Binding of complement regulators to invasive nontypeable *Haemophilus influenzae* is not increased compared to nasopharyngeal isolates, but serum resistance is linked to disease severity.

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Running title: NTHi AND SERUM RESISTANCE

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ABSTRACT

The aim of the present study was to analyse the importance for non-typeable *Haemophilus influenzae* (NTHi) isolated from patients with sepsis (invasive isolates) compared to nasopharyngeal isolates from patients with upper respiratory tract infection to resist the complement-mediated attack in human serum and to correlate this to disease severity. We in detail studied and characterized cases of invasive NTHi disease. All patients with invasive NTHi isolates were adults and 35% had a clinical presentation of severe sepsis according to the ACCP/SCCM classification of sepsis grading. Moreover, 41% of the cases had evidence of immune deficiency. The different isolates were analyzed for survival in human serum, for binding of $[^{125}\text{I}]-$labeled purified human complement inhibitors C4b-binding protein (C4BP), Factor H and vitronectin in addition to binding of regulators directly from serum. No significant differences were found when blood and nasopharyngeal isolates were compared, suggesting that interactions with the complement system are equally important for NTHi strains irrespectively of isolation site. Interestingly, a correlation between serum resistance and invasive disease severity was found. The ability to resist the attack of the complement system seems to be important for NTHi strains infecting the respiratory tract as well as the blood stream.

**Keywords:** complement regulators; invasive disease; nontypeable *Haemophilus influenzae*; serum resistance
**INTRODUCTION**

*Haemophilus influenzae* is an important human-specific pathogen that can be classified according to the presence of a polysaccharide capsule (20). The encapsulated strains cause invasive diseases, whereas the unencapsulated and hence nontypeable *H. influenzae* (NTHi) are mainly found in local upper and lower respiratory tract infections (2, 35). However, NTHi is after *Streptococcus pneumoniae* the most common microbe found in children with acute otitis media and is the main cause of exacerbations in patients suffering from chronic obstructive pulmonary disease and bronchiectasis (3, 4, 13, 28, 32, 34). NTHi can also cause sinusitis, conjunctivitis and pneumonia in children (25). Thus, NTHi is a heterogeneous species, capable of great variation in virulence and is found in the airway as either a commensal or as a pathogen with capacity to invade the airway epithelium.

Invasive disease caused by *H. influenzae* type b (Hib) mainly affects infants and children, causing potentially life-threatening conditions such as meningitis, epiglottitis, and severe sepsis. After introduction of the conjugate vaccine against Hib in the early 1990s, the incidence of invasive disease caused by Hib has decreased substantially in the Western hemisphere (5). In contrast, it has been suggested that the incidence of NTHi septicemia is increasing (36). Most clinical studies on invasive *Haemophilus* infections have been about Hib, and less is known about the clinical characteristics of invasive disease caused by NTHi.

In relation to its high presence in nasopharyngeal and sputum cultures, NTHi is infrequently found in the bloodstream and it seems likely that host factors are equally important as specific bacterial virulence in cases with NTHi sepsis.

The complement system is the first line of defense against pathogenic microorganisms (7). Activation of the complement system leads to a cascade of protein activation and deposition on the surface of the pathogen, resulting in formation of the membrane attack complex (MAC) and opsonization of the pathogen followed by
The classical pathway of the complement system is activated by target bound antibodies and C-reactive protein (40), whereas the alternative pathway is spontaneously activated through direct contact with foreign particles or cells (37). Both pathways lead to the formation of the C3 convertases, with subsequent cleavage of C3 to C3a and C3b. Thereafter the C5 convertases are formed and the terminal pathway is activated, which results in the formation of the MAC and lysis of the cell. To prevent non-specific damage from excess complement activation, the complement cascade is tightly regulated. Important regulators of the complement system are C4b-binding protein (C4BP) (governing the classical/lectin pathway) (6), Factor H and Factor H like protein-1 (alternative pathway) (41), vitronectin and complement Factor H related protein 1 (terminal pathway) (18, 33).

The complement system is classified as a part of serum, but there are several studies demonstrating the presence of complement in various sites of the body. Reports of the presence of complement components in the respiratory tract of healthy individuals are scarce. There are several studies, however, indicating the importance of complement in the respiratory tract during infections. The permeability of the mucosa increases during inflammation, and plasma, including complement proteins and immunoglobulins, enters the airway lumen (11, 12, 29). This process designated plasma exudation has been suggested to be the first line of the mucosal defense.

The pathogenesis of many microorganisms relies on their capacity to avoid, resist or neutralize the host defense including the complement system. Therefore, many pathogens have evolved different mechanisms to avoid complement-mediated killing. A frequent strategy used by some pathogens is binding of complement inhibitors such as C4BP, Factor H, and vitronectin, which all protect from complement-mediated attacks (7, 22, 31). These inhibitors are captured on the bacterial surface in such a way that they are still functionally active. In previous studies we have shown that NTHi binds C4BP and Factor H,
and that these interactions significantly contributes to bacterial serum resistance (15, 17). In addition, *Haemophilus* surface fibrils that solely can be found in Hib, and protein E, which exists both in encapsulated and nontypeable strains (30) interact with vitronectin and thereby prevents complement-induced lysis resulting in increased bacterial survival in normal human serum (NHS) (14, 16).

In the present study, the characteristics of invasive NTHi infections, including evidence of immune deficiency of the individual patient and the clinical presentation of the septic event were studied. We correlated these findings with the capacity to bind specific complement regulators and *in vitro* serum resistance of the individual isolates. The invasive NTHi isolates obtained from patients with sepsis were compared to nasopharyngeal strains from patients with upper respiratory tract infection. There was no clear difference in serum resistance or binding to complement inhibitors between the two groups of NTHi. Our findings also demonstrate that binding of complement regulators and resistance to human serum is important for NTHi isolated from the upper respiratory tract as well as from blood. Furthermore, a significant correlation between disease severity and *in vitro* serum resistance was identified in cases of NTHi invasive disease.
MATERIAL AND METHODS

Patient data. Complete medical records, including clinical presentation, patient history and laboratory results from 17 out of 21 identified patients with invasive NTHi disease (bacteremia or meningitis) were studied and registered. The severity of the sepsis was graded according to the ACCP/SCCM classification of sepsis grading (8). Randomly selected nasopharyngeal cultures positive for NTHi (n=21) were used as controls. All of these cultures had a referral history of airway infection.

Bacterial strains and culture conditions. NTHi blood isolates (n=21) were from patients with invasive NTHi disease (bacteremia or meningitis) in the county of Southwest Skåne, Sweden 2001-2007. In addition, NTHi strains isolated from nasopharynx (n=21) of patients (Southwest Skåne in 2007) suffering from upper respiratory tract infection were included for comparison. Bacteria were routinely cultured in brain heart infusion (BHI) liquid broth supplemented with NAD and hemin (both at 10 µg/ml), or on chocolate agar plates at 37°C in a humid atmosphere containing 5 % CO₂. All strains were characterized by standard bacteriological techniques including oxidase, fermentation, satellite and XV tests. Thereafter, the H. influenzae strains were typed by PCR. To exclude that H. haemolyticus was among the isolates, PCR and 16S rRNA sequencing was done (24).

Proteins and antibodies. C4BP and Factor H from human plasma were purchased from Complement technology (Tyler, TE) and vitronectin from human plasma was from Sigma (Sigma-Aldrich, St. Louis, MO). The polyclonal rabbit anti-C4BP and goat anti-human Factor H antiserum were purchased from Complement technology or Calbiochem (La Jolla, CA). The horseradish peroxidase (HRP)-conjugated donkey anti-goat pAb was obtained from Serotech (Oxford, UK). The goat anti-human vitronectin and FITC-conjugated donkey anti-
goat pAb were from Serotec (Oxford, UK). An antiserum obtained from a rabbit immunized
with the clinical isolate NTHi 772 for 3 times with 2 week intervals was also included as a
control (1).

Serum bactericidal assay. Pooled NHS was obtained from healthy blood donors (n=5) with
informed consent. The NTHi strains were diluted in DGVB++ (2.5 mM Veronal buffer, pH 7.3
containing 70 mM NaCl, 140 mM glucose 0.1% (wt/vol) gelatin, 1 mM MgCl₂ and 0.15 mM
CaCl₂). Bacteria (10⁴ colony forming units (CFU)) were incubated in NHS or heat-inactivated
NHS (hiNHS) in a final volume of 100 µl at 37°C. After 20 min, 10 µl aliquots were removed
and spread onto chocolate agar plates. After 18 h of incubation at 37 °C, CFU were
determined.

Flow cytometry. To analyze whether the NHS contained IgG directed against NTHi and if
there were any differences in IgG deposition on NTHi isolates from blood or nasopharynx,
NHS was tested for reactivity by flow cytometry using a FITC-conjugated mouse anti-human
IgG pAb (Dakopatts). A rabbit anti-NTHi pAb was included as a positive control, and in these
experiments, a FITC-conjugated goat anti-rabbit pAb (Dakopatts) was used as a secondary
layer. Briefly, bacteria (10⁷) were incubated with 50 % hiNHS for 1 h on ice. Thereafter,
bacteria were washed followed by addition of the FITC-conjugated detection antibodies,
additional washes and finally flow cytometry analysis. All incubations were done in PBS
containing 2 % BSA and washings were with the same buffer. Secondary antibodies were
added separately as negative controls for each strain analyzed.

Protein labeling and direct binding assay. Purified C4BP, Factor H, and vitronectin were
labeled with 0.05 mol iodine (GE Healthcare, Buckinghamshire, UK) per mol protein, using
the Chloramine T method (10). The different *H. influenzae* strains were grown in BHI liquid broth overnight and washed in phosphate-buffered saline (PBS) containing 1 % bovine serum albumin (BSA) (Saveen Werner, Malmö, Sweden) (PBS-BSA). Bacteria (2x10⁷) were incubated with [¹²⁵I]-labeled complement regulators at 37 °C for 1 h. After incubation, bacteria were centrifuged (10,000xg) through a 20 % sucrose column. The tubes were frozen, cut and radioactivity in the pellet respectively supernatant was measured in a gamma counter. Binding was calculated as amount of bound radioactivity (pellet) versus total radioactivity (pellet + supernatant).

**Serum binding assay.** To analyze whether NTHi from different isolation sites bound C4BP or Factor H directly from NHS, bacteria were grown overnight in BHI broth. NTHi (10⁹) were incubated with hiNHS and buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4) for 1 h at 37°C. To remove unbound proteins, bacteria were washed 5 times with the same buffer. Thereafter, the bacterial pellet was resuspended in 150 µl of 0.1 M glycine-HCl, pH 2.0, in order to elute bound proteins (15, 17, 21). Bacteria without NHS were used as a negative control. After 15 min incubation at 37°C with shaking, bacteria were centrifuged and the supernatants were subjected to SDS-PAGE (10 %).

To analyze whether NTHi bound vitronectin from NHS, bacteria were grown overnight and incubated with hiNHS and PBS. To remove unbound proteins, NTHi were washed 5 times with the same buffer. Thereafter, the bacterial pellet was resuspended in 50 µl of 0.1 % Triton X-100 (Darmstadt, Germany) and protease inhibitors (Complete, Roche, Mannheim, Germany). After 30 min incubation at 4°C, bacteria were centrifuged and the supernatants were subjected to SDS-PAGE (10 %). Electrophoretical transfer of protein bands from the gel to an Immobilon-P membrane (Millipore, Bedford, MA) was done at 35 V for 2 h. After
transfer, the Immobilon-P membrane was blocked in PBS with 0.1 % Tween 20 (PBS-Tween) containing 5 % milk powder. After several washings, the membrane was incubated with rabbit anti-human C4BP, goat anti-human Factor H, or goat anti-human vitronectin pAb followed by incubation with HRP-conjugated swine anti-rabbit or donkey-anti-goat pAb. After incubation and additional washings in PBS-Tween, development was performed with ECL Western blotting detection reagents (Pierce, Rockford, IL). No cross-reactivity of the anti-C4BP, anti-Factor H or anti-vitronectin pAbs was seen with bacteria incubated in the absence of NHS.

Statistical analysis. To test for differences in serum survival and levels of complement inhibitor binding between the groups, a two-sided Mann-Whitney U-test was used. This was done considering small sample sizes and non-parametric data.

RESULTS

Clinical characteristics of invasive NTHi disease. Twenty-one cases of invasive NTHi were identified during the period 2001-2007, and complete medical records were obtained from seventeen cases as shown in Table 1. In contrast to invasive Hib disease that in unvaccinated populations mainly is associated with younger patients, all patients with invasive NTHi disease were adults. The cases were divided into separate groups by severity of the clinical disease according to the ACCP/SCCM classification (Table 1). Eight of the cases (47 %) (patient 1-8) had a clinical presentation of sepsis, while nine (53 %) (patient 9-17) had a presentation of sepsis with meningitis or severe sepsis. Interestingly, seven of the patients (41 %) had evidence of immune deficiency. Out of these 7 patients, 6 presented secondary immune deficiencies such as leukemia and treatment-induced neutropenia. However, severe
clinical presentations were not linked to immune deficiency. One patient died from the septic episode (28-day mortality 6%) while 5 patients died within a year (1-year mortality 29%). All but one of the patients that died within a year from their septic episode presented a mild clinical picture, suggesting the importance of underlying disease.

Serum resistance correlates to sepsis severity. Eight strains isolated from blood were studied regarding survival at increasing concentrations of pooled NHS from 5 donors to determine the appropriate serum concentration for further serum resistance experiments (Fig. 1, and data not shown). When a titration of the serum concentration was performed, all strains except the isolate from patient 13 (Fig. 1C) were killed by 15 % NHS after 20 min of incubation. All strains survived in 5 % NHS, and therefore this concentration was used for further studies.

When the cases of invasive disease were sorted according to disease severity, we considered the cases of sepsis with meningitis as clinically severe, and pooled these with the cases of severe sepsis according to the ACCP/SCCM grading. All NTHi strains were tested for serum resistance in 5 % NHS for 20 min. Interestingly, NTHi isolated from cases with severe sepsis and meningitis (n=9) had a significantly (p=0.04) higher degree of serum resistance (mean 63 %) as compared to NTHi isolates from those with sepsis (n=8) (mean 31 %) (Table 1). When we excluded the cases of sepsis with meningitis from the analysis, the serum resistance from the cases of severe sepsis (n=6) (mean 72%) was still significantly higher than those with sepsis (p=0.04).

No difference in serum resistance between invasive or nasopharyngeal NTHi isolates. To test whether there was a difference regarding serum resistance between NTHi isolated from separate patients suffering from sepsis (blood isolates) (Table 1) or upper respiratory tract
infection (nasopharyngeal isolates) (Table 2), bacteria from the two sites of isolation were compared in a serum bactericidal assay. The nasopharyngeal strains were isolated from immunocompetent patients with suspected bacterial upper respiratory tract infection. Primary viral infection could, however, not be excluded. The age distribution of the patients from whom the nasopharyngeal control isolates were obtained slightly differed from the patients with invasive NTHi infection, reflecting the local clinical tradition of taking nasopharyngeal swabs primarily from children. After incubation in NHS (5 %), the survival of the different NTHi was determined (Fig. 2A-B). Survival varied between 2 to 89 % for the blood isolates and 4 to 94 % for the nasopharyngeal isolates. No significant difference in survival was, however, found between the two different sites of isolation. All strains included in the study were resistant to hiNHS (Fig. 2C).

To confirm that the NHS used in this study contained antibodies directed against NTHi isolated from both blood and nasopharynx and whether there was a difference in binding of IgG between strains from blood or nasopharynx, we analyzed IgG deposition on randomly selected blood (n=3) and nasopharyngeal isolates (n=3). Two typical strains are shown in Fig. 2D. Human IgG was deposited on the blood isolates and the nasopharyngeal isolates to a similar extent, suggesting that there were no significant difference in antibody binding to strains from the two anatomical sites of isolation. The anti-NTHi 772 rabbit antiserum was included as a positive control and bound all strains tested. Thus, invasive NTHi are not more serum resistant as compared to strains isolated from the upper respiratory tract.

**NTHi isolated from blood or nasopharynx bind complement regulators.** Several bacterial species have been shown to bind complement regulators and thereby counteract the different pathways of the complement system (7, 22, 31). To determine whether there was a difference
in binding of various complement inhibitors between NTHi isolated from patients with invasive disease (blood isolates) or from patients with upper respiratory tract infection (nasopharyngeal isolates), a collection of NTHi strains were incubated with $[^{125}\text{I}]$-labeled C4BP, Factor H, or vitronectin followed by separation of unbound ligand. Binding was calculated as the ratio of bound radioactivity versus total radioactivity added. Interestingly, there was no significant difference in binding between blood and nasopharyngeal isolates (Fig. 3). Binding of the complement regulators varied between different strains independently of isolation sites. The highest C4BP-binding blood and nasopharyngeal strains bound 19.0% and 21.3%, respectively (Fig. 3A). When Factor H was added, the highest Factor H-binding blood and nasopharyngeal strains bound 16% and 30.4% Factor H, respectively (Fig. 3B). Finally, the highest vitronectin-binding blood strain bound 28.0% vitronectin and the highest binding nasopharyngeal strain bound 24.1% vitronectin.

In order to analyze whether the strains isolated from blood or nasopharynx also bound the three different complement inhibitors directly from NHS, bacteria were incubated with hiNHS for 1 h at 37 °C. Thereafter, bound proteins were eluted, separated by SDS-PAGE and analyzed by Western blot using specific antibodies directed against human C4BP, Factor H, or vitronectin. In these experiments, binding of the three different regulators corresponded with the results from the direct binding assay. Moreover, no significant difference in binding of complement inhibitors could be detected when blood or nasopharyngeal NTHi isolates were compared (data not shown). Taken together, there was no significant difference in binding of the three different complement regulators between blood isolates and nasopharyngeal strains from patients with upper respiratory tract infection, suggesting the importance of complement regulator binding capacity also of NTHi in the upper respiratory tract.
The results in this study imply that invasive disease caused by NTHi is heterogeneous in character. Although it has been reported that NTHi sepsis can have a severe clinical presentation mimicking that of encapsulated strains (39), the proportion of cases in the present study with a severe clinical picture was surprisingly high. The patients affected were all adults and most cases could be sorted into one of two typical presentations; patients with evidence of immune deficiency with a mild clinical presentation or adults with no prior evidence of immune deficiency with a severe clinical presentation. In the first category, host factors seem decisive for infection, but in the second category virulence of specific bacterial strains could be important, supported by the higher degree of serum resistance in vitro in this group. Thus, invasive NTHi disease differs from invasive disease caused by Hib in epidemiology as well as general clinical presentation. NTHi can, however, still readily cause severe sepsis in priorly healthy individuals. When the individual cases of sepsis were graded by severity according to ACCP/SCCM grading system, a correlation between severe clinical sepsis and the bacterial in vitro serum resistance was found. This indicates that serum resistance is great importance for the severity of the invasive disease. This finding is consistent with the theory that resistance to human serum facilitates spread of bacteria in the body, but has not been demonstrated in NTHi sepsis prior to this study.

An analysis of the clinical data from the sepsis patients showed that many of the cases of NTHi sepsis occurred in immuno-compromised hosts. The finding that the severity of the sepsis cases was significantly related to the in vitro serum survival of the NTHi strains implies that while NTHi seems to act as an opportunistic agent in many cases of invasive disease, once it has caused invasive disease, a higher level of complement resistance and serum survival is related to a more clinically severe sepsis. Our data suggest that NTHi septic
disease is heterogeneous, possibly reflecting the NTHi cluster in general. The number of cases of invasive disease in this study was limited, however, and this has to be taken into account in the interpretation of the results.

Studies using pulse-field gel electrophoresis have shown that the dynamics of NTHi carriage in the airways is rapid, and that one individual patient can carry more than a dozen different subtypes of NTHi at the same time (38). This raises the issue of selection bias in nasopharyngeal cultures. However, antibodies raised against one NTHi strain gives considerable cross-protection against other strains (23). This could partly explain why the incidence of bloodstream infections caused by NTHi is low. In parallel, a decreased humoral immune competence caused by a disease such as leukemia or by immune-modulating pharmaceutical agents could explain some of the cases of NTHi sepsis seen and presented in this study.

In our initial experiments, we incubated invasive isolates with increasing concentrations of NHS and analyzed survival. All strains except one were killed by 15% NHS (Fig. 1 and data not shown). However, even if the majority of a population is sensitive to NHS it is likely that just a few bacteria may survive and that this particular sub-population is able to multiply and initiate an infection. Importantly, no significant difference in survival between the various clinical strains from blood or nasopharynx was found when serum resistance was determined, suggesting the need for NTHi, irrespectively of isolation site, to resist the bactericidal activity of human serum.

Previous studies showed that the NTHi strain R2866, which was isolated from a child with meningitis, had a high degree of serum resistance depending on the lipooligosaccharide biosynthesis gene \textit{lgtC} expression (9, 19, 39). The phase-variable \textit{lgtC} expression was demonstrated to inhibit C4b deposition and render the bacteria more resistant to human serum (19). Phase variation of outer membrane proteins and lipooligosaccharides
also contributes to virulence of *H. influenzae* and is involved in the evasion of the immune system (38). Intriguingly, phase variation can affect binding of regulatory proteins and have been shown to affect serum resistance (19). Since phase variation is a common phenomenon and depends on the current bacterial environment it cannot be excluded that it is a factor affecting the *in vitro* results of our study.

Another aim was to analyze the difference in binding of complement inhibitors and serum resistance between invasive and nasopharyngeal isolates. We have recently demonstrated that NTHi binds C4BP (15), and that both NTHi and Hib bind Factor H (17) and vitronectin (14, 16). However, no major differences in binding of complement regulators were detected between strains from the two isolation sites. This indicates that binding of complement regulators is important and possibly facilitates survival and colonization of NTHi in both the airway and in the bloodstream. However, the variation of complement regulator binding capacity and ability to survive in human serum between the different invasive isolates, suggests that additional factors are involved in the ability of NTHi to survive in the respiratory tract and in human serum.

The ability of nasopharyngeal isolates to bind Factor H, C4BP and vitronectin suggests that complement regulators may be present in the nasopharyngeal tract and thus would be utilized by NTHi. In fact, during inflammation, complement proteins as well as immunoglobulins and components of the coagulation system enter the airway lumen (11, 12, 29). In patients with chronic otitis media with effusion, local complement activation in the middle ear mucosa has been observed, including an intense deposition of C3 (27). In addition, Factor H, Factor H like protein-1 and Factor H related proteins are complement components found in middle ear effusions of patients with otitis media (26).

In conclusion, NTHi isolated from cases with severe sepsis have a higher capacity to resist the bactericidal effect of human serum compared to NTHi isolated from
cases of mild clinical sepsis. Our results also show that it is of importance for NTHi to bind specific complement regulators irrespectively of whether the bacteria are located in the blood stream or in the nasopharynx, suggesting this being an adaption to the innate immunity in the upper respiratory tract. In addition to a variation in host factors, other virulence factors than bacterial components binding complement inhibitors are required for determining the invasive capacity of a particular NTHi strain.
This work was supported by grants from the Alfred Österlund, the Anna and Edwin Berger, the Marianne and Marcus Wallenberg, and the Greta and Johan Kock Foundations, the Swedish Medical Research Council, the Swedish Society of Medicine, and the Cancer Foundation at the University Hospital in Malmö, and Skane county council’s research and development foundation.
REFERENCES


FIGURE LEGENDS

FIG. 1. Bactericidal activity against a series of randomly selected blood isolates. NTHi were incubated in the presence of increasing concentrations of NHS (5-20%). Four typical strains are shown in panels (A) to (D). After incubation for 20 min, bacteria were spread on chocolate agar plates to allow determination of the number of surviving bacteria. Numbers of bacteria (CFU) at the initiation of the experiment was defined as 100%. The mean values of three independent experiments are shown with error bars indicating SD.

FIG. 2. NTHi isolated from blood (invasive disease) or nasopharynx of patients with upper respiratory tract infection show variation in serum resistance independently of presence of IgG in the NHS. (A and B) No significant difference in survival in NHS was observed between NTHi isolated from blood or nasopharynx. (C) All strains were resistant to hiNHS. NTHi isolates from blood (n=21) (A) and nasopharynx (n=21) (B) were analyzed for survival in human serum. The strains were incubated in the presence of 5% NHS or 5% hiNHS for 20 min. Thereafter, bacteria were spread on chocolate agar plates to allow determination of the number of surviving bacteria. Numbers of bacteria (CFU) at the initiation of the experiment was defined as 100%. (D) The NHS contained IgG directed against NTHi independently of isolation from blood or nasopharynx. Flow cytometry profiles showing the deposition of IgG and the binding of a rabbit anti-NTHi antiserum to the surface of the NTHi strains from patients nos. 11 (blood) and 4 (nasopharynx). Bacteria were incubated with mouse anti-human IgG or rabbit anti-NTHi antiserum followed by FITC-conjugated anti-mouse pAb or anti-rabbit pAb. One representative experiment out of three independent ones performed is shown.
FIG. 3. Both NTHi blood and nasopharyngeal isolates bind complement regulators. A series
of clinical strains isolated from blood ($n=21$; cases with invasive disease and an available
patient history are defined in Table 1) or nasopharynx ($n=21$; patients with upper respiratory
tract infection) was tested for C4BP (A), Factor H (B), and vitronectin (C) binding. The
different NTHi strains were grown overnight and incubated with $[^{125}\text{I}]-$labeled C4BP, Factor
H, or vitronectin. Binding was determined as percentage of bound radioactivity versus added
radioactivity measured after separation of free and bound $[^{125}\text{I}]-$labeled protein over a sucrose
column. The mean values out of three independent experiments are shown. The mean value
out of all isolates in the same group is indicated by a line.
TABLE 1. Clinical and epidemiological data from 17 cases of invasive NTHi infection and serum survival of isolates in vitro

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Gender</th>
<th>Immune status at time of sepsis</th>
<th>Sepsis grading1</th>
<th>Serum survival of NTHi strain in vitro (%)2,3</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>Male</td>
<td>Agammaglobulinemia</td>
<td>Sepsis</td>
<td>5 ± 3</td>
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<tr>
<td>2</td>
<td>69</td>
<td>Male</td>
<td>No sign of immune deficiency</td>
<td>Sepsis</td>
<td>2 ± 3</td>
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<tr>
<td>3</td>
<td>60</td>
<td>Female</td>
<td>Metastatic breast cancer</td>
<td>Sepsis</td>
<td>18 ± 14</td>
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<tr>
<td>4</td>
<td>75</td>
<td>Male</td>
<td>Hypogammaglobulinemia</td>
<td>Sepsis</td>
<td>66 ± 31</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>Male</td>
<td>No sign of immune deficiency</td>
<td>Sepsis</td>
<td>8 ± 7</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>Female</td>
<td>Chemotherapy</td>
<td>Sepsis</td>
<td>23 ± 7</td>
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<tr>
<td>7</td>
<td>58</td>
<td>Male</td>
<td>Leukemia, neutropenia</td>
<td>Sepsis</td>
<td>63 ± 15</td>
</tr>
<tr>
<td>8</td>
<td>74</td>
<td>Female</td>
<td>No sign of immune deficiency</td>
<td>Sepsis</td>
<td>62 ± 0</td>
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<tr>
<td>9</td>
<td>36</td>
<td>Male</td>
<td>No sign of immune deficiency</td>
<td>Sepsis/ meningitis</td>
<td>17 ± 8</td>
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<tr>
<td>10</td>
<td>73</td>
<td>Male</td>
<td>NA4</td>
<td>Sepsis/ meningitis</td>
<td>44 ± 10</td>
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<tr>
<td>11</td>
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<td>Male</td>
<td>No sign of immune deficiency</td>
<td>Sepsis/ meningitis</td>
<td>79 ± 24</td>
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<td>42</td>
<td>Female</td>
<td>No sign of immune deficiency</td>
<td>Severe sepsis</td>
<td>89 ± 2</td>
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<tr>
<td>13</td>
<td>39</td>
<td>Male</td>
<td>No sign of immune deficiency</td>
<td>Severe sepsis</td>
<td>81 ± 25</td>
</tr>
<tr>
<td>14</td>
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<td>Female</td>
<td>No sign of immune deficiency</td>
<td>Severe sepsis</td>
<td>52 ± 4</td>
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<tr>
<td>15</td>
<td>89</td>
<td>Female</td>
<td>Chronic Leukemia</td>
<td>Severe sepsis</td>
<td>86 ± 19</td>
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<tr>
<td>16</td>
<td>83</td>
<td>Male</td>
<td>No sign of immune deficiency</td>
<td>Severe sepsis</td>
<td>64 ± 40</td>
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<tr>
<td>17</td>
<td>74</td>
<td>Female</td>
<td>No sign of immune deficiency</td>
<td>Severe sepsis</td>
<td>58 ± 12</td>
</tr>
</tbody>
</table>

1Graded according to the ACCP/SCCM classification of sepsis grading.
2After 20 min incubation with 5 % NHS
3Mean values including SD out of three experiments performed
4Abbreviation: Not Available
TABLE 2. Demographics of 21 cases of upper respiratory tract infection and serum resistance of NTHi nasopharyngeal isolates in vitro

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Gender</th>
<th>Serum survival of NTHi strain in vitro (%)</th>
</tr>
</thead>
<tbody>
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1 After 20 min incubation with 5% NHS
2 Mean values including SD out of three experiments performed
Figure 1. Hallström et al.
Figure 2. Hallström et al.

A) Blood
B) Nasopharynx
C) Inactivated NHS

D) NHS

Patient 11 (blood isolate)

Patient 4 (nasopharynx isolate)
Figure 3. Hallström et al.

A

B

C

Blood
Nasopharynx
C4BP-binding (%)
Factor H-binding (%)
Vitronectin-binding (%)

0
5
10
15
20
25

8.8
10.2

11.5
12.2