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Host-pathogen Interplay of *Haemophilus ducreyi*

Diane M. Janowicz[a,*], **Wei Li**[a,*], and **Margaret E. Bauer**[b]

[a] Department of Medicine, Indiana University School of Medicine, Indianapolis, IN USA

[b] Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN USA

Abstract

Purpose of review—*Haemophilus ducreyi*, the causative agent of the sexually transmitted infection chancroid, is primarily a pathogen of human skin. During infection, *H. ducreyi* thrives extracellularly in a milieu of professional phagocytes and other antibacterial components of the innate and adaptive immune responses. This review summarizes our understanding of the interplay between this pathogen and its host that leads to development and persistence of disease.

Recent findings—*H. ducreyi* expresses key virulence mechanisms to resist host defenses. The secreted LspA proteins are tyrosine-phosphorylated by host kinases, which may contribute to their antiphagocytic effector function. The serum resistance and adherence functions of DsrA map to separate domains of this multifunctional virulence factor. An influx transporter protects *H. ducreyi* from killing by the antimicrobial peptide LL37. Regulatory genes have been identified that may coordinate virulence factor expression during disease. Dendritic cells and natural killer cells respond to *H. ducreyi* and may be involved in determining the differential outcomes of infection observed in humans.

Summary—A human model of *H. ducreyi* infection has provided insights into virulence mechanisms that allow this human-specific pathogen to survive immune pressures. Components of the human innate immune system may also determine the ultimate fate of *H. ducreyi* infection by driving either clearance of the organism or an ineffective response that allows disease progression.

Keywords

Haemophilus ducreyi; chancroid; human model; differential host susceptibility; immunopathogenesis

Introduction

Haemophilus ducreyi, the causative agent of the sexually transmitted genital ulcer disease (GUD) chancroid, is an obligate human pathogen that primarily infects epithelial surfaces. In this niche, *H. ducreyi* persists extracellularly despite generating vigorous innate and adaptive innate immune responses. A human model of *H. ducreyi* infection has provided more detailed experimental analyses of the interactions between humans and *H. ducreyi* than is available for most bacterial pathogens. Recent studies have revealed host effects on the outcome of *H. ducreyi* infection; current research focuses on understanding key virulence factors of *H. ducreyi* that protect the organism from antibacterial mechanisms of innate

Correspondence to Margaret E. Bauer, PhD, Department of Microbiology and Immunology, Indiana University School of Medicine, 635 Barnhill Drive, Room MS 420, Indianapolis, IN 46202-5124. Tel: 317-274-8143; fax: 317-274-4090; mebauer@iupui.edu.
*These authors contributed equally to this paper.

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immunity and on elucidating the roles of several leukocyte populations in developing immune responses to *H. ducreyi*.

Human model of *H. ducreyi* infection

To better define the biology of *H. ducreyi* infection, a human model of experimental chancroid infection was developed during the 1990s [1,2]. In this model, volunteers are inoculated with *H. ducreyi* at multiple sites on the skin overlying the upper arm. The estimated infectious dose is as little as 1 CFU, and isease progresses similarly to natural infection. Within 24 h, papules develop at the site of inoculation and then either spontaneously resolve or evolve into pustules. Volunteers are observed until they achieve a clinical endpoint: resolution of disease at all sites; development of a pustule that is either painful or > 4 mm in diameter; or 14 days after inoculation. Although the model is limited to the pustular stage of disease, experimental pustules and natural ulcers are histologically almost identical [3,4].

Since the model was established, 267 healthy adult volunteers have been infected at least one time with the parent strain 35000HP or with isogenic mutants of 35000HP, and 41 volunteers were infected a second time with the parent only [5**]. Based on analysis of papule and pustule formation rates, sites do not act independently and pustule formation is influenced by gender and other host effects (discussed below) [5**]. Two phenotypes within the context of the model have subsequently been defined. Subjects who develop at least 1 pustule are defined as pustule formers, while those who resolve all sites of infection are resolvers. When challenged a second time, these subjects tend to segregate towards their initial outcomes, lending additional credence to host effects determining outcome. Data collected from these trials have contributed to the discovery of immune mechanisms that help explain the differential host susceptibility to experimental *H. ducreyi* infection [6**] and the roles that dendritic cells (DC) and natural killer (NK) cells play in the host response to the pathogen [7*,8*].

Mutant-parent comparison trials permit the identification of putative virulence factors of *H. ducreyi*. These trials are double-blinded, multi-stage, dose ranging studies whose primary endpoint is the pustule formation rate [9,10]. Subjects serve as their own control for host effects. Mutants are categorized as attenuated (unable to form pustules), partially attenuated (form pustules at higher doses than the parent) or virulent (form pustules at doses similar to the parent). To date, 21 mutants have been tested in the human model: 6 were attenuated, 4 were partially attenuated, and 11 were virulent [5**]. The strength of these trials lies in their abilities to validate key steps in pathogenesis and to identify vaccine candidates. A weakness of the trials is that the mechanism for clearance of attenuated mutants cannot be directly studied in vivo, due to the low inoculum and the fact that these mutants are cleared.

Pathogenesis

Upon *H. ducreyi* infection of human skin, the host responds with a robust infiltrate of polymorphonuclear leukocytes (PMNs) and macrophages that leads to development of lesions which progress from papules to pustules to ulcers. Throughout experimental and natural disease, the bacteria are surrounded by PMNs and macrophages but remain extracellular, where they colocalize with phagocytes, collagen, and fibrin. Much of the recent research on *H. ducreyi* pathogenesis focuses on how the organism resists phagocytosis and survives the bactericidal activity of serum components and antimicrobial peptides.

Pathogenic mechanisms

Antiphagocytic mechanisms are critical to survival of extracellular pathogens. In *H. ducreyi*, resistance to phagocytosis is mediated by two homologous, large supernatant proteins, LspA1 and LspA2 [11]. These proteins are required for virulence in experimental infection, and recent studies have focused on their mechanism of action [10,11]. Specifically, LspA1 and LspA2 block Fcγ receptor-mediated uptake by inhibiting phosphorylation of Src family tyrosine kinases in phagocytes [12]. Although a number of other extracellular pathogens prevent phagocytosis by inhibiting signal-transducing phosphorylation events in the phagocyte, the LspA proteins were the first reported to affect the Src family proteins which, after Fcγ receptor activation, provide the initial signal event that triggers phagocytosis [12]. How the LspA proteins enter host cells is unknown. Interestingly, during macrophage-*H. ducreyi* co-incubations, macrophage kinases tyrosine-phosphorylate LspA proteins [13*]. Although the relevance of tyrosine phosphorylation to the function of LspA1 and LspA2 is unknown, it is intriguing to consider that macrophage-encoded enzymes may activate *H. ducreyi*-encoded proteins which, in turn, block signalling events in order to shut down phagocytosis.

H. ducreyi is also resistant to serum-mediated bactericidal activity, primarily through expression of the outer membrane protein *ducreyi* serum resistance protein A (DsrA) [14]. DsrA is a member of the oligomeric coiled adhesion (Oca) protein family, which forms homomeric trimers on bacterial surfaces and provides adherence to host tissues and protection from serum complement. As with the *lspA1 lspA2* mutant, a *dsrA* mutant of *H. ducreyi* was fully attenuated in the human model of infection [15]. The mechanism of DsrA-mediated serum resistance is not well understood but appears to protect the organism from the classical complement cascade by blocking surface deposition of IgM and complement components [16]. DsrA also binds vitronectin and fibronectin, two extracellular matrix components [17,18]. A structure-function analysis demonstrated that the matrix binding and serum resistance activities are mediated by separate domains of DsrA [19*]. The authors hypothesize that, independent of its interactions with extracellular matrix components, DsrA may physically shield bacterial surface components from interacting with host antibodies, thereby preventing complement deposition [19*].

Another extracellular matrix binding protein was recently described in *H. ducreyi*. Fibrinogen binder A (FgbA) binds fibrinogen in vitro and contributes to virulence in vivo [20*]. Although the mechanism of action of FgbA in vivo is unknown, *H. ducreyi* is often surrounded by fibrin during infection [3,4]. Surface deposition of fibrin protects other extracellular pathogens from phagocytes, complement, and antibodies [21,22]; thus, FgbA could initiate fibrin deposition to shield the bacterial surface from host-mediated attack.

Another important antibacterial component of the innate immune system is the production of cationic antimicrobial peptides (APs) such as defensins and cathelicidin [recently reviewed in 23]. Several AP-secreting cells are present during *H. ducreyi* infection, including keratinocytes, PMNs, and macrophages. Recent studies have shown that *H. ducreyi* resists killing by several human APs, including α-defensins, β-defensins, and the cathelicidin LL37 [24*]. *H. ducreyi* expresses a sensitive to antimicrobial peptides (Sap) influx transporter; in other pathogens, Sap transporters confer resistance to APs and contribute to virulence. A *sapA* mutant of *H. ducreyi* is more sensitive than the isogenic parent strain to killing by cathelicidin but not by defensins [Bauer ME, et al., unpublished data]. Thus, the Sap transporter provides one mechanism of AP resistance for *H. ducreyi*. However, the organism also expresses other, currently undefined mechanisms for resistance to defensins.

Regulation of virulence factors in *H. ducreyi*

Another active area of investigation is how *H. ducreyi* regulates expression of virulence factors during infection. In a recent study, RNA isolated from experimentally infected pustules was subjected to selective capture of transcribed sequences (SCOTS) to identify genes expressed in vivo. The study identified 531 *H. ducreyi* genes preferentially expressed during human infection compared with broth-grown bacteria [25*]. Among the in vivo expressed genes were *lspA1* and *lspA2*, providing the first evidence that *H. ducreyi* regulates virulence factors during human infection [25*]. In subsequent studies, expression of *lspA2* was increased during stationary phase growth and enhanced by addition of serum to the medium [26*]. Serum affected the expression of many *H. ducreyi* genes, including downregulating genes encoding the two-component regulator CpxRA [26*]. Overexpression of CpxR, in turn, repressed expression of both *lspA2* and *lspB*, which encodes the transporter required for secretion of the LspA proteins [26*]. This is the first study identifying a specific regulator of virulence in *H. ducreyi*. The full regulon of CpxRA-controlled genes, likely including other virulence factors, has not been defined. Although serum downmodulates CpxR in vitro, serum was present in both in vitro and in vivo conditions compared in the SCOTS study [25*]; the signals triggering CpxRA regulation in vivo remain unknown.

Another important study examined the role of the *H. ducreyi luxS* homolog in virulence. LuxS synthesizes an autoinducer molecule used in quorum sensing, a mechanism by which bacteria sense the density of their own populations and respond by altering gene expression. Among pathogens, quorum sensing affects expression of various virulence factors and is notably associated with biofilm formation. Although *H. ducreyi* is not known to form biofilms, the organism does form microcolonies, a precursor to biofilm development. *H. ducreyi* produces an autoinducer in a *luxS*-dependent manner [27*]; furthermore, a *luxS* mutant is partially attenuated for virulence in the human model of *H. ducreyi* infection [27*]. Although *H. ducreyi*'s transcriptional response to increasing autoinducer concentrations is undefined, the fact that LuxS contributes significantly to virulence suggests that the autoinducer may regulate virulence factors during human infection.

Human immune responses to *H. ducreyi*

H. ducreyi infection induces rapid recruitment of innate and adaptive immune cells to infected sites [7*,8*,28]. Infiltrating PMNs coalesce to form an epidermal abscess, and macrophages form a collar at the base of the abscess. Below the collar, there is a dermal infiltrate of memory/effector CD4 and CD8 T cells, NK cells, myeloid DC, and macrophages. There are few B cells and no serum antibody induction during experimental infection, although non-bactericidal antibodies are generated late in the ulcerative stage of natural disease [29,30,31]. The cutaneous immune responses to *H. ducreyi* could promote phagocytosis and disease resolution or could contribute to phagocytic failure [30]. Here, we focus on recent studies of DC and NK cells obtained from experimental pustules and on different host susceptibilities to *H. ducreyi*.

Dendritic cells

DC serve as sentinels for detecting invading pathogens and play an essential role in initiating both innate and adaptive immune responses. Normal human skin contains several types of DC, including epidermal Langerhans cells, dermal myeloid DC, and dermal plasmacytoid DC (pDC). *H. ducreyi* infection leads to an increased accumulation of Langerhans cells in the epidermis, hair follicles, and eccrine ducts [30] and a 2.8-fold increase in the ratio of $CD11c^+$ myeloid DC to $CD123^+$ pDC at the infected site [7*]. Some lesional DC are activated, expressing CD83 and DC lysosome-associated membrane protein

[32]. Despite LspA activity, monocyte-derived myeloid DC ingest and kill the bacterium through an unknown uptake pathway [7*]. DC exposed to *H. ducreyi* secrete proinflammatory cytokines, including IL-6, IL-1β, IL-8, IL-12 and TNF-α, as well as the anti-inflammatory cytokine IL-10 [7*,33]. Live *H. ducreyi* leads to partial upregulation of DC surface activation markers relative to heat killed *H. ducreyi* [7*]. Partially-activated DC, together with IL-10, may promote the generation of suppressive regulatory T (Treg) cells and compromise the establishment of a full Th1 response and effective phagocytic activity. The marked donor-to-donor variations observed in cytokine production of *H. ducreyi*-exposed DC suggest differences in the DC response to the organism, which could contribute to the differential host outcomes discussed below [7*].

Natural killer cells

NK cells play an essential role in controlling infection by killing infected cells and producing immunoregulatory cytokines. Human NK cells are $CD56^{+}CD3^{-}$ and can be divided into $CD56^{dim}$ and $CD56^{bright}$ subsets, which differ in their homing properties and effector functions [34]. $CD56^{+}CD3^{-}$ NK cells represent 10% of the lymphocyte infiltrate at infected sites, which is similar to the proportion of NK cells in peripheral blood mononuclear cells (PBMC) [8*]. Using PBMC from uninfected subjects as surrogates for lesional cells, *H. ducreyi* was found to activate NK cells indirectly through antigen presenting cells (APC) such as monocytes, macrophages and DC. *H. ducreyi*-infected monocytes and macrophages provide IL-18 and unidentified contact-dependent signals for NK cell activation, while DC activate NK cells through IL-12. Additionally, monocytes/macrophages must phagocytose *H. ducreyi* to activate NK cells. Consistent with these findings, NK cells from *H. ducreyi*-infected sites secrete IFN-γ when stimulated with IL-12 and IL-18. The role of NK cells in differential host responses to *H. ducreyi* is being investigated.

Differential host responses to *H. ducreyi* infection

In the human challenge model, gender and other host effects influence outcome [5**,9]. Men are twice as likely as women to form pustules; the mechanism for gender differences in susceptibility is unknown. Among resolvers and pustule formers, there are no differences in serum bactericidal activities, abilities of PMNs and macrophages to phagocytose *H. ducreyi*, proliferative T cell responses to *H. ducreyi* antigens, or the populations of immune cells recruited to infected skin [6**,9]. However, differences in the two groups are reflected in differential transcriptional responses to *H. ducreyi* [6**]. Susceptibility to *H. ducreyi* infection correlates with distinct transcriptional responses of myeloid-derived DC [6**]. RR DC express transcripts that should induce protective Th1 and Th17 T cell development, which could enhance phagocytic activity; in contrast, PP DC have a transcript profile that should promote hyperinflammatory Th1 and inhibitory Treg cell responses, which can suppress anti-*H. ducreyi* immunity and prevent bacterial clearance. Indeed, T cell lines with characteristics of the Tr1 subset of Treg cells have been isolated from pustules. The $CD4^{+}FOXP3^{+}$ subset of Treg cells are also found in *H. ducreyi*-infected pustules and can suppress *H. ducreyi*-specific CD4 T cell responses [Li W, et al., unpublished data]. Further study is needed to determine whether DC and other APC from pustule formers and resolvers could differentially regulate NK cell activation, T cell differentiation, and phagocytic activity. Identifying genetic determinants that affect Toll-like receptor (TLR) expression levels, polymorphisms in genes encoding TLRs, cytokines, and other immune modulators could also provide insight into mechanisms underlying differential host susceptibility to *H. ducreyi* infection.

Vaccine development

Although natural infection does not confer immunity or promote development of bactericidal antibodies, *H. ducreyi* remains extracellular throughout disease, and a vaccine that elicits bactericidal antibodies could be protective in humans. Mutant-parent comparison trials in the human model have identified several virulence factors that are potential vaccine candidates. The well-characterized hemoglobin-binding protein HgbA is required for pustule formation in humans, is necessary and sufficient for heme/iron acquisition, and contains a central domain important for hemoglobin binding [35*,36*]. Thus, HgbA is being targeted for vaccine development. Immunization with purified HgbA elicited bactericidal antibodies and protection against infection in the swine model [37]. Vaccine candidates have only been studied in animal models to date, and further studies are needed, given the differences between the animal and human models [28]. Vaccine development remains a primary focus of research, given that chancroid, like other GUD, increases the transmission of HIV-1.

Conclusion

During human infection, *H. ducreyi* utilizes several virulence factors to survive host antibacterial mechanisms. How the organism regulates expression of these and other virulence factors in response to the host environment is an active area of investigation. Similarly, the host responds to *H. ducreyi* by producing an immune response that can be effective or ineffective; the host factors underlying these differential responses are not yet understood. Ultimately, development of novel therapies or vaccines against *H. ducreyi* will require further understanding of the complex interactions between *H. ducreyi* and the human host.

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