Secretory Leukocyte Protease Inhibitor
A Secreted Pattern Recognition Receptor for Mycobacteria

Sonia A. Gomez1,2, Claudia L. Argüelles1, Diego Guerrieri1, Nancy L. Tateosian1, Nicolás O. Amiano1, Rut Slimovich1, Paulo C. Maffía1, Eduardo Abbate4, Rosa M. Musella4, Verónica E. García2*, and H. Eduardo Chuluyan1*  
1Department of Pharmacology, University of Buenos Aires School of Medicine, Buenos Aires, Argentina; 2Department of Biological Chemistry, University of Buenos Aires School of Sciences, Buenos Aires Argentina; 3Servicio Derivados de Micobacterias, Instituto Nacional de Producción de Biológicos, Administración Nacional de Laboratorios e Institutos de Salud Dr. Carlos G. Malbrán, Buenos Aires, Argentina; and 4División de Tisoneumonología, Hospital F. J. Muñiz, Buenos Aires, Argentina

Rationale: Human secretory leukocyte protease inhibitor (SLPI) displays bactericidal activity against pathogens such as Escherichia coli and Streptococcus. Furthermore, it has been reported that murine SLPI shows potent antimycobacterial activity.

Objectives: The aim of the present study was to investigate whether human recombinant SLPI not only kills mycobacteria but also acts as a pattern recognition receptor for the host immune system.

Methods: For the in vivo experiment, BALB/c mice were infected by intranasal instillation with Mycobacterium bovis BCG and viable BCG load in lung homogenates was later determined. For the in vitro experiments, SLPI was incubated overnight with a suspension of M. bovis BCG or the virulent strain Mycobacterium tuberculosis H37Rv, and the percentage survival as well as the binding of SLPI to mycobacteria was determined. Furthermore, bacteria phagocytosis was also determined by flow cytometry.

Measurements and Main Results: Intranasal SLPI treatment decreased the number of colony-forming units recovered from lung homogenates, indicating that SLPI interfered with M. bovis BCG infection. Moreover, SLPI decreased the viability of both M. bovis BCG and H37Rv. We demonstrated that SLPI attached to the surface of the mycobacteria by binding to pathogen-associated molecular pattern mannann-capped lipoarabinomannans and phosphatidylinositol mannoside. Furthermore, we found that the spumt of patients with tuberculosis, mycobacteria were coated with endogenous SLPI. Finally, we showed that phagocytosis of SLPI-coated mycobacteria was faster than that of uncoated bacteria.

Conclusions: The present results demonstrate for the first time that human SLPI kills mycobacteria and is a new pattern recognition receptor for them.

Keywords: tuberculosis; secretory leukocyte protease inhibitor; phagocytosis

Tuberculosis, the oldest of the present world’s pandemics, remains a substantial worldwide health problem despite the current drug treatments. The disease produced by Mycobacterium tuberculosis causes nearly 9 million new cases and 1.7 million deaths annually (1). Moreover, tuberculosis is among the most common causes of morbidity and mortality in patients with HIV (2, 3). The resurgence of tuberculosis and the appearance of multidrug-resistant clinical isolates of M. tuberculosis have reaffirmed this disease as a primary public health problem globally. BCG, the only available vaccine, is of variable efficacy, especially in tuberculosis-endemic regions. Thus, to develop a more effective vaccine it is necessary to gain a better understanding of the human immune response to this pathogen.

During the course of M. tuberculosis infection, innate immune responses control the spread of the bacteria, but T-lymphocyte recruitment to the lung is later required for containment of the bacteria in the granulomas (4). The fate of an individual exposed to the pathogen will be determined by several factors including genetic background and the strength and specificity of the endogenous defense mechanisms mounted against the pathogen. To control the spread of bacteria, the innate immune system of the host detects conserved molecular structures, called pathogen-associated molecular patterns (PAMPs), produced exclusively by microorganisms and that are essential for the physiology of the microbes (5). PAMPs are recognized by germline-encoded pattern recognition receptors (PRRs) of the host, some of them (e.g., Toll-like receptor-2 [TLR2] and TLR4) present on the surface of cells from the innate immune system. In contrast, other PRRs, such as TLR7, are found inside the cells. Finally, the last group of known PRRs, such as surfactants and C-reactive protein, are soluble and present in extracellular body fluids. The recognition of PAMPs by PRRs facilitates uptake of the pathogen or the signaling needed for induction of the appropriate immune response of the host (5, 6). Virulent M. tuberculosis and Mycobacterium bovis BCG display different PAMPs, such as lipoproteins (19 kD) and glycoproteins (mannosylated lipoarabinomannan [ManLAM] and phosphatidylinositol mannoside [PIM]) (7) present on the cell envelope of the bacteria. These mycobacterial PAMPs are detected by different PRRs, such as mannose receptor, complement receptors, TLR2, and TLR4 (8).

Originally Published in Press as DOI: 10.1164/rccm.200804-615OC on November 14, 2008
Internet address: www.atsjournals.org

Correspondence and requests for reprints should be addressed to H. Eduardo Chuluyan, M.D., Ph.D., 3ra Cátedra de Farmacología, Piso 9, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, CP 1121, Buenos Aires, Argentina. E-mail: echuluyan@fmed.uba.ar

This article has an online supplement, which is accessible from this issue’s table of contents at www.atsjournals.org

Received in original form April 25, 2008; accepted in final form November 13, 2008

* These authors contributed equally to this work.

Supported by a grant from the Universidad de Buenos Aires (UBACYT2004-2007, M019; UBACYT2008-2010, M011) and Agencia (BID 1728/OC-AR PICT 15069, 1476, and 25996).

* These authors contributed equally to this work.
Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor with activity against cathepsin G, trypsin, and chymotrypsin, but primarily against neutrophil elastase (9). It is constitutively produced by several epithelial cell types and it is induced in response to diverse inflammatory stimuli by various types of cells, such as neutrophils and alveolar macrophages (9–11). On the other hand, the expression and secretion of SLPI are down-modulated by adenoviral infection, transforming growth factor-β1 cytokine production, and during chronic obstructive lung disease (12–14). In addition, cathepsins B, L, and S and cigarette smoke exposure result in the cleavage and inactivation of SLPI (15, 16). Remarkably, the exposure of murine peritoneal macrophages to heat-killed M. tuberculosis (HK-Mtb) led to an increase in SLPI gene expression and protein secretion (17). This protein may function as an endogenous immunomodulatory, antiinflammatory, and/or antimicrobial substance (9, 18). In fact, the antimicrobial effects of SLPI against fungi, viruses, and several bacteria have been demonstrated (9). Specifically, the role of SLPI has been shown in vivo and in vitro against Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, and Group A Streptococcus (9). Moreover, Nishimura and colleagues described that recombinant mouse SLPI inhibited the growth of bacillus Calmette-Guérin and M. tuberculosis through the disruption of the mycobacterial cell wall structure, even demonstrating that the N-terminal domain of SLPI was required for the disruption (19). In the present work, we investigated the role of human SLPI on mycobacteria. Our results demonstrated that human SLPI would be a novel soluble PRR that might bind and kill virulent mycobacteria through the direct interaction with ManLAM and PIM, two well known mycobacterium PAMPs.

Some of the results of these studies have been previously reported in the form of an abstract (20).

METHODS

Reagents

Recombinant human SLPI (rhSLPI) was cloned and expressed as described previously (21).

Murine Model of M. bovis BCG Pulmonary Infection

BALB/c mice were anesthetized and intranasally infected with M. bovis BCG (1 × 10^6 CFU/mouse). Mice were then intranasally instilled with 50 μl of rhSLPI (150 μg/ml per mouse) and killed according to the protocol described in each figure. Bronchoalveolar lavage fluid and lung tissue were processed as described previously (22) and analyzed for bacterial load. See the online supplement for additional details on mice and bacterial load detection.

Antibacterial Assays

The antibacterial activity of rhSLPI was determined with mid-log-phase–grown M. bovis BCG-Pasteur and M. tuberculosis H37Rv (see the online supplement for additional detail on bacterial culture). Bacterial suspensions (1 mg/ml) were treated (16, 37°C) with rhSLPI, heat-denatured SLPI (SLPI-Ht), pH-denatured SLPI (SLPI-pH), or control buffer (300 mM NaCl, 50 mM NaH2PO4, 2.5 mM imidazole). For kinetic studies, samples were taken every 24 hours for colony-forming unit counts and/or stained with Ziehl-Neelsen. In appropriate dilutions of lung homogenates were seeded onto agar plates for colony-forming unit counts and/or stained with Ziehl-Neelsen. In "A" colony-forming units per lung and "B" percentage of alveolar macrophages (AM) positive for M. bovis BCG are shown. The average of 10 fields of vision per lung under oil immersion is shown. *P < 0.05, **P < 0.001 (n = 6 per group) by Student t test.

SLPI Binding to M. bovis BCG

M. bovis BCG suspensions were incubated with rhSLPI, rhSLPI-pH, or rhSLPI-Ht (2 h, 37°C) and then incubated with Penta-His Alexa Fluor 647 conjugate (30 min, 4°C) to detect rhSLPI histidine-tagged protein (21). After washing, the cells were fixed (1.5% paraformaldehyde) and binding was detected by flow cytometry (see the online supplement for additional detail on flow cytometry).

SLPI Binding to Mycobacterium PAMPs

Tripalmitic acid, trehalose dimycolate (TDM; see the online supplement for additional detail on lipid extractions), mannan-capped lipasarabinomannan (ManLAM), phosphatidylinositol mannoside (PIM), mycolic acids, and 19-kD lipoprotein were coated in the wells of a 96-well microplate. After blocking the nonspecific binding sites, rhSLPI (50 ng/ml), rabbit polyclonal anti-human SLPI antibody, goat anti-rabbit peroxidase-conjugated antibody, and colorimetric reactant were sequentially added. The reaction was stopped with H2SO4 and the A450 was measured with a Multiskan ELISA plate reader (Rayto, Buenos Aires, Argentina).

Phagocytosis of BCG and H37Rv Particles

THP-1 macrophages and murine alveolar macrophages (AMs) were fed with rhSLPI-opsonized fluorescein isothiocyanate (FITC)-conjugated BCG and H37Rv, respectively. Cells were washed and fixed. Fluorescent THP-1 macrophages (i.e., cells that had incorporated BCG–FITC) were analyzed by flow cytometry, whereas AMs were stained with Ziehl-Neelsen to analyze the phagocytosis of H37Rv mycobacteria by microscopy (see the online supplement for additional detail on phagocytosis assays, macrophage culture, and labeling of mycobacteria).
Detection of SLPI Bound to Mycobacteria in Sputum from Patients with Active Tuberculosis

Sputum samples from 11 patients with tuberculosis (see the online supplement for additional detail on patients with active tuberculosis) were heat fixed on glass slides and divided into two sections. After blocking, one section of each glass was incubated with murine IgG1 isotype control and the other with mouse IgG1 anti-human SLPI monoclonal antibody (2 h, 4°C). After washing and further blocking, the sputum samples were incubated with anti-mouse–FITC polyclonal antibody. Last, the slides were fixed and fluorescent bacilli were photographed as described in the figures.

Statistical Analysis

Student t test, analysis of variance, and post hoc Bonferroni and Student-Newman-Keuls were used for statistical analysis of the data, as indicated in each figure.

RESULTS

Murine Model of Pulmonary Infection with M. bovis BCG-Pasteur

Because SLPI is an endogenous antiinflammatory and antimicrobial substance present in the respiratory tract (10), we investigated the potential role of SLPI during pulmonary mycobacterial infection. Thus, we first analyzed whether SLPI treatment might participate in the outcome of M. bovis BCG infection in BALB/c mice. Mice receiving SLPI since Day 13 (twice per day) were killed on Day 14 postinfection and lung bacterial load was determined. (Figure 1A) shows that the number of colony-forming units recovered from BCG-SLPI-treated mice was significantly lower compared with BCG control mice. Furthermore, fewer AM from BCG-SLPI–treated mice had phagocytized mycobacteria compared with control BCG-treated animals (Figure 1B). Together, these results demonstrate that SLPI decreases the M. bovis BCG burden once the pulmonary infection has been established.

In Vitro Antimycobacterial Effect of SLPI

We hypothesized that SLPI could act on the pathogen and/or on cells of the immune system of the host. To test this hypothesis, we first analyzed the potential direct effect of SLPI on Mycobacterium viability in vitro. For this, mid-log–phase–grown M. bovis BGC was incubated with various concentrations of SLPI and the bacteria were plated and incubated at 37°C. At various time points M. bovis BCG viability was assessed by colony-forming unit counting. Figure 2A shows that SLPI reduced the viability of M. bovis BCG in a dose-dependent manner, indicating that SLPI would be in fact acting on the pathogen. Next, we performed time course experiments to determine whether SLPI was also interfering with bacterial growth. Thus, bacterial suspensions (10^5/ml) were incubated with SLPI for 7 days. Samples were taken from BCG cultures every day and bacterial growth was analyzed on agar plates as described previously. As shown in Figure 2B, SLPI gradually and progressively decreased the number of colony-forming units throughout the 7 days studied. Remarkably, the effect of SLPI on mycobacterial viability was also observed with the virulent strain M. tuberculosis H37Rv (Figure 2C), further demonstrating that SLPI effectively kills Mycobacterium species. The effect of SLPI on mycobacterial viability was not observed when heat-denatured SLPI was used (Figure 2D). However, we still observed that pH-treated SLPI decreased bacterial viability (Figure 2D). Interestingly, both pH- and heat-denatured SLPI lose their ability to inhibit trypsin serine protease activity (data not shown). Thus, these data indicate that the effect of SLPI on mycobacterial viability does not require the COOH-terminal domain, where the serine protease inhibitory activity resides.

Figure 2. In vitro effect of rhSLPI on M. bovis BCG and H37Rv viability. (A) Fifty-microliter volumes of mid-log–phase bacteria at 1 mg/ml in Sauton buffer were added to 96-well polystyrene microplates in triplicate, together with 50 μl of various concentrations of rhSLPI or control buffer. After overnight incubation at 37°C, the number of colony-forming units was determined by plating serial dilutions onto 7H11 agar plates. For the kinetics study (B), 1-ml aliquots of bacterial suspension were incubated with equal volumes of rhSLPI (5 μM) and incubated at 37°C for 7 days. Aliquots (20 μl) were taken every 24 hours to perform colony-forming unit counts. After 2 weeks of incubation, colony-forming unit counts were assessed by visualization under a magnifying glass. The same protocol described in (A) was used for experiments with virulent strain M. tuberculosis H37Rv (C). To examined the effect of pH- and heat-denatured rhSLPI (SLPI-pH and SLPI-Ht, respectively), the peptides were incubated with bacteria for 7 days at 5 μM (D). Data represent the mean ± SEM of three separate experiments. *P < 0.05, **P < 0.001, ***P < 0.0001 compared with control groups, using a Student t test (A–C) and analysis of variance (ANOVA) with Bonferroni test (D).
Direct Interaction between SLPI and *M. bovis* BCG Cell Envelope

Cationic peptides, like SLPI, kill bacteria by altering the stability of the cell envelope. Thus, we next investigated by flow cytometry the ability of SLPI to attach to the BCG surface. As shown in Figures 3A and 3B, SLPI and SLPI-pH bound to the surface of BCG particles, as demonstrated by a marked increase in mean fluorescence. However, SLPI-Ht was not able to bind to the surface of the bacteria (Figures 3A and 3B). Remarkably, SLPI binding to the *M. tuberculosis* surface was also observed in sputum samples from patients with active tuberculosis (Figures 3D and 3E). To confirm the staining of bacteria, the same slides were analyzed for acid- and alcohol-resistant bacilli by Ziehl-Neelsen (ZN) staining. As shown in Figure 3C, the SLPI fluorescence pattern was quite similar to that of ZN staining. Together, these results clearly demonstrate that SLPI binds directly to bacteria.

Mycobacteria display some well-defined PAMPs required by the host immune system to detect the presence of the bacillus and to trigger an effective immune response (23). Therefore, we next investigated whether SLPI might bind to any of the already known mycobacterial PAMPs. Interestingly, our results showed that SLPI displayed a high binding capacity for TDM and ManLAM, and a lower binding capacity for PIM (Figure 4). In contrast, SLPI did not bind 19-kD lipoprotein or mycolic acids (Figure 4). Together, these results further confirm the capacity of SLPI to specifically bind to known PAMPs from virulent mycobacteria, strongly suggesting that SLPI could act as a PRR during the host immune response against *Mycobacterium* infection.

SLPI Facilitates Phagocytosis of Mycobacterial Strains

To confirm that SLPI is a PRR, we next analyzed the capacity of SLPI to facilitate THP-1 macrophage and murine AM phagocytosis of BCG particles and H37Rv, respectively. BCG particles were first stained with FITC and then coated with rhSLPI.
In this way, rhSLPI-coated BCG particles were fed to macrophages and uptake was evaluated by flow cytometry. As shown in Figure 5A, over a time period of 120 minutes, phagocytosis of SLPI-coated M. bovis BCG was faster than that of uncoated mycobacteria. Figure 5B also shows that phagocytosis of H37Rv by murine AMs was higher when the virulent strain was coated with SLPI or SLPI-pH compared with uncoated H37Rv. These data demonstrated that SLPI is not only able to kill mycobacteria, but that it also can facilitate clearing of the pathogen by the immune system. The role of SLPI as a PRR was also tested and confirmed with an in vivo model of murine BCG infection. For this, we performed experiments similar to those described in Figure 1, with modifications. Briefly, SLPI was administered (twice per day) to animals 24 hours after M. bovis BCG mouse infection. On Day 3, mice were killed and the number of infected AMs was determined. In contrast to our findings shown in Figure 1, Figure 5C shows that significantly higher numbers of AMs containing BCG were detected in BCG-SLPI- and BCG-SLPI-pH-treated mice compared with control animals. This effect was not observed in BCG-SLPI-Ht–treated mice (Figure 5C). Thus, SLPI increased not only in vitro but also in vivo bacterial phagocytosis, and the effect does not require the activity of serine protease inhibitory domain.

**DISCUSSION**

Our results demonstrated that human SLPI displays antimycobacterial activity, therefore confirming that SLPI is an important endogenous agent as such. Moreover, we showed that SLPI is able to attach to and interact with the surface of mycobacteria, increasing the phagocytosis of the pathogen. Furthermore, the present data sustain our hypothesis that SLPI is a novel secreted PRR that might participate in the early phases of a pulmonary infection with virulent mycobacteria.

Many species contain antimicrobial cationic peptides that have a broad spectrum of activity not only against gram-negative and gram-positive bacteria but also against fungi, viruses, and parasites (24). SLPI is a polycationic nonglycosylated peptide that displays antimicrobial properties in vivo and in vitro (25). The antimicrobial activity of human SLPI has been described for various bacteria as well as Aspergillus fumigatus, Candida albicans, and HIV (25). The antimycobacterial activity of SLPI resides in the whey-acidic-protein domains of the molecule and is similar to that of other cationic peptides, such as defensins (26). In an attempt to localize the antimycobacterial activity of SLPI, we performed studies with denatured SLPI. In our experiments, SLPI denatured either by heat or pH renders a protease that loses antiprotease activity (data not shown). However, this antiprotease activity is not directly related to the antimycobacterial activity and binding capacity of the protein because pH-treated rhSLPI, but not heat-inactivated rhSLPI (Figure 3B), is able to bind to the mycobacterial surface and maintain the antimycobacterial activity (Figure 2D). It is worth noting that heat inactivation disrupts hydrogen bonds and nonpolar hydrophobic interactions, whereas acids disrupt salt bridges. Within a physiological context, it is of relevance that even after acid pH denaturation SLPI retained its activity against bacteria, indicating that the effect of SLPI on mycobacteria might persist even at inflammatory sites, where the extracellular pH is often decreased (27).

Although we showed the binding of SLPI to the surface of mycobacteria in vitro, an important aspect of our study was the ability to demonstrate the attachment of SLPI to M. tuberculosis observed in sputum samples of patients with active disease. These results confirm that endogenous SLPI is able to bind to the surface of virulent strains of *Mycobacterium*, although we cannot confirm the origin of the SLPI present in sputum from...
patients with tuberculosis, in particular considering that SLPI is found in high concentration in saliva (28).

In the lung, SLPI is an alarm-mediating acute-phase reactant secreted in response to LPS, IL-1, tumor necrosis factor-α, epidermal growth factor, defensins, and human neutrophil elastase (9). The SLPI concentration found in respiratory epithelial lining fluid is about 1 μM (29), a value that is close to the concentration we used to detect the antimicrobial activity of rhSLPI (2.5 μM protein). However, this concentration is higher than the concentration that was used to demonstrate the antimycobacterial activity of mouse SLPI (19). It is probable that the discrepancy in effects of human and mouse SLPI are related to structural differences between the peptides (19). In the present study, we showed that low concentrations of rhSLPI were enough to bind, kill, and induce phagocytosis against mycobacteria. Thus, although the concentration required for the antimicrobial activity of rhSLPI is higher than in mice, it is likely that the binding of SLPI to mycobacteria does not require a high protein concentration.

Pattern recognition receptors evolved to recognize PAMPs on pathogens, and therefore to detect the presence of infection. Several PAMPs have been recognized on M. tuberculosis (30). Binding of SLPI to Mycobacterium seems to be specific and not related to the electric charge because we observed specific binding to TDM, ManLAM, and PIM but not to mycolic acids and 19-kD lipoprotein. The 19-kD lipoprotein has been identified as a major PAMP, considering its activity to inhibit MHC-II antigen processing. Then, our results clearly indicate that SLPI is a secreted PRR that binds to other major PAMPs on the surface of Mycobacterium and that this binding facilitates the clearing by macrophages and subsequent killing of the pathogen. The clearance of bacteria by macrophages is also supported by treating mice with SLPI shortly after BCG challenge (Figure 5C). In fact, this protocol allowed us to detect higher numbers of AM containing BCG compared with AM recovered from control mice. On the other hand, when SLPI was given once infection had been established (Figure 1B), and probably most of the mycobacteria were inside the macrophages, a lower number of AM containing mycobacteria was observed. Besides, the ability of SLPI to function as a PRR for mycobacteria is lost when the protein is denatured with heat but not with pH treatment, either for in vitro (Figure 3A) or in vivo (Figure 5C) experiments. Moreover, similar to SLPI microbicidal activity, SLPI PRR ability does not depend on the COOH-terminal domain, where the serine protease inhibitory activity resides, because SLPI-ph lacks serine protease activity (data not shown).

Herein we have described for the first time that human SLPI is a secreted PRR for mycobacteria that increases both the phagocytosis and killing of the pathogen. Further experiments are required to unravel the role of SLPI in the pathophysiology of tuberculosis, before it may be considered therapeutically useful in the management of the disease.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Dr. Martha Ambrogi for expert assistance with the samples from patients with tuberculosis. The authors also thank Marcela Amoroso and María Luisa Breto (Bioterio Central del INPB-ANLIS Dr. Carlos G. Malbrán) for technical assistance.

References


