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Human lactoferrin receptor activity in non-encapsulated *Haemophilus influenzae*

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Abstract

Since the ability of bacteria to compete with lactoferrin for iron contributes to the pathogenesis of mucosal infections, the presence of lactoferrin receptor activity in non-encapsulated *Haemophilus influenzae* was investigated. The growth of 18 *H. influenzae* isolates from the sputum samples of chronic bronchitis patients and of six of seven *H. influenzae* throat isolates from healthy adults was stimulated by iron saturated human lactoferrin. Apo-lactoferrin did not stimulate the growth of *H. influenzae*. Human lactoferrin binding to iron limited bacteria was detected for 16 *H. influenzae* strains from chronic bronchitis patients and for five of seven isolates from healthy adults. We conclude that the majority of *H. influenzae* isolates tested bind human lactoferrin and that the iron from lactoferrin is used for growth.

Keywords: Lactoferrin; *Haemophilus influenzae*

1. Introduction

Iron is an essential growth supplement of many bacteria. Since in the circulation iron is bound to transferrin (TF) and on mucosal surfaces to lactoferrin (LF) [1], the ability of many pathogenic bacteria to compete with LF and TF for iron is essential in the pathogenesis of infections [2–4]. To acquire iron from TF and LF, several bacterial species including *Neisseria* have been shown to express spe-

cific receptors for these proteins under iron limitation [5–7].

Since non-encapsulated *Haemophilus influenzae* persists in the lower respiratory tract of patients with chronic bronchitis [8,9], LF can be expected to be an important source of iron for the bacteria. Therefore, we investigated whether iron saturated LF bound to non-encapsulated *H. influenzae* isolated from the sputum of chronic bronchitis patients and supplied the bacteria with iron for growth.

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2. Materials and methods

2.1. Bacterial strains

Eighteen non-encapsulated *H. influenzae* strains were isolated from sputum samples of patients with chronic bronchitis having an infection. The diagnosis chronic bronchitis was according to the definition of the Medical Research Council [10]. Seven *H. influenzae* strains were isolated from throat swabs taken from seven healthy adults for comparison. *H. influenzae* strain KC548, kindly provided by Dr. W. Albritton, was included since it was the only *H. influenzae* strain reported to be positive for a LF receptor [6]. *Neisseria mucosa* Heidelberg was used as a negative control for LF as well as TF binding and *N. meningitidis* MAC A was a positive control for LF as well as TF receptor activity in binding activity and growth stimulation experiments [5].

2.2. Growth conditions

H. influenzae and *Neisseria* strains were grown on chocolate agar plates supplemented with NAD (10 mg l⁻¹) and hemin (10 mg l⁻¹) at 37°C in a humid atmosphere containing 5% CO₂. Iron sufficient liquid cultures were grown in brain heart infusion broth supplemented with NAD (10 mg l⁻¹) and hemin (10 mg l⁻¹) at 37°C under shaking.

Iron deficient medium was prepared by the addition of 100 µM ethylenediamine di-*ortho*-phenylacetic acid (EDDA) and of protoporphyrin IX (2 µg ml⁻¹). The iron deficiency of the medium was reversed by the addition of ferric chloride to a final concentration of 120 µM. To study the growth of the *H. influenzae* strains in the presence of LF (Sigma Chemical Co., St. Louis, MO, L-3770) and TF (Sigma, T-2158), strains were cultured in iron deficient medium at 37°C, 5% CO₂ for at least 2 h under vigorous shaking, and were suspended at an OD of 0.01 at 530 nm in iron deficient medium before they were further incubated in either iron replete medium, iron deficient medium containing 2 or 4 µM LF or TF, or iron deficient medium without any addition. All cultures were performed in triplicate in 100 µl liquid medium in microtiter plates for 16 h. Within 16 h the stationary growth phase was reached [11]. Growth was measured at 540 nm with an ELISA

reader (Titertek Multiscan). With this method the OD is about 5-fold lower than normal due to a shorter length of the light path.

Increases in OD₅₄₀ were correlated with increases in the number of colony forming units (CFUs) for *H. influenzae* strain A950004.

2.3. Enzyme-linked ligand binding assay

The binding of LF and TF to *H. influenzae* and *Neisseria* strains was measured with an enzyme-linked ligand binding assay (ELBA) essentially as described by Kishore et al. [12]. The titer was defined as the dilution of LF or TF which resulted in an OD of 0.2 above the background as extrapolated from the slope of the curve.

The binding of LF was expressed as the ratio of the titer of LF binding to bacteria grown in iron deficient medium over that to bacteria grown in free iron containing medium (titer ratio).

2.4. Statistics

The significance of growth stimulation and LF binding was determined with Student's *t*-test.

3. Results and discussion

3.1. Effect of lactoferrin as source of iron on the growth of non-encapsulated *H. influenzae*

The effect of various iron sources on the growth of *H. influenzae* under iron limitation was studied with strains A850075 and 6653 isolated from the sputum of patients with chronic bronchitis and strain A920022 from the throat of a healthy adult. The results shown in Fig. 1 indicate that LF with bound iron, in contrast to apo-LF, stimulated the growth of most isolates of *H. influenzae*. The growth stimulation by Fe-LF was less than by Fe-TF, indicating that Fe-LF is a less efficient iron donor. The growth of strain KC548 was also stimulated by 2 µM Fe-LF (increase in OD₅₄₀ 0.082 ± 0.008), but not by apo-LF (difference in OD₅₄₀ 0.002 ± 0.008). This strain was previously shown to be positive for the LF receptor [6] although this was overlooked later [13]. The growth of the LF receptor positive control *N. me-*

Fig. 1. Growth expressed as OD₅₄₀ of *H. influenzae* strains A850075, 6653 and A920022 under iron limiting conditions and in the presence of FeCl₃, human 2 µM Fe-LF, 2 µM Fe-LF with an excess of apo-LF (60 µM), apo-LF (2 µM and 4 µM) and Fe-TF (2 µM) at 37°C for 16 h.

ningitidis strain MAC A was stimulated by human 2 µM Fe-LF (increase in OD₅₄₀ 0.188 ± 0.034) in contrast to the negative control *N. mucosa* (difference in OD₅₄₀ 0.002 ± 0.003). This result shows that the growth stimulation was specific for LF containing bacteria, and that the growth of the *H. influenzae* strains in the presence of human Fe-LF was not caused by contaminating iron in the LF preparation.

Next, the growth stimulatory effect of 2 µM Fe-LF on 18 strains from sputum samples of patients with chronic bronchitis and seven throat isolates from healthy carriers was determined (Fig. 2). The growth of all strains, except strain A920022, was significantly stimulated in the presence of 2 µM human Fe-LF as the sole source of iron compared to iron deficient medium as measured as increased OD₅₄₀. The growth of 18 strains was even significantly more stimulated in the presence of 4 µM Fe-LF compared to 2 µM Fe-LF. The increase in OD was caused by a significant increase in the number of CFUs since for strain A950004 we found that the number of CFU ml⁻¹ in iron limited medium was $(4.1 \pm 0.6) \times 10^6$, in the presence of 2 µM Fe-LF $(20.8 \pm 1.2) \times 10^6$ and in the presence of 4 µM Fe-LF $(74.1 \pm 14.9) \times 10^6$.

Our results are in contrast to those reported by Herrington and Sparling [11] and Pidcock et al. [14] who found that non-encapsulated *H. influenzae* strains did not use human LF as a sole source of iron

for growth. However, they measured growth on solidified medium. Their method may be less sensitive than our method using liquid medium, since LF in low concentrations probably diffuses slowly and since LF shows aspecific binding. In addition, the different results from our study and those of Herrington and Sparling [11] and Pidcock et al. [14] may be due to strain differences.

3.2. Binding of human lactoferrin to *H. influenzae*

Typical binding curves for LF labeled with horseradish peroxidase (HRPO) to *H. influenzae* strains KC548 and A920022 are shown in Fig. 3a,b. The binding of LF-HRPO to both strains grown in iron sufficient medium was considerable. This 'background' binding was likely caused by the hydrophobic nature of LF or by the binding of LF to lipopolysaccharide [15]. This type of binding was not related to the presence of LF receptor activity since it was also observed for *N. mucosa* Heidelberg, lacking LF receptor activity. Attempts to reduce the background by Tween, Triton, albumin and more extensive washing failed.

LF-HRPO bound better to strain KC548 grown under iron limitation than to bacteria grown in iron saturated medium. The increased binding of LF-HRPO to iron limited bacteria was specific for the LF in the conjugate since the increased binding was inhibited in the presence of a 50-fold excess of unlabeled LF. LF binding to bacteria of strain A920022 grown under iron limitation was not ob-

Fig. 2. Growth stimulation by 2 or 4 µM human Fe-LF, expressed as increase in OD₅₄₀, of iron limited cultures of non-encapsulated *H. influenzae* isolated from the sputum of patients with chronic bronchitis (■) or from the throat of healthy adults (▼).

Fig. 3. Human LF-HRPO binding to *H. influenzae* as detected in the ELBA. Strains KC548 (a) and A920022 (b) were grown in iron sufficient medium or under iron limiting conditions and incubated with and without a 50-fold molar excess of unlabeled Fe-LF.

served (Fig. 3b). Strain A920022 is probably lacking a LF receptor since the growth of this strain was also not stimulated. Even more binding to iron limited bacteria might have been detected if a chemically defined iron depleted medium had been used, as was described for the binding of TF to *H. influenzae* [16].

Binding experiments with strain KC548 performed on various days showed that the difference between the titer of the iron limited bacteria and the bacteria grown in iron sufficient medium varied from day to

day (mean: 260 ± 149 (S.D.) units, $n=21$). This problem was overcome by using the ratio of the titer of the bacteria grown in iron deficient medium over the titer of the LF binding to bacteria grown in iron sufficient medium (titer ratio). This titer ratio was fairly reproducible for strain KC548 (mean: 2.1 ± 0.6 (S.D.), $n=21$). Although no quantitative information is provided by this titer ratio about the binding of LF to bacteria, a ratio significantly higher than 1 indicates that the LF binding was induced upon iron starvation during growth of the bacterial strain.

The specificity of the binding of human LF to *H. influenzae* was analyzed with competitive binding assays, in which the effect of an 10-fold molar excess of various iron binding proteins on the binding of human Fe-LF-HRPO ($0.17 \mu\text{M}$) was determined. The binding ratio of strain KC548 in iron limited medium over iron sufficient medium was 1.75 ± 0.57 . Ten-fold excesses of human Fe-TF and bovine Fe-LF were unable to block the binding of human Fe-LF-HRPO, since the binding ratio of iron limited bacteria of strain KC548 over bacteria grown in iron sufficient medium was 1.85 ± 0.04 and 2.49 ± 0.01 respectively. This result demonstrates the specificity of the human Fe-LF binding and also indicates that the LF preparation was not contaminated with TF. The specificity of binding is in

Fig. 4. Fe-LF-HRPO binding to non-encapsulated *H. influenzae* strains in the ELBA expressed as the ratio of the titer of LF binding to the iron limited bacteria over the titer of LF binding to the bacteria grown in iron sufficient medium (titer ratio, $\pm$ S.D.). A ratio significantly higher than 1 indicates induction of the Fe-LF binding upon iron starvation. Isolates from the sputum of patients with chronic bronchitis, $n=18$; throat isolates from healthy adults, $n=7$.

agreement with the findings of Schryvers [6], using strain KC548 too. However, this result was disputed later since it had not been reproduced [13]. Competitive binding experiments in which 10-fold molar excesses of apo-LF and Fe-LF-HRPO (0.17 μ M) were mixed resulted in a reduction of the binding of Fe-LF-HRPO to a titer ratio of 0.91 ± 0.27 . This result demonstrates that apo-LF and Fe-LF competed for the same binding site on *H. influenzae*.

The results of LF binding assays with all tested *H. influenzae* strains, summarized in Fig. 4, showed that all strains except four bound human LF under iron limitation (titer ratio significantly higher than 1, $P < 0.05$). Three strains (A860514, 5641, A840162) of the four that did not bind LF were stimulated in their growth by human Fe-LF. The expression of LF receptor activity may be weak but sufficient for growth stimulation by human Fe-LF.

On the mucosal surfaces iron is largely bound to LF. We have found that in patients with chronic bronchitis the levels of LF in the airway secretions were increased compared to those in healthy subjects [17] (L. Vogel, D. Schoonbrood, F. Geluk, F. Hoek, P. Bresser, T. Out, H. Jansen, J. Dankert and L. van Alphen, unpublished results). The degree of iron saturation of LF present in the sputum of patients with chronic bronchitis is not known. At low pH, LF can obtain iron by exchange from TF [18], since LF retains high affinity for iron at pH values below 4.5, and TF loses its affinity for iron at pH < 5.0 [4]. In this way, apo-LF bound to *H. influenzae* may obtain Fe, which subsequently may be used by *H. influenzae* for its growth. Increased levels of transferrin in the sputum of patients with chronic bronchitis may indirectly cause increased iron saturation of LF, thereby contributing to growth of *H. influenzae* in the inflamed respiratory tract.

In conclusion, the majority of non-encapsulated *H. influenzae* isolates tested bound human LF specifically and used LF bound iron as growth supplement.

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