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Prokaryotic Expression and *In vitro* Functional Analysis of IL-1 and MCP-1 from Guinea Pig

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Abstract

The Guinea pig (*Cavia porcellus*) is an excellent animal model for studying human tuberculosis (TB) and also for a number of other infectious and non-infectious diseases. One of the major roadblocks in effective utilization of this animal model is the lack of readily available immunological reagents. In order to address this issue, guinea pig interleukin 1 beta (IL-1 β) and monocyte chemo attractant protein-1 (MCP-1) were efficiently cloned and expressed in a prokaryotic expression vector (pET-30a) and the expressed proteins in soluble form from both the genes were confirmed by N-terminal sequencing. The biological activity of recombinant guinea pig IL-1 β was demonstrated by its ability to drive proliferation in thymocytes and the recombinant guinea pig MCP-1 exhibited chemotactic activity for guinea pig resident peritoneal macrophages. These biologically active recombinant guinea pig proteins will facilitate an in-depth understanding of the role they play in the immune responses of the guinea pig to TB and other diseases.

Keywords

assays; guinea pig; IL-1 β ; MCP-1; proteins

Introduction

Tuberculosis (TB) remains a major global health challenge since time immemorial with an estimated annual death toll of 1.3-1.6 million in 2010, making TB the second leading cause of death from an infectious disease worldwide [1]. Although the only existing vaccine 'BCG' has been administered to combat tuberculosis for the past 90 years, the major concern is its variable efficacy [2]. Global initiatives to develop better TB vaccines to prevent disease caused by *Mycobacterium tuberculosis* are currently underway. These approaches are aimed at replacing, modifying or boosting the existing BCG vaccine [3]. The guinea pig has been extensively used as an excellent animal model for testing novel vaccine candidates during pre-clinical development because it mimics human tuberculosis in the formation of granulomatous lesions and subsequent necrosis, its ability to be infected by a very small number of tubercle bacilli via the respiratory route, a very good response to anti-TB drugs and to vaccination [4, 5, 6]. Although the guinea pig is a promising model for preclinical vaccine development and other research applications in comparison with other animal models such as mice, the shortage of readily available immunological reagents has severely limited its use in TB and many other important diseases [7].

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The proteins derived by subcloning and expressing guinea pig cytokine and chemokine genes remain few in number [8] and this is one of the prime reasons that most of the cytokine studies published to date have reported mRNA expression analysis by real time PCR. The availability of recombinant guinea pig proteins and antibodies to those proteins would allow a deeper understanding of the host response to vaccination and infection including the evaluation of novel vaccines. These guinea pig proteins will also facilitate *in vitro* as well as *in vivo* studies for a number of other infectious and non-infectious diseases. Our laboratory has been actively involved in developing molecular and immunological reagents for guinea pig cytokine and chemokines such as interleukin-8 (IL-8) [9], regulated upon activation, normal T-cell expressed, and secreted (RANTES) [10], tumor necrosis factor-alpha (TNF- α) [11], interferon-gamma (IFN- γ) [12], interleukin-4 (IL-4) [13] and interleukin-10 (IL-10) [14]. Realizing the potential of guinea pig model of pulmonary tuberculosis, other groups also started to actively contribute to the wealth of guinea pig immunological reagents for analyzing cellular immune responses [15].

The ubiquitous cytokine IL-1 β has been shown to play an important role in host resistance to mycobacteria [16]. IL-1 β is released from cells after cleavage of a pro-form by caspase 1 in response to many pathogens [17]. IL-1 β activates many different cell types and produces a range of inflammatory activities as it is often involved in immune responses [18]. Increased gene expression of IL-1 β was observed in bronchoalveolar lavage (BAL) cells from tuberculosis patients when compared with cells from healthy individuals [19]. Decreased levels of IL-1 β expression were observed in patients who responded early (early responders) to anti-TB therapy [20]. Monocytes are crucial in containing *M. tuberculosis* infection, and MCP-1 plays a role in their recruitment to the site of infection [21]. A recent study has shown that MCP-1 responses were very important in distinguishing people with active tuberculosis from people with latent tuberculosis [22].

Guinea pig IL-1 β was previously cloned [23] but has not been expressed. In this study, we subcloned rgp IL-1 β in the pET-30a vector, expressed it in *Escherichia coli* and analyzed the recombinant protein for its ability to drive proliferation in thymocytes. MCP-1 was previously expressed using COS cells and the biological activity was determined using peritoneal exudate macrophages [24]. In this study, we expressed recombinant guinea pig MCP-1 in *E. coli* and determined its biological activity using resident peritoneal macrophages. Here we report expression of biologically active recombinant guinea pig (rgp) IL-1 β and MCP-1 proteins that can be used for a wide range of research applications.

Materials and Methods

Cloning of guinea pig IL-1 and MCP-1 genes

The cloning of full-length guinea pig IL-1 β (Accession number-AF119622) and MCP-1 (Accession number-NM_001172926) genes into the pBlueScript vector was accomplished by using the ConA-stimulated guinea pig spleen cDNA library [25, 26]. The genes residing in the pBlueScript vector were obtained from Dr. Yoshimura at National Cancer Institute, Frederick, USA.

Sub-cloning of guinea pig IL-1 β and MCP-1 genes into the prokaryotic expression vector

The mature peptide regions of guinea pig IL-1 β and MCP-1 genes residing in pBlue Script vectors were amplified by PCR with primer sequences (Invitrogen, Carlsbad, CA) containing *Bam*HI and *Hind*III recognition sites to facilitate cloning. The forward and reverse primer sequences used for amplification of the IL-1 β gene were 5'-TAGGATCCACGCCTGTCCCATCACGGAA-3' and 5'-TAAAGCTTCTGGCCATTGTTATTTCCCA-3' and for MCP1 gene were 5'-

TAGGATCCAGCCGGATGGAGTTAATAC-3' and 5'-
TAAAGCTTTTAGCTACGGTTCTTGGGGT-3'. The underlined parts of primer sequences represent the nucleotide sequences of the gene whereas 5' overhangs (italicized) are restriction sites designed to facilitate cloning.

The conditions for PCR amplification of the IL-1 β or MCP-1 genes were the same as that described for one of the guinea pig genes in our previous report [14]. PCR products that were obtained for both IL-1 β and MCP-1 genes were digested with *Bam*HI and *Hind*III restriction enzymes and ligated into the pET-30a(+) vector (Novagen, Madison, WI). The ligation mixtures were transformed with chemically competent Novablue cells (Novagen). Five transformants were randomly selected for plasmid DNA isolation and analyzed for the presence of inserts by restriction analysis. Bidirectional sequencing of the transformants was performed using fluorescent-labeled dideoxy nucleotide terminators with Big Dye version 3.1 and ABI 3130 xI automated sequencers (Applied Biosystems, Foster City, CA). Plasmid DNA from two of the confirmed transformants was transformed into chemically competent Rosetta 2(De3) cells (Novagen).

Expression of IL-1 β and MCP-1 genes and determination of target protein solubility

The colonies were grown in 3 milliliters (ml) of LB broth containing kanamycin (15 μ g/ml) and chloramphenicol (34 μ g/ml) overnight at 37 °C and was added to 50 ml of identical culture medium and then cultured at 37 °C. When the OD₆₀₀ of the cultures reached 0.6–0.8, protein expression was induced by adding Isopropyl- β -d-thiogalactoside (IPTG) (Sigma, St. Louis, MO) to a final concentration of 1.0 mM. After induction with IPTG, the incubation continued for five hours at 37 °C. A small fraction of the sample was collected at different time intervals to determine the pattern of protein expression. The induced/expressed proteins were analyzed by Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a pre-cast Novex 10-20% Tricine gel (Invitrogen).

The cells were harvested by centrifugation and the pellet was resuspended in 5 mL lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM Imidazole, pH 8.0) followed by addition of lysozyme (Sigma) to 1mg/ml and incubation on ice for 30 minutes. The sample was subjected to sonication (Misonix Inc., Farmingdale, NY) at 30 W for a total of 6 times (10 seconds each time with a pause of 10 seconds). The sonicated sample was centrifuged to obtain the cleared lysate and the pellet. A fraction of the cleared lysate or pellet was analyzed on Novex 10-20% Tricine gel (Invitrogen) to determine the solubility of the target protein. Both the target proteins were obtained in the soluble form and purified under native conditions.

Protein purification for soluble proteins

The cleared lysates obtained after sonication were purified by immobilized metal affinity chromatography under native conditions. In brief, 1 ml of Ni-NTA agarose resin was added to 4 ml of cleared lysate in a polypropylene column (Qiagen) and was placed on an orbital shaker at 4°C for 1 hour. After allowing the flow-through to pass, the column was equilibrated with 5 ml of lysis buffer, and was washed twice with 10 ml of wash buffer, which was followed by finally eluting the His-tagged protein with the elution buffer to the column in 0.5 ml aliquots. The eluted fractions containing the protein of interest were pooled and concentrated using Amicon Ultra-15 centrifugal filter devices (Millipore, Billerica, MA). The protein concentrations were determined using the Protein assay kit (Bio-Rad, Hercules, CA).

N-terminal sequencing of proteins

The eluted and concentrated IL-1 β and MCP-1 fractions (approximately 100 μ g) were digested with 3-4 units of enterokinase enzyme (Novagen) and were monitored for digestion at 6 h and 14 h. The enterokinase digested protein products were run on Novex 10-20% tricine gels (Invitrogen). They were blotted onto PVDF membranes using the Mini-trans blot electrophoretic transfer cell apparatus (Biorad), and were then stained with Coomassie Brilliant Blue. The proteins of interest that appeared as bands on the membrane were excised and subjected to Edman protein sequencing method on a Procise 492 protein sequencer (Applied biosystems) at the Protein Chemistry Laboratory of Texas A&M University. The LPS contamination in the protein samples was analyzed by Toxin sensor chromogenic LAL endotoxin assay kit (Genscript, Piscataway, NJ).

Animals used in this study

Specific pathogen-free, random-bred Hartley strain guinea pigs weighing approximately 250-350g were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA, USA). The animals were housed individually in polycarbonate cages in a temperature and humidity controlled environment with a 12-h light-dark cycle. All procedures were reviewed and approved by the Texas A & M University Laboratory Animal Care Committee.

Determining the biological activity of rgpIL-1 activity

The activity of rgpIL-1 was measured using a proliferation assay as described previously [25]. Briefly, non-adherent guinea pig thymocytes were obtained and purified on a nylon wool column. The cell viability was measured by Trypan blue exclusion [26]. The cells were adjusted to 5×10^5 cells/ml, seeded onto a 96-well microtitre plate (Becton Dickinson Labware, Franklin Lakes, NJ), and stimulated with 5 g/ml rgpIL-1 or 5 g/ml Concanavalin A (Con A; Sigma) or a combination of both (mix of 5 g/ml rgpIL-1 and 5 g/ml Con A). The cells were incubated for 48 h at 37 C in the presence of 5% CO₂. The cells were then labeled with [³H] thymidine (1 Ci/well) for the final 5 hours of incubation and harvested by a multiple automated cell harvester (Packard). The radioactivity in the collecting filter disks was measured by a Beckman scintillation counter. The counts are expressed as a stimulation index (SI), which was obtained by dividing the counts per minute (cpm) of stimulated cells by the cpm of unstimulated cells. The levels of IL-1 activity were expressed by the mean counts per minute (CPM) values $\pm$ SEM.

Determining the biological activity of rgpMCP-1

To obtain resident peritoneal macrophages, guinea pigs were euthanized by intramuscular injection of Sodium pentobarbital (Sleepaway; Fort Dodge Laboratories, IA). The peritoneal cavity was opened aseptically and flushed three to four times using a wash solution consisting of 10 U/ml heparin and 2% FBS in 1XPBS. Red blood cells were lysed using ACK lysing buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.2). Macrophages were resuspended in complete media (RPMI 1640, 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 0.01 mM β -ME) and allowed to adhere to tissue culture plates for 1.5 hours at 37 C in the presence of 5% CO₂. Nonadherent cells in suspension were removed. Adherent cells were gently scraped, and cell viability was measured by Trypan blue exclusion [24] and adjusted to a concentration of 1×10^6 cells/ml to be used in the assay.

The rgpMCP-1 was serially diluted from 10 ng/ml to 20 μ g/ml and added to the lower chambers of a 96-well chemotaxis plate (Corning; 5 μ m pore-size, PVP-free polycarbonate filter). Formyl-Met-Leu-Phe (fMLP; Sigma) was also serially diluted from 10 ng/ml to 20 μ g/ml and used as a chemoattractant positive control. Adherent macrophages were added to

the upper wells of the chamber and incubated for 3 h at 37 C in the presence of 5% CO₂. After incubation, any cells that did not migrate from the top of the filter were scraped off. The filter was stained with Diff-Quik to ensure that no cells were left on the filter. The samples were transferred to a new 96-well plate and 5mg/ml of MTT (Sigma; 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) was added to each sample and incubated for 4 hours at 37 C in the presence of 5% CO₂. Finally, 100 l of solubilization buffer containing 20% SDS was added to each well and incubated for another 4 hours at 37 C in the presence of 5% CO₂. Samples were read on an automated microtiter plate reader (Dynatech MR 5000; Molecular Dynamics, Chantilly, VA) at 540nm/690nm and average optical density at 540nm (OD 540) $\pm$ SEM was expressed.

Statistical analysis

ANOVA was used to determine statistical significances between groups at 95% confidence interval using Duncan *post hoc* analysis. The statistical tests were performed using SAS software (Release 8.01, SAS institute, Cary, NC)

Results

Confirmation and expression of IL-1 β and MCP-1

All the five putative transformants randomly selected for the IL-1 β or MCP-1 contained the gene of interest when analyzed by restriction analysis with a recombinant percentage of 100%. The two transformants corresponding to the pET-30a(+) vector containing IL-1 β or MCP-1 upon induction with IPTG resulted in the generation of proteins of interest that were visible as a 27 kDa band for IL-1 and 19 kDa for MCP-1 (Figure 1). Obviously the tags and cleavage sites that were present in the expression vector contributed to the increased size of the proteins. Unlike the rgp IL-10 and IFN- γ proteins that were obtained in the insoluble form [12, 22], target protein solubility for both IL-1 and MCP-1 proteins showed that they were obtained in the soluble form and were purified under native conditions by immobilized metal affinity chromatography (IMAC) using Ni-NTA columns.

Sequencing of the proteins

Both the IL-1 β and MCP-1 proteins were efficiently cleaved (Figure 2) and there was no significant difference in the extent of cleavage at 6 or 14 hours (Figure 2). The cleaved products were transferred onto the PVDF membrane by electroblotting and then stained with Coomassie brilliant blue. Bands corresponding to IL-1 β and MCP-1 were excised from the PVDF membrane and when subjected to N-terminal sequencing yielded the sequences AMADIGSQPDGV and AMADIGSSQG, respectively. AMADIG refers to the part of the vector sequence that is translated along with the protein of interest. These sequences corresponded to the predicted N-terminal sequence of guinea pig IL-1 β and MCP-1 based on the cDNA sequences published earlier [23,24] that confirms the identity of the purified proteins.

Proliferation of guinea pig thymocytes by rgpIL-1 β

To determine the biological activity of rgpIL-1, the proliferative response of guinea pig thymocytes was assessed (Figure 3). In the presence of ConA, thymocyte proliferation was significantly increased by 55-fold when compared to media alone ($p < 0.01$). When thymocytes were stimulated with IL-1 alone, proliferation was significantly higher than media alone (RPMI, 380 ± 53.95 CPM; IL-1, 838 ± 31.22 CPM) ($p < 0.01$). The presence of both ConA and rgpIL-1 had a slight additive effect as thymocyte proliferation was significantly upregulated by about 60-fold. These results clearly demonstrate the biological activity of rgpIL-1.

Chemotactic activity of rgpMCP-1

To determine the chemotactic activity of rgpMCP-1, resident peritoneal guinea pig macrophages were allowed to migrate with various concentrations of the chemoattractant, f-Met-Leu-Phe, or the recombinant protein (Figure 4). The migration of macrophages was dose-dependent. Peak activity was achieved at 1.25 µg/ml for f-Met-Leu-Phe and 5 µg/ml for rgpMCP-1, while chemotactic activity declined at higher concentrations. Migratory activity was similar in response to both stimuli as no significant differences were found between groups at any of the concentrations.

Discussion

Although the guinea pig IL-1β sequence was reported earlier [24], it has not been expressed. This study is the first to report guinea pig IL1β protein expression and demonstrate its biological activity. Other studies that are in progress for further functional characterization of rgpIL-1β involves *in vitro* assays such as its ability to induce macrophage activation (e.g., MHC class-2 expression), to overcome the arrested maturation of the *M. tuberculosis* containing phagosome [17, 28, 29], augmentation of T cell proliferation [30], and upregulation of IL-8 mRNA expression in the guinea pig fibroblast cell line 104C1 [31].

Although guinea pig MCP-1 has been previously expressed using the eukaryotic expression system (COS cells) [24], a disadvantage with the eukaryotic expression system is that less protein will be produced in comparison to a prokaryotic expression system [32]. Hence, expression of guinea pig MCP-1 in the prokaryotic expression system serves two important purposes. First, a large amount of protein is produced economically for downstream applications such as monoclonal antibody development, biological assays, etc [33, 34]. Second, since the protein was expressed in soluble form, it retained its biological activity which is not the case with the majority of eukaryotic proteins produced using prokaryotic expression systems [35]. Although several strategies exist to circumvent this problem [36], the approach of refolding proteins is considered to be a laborious and uneconomical process for the production of large quantities of recombinant proteins [37].

It was not apparent from the previous study whether rgpMCP-1 induced any response in resident (as opposed to exudate) peritoneal macrophages [24]. From our results, it is clear that rgpMCP-1 induces a chemotactic response by peritoneal resident macrophages. The *in vivo* effect of rgpMCP-1 on infiltration of macrophages in the guinea pig skin will be assessed as reported before [24]. We previously reported the *in vivo* effects of rgpTNF-α [38]. Future studies will focus on generation of polyclonal antibodies for both rgpIL-1β and MCP-1 and *in vivo* neutralization experiments involving anti-IL-1β and anti-MCP-1 will be performed in a similar manner as that done for anti-TNF-α by our group [12, 39].

Expression of recombinant proteins in soluble form is an important goal, because downstream processing of the insoluble proteins is a complex process even with established procedures. Our strategy was to clone IL-1β and MCP-1 genes into the pQE-30 vector and determine the target protein solubility. If the protein was expressed in insoluble form, our next strategy was to clone IL-1β and MCP-1 genes into appropriate vectors containing soluble fusion tags [40], cleave off those tags and obtain the soluble form of protein. Although a widely used alternative, the disadvantage with the method is that it results in low yields after cleavage and the proteins of interest may remain functionally inactive if they remain as fusion proteins [41]. Initial efforts to sub-clone the guinea pig IL-1 and MCP-1 genes into pQE-30 vector were successful, however little or no protein expression was observed after induction (data not shown). We hypothesized that the presence of rare codons in both the IL-1β and MCP-1 genes (approximately 10%) as analyzed by Graphical Codon Usage Analyzer [42] was detrimental to the expression of the proteins. Therefore, we chose

the Rosetta 2(DE3) containing the pRARE plasmid that will enhance the expression of eukaryotic proteins that contain codons rarely used in *E.coli*. In order to determine whether the presence of rare codons affected the expression of IL-1 β and MCP1 genes, we also transformed the pET-30a(+) vector construct containing the genes of interest (IL-1 β and MCP-1) with BL21(DE3) cells that didn't contain the pRARE plasmid and efficient protein expression was observed (data not shown). Thus, it appears that rare codons were not involved in the failure to express these genes in pQE-30. Some other factors may be involved in the successful expression of both the IL-1 β and MCP-1 genes in the pET-30a(+) vector and their failure to express in pQE-30. These are issues worthy of further investigation.

In conclusion, we describe an efficient strategy for generating biologically active recombinant guinea pig IL-1 β and MCP-1 proteins in a prokaryotic expression system which can serve as invaluable tools for the study of TB and other infectious and non-infectious diseases.

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Fig. 1. Coomassie blue-stained SDS-PAGE analysis of recombinant IL-1 β and MCP-1 from each clone after IPTG induction at 2, 4, and 5 hours interval. MM, molecular weight marker; UI, uninduced samples; I, Induced samples; h, Hours of induction.

Fig. 2.

Cleavage of additional tags (Histidine, thrombin, S, enterokinase) from recombinant IL-1 β and MCP-1 proteins. The recombinant IL-1 β and MCP-1 proteins were digested with enterokinase enzyme at different time intervals for analysis to subject them to N-terminal sequencing in the next step. MM, molecular weight marker; UDIL-1 β , Undigested IL-1 β ; DIL-1 β (6h), IL-1 β digested with enterokinase enzyme for 6 hours; DIL-1 β (14h), IL-1 β digested with enterokinase enzyme for 14 hours; DMCP-1 (6h), MCP-1 digested with enterokinase enzyme for 6 hours; DMCP-1 (14h), MCP-1 digested with enterokinase enzyme for 14 hours.

Fig. 3.

Proliferation of thymocytes induced by rgp IL-1 β . Guinea pig thymocytes (5×10^6 cells/ml) were stimulated with rgp IL-1 β (5 g/ml) alone, ConA (5 g/ml) alone and rgp IL-1 β (5 g/ml) with ConA (5 g/ml) together for 2 days. Cells were harvested after the addition of [3 H]-thymidine 5h earlier. **, Thymocyte proliferation was significantly upregulated ($P<0.01$) by IL-1 β and ConA, when compared with media alone.

Fig. 4.

Chemotactic activity of rgp MCP-1 was assessed by determining the migration of resident peritoneal macrophages to various concentrations of recombinant protein and fMLP compared to medium (RPMI) alone. Cell migration was determined using the MTT viability assay after the cells were scraped from the filter. Results are displayed as the mean $\pm$ SEM. *, Significant differences ($P < 0.05$) were seen in migration of chemotactic agents (rgpMCP-1 & fMLP) compared with media alone.