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Published in:
Journal of interferon & cytokine research

DOI:
10.1089/jir.2012.0017

2012

Link to publication

Citation for published version (APA):

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Characterization of Released Polypeptides During an Interferon-γ-Dependent Antibacterial Response in Airway Epithelial Cells

Mette Eliasson,1 Anders I. Olin,1,2 Johan A. Malmström,3 Matthias Mörgelin,2 Mikael Bodelsson,4 Mattias Collin,2 and Arne Egesten1

When pathogenic bacteria breach the epithelial lining at mucosal surfaces, rapidly available innate immune mechanisms are critical to halt the infection. In the present study, we characterized the production of antibacterial polypeptides released by epithelial cells. IFN-γ, but neither TNF nor IL-1β alone, induced release of antibacterial activity to a cell culture medium, causing a lytic appearance of killed bacteria as revealed by electron microscopy. Addition of the protein streptococcal inhibitor of complement, derived from Streptococcus pyogenes, known for its ability to neutralize antimicrobial polypeptides (AMPs), reduced the antibacterial activity of the medium. Characterization of the antibacterial incubation medium using mass spectrometric approaches and ELISAs, displayed presence of several classical AMPs, antibacterial chemokines, as well as complement factors and proteases that may interfere with bacterial killing. Many were constitutively produced, that is, being released by cells incubated in a medium alone. While a combination of IFN-γ and TNF did not increase bacterial killing, the presence of TNF boosted the amounts and detectable number of AMPs, including antibacterial chemokines. However, the methods applied in the study failed to single out certain AMPs as critical mediators, but rather demonstrate the broad range of molecules involved. Since many AMPs are highly amphiphatic in nature (i.e., cationic and hydrophobic), it is possible that difficulties in optimizing recovery present limitations in the context investigated. The findings demonstrate that epithelial cells have a constitutive production of AMPs and that IFN-γ is an important inducer of an antibacterial response in which is likely to be a critical part of the innate host defense against pathogenic bacteria at mucosal surfaces.

Introduction

When pathogens start colonizing a mucosal surface, readily available host defense mechanisms at the epithelial lining are crucial to prevent disease. Normally, pathogens are eliminated, but if the defense is insufficient, the pathogen can invade underlying tissues and the disease may evolve from a local infection into a systemic, life-threatening disease (Bisno and others 2003; Sansonetti 2004). Many individuals are colonized by potentially virulent bacteria without having clinical signs of infection (Zwart and others 2003). However, if the epithelial lining is injured, an inflammatory response is initiated through activation of immune cells residing in submucosal tissues. This response generates production of proinflammatory cytokines that orchestrate appropriate host responses against the invading bacteria. Interferon (IFN)-γ is a key regulator of Th1-polarized immune responses seen during bacterial infections (Kolls and others 2008), and can affect epithelial cells of the mucosal layer, resulting in an activated phenotype (Sauty and others 1999; Strieter and others 2002; Sansonetti 2004).

The activated epithelium can elaborate antimicrobial polypeptides (AMPs), among them human β-defensins (hBD) and antibacterial chemokines that play important roles during early stages of bacterial infection (Sauty and others 1999; Egesten and others 2007). Distinguished features of AMPs are that they are mostly small (molecular weights of less than 15 kDa) and cationic (pI often above 9). The latter is a feature that, together with intermingled hydrophobic amino acid residues, results in domains with an amphipathic secondary
structure. This structure provides a propensity to disrupt bacterial membranes (Hancock and Diamond 2000; Boman 2003; Ganz 2004). However, there may be other targets in bacteria, for example, mechanisms resulting in bacteriostatic effects, such as depletion of iron in the case of neutrophil gelatinase-associated lipocalin (NGAL) (Borregaard and Cowland 2006). Most AMPs are multifunctional and many possess chemotactic activity and some act as growth factors (De and others 2000; Malmsten and others 2007). Some AMPs belong to the large family of chemokines that are peptides sharing glycosaminoglycan (GAG)-binding properties, and that attract and activate leukocytes during immune responses. In addition, some chemokines act as growth factors and yet some possess antibacterial activity (Baggiolini 2001; Cole and others 2001; Yang and others 2003; Egesten and others 2009). In parallel with AMPs, other arms of innate immunity include, for example, the complement system, ficolins, unstable oxygen derivatives, and cell-mediated responses executed by neutrophils and NK cells (Rada and Leto 2008; Matsumita 2010).

Pharyngitis is one of the most common mucosal infections in humans, often caused by the Gram-positive bacterium *Streptococcus pyogenes* (Cunningham 2000). In most cases, the infection is resolved without the use of antibiotics, indicating that robust local host defense mechanisms normally eradicate the bacteria (Zwart and others 2003). In a previous study, we used the cDNA microarray technique to analyze the differential regulation of AMP gene expression in pharyngeal epithelial cells (Eliasson and others 2007). Previously, we have also showed that bactericidal activity is retained on the surface of pharyngeal epithelial cells stimulated by a combination of IFN-γ and tumor necrosis factor (TNF), and dependent on the chemokine CXCL9/MIG (monokine induced by IFN-γ), possibly associated with GAGs (Egesten and others 2007). We also observed a decreased bactericidal activity in a medium from IFN-γ-stimulated pharyngeal epithelial cells where CXCL9/MIG expression was subjected to siRNA knockdown. In *vivo*, neutralization of CXCL9/MIG, CXCL10/IP-10, and CXCL11/1-TAC using antibodies, impaired host defense in an anthrax-model of airway infection (Crawford and others 2010). Furthermore, using genetically modified mice, IFN-γ-induced responses were required to prevent local dissemination of *S. pyogenes*, the draining lymph nodes (Hyland and others 2009). Taken together, these findings suggest important roles for IFN-γ-dependent antibacterial chemokines in hot defense at epithelial surfaces.

In the present study, proteomic approaches were used to characterize the production of AMPs and antibacterial chemokines. We demonstrate that epithelial cells alone, after appropriate stimuli, can produce a broad range of AMPs and antibacterial chemokines, resulting in antibacterial activity. This bactericidal response is likely to be important for the outcome of mucosal infections.

**Materials and Methods**

**Reagents**

Human recombinant IFN-γ, TNF, interleukin (IL)-1β, CXCL9/MIG, CXCL10/IP-10, CXCL11/1-TAC, CXCL1/

**Cell culture and stimulation with cytokines**

The human pharyngeal epithelial cell line Detroit 562 (ATCC CCL 138; ATCC) was cultured in 25-cm² cell culture flasks (NUNC, Slangerup, Denmark) using the Minimal Essential Medium (MEM) with Earle’s salt (Invitrogen, Stockholm, Sweden) supplemented with 2 mM glucose (Invitrogen), 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), amphotericin B (0.25 µg mL⁻¹; Invitrogen), penicillin/streptomycin (100 U mL⁻¹ and 0.1 ng mL⁻¹, respectively; Invitrogen), referred to as the “culture medium,” at 37°C in an atmosphere containing 5% CO₂ with 100% relative humidity. To investigate conditions resulting in soluble bactericidal activity, Detroit cells were cultured to near confluence in 96-well plates, washed 3 times followed by stimulation in the “incubation medium” (MEM with Earle’s salt supplemented with 2 mM glucose) for 24 h as follows: IFN-γ (100 U mL⁻¹), IFN-γ+TNF (10 ng mL⁻¹), IFN-γ+IL-1β (10 ng mL⁻¹), IFN-γ+TNF+IL-1β, TNF alone, and IL-1β alone. In addition, cells were stimulated with IFN-γ (100 U mL⁻¹) for 1, 2, 4, 8, 16, and 24 h, respectively, to investigate a possible time-dependency with respect to bactericidal activity. The incubation medium was collected and used in a viable count assay and characterization of AMP content with ELISA and liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described below.

**Bacteria**

The *S. pyogenes* strain API of the M1 serotype was from the WHO Collaborating Centre for Reference and Research on Streptococci, Institute of Hygiene and Epidemiology, Prague, Czech Republic.

**Bacterial killing assay (viable count)**

The incubation medium, collected as described above, was used in a viable count assay. API bacteria were grown at 37°C, 5% CO₂, to mid-logarithmic phase (OD₆₀₀=0.4) in the Todd-Hewitt broth (TH; Difco, Stockholm, Sweden), washed, and diluted in 10 mM Tris (pH 7.5) containing 5 mM glucose (Tris-glucose) to a concentration of 1% [2×10⁶ GRO-α, CXCL2/GRO-β, CXCL3/GRO-γ, CCL20/MIP-3α, CCL28/MEC, and ELISAs for the detection of hBD-1, hBD-2, and hBD-3 were from Peprotech, Rocky Hill, NJ. Recombinant secretory leukocyte protease inhibitor (SLPI) and ELISAs for the detection of CXCL9/MIG, CXCL10/IP-10, CXCL11/1-TAC, CXCL1/GRO-α, CXCL2/GRO-β, CCL20/MIP-3α, CCL28/MEC, and SLPI were purchased from R&D Systems, Abingdon, United Kingdom. Additional ELISAs were as follows: CXCL3/GRO-γ (Antigenix America, Huntington Station, NY), lactotransferrin (Kamiya Biomedical, Seattle, WA), and lysozyme (Calbiotech, Spring Valley, CA). Protein streptococcal inhibitor of complement (SIC) of the strain API1 was purified as described (Åkesson and others 1999). A cellular luminescence enhancement system for superoxide detection (Diogenes) was from National Diagnostics, Atlanta, GA. L-NAME was from Cayman Chemical, Ann Arbor, MI. Diphenyleneiodonium chloride, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADPH, nitrate reductase, and Griess reagent, were from Sigma-Aldrich, Stockholm, Sweden. Centrifugal filter devices (Nanosep; cutoff 100 kDa) were from PALL Life Sciences, Port Washington, NY.
colony-forming units (cfu) mL$^{-1}$. About 25 μL of the incubation medium was incubated with 25 μL of suspended bacteria for 1 h at 37°C (in an atmosphere containing 5% CO$_2$ with 100% relative humidity). To quantify bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, and the number of cfu was determined.

To determine the bactericidal activity of recombinant peptides against *S. pyogenes*, 50 μL of API bacteria (2 × 10$^7$ cfu mL$^{-1}$) was incubated with peptides, at various concentrations or Tris-glucose alone (control) for 1 h at 37°C. To quantify bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, and the number of cfu was determined.

To investigate if the protein SIC of *S. pyogenes* interfered with the bactericidal activity, the incubation medium from IFN-γ-stimulated cells were preincubated with SIC at increasing concentrations (0, 0.01, 0.1, 0.5, 1 μM) or heparin-Sepharose (GE-Healthcare, Uppsala, Sweden) for 20 min, and then incubated with API bacteria for 1 h followed by measurement of bactericidal activity using the viable count assay.

**Electron microscopy**

API bacteria were grown to the mid-logarithmic phase followed by incubation for 1 h at 37°C in the incubation medium from nonstimulated or IFN-γ-stimulated (24 h) Detroit cells. To determine the bactericidal activity, part of the sample was plated on TH agar plates, incubated at 37°C overnight, and the cfu were counted the following day. The remaining parts of the samples were processed for electron microscopy as described (Bengtson and others 2008). In short, samples (5 μL) were absorbed for 45 s onto the grids followed by 2 washes with distilled water. The samples were stained for 3 s with 0.75% uranyl formate droplets followed by staining for an additional 15-20 s with 0.75% uranyl formate. Samples were examined using a Jeol JEM 1230 EX transmission electron microscope operated at 60 kV accelerating voltage.

**Microcapillary reversed-phase liquid chromatography and MS/MS**

Fifty microliters of the incubation medium (nonstimulated and stimulated) were mixed with 50 μL 8 M urea and 100 mM ammonium bicarbonate. The proteins were reduced with 5 mM TCEP, and alkylated with 10 mM iodoacetamide, before diluted with 100 mM fresh ammonium bicarbonate. The polypeptides were digested using porcine trypsin (Promega, Madison, WI) 1 μg over night. After digestion, the polypeptides were cleaned-up using spin columns from Harvard Apparatus (Holliston, MA) according to the manufacturer’s instructions. The setup of the microcapillary reversed-phase liquid chromatography (μRPLC) system was as described previously (Yi and others 2003). ESI-based LC–MS/MS (LTQ Thermofinnigan, San Jose, CA) analysis was carried out using an Agilent 1100 series (Agilent Technologies, Palo Alto, CA) on a 75-μm×10.5-cm fused silica microcapillary reversed-phase column as described previously (Malinström and others 2006a). MS/MS spectra were searched using the XTandem search tool (Craig and Beavis 2003) against the human Swissprot database version 57.4. The search was performed allowing for unconstrained enzyme search, specifying monoisotopic mass and mass tolerance of 3 Da, and with methionine oxidation as a variable modification. The database search results were first processed using the PeptideProphet program (Keller and others 2002). Peptides were assembled into proteins and the probabilities at the protein level were computed by the protein inference program ProteinProphet (Nesvizhskii and others 2003) and a reversed database search strategies (Elias and Gygi 2007). The selected cutoff was at 1% false discovery rate. The spectral counting was achieved using the 2DDB software (Malinström and others 2002, 2006b).

**Results**

**IFN-γ induces a soluble bactericidal response in epithelial cells**

Pharyngeal epithelial cells were stimulated with cytokines, and the bactericidal activity of the incubation medium
was determined using a viable count assay. Significant bactericidal activity, resulting in ~50% killing of S. pyogenes, was seen in the presence of IFN-γ, either alone or in combination with TNF or IL-1β. Neither TNF nor IL-1β alone induced bactericidal activity in the cell incubation medium (Fig. 1A). Investigation of possible time-dependence of the response revealed that the bactericidal effect against S. pyogenes became evident with a threshold-effect after 24 h of stimulation with IFN-γ (Fig. 1B). IFN-γ itself, at the concentration used, showed no bactericidal effect (data not shown). Electron microscopy was used to investigate the morphologic appearance of S. pyogenes after exposure to the incubation medium from nonstimulated cells and IFN-γ-stimulated epithelial cells, respectively. Bacteria exposed to the incubation medium from IFN-γ-stimulated cells showed disrupted integrity and leakage of intracellular contents, whereas bacteria incubated with the incubation medium from nonstimulated cells were unaffected (Fig. 1C).

**Reduced bactericidal activity after addition of protein SIC or heparin**

Protein SIC is a protein produced in large amounts by the most prevalent serotypes of S. pyogenes and it is released to the environment (Åkesson and others 1996). It is a strong inhibitor of several AMPs, for example, β-defensins, CXCL9/MIG, and lysozyme (Frick and others 2003; Fernie-King and others 2004; Egesten and others 2007). Addition of the protein SIC dose-dependently reduced the bactericidal effect of the incubation medium from IFN-γ-stimulated epithelial cells (Fig. 2). However, the reduction reached a plateau, demonstrating that not all bactericidal activity was sensitive to inhibition by SIC. Heparin-binding property is a common feature of many AMPs and soluble GAGs can inhibit their activity (Schmidtchen and others 2001; Andersson and others 2004). Therefore, the GAG-equivalent heparin (i.e., heparin-Sepharose) was added to the incubation medium from IFN-γ-stimulated cells, resulting in a reduction of the bactericidal activity, similar to that of the protein SIC (Fig. 2B). Taken together, a significant proportion of the bactericidal response is likely to be mediated by cationic polypeptides.

**Detection of AMPs by SDS-PAGE followed by LC-MS/MS**

Most AMPs are highly cationic and have a size of less than 15 kDa, making it difficult to perform protein-separation using 2D-electrophoresis. Therefore, SDS-PAGE followed by silver staining was chosen to compare the patterns of produced polypeptides present in the cell incubation medium during nonstimulated and cytokine-stimulated conditions, respectively. To improve the recovery of polypeptides, it proved necessary to use acetone precipitation and an extraction procedure with urea. The separated polypeptides were further characterized and identified using LC-MS/MS. Several previously characterized AMPs, antibacterial chemokines, and additional molecules that could interfere with bacteria could be identified using this approach, including SLPI, adrenomedullin, lactotransferrin, short palate, lung, and nasal epithelium clone 1 (SPLUNC1), elafin, dermcidin, CXCL10/IP-10 (IFN-γ-induced protein 10 kDa), the lipocalin NGAL, proteins S100-A8 and S100-A9. NGAL, SLPI, and

![FIG. 1. IFN-γ-dependent bactericidal activity in the incubation medium from pharyngeal epithelial cells. (A) The incubation medium from pharyngeal epithelial cells, incubated in a medium alone (control) or stimulated with IFN-γ (100 U mL⁻¹), and/or TNF (10 ng mL⁻¹) and/or IL-1β (10 ng mL⁻¹) for 24 h, was incubated with S. pyogenes (API strain) for 1 h. Bacterial killing was measured using a viable count assay. The number of colony-forming units (cfu) in a medium alone (control) was set to 0% killing and compared with the stimulated conditions. The data represent mean ± SEM of 3 independent experiments. (B) The IFN-γ-induced bactericidal response in the incubation medium is time-dependent. The incubation medium from pharyngeal epithelial cells stimulated for 0–24 h with IFN-γ (100 U mL⁻¹) was used in a viable counts assay against S. pyogenes. The data represent mean ± SEM of 2 independent experiments. The control medium (without IFN-γ) was set to 100% survival. (C) Electron micrographs showing the morphology of S. pyogenes (API strain) after exposure to the cell incubation medium from nonstimulated and IFN-γ-stimulated pharyngeal epithelial cells, respectively. Bacteria were incubated for 1 h in the cell incubation medium from nonstimulated cells (I) or a medium from cells stimulated with IFN-γ (I; 100 U ml⁻¹; 24 h). In the latter case, protrusions and leakage of intracellular contents from the bacteria are seen (micrographs II and IV), which was not the case in a bacteria-exposed medium from nonstimulated conditions (micrographs I, III). Bar in (II) is 1 μm and bar in micrograph (IV) is 100 nm. IFN, interferon; TNF, tumor necrosis factor; IL, interleukin.
adrenomedullin were visible also in a medium from non-stimulated epithelial cells, and the intensity of the bands using SDS-PAGE was similar to that of stimulated conditions, suggesting a constitutive production of these polypeptides. However, in a particular protein S100-A8 and S100-A9 appeared after stimulation (Fig. 3). S100-A8 and S100-A9 forms a hetero tetramer in the cytoplasm of keratinocytes, and it is possible that the presence of these molecules reflects disturbed epithelial cell integrity due to necrosis or apoptosis. However, we could not see decreased cell viability as deduced by Trypan Blue exclusion or disturbed morphologic appearances during cell culture.

**Semiquantitative detection of AMPs using μRPLC followed by MS/MS**

Using μRPLC followed by MS/MS, a sensitive and semiquantitative detection of antibacterial polypeptides was achieved. The incubation medium from nonstimulated cells and cells stimulated with either IFN-γ alone, or a combination of IFN-γ and TNF for 24 h were compared (Table 1). The semiquantitative estimation was based on the number of scans detecting the respective polypeptides. In addition, this method increased the sensitivity and additional AMPs could be detected compared with polypeptide precipitation and urea extraction followed by SDS-PAGE and LC-MS/MS. The AMPs lysozyme C, dermcidin, lactotransferrin, SLPI, and elafin, the antibacterial chemokines CXCL9/MIG and CXCL10/IP-10, midkine, protein S100-A8, and NGAL were identified. Most of them could be detected after stimulation with IFN-γ in combination with TNF. In addition, several complement factors and proteases were detected during stimulation.

**Detection of AMPs by ELISA, and determination of their bactericidal activity**

Since the mass-spectrometric procedures used only provided qualitative and semiquantitative information of the polypeptide content, ELISA was used to estimate the quantitative amounts of selected AMPs and antibacterial chemokines. In addition, ELISA was used to try to detect some AMPs and antibacterial chemokines that were expected, but did not show up using the mass-spectrometric methods.

The amount of the chemokines CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC (IFN-inducible T-cell α-chemoattractant), CXCL1/GRO-α (growth-related oncogene), CXCL2/GRO-β, CXCL3/GRO-γ, CCL20/MIP-3α (macrophage inflammatory protein 3-α), CCL28/MEC (mucosae-associated epithelial chemokine), and the polypeptide SLPI, as detected by ELISAs are shown in Table 2. Stimulated conditions showed relatively high, but not bactericidal, amounts of the IFN-γ inducible chemokines CXCL9/MIG and CXCL10/IP-10 and SLPI (Table 2). Neither the hBD-1, hBD-2, and hBD-3, nor lysozyme and lactotransferrin, could be detected by ELISA. To determine and compare the bactericidal activity of the polypeptides detected using ELISA, viable count assay with recombinant peptides and API bacteria was performed. Of the polypeptides detected, CXCL9/MIG was the most efficient in killing *S. pyogenes*, with a MBC₉₀ value of 0.1 μM (Table 2).

**Discussion**

In the present study, we show that pharyngeal epithelial cells stimulated with IFN-γ, a key regulator of the host response during bacterial and viral infections, cause release of soluble bactericidal activity.

There are several lines of evidence, showing that IFN-γ is important during *S. pyogenes* infection in vivo. In an animal model, IFN-γ responsive genes were highly upregulated 24 h after infection (Hyland and others 2009). Furthermore, both neutralization of IFN-γ in vivo and IFN-γ knockout mice, showed increased susceptibility to *S. pyogenes* at sites of infection compared with control/wild-type mice (Raeder and others 2000). Taken together, IFN-γ seems to be a key regulator of both the Th1 response in our model in general and, in directing a local innate bactericidal response mediated by epithelial cells. Interestingly, TNF amplified the IFN-γ-induced response with respect to several AMPs, without a parallel increase in the antibacterial activity. The reason for this is unclear, and several explanations are possible. One possible explanation could be that the addition of TNF induces production of molecules that bind

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**FIG. 2.** Protein streptococcal inhibitor of complement (SIC) of *S. pyogenes* and heparin reduce the antibacterial activity present in the IFN-γ-stimulated incubation medium from pharyngeal epithelial cells. (A) The incubation medium from IFN-γ-stimulated pharyngeal epithelial cells was preincubated with protein SIC for 20 min at the concentrations indicated before incubation with *S. pyogenes* for 1 h. The resulting numbers of cfu were determined using a viable count assay. The reduction of bacterial killing is shown. The data shown represent mean±SEM from 3 separate experiments. (B) The incubation medium from IFN-γ-stimulated pharyngeal epithelial cells was preincubated with heparin-Sepharose for 20 min, followed by removal of the beads and subsequent incubation with *S. pyogenes* for 1 h. The data shown are a representative out of 3 experiments.
AMPs and interfere with their antibacterial activity, still allowing the AMPs to be detected by the methods used. One example is GAGs where the synthesis of some is increased by TNF (Nelimarkka and others 1997). GAGs can bind and inactivate AMPs (Schmidtchen and others 2001). The urea-purification step may release AMPs that are in complex with different, possibly neutralizing carriers, for example, anionic proteins, GAGs, and DNA. Another possible explanation is that the marked increase in the presence of proteases after incubation, with a combination of IFN-γ and TNF, modifies the AMPs in a manner that impair their antibacterial activity, but still allow their detection (Steffen and others 2006).

Several findings support that the bactericidal activity found in the present study is mediated by polypeptides. The time-dependent threshold effect with respect to bactericidal activity, suggests that antibacterial agents accumulate in the medium, reaching a critical concentration with time, kinetics typical of AMPs. The relatively long time necessary to reach antibacterial activity in the medium after stimulation of cells with IFN-γ and TNF, modifies the AMPs in a manner that impair their antibacterial activity, but still allow their detection (Steffen and others 2006).

Protein SIC, released from S. pyogenes, binds and inhibits the effect of many AMPs, for example, lysozyme C, CXCL9/MIG, hBD, and SLPI (Frick and others 2003; Fernie-King and others 2004; Egesten and others 2007). Similarly, the killing of S pyogenes was reduced dose-dependently after addition of the protein SIC to the incubation medium from cells stimulated with IFN-γ. However, the reduction reached a plateau, demonstrating that not all bactericidal activity was sensitive to inhibition by the protein SIC. In addition, many AMPs have heparin-binding properties and their activities are inhibited by released sulfated GAGs (Schmidtchen and others 2001; Andersson and others 2004). In line with this, addition of heparin to the incubation medium from IFN-γ-stimulated pharyngeal epithelial cells, reduced the bactericidal activity, similar to that of the protein SIC. Contribution of nonpeptide effector molecules cannot be entirely ruled out. Epithelial cells can produce reactive oxygen species and nitric oxide during inflammation. These may form antibacterial agents, especially in the presence of a combination of peroxidase activity and halides (Hampton and others 1998; Moskwa and others 2007).

Characterization of the polypeptide content in the incubation medium after cytokine stimulation demonstrated a cumulative presence of several AMPs. Lysozyme C acts...
Table 1. Detection of Antimicrobial Polypeptides and Molecules That Putatively May Interact with Innate Immune Functions in the Incubation Medium Using Microcapillary Reversed-Phase Liquid Chromatography and Tandem Mass Spectrometry

<table>
<thead>
<tr>
<th>Distribution of scans (%)</th>
<th>Acc. No.</th>
<th>Control</th>
<th>IFN-γ</th>
<th>IFN-γ+TNF-α</th>
<th>(Total scans)</th>
<th>References</th>
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<td><strong>Antimicrobial polypeptides</strong></td>
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<tr>
<td>Lysozyme C</td>
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<td>0</td>
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<td>Dermcidin</td>
<td>P81605</td>
<td>22</td>
<td>22</td>
<td>54</td>
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<td>Schittek and others (2001)</td>
</tr>
<tr>
<td>Lactotransferrin</td>
<td>P02788</td>
<td>33</td>
<td>33</td>
<td>33</td>
<td>(6)</td>
<td>Arnold and others (1977)</td>
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<td>CXCL9/MIG</td>
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<tr>
<td>Complement factor B</td>
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<td>19</td>
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<td>0</td>
<td>100</td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>Cathepsin Z</td>
<td>Q9UBR20</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>P03956</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>(2)</td>
<td></td>
</tr>
<tr>
<td>MMP-10</td>
<td>P09238</td>
<td>36</td>
<td>18</td>
<td>45</td>
<td>(33)</td>
<td></td>
</tr>
<tr>
<td><strong>Protease inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calgranulin a (Protein S100-A8)</td>
<td>MR8</td>
<td>36</td>
<td>27</td>
<td>36</td>
<td>(11)</td>
<td>Steinbakk and others (1990)</td>
</tr>
<tr>
<td>Cystatin C</td>
<td>P01034</td>
<td>33</td>
<td>0</td>
<td>67</td>
<td>(3)</td>
<td>Björck and others (1989)</td>
</tr>
<tr>
<td>Elafin</td>
<td>P19957</td>
<td>57</td>
<td>14</td>
<td>29</td>
<td>(7)</td>
<td>Simpson and others (1999)</td>
</tr>
<tr>
<td>SLPI</td>
<td>P03973</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>(5)</td>
<td>Hiemstra and others (1996)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombospondin-1</td>
<td>P07996</td>
<td>41</td>
<td>15</td>
<td>44</td>
<td>(27)</td>
<td>Malmsten and others (2006)</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>P00450</td>
<td>18</td>
<td>30</td>
<td>52</td>
<td>(33)</td>
<td>Klenkoff (1992)</td>
</tr>
<tr>
<td>NGAL</td>
<td>P80188</td>
<td>31</td>
<td>31</td>
<td>38</td>
<td>(45)</td>
<td>Goetz and others (2002)</td>
</tr>
</tbody>
</table>

Polypeptides in the incubation medium were denatured using urea (8 M), reduced, and digested with trypsin. After μRPLC and obtaining MS/MS spectra, searches were performed against the human Swissprot database version 57.4. Peptides were assembled into proteins and the probabilities at the protein level were computed by a protein inference program and a reverse database search strategy. The selected cutoff was at 1% false discovery rate. References reporting antibacterial activity are indicated on the right.

IFN, interferon; μRPLC, microcapillary reversed-phase liquid chromatography; NGAL, neutrophil gelatinase-associated lipocalin; SLPI, secretory leukocyte protease inhibitor; TNF, tumor necrosis factor.

Table 2. Detection of Antimicrobial Polypeptides in the Incubation Medium from Pharyngeal Epithelial Cells Using ELISA

<table>
<thead>
<tr>
<th>Control</th>
<th>IFN-γ</th>
<th>IFN-γ+TNF-α</th>
<th>MBC90</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL9/MIG</td>
<td>N.D.</td>
<td>0.5±0</td>
<td>11±1.4</td>
<td>0.1</td>
</tr>
<tr>
<td>CXCL10/IP-10</td>
<td>N.D.</td>
<td>0.1±0</td>
<td>15±4</td>
<td>0.7</td>
</tr>
<tr>
<td>CXCL11/TAC</td>
<td>N.D.</td>
<td>0.7±0.2</td>
<td>0.8</td>
<td>Cole and others (2001)</td>
</tr>
<tr>
<td>CXCL1/GRO-α</td>
<td>0.9±0.3</td>
<td>0.3±0.1</td>
<td>1.0</td>
<td>Yount and others (2007)</td>
</tr>
<tr>
<td>CXCL2/GRO-β</td>
<td>0.05±0</td>
<td>0.03±0</td>
<td>0.9</td>
<td>Present study</td>
</tr>
<tr>
<td>CXCL3/GRO-γ</td>
<td>0±0.2</td>
<td>0.2±0.06</td>
<td>0.9</td>
<td>Present study</td>
</tr>
<tr>
<td>CCL20/MIP-3α</td>
<td>0.01±0</td>
<td>0.01±0</td>
<td>0.9</td>
<td>Hoover and others (2002)</td>
</tr>
<tr>
<td>SLPI</td>
<td>9±3</td>
<td>11.9±3</td>
<td>14±2.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

The cell culture medium from pharyngeal epithelial cells, incubated in a medium alone (control) or stimulated with IFN-γ (100 U mL⁻¹), and/or TNF-α (10 ng mL⁻¹) for 24 h, were analyzed using specific ELISAs for their content of antibacterial polypeptides. The values are expressed as mean (μM)±SEM from 3 separate experiments.

The bactericidal activity of the detected polypeptides against *S. pyogenes* was determined by incubating recombinant peptides with AP1 bacteria followed by a viable count assay. MBC90 values show the peptide concentrations (μM) at which 90% of the bacteria were killed. The values shown represent mean from at least 3 separate experiments.

N.D., not detectable.

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The finding of many molecules belonging to the complement system is intriguing. This suggests that locally produced complement can participate in both bacterial killing and in regulating the inflammatory process. It has been shown that the complement fragment C3a and C4a have strong bactericidal activity (Nordahl and others 2004). The production of several endogenous proteases may play a role in producing antibacterial polypeptides, as has been shown. Another possibility is that they play a role modulating and downregulating the inflammatory response (Malmsten and others 2007).

Taken together, the results show that stimulation of pharyngeal epithelial cells with IFN-γ, results in a bactericidal innate response against S. pyogenes, constituted of a spectrum of AMPs and antibacterial chemokines. The findings strongly emphasize an important role for the IFN-γ-stimulated epithelium in the first line of defense against pathogenic bacteria at mucosal surfaces.

Acknowledgments

The work was supported by grants from the Swedish Research Council (project # 20674), the Swedish Heart and Lung Foundation (project # 20100164), the Medical Faculty at Lund University Swedish Government Funds for Clinical Research (ALF), the Foundations of Bergh, Hierta, Kock, Marcus & Marianne Wallenberg, Osterlund, and the Royal Physiographic Society in Lund.

Author Disclosure Statement

The authors declare no conflict of interests.

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Received 18 February 2012/Accepted 13 June 2012