Introduction

The binding of bacteria to the phagocyte membrane is considered a prerequisite for both internalization and activation of microbicidal mechanisms [5, 7, 12]. Some authors have suggested that, in the absence of serum, the interactions between bacteria and polymorphonuclear leukocytes (PMN) depend on physical, chemical properties of bacteria [17, 19]. Others have described bacterial ligands that, interacting with specific receptors present on the PMN membrane, mediate an efficient binding [1, 2, 9-12, 18]. The role of attachment in sensitivity to phagocytosis is generally studied by the comparison of bacteria binding to blocked PMN (incubated at 4°C or treated with drugs that specifically impair internalization ability) with their sensitivity to phagocytosis by PMN incubated at 37°C.

Efficient phagocytosis of Klebsiella pneumoniae strains that poorly bind to human polymorphonuclear leukocytes

Summary The phagocytosis process of unencapsulated MIAT-negative strains that, although binding very poorly to human polymorphonuclear leukocytes (PMN) at 4°C, are efficiently killed by these cells at 37°C, was studied. At 37°C the number of bacteria bound to the PMN external surface was similar to that observed at 4°C (about 100 bacteria/100 PMN after 60 min); on the contrary the number of internalized bacteria was much higher (from 500 bacteria/100 PMN after 60 min). Interactions between phagocytosis-sensitive Klebsiella pneumoniae strains (PSK) and PMN were then compared with those of two isogenic Escherichia coli strains with and without type 1 fimbriae. Whereas PSK strain binding to blocked PMN was very slow and became significant only after 5–6 h, that of phagocytosis-sensitive fimbriated E. coli was rapid and efficient. Phagocytosis-resistant, non fimbriated E. coli strain bound with an efficiency that, within the first 60 min, was not very different from that of the PSK strains. However, longer incubations led to increases in PSK binding, whereas unfimbriated E. coli remained constant. PSK and fimbriated E. coli strains were efficiently internalized and killed, whereas the unfimbriated E. coli strain was not. It is suggested that PMN can phagocytize unopsonized bacteria through two different mechanisms. By one mechanism, observed with the fimbriated E. coli strain, PMN bind many more bacteria than those they can internalize. By the other, observed with PSK strains, PMN bind only the bacteria they can immediately internalize.

Key words Klebsiella pneumoniae · Non-opsonic phagocytosis · Cell binding · Internalization · Fimbriated strains
Table 1 Strains of Klebsiella pneumoniae and Escherichia coli used in this work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
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<tr>
<td>K. pneumoniae</td>
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<tr>
<td>PSK-TRI</td>
<td>Spontaneous mutant selected from K59 [15] for the absence of the MIAT adhesin [13–15]; unfimbriated and unencapsulated</td>
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<tr>
<td>PSK5</td>
<td>Wild-type, unfimbriated, unencapsulated, MIAT-negative strain isolated from human urine</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>MG1655</td>
<td>(CGSC6300) wild-type strain carrying type 1 fimbriae [6]</td>
</tr>
<tr>
<td>AAEC072</td>
<td>*fim; MG1655 isogenic derivative without type 1 fimbriae [3]</td>
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*K. pneumoniae* strains (here indicated as PSK). We noted that PSK strains are indeed able to bind normal and blocked PMN, but such binding, compared with that of other previously described bacteria that are efficiently phagocytized, is very inefficient and characterized by very slow kinetics. This binding is mediated by a previously undetected receptor-ligand system that, contrarily to those previously described, allows efficient internalization in spite of the very inefficient bacteria-PMN binding. We suggest that unopsonized bacteria could be phagocytized by at least two mechanisms. Through the former, previously described by others, PMN rapidly bind more bacteria than they can immediately engulf. By the latter, herein described, phagocytes bind only the bacteria that they can immediately internalize.

**Materials and methods**

*Strains, media and buffers* All strains used are listed in Table 1. Luria-Bertani (LB) broth and agar [16] were employed throughout this study. The Minimal Medium described by Clowes and Hayes [4] was used for radioactive labelling of bacteria. Phosphate-buffered saline (PBS, 0.1 M KH₂PO₄, 0.1 M Na₂HPO₄, 0.15 M NaCl, pH 7.2–7.4) and Hank’s balanced salt solution (HBSS) were utilized for the preparation of PMN and in phagocytosis experiments. Bacteria were grown in LB broth at 37°C for 24 h under static conditions. For phagocytosis experiments, bacteria were washed twice in PBS and resuspended in HBSS.

**Radioactive labelling of bacteria and PMN preparation**

³H labelled bacteria were prepared by inoculating 0.2 ml of an overnight bacterial culture into 20 ml of Minimal Medium containing 20 mg/ml of cold thymidine, 1 mg/ml of cold uridine, and 1 mCi/ml of ³H thymidine. When cultures reached the optical density of 1.2 OU, bacteria were washed 3 times with PBS and diluted to a final concentration of 5 × 10⁸ colony-forming units (CFU)/ml using a spectrophotometric method confirmed by pour plate colony counts.

PMN were obtained from heparinized blood of healthy adults using the Mono Poly Resolving Medium (Flow Laboratories Inc., Irvine, UK). PMN counts were performed using standard methods and the final leukocyte pellet was resuspended in HBSS at a concentration of about 5 × 10⁶ PMN/ml.

**Bacteria-PMN interactions** Bacteria associated (i.e. bound plus internalized) with PMN monolayer were examined as described by Mangan and Snyder [8]. Briefly, a drop of PMN suspension was pipetted onto glass cover slips (20 × 22 mm). The cover slips were incubated at 37°C for 1 hour. Non-adherent PMN were removed by dipping the cover slips into PBS. Cover slips were then placed in plastic culture dishes containing 1 ml of bacterial suspension (1 × 10⁶ bacteria/ml). Triplicate preparations were made for each sampling time. The dishes were incubated at either 4°C or 37°C for different periods, then cover slips were rinsed in cold PBS, allowed to air dry, Giemsa stained and examined under oil immersion (1000×) in a Leitz standard microscope. In each cover slip bacteria associated with 100 PMN were counted and averaged from the triplicate preparations. The same experiments were also performed treating PMN, at 4°C or 37°C, with cytochalasin D (CD) at the final concentration of 5 mg/ml. In other experiments, PMN were fixed in PBS containing glutaraldehyde (GA, 0.25% v/v) at 4°C and 37°C, washed twice with 0.1 M glycine and twice with PBS. Where not otherwise specified, unopsonized bacteria were used. Opsonized cells were obtained by incubating a bacterial suspension of 10⁶ bacteria/ml with 50% normal human serum at 37°C for 30 min; after three washes with PBS, bacteria were suspended in HBSS. Ingested bacteria were differentiated from those simply bound by the fluorescence quenching method. Bacterial survival during incubation with PMN was assayed as previously described [8]; the bacteria:PMN ratio was 100:1.

Sugars were added to the phagocytosis mixtures at the final concentration of 2 mg/ml. The added carbohydrates were: D-mannose, D-glucose, D-galactosamine, D-sorbitol, D-fucose, L-fucose, D-galactose, D-ribose, D-xylose, D-fructose, D-arabinose, L-rhamnose, N-acetyl-D-glucosamine, D-glucosamine, lactose, D-trehalose, N-acetyl-D-galactosamine.

**Statistical calculations** Differences in binding, internalization and killing efficiency between sugar-treated samples and their control were analyzed for significance by Student *t* test. Differences were considered significant at *p* < 0.05.
Results

Ability of PSK strains to bind PMN at 4°C and 37°C To explain the unusual behavior of PSK strains described above (see Introduction), we analyzed bacteria-PMN interactions at both 4°C and 37°C by differentiating bound bacteria from those internalized. Fig. 1 shows that at 4°C, PSK bacteria bind very poorly to PMN (less than 100 bacteria/100 PMN after 60 min) and, as expected, virtually no bacteria were internalized. In contrast, at 37°C, whereas the number of bacteria bound to the external surface was similar to that observed at 4°C (100 bacteria/100 PMN after 60 min), the number of those internalized was much higher (from 505 to 540 bacteria/100 PMN after 60 min). Therefore, the average number of bacteria bound (and non engulfed) per cell is virtually equal at 4 and 37°C.

To exclude potential effects of incubation temperature on physico-chemical properties of PMN surface, which in turn may influence interactions with the tested bacteria, experiments similar to those described above were performed blocking PMNs at 37°C with either GA or CD. The obtained results (not shown) confirmed those obtained at 4°C, indicating that incubation temperature does not modify the binding of PSK bacteria with PMN.

Effect of PMN preincubation with PSK bacteria on phagocytic ability to express new receptors The finding that at 37°C PSK bacteria are efficiently internalized in spite of the low binding efficiency, prompted us to investigate the hypothesis that PMN, following the binding of the first few bacteria, rearrange their surface and expose additional receptors. To evaluate this, PMN were preincubated at 37°C with a PSK strain (bacteria-PMN ratios from 2 to 100) to allow the possible induction of additional binding sites, and were then treated with GA or CD. Afterwards, the ability of 3H labelled PSK bacteria to bind such cells was determined. It was found that when PMN were pretreated with the PSK-TR1 strain, the percentage of bound 3H labelled bacteria was very similar to that observed with untreated PMN, ranging in all cases from 13 to 20% of the inoculum.

Binding kinetics of *K. pneumoniae* and *E. coli* strains The behavior of PSK bacteria was then compared with that of two isogenic *Escherichia coli* strains fimbriated and unfimbriated. The type 1 fimbriated MG1655 strain was sensitive to phagocytosis and killing by PMN as PSK strains, whereas the unfimbriated AAEC072 strain was resistant. As shown in Fig. 2, PSK strain binding to blocked PMN (incubated at 4°C) was very slow and became significant only after 5–6 h, demonstrating that PSK bacteria can bind to PMN, but have a very low binding efficiency. *E. coli* MG1655 strain bound to blocked PMN much more rapidly than the PSK bacteria (about 490 bacteria/100 PMN after 1 hour). In contrast, the binding efficiency of the non fimbriated *E. coli* AAEC072 strain, which is not killed by PMN, was not very different from that of the PSK strains (80–100 bacteria/100 PMN) within the first 60 min. However, when extending incubation, PSK binding increased continuously, whereas binding of the unfimbriated AAEC072 strain did no longer increase.

Ability of PSK and fimbriated and unfimbriated *E. coli* strains to internalize into PMN To better evaluate the results obtained, the efficiency of internalization of strains demonstrating different abilities to bind PMN was compared.
To this goal, in addition to the unopsonized Klebsiella and E. coli strains described above, opsonized PSK bacteria were also analyzed. Fig. 3 shows that opsonized or unopsonized PSK-TR1 strain, and fimbriated and unfimbriated Escherichia coli strains. Adhering and internalized bacteria were determined at 37°C. Results are expressed as mean number of bacteria per 100 PMN and represent an average of three experiments. Bars represent standard deviation. Results similar to those shown for PSK-TR1 were obtained with PSK5; ■ fimbriated E. coli MG1655; ○ unfimbriated E. coli AAEC072; ▲ opsonized PSK-TR1; ○ unopsonized PSK-TR1

maximum and then started to decrease. In all cases, the number of internalized bacteria increased with time, reaching values of about 600 bacteria/100 PMN for unopsonized bacteria and 750–780 bacteria/100 PMN for the opsonized ones.

Identification of the receptor-ligand system mediating phagocytosis of PSK strains To identify the ligand-receptor system involved in the binding of PSK strains to PMN, the interactions of these bacteria with phagocytes were studied in the presence of the different sugars listed in Materials and methods. Only D-trehalose and D-galactosamine prevented significantly the adherence, association and killing ($p < 0.05$). As shown in Fig. 4, after 60 min of incubation, D-trehalose and D-galactosamine inhibited adherence from 58 to 68%, association from 63 to 72% and killing from 64 to 73%. Note that inhibition was stronger when both sugars were added; in fact, the combination caused the inhibition of the studied properties from 85 to 88%. Pretreatment of bacteria, but not of cells, with D-trehalose and D-galactosamine, either alone or in combination, caused an inhibition of adherence, association and killing similar to that obtained when the sugars were added to the phagocytosis mixture (data not shown). These results suggested that the sugar moiety should be recognized by a bacterial surface structure.

Discussion

This study shows that rapid phagocytosis of unopsonized bacteria can occur in spite of an inefficient binding between bacteria and PMN. Some experiments have been crucial for the comprehension of the phenomenon. Experiments in which bound and internalized PSK bacteria were counted separately indicated...
that, although the bound PSK bacteria were constantly few, the number of internalized PSK bacteria increased continuously. Furthermore, when the contact between PSK cells and blocked PMN was extended up to 6 h, the number of bound bacteria reached high values. These results demonstrated the presence of low affinity-binding sites for PSK bacteria on the PMN membrane. On the other hand, the unimbribated E. coli that is resistant to phagocytosis did not bind PMN in a significant proportion even after 6 h of contact with them. Competition experiments demonstrated that t-trehalose and t-galactosamine specifically prevent the binding of PSK bacteria to blocked PMN and they inhibit their killing.

The results show that efficient phagocytosis and killing of bacteria can be mediated by a ligand-receptor system causing relatively inefficient binding. In this mechanism, rapid and extended binding is not always necessary to achieve the fastest engulfment kinetics. Therefore, the major factor limiting the efficiency of phagocytosis of PSK bacteria is the kinetics of internalization. This conclusion does not imply that efficient binding of bacteria is not significant in phagocytosis. Extended, rapid binding results in bacteria trapping, and may lead to a more efficient antibacterial activity. The two different mechanisms for phagocytosis of unencapsulated bacteria, although having similar kinetics of engulfment and killing, may differ for efficiency in general antimicrobial activity. By the previously described mechanism PMN block, per time unit, more bacteria than they can engulf and kill. By the mechanism here presented, PMN block, per time unit, only the bacteria they can internalize. The efficiency of phagocytosis is linked, in the former mechanism, to bacteria attachment efficiency, while in the latter is linked to PMN engulfment capability. The finding that, when internalization was maximal, the number of bound PSK bacteria did not decrease, whereas that of fimbriated E. coli diminished, supports our proposal. This suggests that, in the latter case, when free bacteria begin to be scarce, the internalized microorganisms would increase at the expense of the excess of bacteria that had previously been bound. Due to the fact that bacteria are in excess, the possibility of the efficiency of binding depending also on the kinetics of dissociation should be considered.

In conclusion, it seems reasonable to assume that the ability to bind bacteria in excess might have been developed by PMN to improve the efficiency of their antibacterial activity. Such a system is, from an evolutionary point of view, simpler than those based on more efficient internalization or killing of bacteria. The latter, in fact, would involve changes in several cellular components and a high energy consumption, while the former could simply imply expression of additional surface receptors (or ligands).

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References