**Haemophilus influenzae** Resides in Tonsils and Uses Immunoglobulin D Binding as an Evasion Strategy

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**Haemophilus influenzae** (Hi) causes respiratory tract infections and is also considered to be a commensal, particularly in preschool children. Tonsils from patients (n = 617) undergoing tonsillectomy due to chronic infection or hypertrophy were examined. We found that 51% of tonsils were positive for Hi, and in 95% of cases analyzed in detail (n = 39) Hi resided intracellularly in the core tonsillar tissue. Patients harbored several intracellular unique strains and the majority were nontypeable Hi (NTHi). Interestingly, the isolated NTHi bound soluble immunoglobulin (Ig) D at the constant heavy chain domain 1 as revealed by recombinant IgD/IgG chimeras. NTHi also interacted with B lymphocytes via the IgD B-cell receptor, resulting in internalization of bacteria, T-cell–independent activation via Toll-like receptor 9, and differentiation into non-NTHi-specific IgM-producing cells. Taken together, IgD-binding NTHi leads to an unspecific immune response and may support the bacteria to circumvent the host defense.

**Keywords.** B cells; Haemophilus influenzae; immunoglobulin D.
Immune system is redirected, preventing the production of antibodies directed against the pathogen itself [13]. Interestingly, B-cell activation triggered by *M. catarrhalis* was shown to be a result of a synergistic effect of IgD B-cell receptor (BCR) cross-linking and activation of mainly Toll-like receptor (TLR) 9 [14, 15]. TLRs are host pathogen recognition receptors that efficiently sense conserved microbial patterns [16, 17].

Respiratory pathogens such as *H. influenzae* and *M. catarrhalis* encounter different B-cell populations in the lymphoid tissues associated with the pharynx, including the nasopharyngeal and palatine tonsils [18]. Tonsils are mainly lymphoepithelial organs, which are important in the defense against inhaled pathogens. The subepithelial region is represented by lymphoid follicles with associated germinal centers, which are the site of active B-cell differentiation; the mantle zone, which harbors recirculating naïve B cells with a characteristic IgD+IgM+ phenotype; and finally the extrafollicular region [19, 20]. Chronic infection including recurrent acute tonsillitis and hypertrophy are the most important manifestations leading to tonsillectomy. A direct relationship between bacterial load and tonsillar disease has been suggested, and in addition the high rate of persistent infections by *H. influenzae* is frequently reported [21–24]. Comparison of bacterial species present in tonsils from patients who suffered from either tonsillar hypertrophy or recurrent tonsillitis has established that *H. influenzae* is isolated at a very high rate from tonsillar core tissues [24]. *Haemophilus influenzae* has also been described as a diffuse bacterial infiltration in tonsils from children up to 15 years of age, but not in adults [25]. Herein we define an IgD-binding NTHi population in tonsillar tissue, and further characterize interactions of these NTHi with B cells. Our findings suggest a novel mechanism by which NTHi may survive in tonsils.

**MATERIALS AND METHODS**

**Tonsils**

Palatine tonsils were obtained at Skåne University Hospital (ethics approval number BD46/2007) from patients undergoing tonsillectomy. Immediately after removal, samples for microbial examination were taken from the surface and the core of the tonsil. The patients were divided into 2 groups based on their clinical diagnosis. One group consisted of patients who were scheduled for tonsillectomy due to idiopathic tonsillar hypertrophy, and had no history of recurrent tonsillitis. The second group consisted of patients with history of chronic infection (>4 episodes of recurrent acute tonsillitis during the year before surgery) as well as patients with a history of recent peritonsillar abscesses.

**Bacteria, Cell Culture Conditions, and Repetitive Polymerase Chain Reaction**

Routine microbiological techniques were used to ascertain that only 1 particular *H. influenzae* clone was growing on the chocolate agar plate. Hence, *Haemophilus* species were passed twice after isolation to ascertain a pure noncontaminated NTHi population. Thereafter, each NTHi isolate was frozen in glycerol at −80°C. Strains were thawed from the same stock and grown on chocolate agar plates the day before each experiment. Bacteria were cultured in brain-heart infusion liquid broth, for NTHi supplemented with nicotinamide adenine dinucleotide and hemin, or on chocolate agar plates at 37°C in 5% CO₂. *Haemophilus parainfluenzae* KR156 was a noninfectious clinical isolate. *Moraxella catarrhalis* BBH18 that expresses *Moraxella* IgD-binding protein (MID) and the corresponding *mid* mutant were as described previously [26]. Polymerase chain reaction (PCR) was performed to verify encapsulated *H. influenzae* among selected isolates [27]. To exclude that *Haemophilus haemolyticus* was among the isolates, PCR and 16S ribosomal RNA sequencing was done [28]. The DiversiLab *Haemophilus* Kit was used for DNA fingerprinting (repetitive PCR [Rep-PCR]; bioMérieux).

**Primary B-Cell Isolation**

B cells were isolated by negative selection (B Cell Isolation Kit II; Miltenyi Biotec), resulting in a purity of ≥98% [29].

**Direct Binding Assays**

*Haemophilus influenzae* in suspension were incubated with recombinant IgD (1 µg) at 4°C [30]. After 1 hour, fluorescein isothiocyanate (FITC)—conjugated antihuman IgD polyclonal antibodies (pAbs; DAKO), 5 µM oligodeoxynucleotides with CpGs (TTAGGG) × 4 (Invivogen), 10 µM chloroquine, or 0.1 µM wortmannin (Sigma) were preincubated with B cells for 40 minutes [31].
Cytokine Array and Enzyme-Linked Immunosorbent Assay
B-cell supernatants were analyzed in a Cytokine Antibody Array (RayBiotech). In brief, B-cell supernatants were added to a filter coated with specific monoclonal antibodies (mAbs) directed against cytokines followed by incubation with a secondary layer of detection antibodies directed against the same cytokines. Interleukin 6 (IL-6) production was determined by enzyme-linked immunosorbent assay (ELISA; R&D Systems), and NTHi-dependent immunoglobulin secretion was analyzed in ELISA using appropriate detection antibodies and a standard serum (DAKO).

Transmission Electron Microscopy
B cells were incubated with NTHi (multiplicity of infection [MOI] 100) in RPMI medium for 1 hour at 37°C. After washing with PBS, samples were fixed in transmission electron microscopy (TEM) fixative and analyzed [29].

Statistical Analysis
Results were assessed by Student’s t test for paired data. P ≤ 0.05 was considered to be statistically significant.

RESULTS
Different H. influenzae Strains Are Isolated From the Tonsillar Core in the Same Patient
We analyzed the presence of H. influenzae and other bacterial species in tonsils obtained from patients undergoing elective tonsillectomy. Among 617 samples analyzed, 356 tonsils were from patients with hypertrophy with no signs of infection. Two hundred sixty tonsils were from patients with chronically infected tonsils, including 95 samples from patients who suffered from recurrent tonsillitis or peritonsillar abscess. As judged by the distribution of bacteria in culture-positive tonsillar samples (n = 441), H. influenzae and S. aureus were the most prevalent bacterial species (Figure 1A).

To determine whether H. influenzae were localized in the core tissue and intracellularly, tonsils from 39 patients were analyzed for the presence of intracellular bacteria. Immediately after surgery, specimens were divided into pieces and homogenized. Extracellular bacteria were subsequently killed using gentamicin and amoxicillin, antimicrobial agents that do not penetrate mammalian cells. Thereafter, aliquots of the samples were spread on chocolate agar plates to determine surviving extracellular bacteria, and in parallel, aliquots were mechanically lysed to open the host cells followed by spreading. Interestingly, we found that in 37 of 39 tonsils analyzed, the H. influenzae intracellular (IC) + extracellular (EC)/EC ratio was >1.0 (Figure 1B), and the mean IC + EC/EC ratio was 3.28.

Several H. influenzae were isolated within the same tonsil, and therefore we wanted to exclude whether these putative different isolates belonged to various clones. In total, 46 intracellular isolates from 5 tonsils displaying the highest IC + EC/EC ratio were genotyped by Rep-PCR. The genotypic analyses displayed 11 unique H. influenzae isolates and indicated that a single patient harbored >1 H. influenzae strain (Supplementary Figure 1). Three H. influenzae isolates from tonsillar sample number 5 were encapsulated, whereas the remaining 8 unique strains obtained from 5 tonsils were NTHi. In conclusion, H. influenzae was one of the predominant species found in patients having chronic tonsillitis or tonsillar hypertrophy. Haemophilus influenzae resided intracellularly in 95% of tonsils analyzed and genetically diverse strains were found within the same tonsil.

Tonsillar NTHi Binds to the Constant Heavy Chain Domain 1 of Soluble IgD
The subepithelial compartment of human palatine tonsils is predominantly composed of B cells (65%) [32]. Because encapsulated H. influenzae type b (Hib) binds human IgD [7, 33], we hypothesized that viable NTHi found in tonsillar core tissues may reside within B lymphocytes and hence use the IgD molecule for entry. To test our hypothesis, we analyzed the binding of soluble IgD to the unique tonsillar NTHi (n = 8; Figure 2A). In addition, encapsulated H. influenzae (n = 3) from tonsillar sample number 5 was included in the analyses. Antigenic recognition of bacteria by the immunoglobulin was avoided by using recombinant IgD with a known specificity [30]. The majority of the intracellular H. influenzae isolates from different patients (numbered tonsil 1 to 5) were positive for binding soluble IgD, albeit at different levels. Two genetically diverse tonsillar isolates from patient number 2 were selected for further experiments (Figure 2A). These were designated NTHi KR402 and KR403 and were considered as low and high IgD-binding isolates, respectively. As can be seen in Figure 2B, the IgD binding of the selected strains were compared with suitable controls. Because M. catarrhalis binds IgD via MID [26], the wild-type MID-expressing M. catarrhalis BBH18 and the isogenic BBH18 Δmid mutant were used as positive and negative controls, respectively. In addition, H. parainfluenzae KR156 was included as a representative of a bacterial species found in the respiratory tract and closely related to H. influenzae, but without IgD-binding capacity.

We have previously reported that Hib binds to the IgD constant heavy chain domain 1 (C{sub}H1) [7]. To determine whether NTHi and Hib share the same binding site on the IgD molecule, we tested IgD/immunoglobulin G (IgG) chimeras for binding. These chimeric immunoglobulins have overlapping sequences in the C{sub}H1 region replaced by corresponding IgG sequences [7]. Replacement of IgD amino acids 157–197 with IgG (IgD 157–197 IgG) did not disrupt the interaction between NTHi and IgD but showed similar binding as full-length IgD, suggesting that these amino acids were not involved in the interaction (Figure 3). However, when IgD amino acids 185–224 (IgD 185–224 IgG) were
replaced by the corresponding IgG sequence, immunoglobulin binding to NTHi was abolished. NTHi thus primarily recognized amino acids 198–224 in the CH1 region of IgD, which is in parallel to Hib [7]. Collectively, tonsillar NTHi clinical isolates bound IgD outside the antigen binding site; that is, the binding region was located within the CH1 region.

**NTHi Interacts With B Lymphocytes via the Surface IgD B-Cell Receptor**

The ability of NTHi to bind soluble IgD prompted us to check whether bacteria also attach to B cells via the surface-bound IgD BCR. We FITC-labeled NTHi KR402, KR403, and the control *H. parainfluenzae* KR156 followed by incubation with...
purified tonsillar B cells. Interestingly, both selected NTHi strains significantly bound to B cells as compared to the negative control H. parainfluenzae KR156 (Figure 4). To confirm that binding of bacteria to B cells was mediated via surface IgD, B lymphocytes were preincubated with rabbit anti-IgD or anti-IgM pAbs. Interestingly, preincubation of B cells with anti-IgD pAb signifi-
cantly decreased the binding of FITC-labeled NTHi (Figure 4). In contrast, NTHi bound equally well to B cells pre-
incubated with anti-IgM pAbs compared to controls in the absence of Abs. Taken together, the IgD-binding NTHi, but not H. parainfluenzae, attached to the IgD BCR on tonsillar B cells.

NTHi Stimulates Primary B Cells via BCR Cross-linking and TLR9 Resulting in a T-Cell–Independent Activation

One of the well-known consequences of cross-linking the BCR is cellular activation. To analyze whether NTHi activates B cells, tonsillar B cells were incubated with fixed bacteria at

Figure 2. Characterization of the immunoglobulin D (IgD)–binding capacity of nontypeable Haemophilus influenzae (NTHi). A, Tonsillar H. influenzae (Hi) isolates bind soluble recombinant IgD. Hi isolates with a unique genomic pattern in repetitive polymerase chain reaction (Supplementary Figure 1; 2 isolates from each patient number 1–3, 1 from patient 4, and 4 unique isolates from patient 5) were analyzed for binding to soluble IgD by flow cytometry. B, IgD binding of nontypeable Hi (NTHi) isolates KR402 and KR403 was compared to other pathogens. The non-IgD-binding Haemophilus parainfluenzae and the Moraxella--IgD-binding protein (MID)–deficient mutant Moraxella catarrhalis BBH18Δmid served as negative controls, and the MID-expressing M. catarrhalis BBH18 wild type as a positive control for IgD binding. Aliquots of bacterial suspensions were incubated with recombinant IgD (1 μg) at 4°C for 1 h followed by FITC-conjugated antihuman IgD polyclonal antibodies for 30 min. After washings, the binding was analyzed by flow cytometry. Binding of IgD to NTHi was unaffected by the bacterial growth phase. Mean values are shown and error bars indicate SD in (A) and SEM in (B). *P ≤ .05. Abbreviations: IgD, immunoglobulin D; Hi, Haemophilus influenzae; M.cat, Moraxella catarrhalis; mfi, mean fluorescence intensity.

Figure 3. Nontypeable Haemophilus influenzae (NTHi) binds to the immunoglobulin D (IgD) constant heavy chain domain 1 (C_{H1}) region within the site spanned by amino acids 198–224. The chimeric IgD 157–197 immunoglobulin G (IgG) has IgD sequences Cys 157 to Ser 197, comprising the C_{H1} loop, replaced by the corresponding IgG sequence. The chimeric IgD 185–224 IgG has IgG sequence replacing the IgD amino acids from Glu 185 to Lys 224 that encompasses the second Cys of the IgD C_{H1} loop. Exchange of residues 157–197 of IgD with IgG did not affect the interaction of NTHi KR402 and KR403 with the chimeric IgD. All chimeric immunoglobulins were recognized to a similar extent by FITC-conjugated anti-IgD antibodies in the control experiments [7]. One representative experiment out of 3 is shown. Abbreviations: FITC, fluorescein isothiocyanate; IgD, immunoglobulin D; IgG, immunoglobulin G; NTHi, nontypeable Haemophilus influenzae.
globulin M; pAb, polyclonal antibody.

To further characterize the NTHi-dependent B-cell activation, we analyzed supernatants from B cells incubated with bacteria for the presence of various proinflammatory mediators using a cytokine array (Figure 5C). The presence of the IgD-binding NTHi KR402 and KR403 lead to an IL-6 increase and a minor upregulation of interleukin 10 (IL-10) as compared to B cells incubated without bacteria. When IL-6 concentrations were measured by ELISA at 48 hours, we found that NTHi KR402 and KR403 elicited a 68- and 57-fold higher IL-6 production, respectively, compared with KR156 and unstimulated controls (Figure 5D).

In the presence of BCR cross-linking, naïve B cells are either sensitized to TLR ligands or upregulate their TLR expression to be more responsive [15, 34]. Unmethylated CpG, recognized via endosomal TLR9, is one of the most potent stimulants for TLR activation [35, 36]. To assess the role of TLR9 in NTHi-dependent B-cell activation, purified B cells were incubated with a dominant-negative TLR9 inhibitory oligonucleotide prior to activation by NTHi. After 96 hours, the proliferation induced by KR402 and KR403 was reduced up to 50% (Figure 6A). A decreased NTHi-mediated B-cell activation was also observed with chloroquine. In addition, we blocked the autophagy by the class III PI3-kinase inhibitor wortmannin, and observed a 10-fold decreased B-cell stimulatory response. Taken together, the NTHi-mediated B-cell activation was suppressed when TLR9 signaling was inhibited.

To analyze whether IgD BCR cross-linking and TLR9-dependent activation lead to differentiation of B cells into antibody-producing plasma cells, supernatants from B cells stimulated with NTHi for 96 hours were analyzed. A strong induction of IgM secretion was observed with NTHi KR402 and KR403 as compared to stimulation with *H. parainfluenzae* KR156 and untreated B cells (Figure 6B). The IgD secretion was increased up to 5-fold as compared to untreated controls (Figure 6C), but was considerably lower as compared to IgM. Finally, no measurable IgG and IgA secretion was detected in the presence of NTHi when compared to untreated controls. To test the specificity of IgM antibodies produced, supernatants corresponding to 30 ng of IgM were incubated with NTHi isolates followed by flow cytometry analysis. The IgMs did not recognize NTHi, suggesting that NTHi cross-linked the IgD BCR outside the antigen-binding site. We conclude that activation of highly purified B cells in response to NTHi lead to differentiation into nonspecific IgM-producing cells, whereas no IgG class switch occurred. Our results thus suggested a T-cell–independent B-cell activation by NTHi.

**NTHi Is Internalized by B Cells in an IgD-Dependent Manner**

Cross-linking of surface immunoglobulin leads to BCR down-regulation and internalization [14, 37]. Because both the IgD BCR and TLR9 were involved in NTHi-mediated B-cell activation, the question arose whether NTHi was internalized. To test this hypothesis, purified tonsillar IgD⁺ B cells were incubated with bacteria at an MOI of 100. Extracellular bacteria were killed by gentamicin, and intracellular bacteria were enumerated after lysis of the B cells. To confirm an IgD-dependent uptake, B cells were preincubated with anti-IgD pAbs, and this treatment resulted in a significantly decreased intracellular recovery of NTHi (Figure 7A). Importantly, the non-IgD-binding *H. parainfluenzae* KR156 was not taken up by B cells.

When the intracellular fate of NTHi was further assessed, a constant decline of viable intracellular bacteria was observed (Figure 7B). However, up to 50% of bacteria (NTHi KR402) were still alive after 7 hours, whereas no viable bacteria were recovered after 24 hours. The uptake of live bacteria was further...
analyzed by TEM. After incubation with IgD⁺ B cells for 1 hour, an active process of NTHi uptake was clearly seen. Different stages of the process, that is, interaction with B cells, initiation of internalization, and eventually engulfed bacteria, were revealed by TEM (Supplementary Figure 2A–C). Using gold-labeled anti-IgD mAbs, the interaction of NTHi with B cells via surface IgD was highlighted. Engulfed bacteria were observed within vacuoles that had IgD molecules clustered on their membranes (Figure 7C–E). To ascertain that NTHi were located intracellularly, we counted bacteria associated with the plasma membrane and the nucleus (Figure 7F). After 1 hour, most bacteria were detected in the vicinity of the cell nuclei as compared to the plasma membrane that proved internalization of bacteria. In conclusion, NTHi utilized the IgD BCR and entered B cells for specific evasion of the host.

**DISCUSSION**

Underlying tonsillar diseases such as tonsillitis and tonsilar hypertrophy are often associated with a high load of bacterial
NTHi is found as a part of the commensal flora colonizing the human nasopharyngeal tract. However, whether NTHi is associated with normal tonsils is still a debatable issue. Some studies suggest absence of recovery of any NTHi strains from normal tonsils, whereas others suggest that NTHi strains colonize normal tonsillar tissue as well, albeit at a concentration much lower than the diseased tonsils [23, 38]. In the present study, we found NTHi to be preferentially associated with tonsillar hypertrophy, where it was coisolated with S. aureus as the second predominant bacterial species. An increased immune cell load is suggested to be a part of various tonsillar diseases [39], and thus we aimed at investigating the effect of NTHi on B lymphocytes.

We show an interaction of NTHi with tonsillar IgD⁺ B lymphocytes via the nonantigen binding site of surface IgD. Membrane-bound IgD is expressed together with IgM at the B-cell surface, and it has been demonstrated that the number of IgD⁺ B cells seems to be increased in diseased tonsils [40]. The immunological role of secreted IgD remains obscure except for detection of aberrant serum levels of soluble IgD in autoimmune diseases and chronic bacterial infections [41]. In addition, IgD-secreting plasma cells and soluble IgD are more frequent in the upper respiratory tract as compared to other anatomical sites [42, 43].

Although a long-term survival of intracellular NTHi within B cells was not established in the present work, it is plausible that a host environment may in some way support survival of intracellularly residing bacteria. Alternatively, the IgD-binding NTHi is sacrificed upon internalization at the cost of an inflammatory response elicited due to the interaction of the IgD BCR. Yet another explanation would be that NTHi resides in B cells for shorter periods of time to escape from the immune system, and after a few hours may return to the extracellular space.

As NTHi infections are known to persist, it has been suggested that invasion of human cells is a survival strategy [44]. NTHi is known to reside in epithelial and monocytic cells as well as in macrophages. It has been demonstrated that NTHi enters the

**Figure 6.** B-cell activation by nontypeable *Haemophilus influenzae* (NTHi) requires costimulation of Toll-like receptor 9 (TLR9) and leads to immunoglobulin M (IgM) and immunoglobulin D (IgD) production. A, B cells were treated with dominant-negative TLR9 ligand (TTAGGG)X4, chloroquine, or wortmannin prior to activation by NTHi and *Haemophilus parainfluenzae*. The B-cell activation was thereafter monitored by [³H]-thymidine uptake at 96 hours. For comparison, the highest value in each experiment was defined as 100 arbitrary units. The mean values (n = 3) were for NTHi KR402 35 742, KR403 26 940, and “B cells only” 189 cpm. Cell viability was not affected by the different inhibitors as determined by Trypan blue exclusion staining. B, As quantified by enzyme-linked immunosorbent assay, B-cell stimulation by NTHi results in significant IgM production in the culture supernatants after 96 hours. C, Minor increased production of IgD antibodies was also observed in response to NTHi. In (A) and (B), mean values are shown and error bars indicate SD from at least 3 different donors. ***P ≤ .001, **P ≤ .01, *P ≤ .05. Abbreviations: cpm, counts per minute; DMSO, dimethylsulfoxide; IgD, immunoglobulin D; IgG, immunoglobulin G.
monocytic and epithelial cells in a β-glucan receptor–mediated nonphagocytic manner [45]. We have also suggested that the outer membrane NTHi protein D enhances the adherence and uptake of NTHi [46].

B cells are conventionally considered nonphagocytic immune cells, although emerging evidence proves that B cells can take up larger particulate antigens including whole bacteria. Indeed, intracellular pathogens have been shown to enter and survive within B lymphocytes [47, 48]. An efficient uptake is, however, suggested to be dependent on recognition of antigen via the BCR [48]. NTHi is normally considered an extracellular pathogen, although a number of reports suggest that NTHi can invade and reside within phagocytic and nonphagocytic cells [45, 49]. Using tonsils as a reservoir, NTHi evades the host immune defense, mounts an inflammatory response, and possibly spreads to other anatomical sites, leading to infection. More work is required to further establish the signaling mechanisms that promote survival of H. influenzae within B lymphocytes and clinical implications for NTHi-dependent colonization and subsequent infection.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.
Notes

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References


