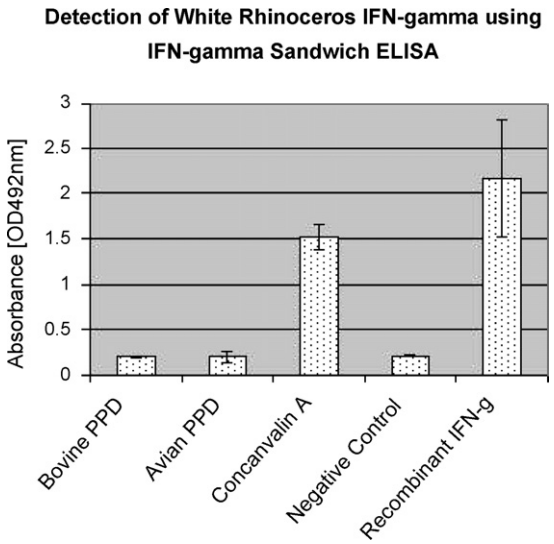

Fig. 4. Detection of white rhinoceros IFN-γ in PBMC stimulated with bovine PPD, avian PPD and concanavalin A using the IFN-γ sandwich ELISA. Results are expressed as the mean value from three rhinoceros. 'I' indicated the standard deviations.

The sandwich ELISA could also detect native IFN-γ, as was demonstrated by the strong signal obtained with supernatants of PBMC of three white rhinoceros that had been stimulated with Con A to induce the expression of this cytokine (Fig. 4).

4. Discussion

M. bovis has been found to have an exceptionally wide host range which includes domesticated ruminants and captive and free-ranging wildlife (Buddle et al., 2000, pp. 1–16; Michel et al., 2006, pp. 91–100). A high bovine tuberculosis prevalence among buffalo herds in the southern region of the KNP has facilitated the spillover of *M. bovis* infection into a number of animal species and poses a real threat on rare species, thus jeopardising both breeding and relocation projects of amongst others, rhinoceros in the context of conservation of biodiversity. Although not yet diagnosed in South Africa, rhinoceros have been reported to be

susceptible to both *M. tuberculosis* and *M. bovis* (Mann et al., 1981, pp. 1123–1129; Oh et al., 2002, pp. 1290–1293; Dalovisio et al., 1992, pp. 598–600; Stetter et al., 1995, pp. 1618–1621), but little is known concerning the pathogenesis of BTB in rhinoceros. Diagnostic tests available for *M. bovis* infection are often limited to certain species or lack validation in others, such as the intradermal tuberculin test in pachyderms. As a consequence the specificity and sensitivity of these tests is unknown in these animal species.

Control of infection in the individual animal or in groups of animals strongly depends on early diagnosis. The IFN-γ test has proven to be highly successful in demonstrating mycobacterial infections in domestic and non-domestic species, including cattle, goats, bison, African buffalo (Bovigam™) (Grobler et al., 2002, pp. 221–227), deer (Cervigam™) and primates (Primagam™) (Waters et al., 2006, pp. 37–44; Garcia et al., 2004, pp. 578–584; Garcia et al., 2004, pp. 86–92). In wildlife species the test is considered practical, as it requires minimal invasion and manipulation.

The present paper reports the successful cloning, sequencing and expression of IFN-γ from white rhinoceros (*C. simum*) and the production of monoclonal antibodies specific for *r*RhIFN-γ, as essential tools for development of assays to detect the IFN-γ response to *M. bovis* infection in rhinoceros.

As expected from the close phylogenetic relationship, the highest homology was observed with the equine IFNγ sequence on the DNA as well as the protein level (both 90%). The RhIFN-γ gene is predicted to encode a signal sequence of 25 aa and a mature protein of 141 aa residues. For expression purposes in *E. coli*, initially the sequence encoding the mature protein was cloned. The forward primer that was used to clone the mature IFN-γ was based on the IFNγ sequence of the horse, because of its relatedness to rhinoceros. Potentially this horse forward primer might have had one or a few mismatches with the rhinoceros sequence, which would consequently result in different amino acids at the N-terminal end of the mature white rhinoceros IFN-γ. The sequence of the 5′-end was therefore cloned separately using forward primers corresponding to equine IFN-γ sequences upstream of the start codon and reverse primers based on the obtained white rhinoceros IFN-γ sequence. Based on the new set of primers derived from those sequences, the final nucleotides at the 5′-end was verified (Fig. 1, Genbank accession no. DQ305037). Indeed, the horse forward primer used to clone the sequence encoding the mature rhinoceros IFN-γ, contained two mismatches with the real rhinoceros sequence. Only the second mismatch results in a different amino acid after translation. This aa, the first of the predicted mature rhinoceros IFN-γ, turned out to be a valine (v), instead of the alanine (a) (Fig. 2). Apparently, the amino acid at this position does not form (an essential) part of the epitope recognized by monoclonal antibody 1H11, as a prototype sandwich ELISA could be developed, using this antibody in combination with polyclonal chicken antibodies, that is able to detect both recombinant and native white rhinoceros IFN-γ.

To date tests have only been performed in white rhinoceros and have yet to be performed in black rhinoceros.

A first confirmation of the specificity of the test was done using Tb free white rhinoceros. No IFN-γ was detected after PPD stimulation although a positive signal was detected after Con A stimulation of PBMC. In conclusion, although optimization of the ELISA with respect to its evaluation for Tb (*M. bovis*/*M. tuberculosis*) infected rhinoceros need to be conducted, the sandwich ELISA as designed demonstrated to be a promising approach towards diagnosis of Tb (*M. bovis*/*M. tuberculosis*) infection in the white rhinoceros.

Acknowledgements

The authors would like to thank the following people and funding institutes for their contributions towards this project: Peter van Kooten, Utrecht University, Department of Immunology and Infectious Diseases, The Netherlands; Peter Buss, Wildlife and Game Capture Unit, Kruger National Park, Skukuza, South Africa; Utrecht Delta Scholarship, Utrecht University, The Netherlands; National Research Foundation (NRF), Thuthuka Grant, South Africa; Belgium Grant, Institutional Collaboration between ITM and DVTD, University of Pretoria (95401) Framework Agreement DGIC-ITM 2003-2007; UP Postgraduate Abroad Programme, University of Pretoria, South Africa.

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