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

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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

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.

We have previously shown that wild-type unencapsulated and unopsonized *Klebsiella pneumoniae* strains are not all equally sensitive to phagocytosis by human PMN. The strains resistant to phagocytosis carry an adhesin (called "Mannose Inhibitable Adhesin/T7 receptor", MIAT) [15] that mediates an efficient attachment to blocked PMN [13]. Nevertheless, bacteria are not phagocytized by metabolically active PMN. The MIAT interaction with its PMN receptors, triggers membrane changes that make phagocytes unable to internalize and kill bacteria [14]. Besides, wild-type unencapsulated *K. pneumoniae* strains naturally lacking the MIAT and MIAT-negative spontaneous mutants are readily phagocytized and killed at 37°C [13]. In contrast to what had been previously described for other phagocytosis sensitive strains, these bacteria adhere very poorly to PMN incubated at 4°C [13].

We analyzed the mechanism involved in the phagocytosis of the unencapsulated MIAT-negative, phagocytosis-sensitive

Table 1 Strains of *Klebsiella pneumoniae* and *Escherichia coli* used in this work

Strain	Properties
K. pneumoniae	
PSK-TRI	Spontaneous mutant selected from K59 [15] for the absence of the MIAT adhesin [13–15]; unfimbriated and unencapsulated
PSK5	Wild-type, unfimbriated, unencapsulated, MIAT-negative strain isolated from human urine
E. coli	
MG1655	(CGSC6300) wild-type strain carrying type 1 fimbriae [6]
AAEC072	^{3}fim; MG1655 isogenic derivative without type 1 fimbriae [3]

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 KH_2PO_4, 0.1 M Na_2HPO_4, 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 ^{3}H 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 ^{3}H 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 $\times$ 10^{8} 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 5×10^{6} 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 $\times$ 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 (1×10^{8} 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 10^{9} 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 ^{3}H 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 ^{3}H labelled bacteria was very similar to that observed with untreated PMN, ranging in all cases from 13 to 20% of the inoculum.

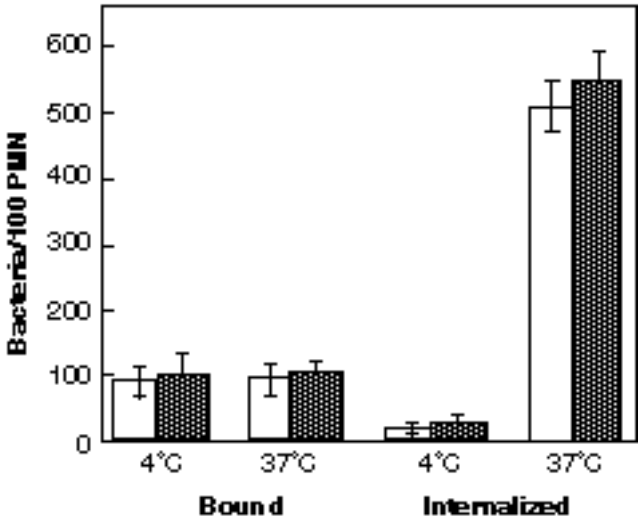

Fig. 1 PSK-TR1 strain binding to and internalization into PMN. Bound and internalized bacteria were evaluated at 4 and 37ÞC after 30 and 60 min of incubation. The results are expressed as mean number of bacteria per 100 PMN and represent an average of three experiments. Bars represent standard deviation. Similar results were obtained with PSK5. ▭ 30 min, ▩ 60 min

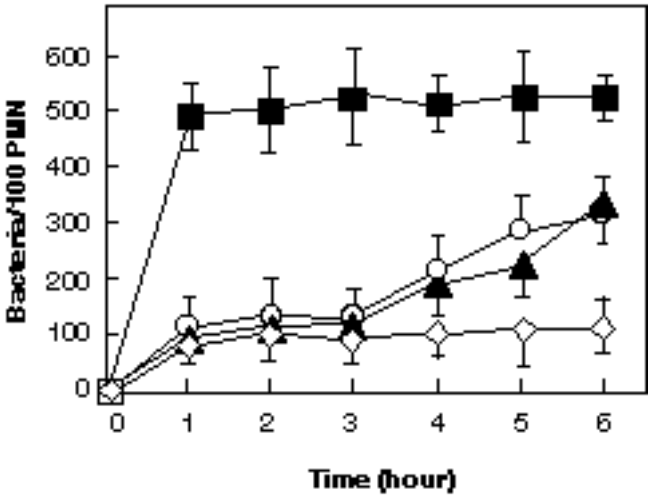

Fig. 2 Kinetics of binding to PMN of PSK and fimbriated and unfimbriated *E. coli* strains. Binding was evaluated at 4ÞC. The results are expressed as mean number of bacteria per 100 PMN and represent an average of three experiments. Bars represent standard deviation. ■Fimbriated *E. coli* MG1655; ◇ unfimbriated *E. coli* AAEC072; ▲ PSK5; ○ PSK-TR1

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.

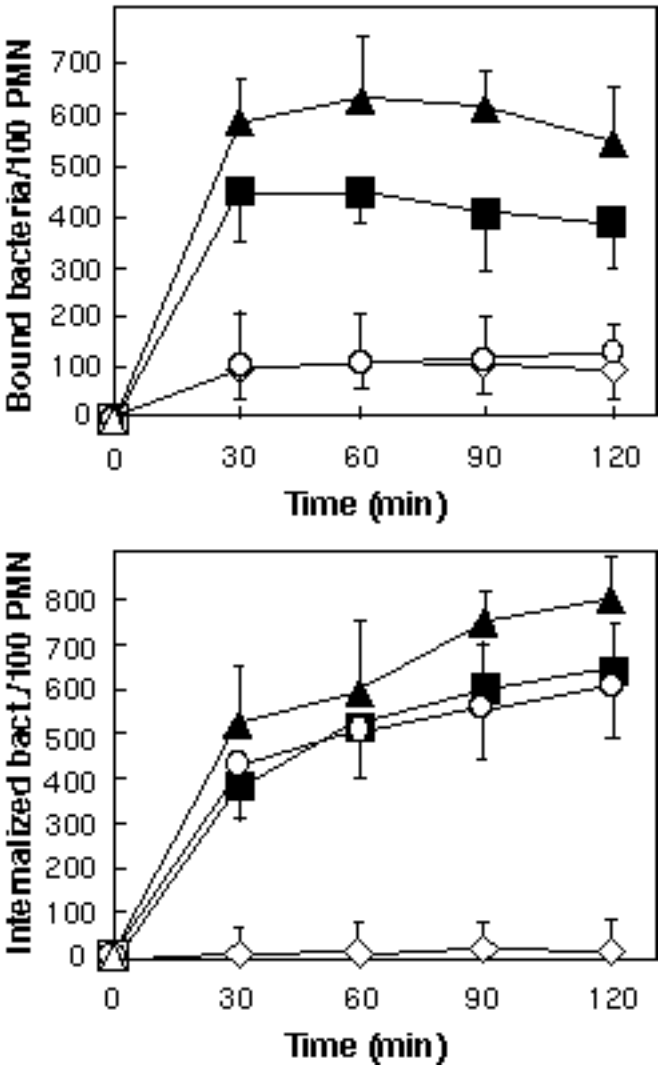

Fig. 3 Binding to and internalization into PMN of opsonized and unopsonized PSK-TR1 strain, and fimbriated and unfimbriated *Escherichia coli* strains. Adhering and internalized bacteria were determined at 37ÞC. Results are expressed as mean number of bacteria per 100 PMN and represent an average of 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

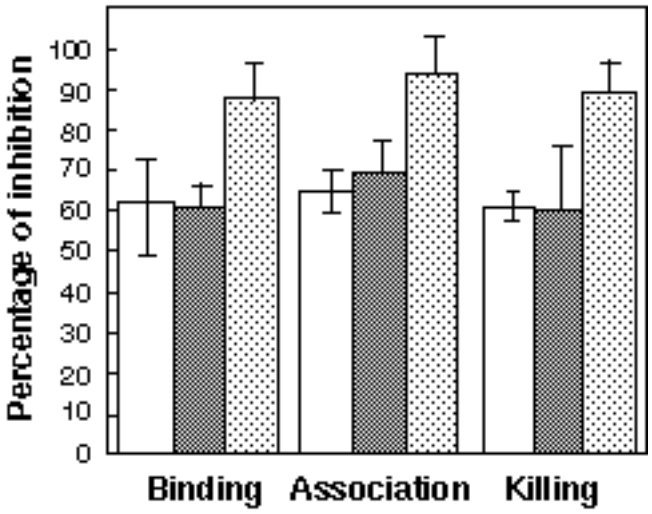

Fig. 4 Effect of D-trehalose and D-galactosamine on PSK-TR1 strain adherence to, association with and killing by human PMN. Results are an average of three experiments. Bars represent standard deviation. ☐ + D-trehalose; ▒ + D-galactosamine; ▒ + a mixture of D-trehalose and D-galactosamine

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 strains and fimbriated *E. coli* MG1655 strain were efficiently internalized, whereas the unfimbriated *E. coli* AAEC072 strain was not. Regarding the efficiency of internalization of the fimbriated *E. coli* and PSK strains (with and without opsonins), some differences were observed in these processes. In fact, the number of fimbriated *E. coli* and opsonized PSK bacteria bound to phagocytes was much higher throughout the experiment. In addition, the number of bound unopsonized PSK bacteria remained approximately constant until the end, whereas that of fimbriated *E. coli* and opsonized PSK bacteria reached a 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 unfimbriated *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 D-trehalose and D-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 the 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

1. Athamma A, Ofek I, Keisari Y, Markowitz S, Dutton GG, Sharon N (1991) Lectinophagocytosis of encapsulated *Klebsiella pneumoniae* mediated by surface lectins of guinea pig alveolar macrophages and human monocyte-derived macrophages. Infect Immun 59:1673-1682
2. Beachey EH (1981) Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J Infect Dis 143:325-345
3. Blomfield IC, McClain MS, Eisenstein BI (1991) Type 1 fimbriae mutants of *Escherichia coli* K12: characterization of recognized afimbriate strains and construction of new fim mutants. Mol Microbiol 5: 1439-1445
4. Clowes RC, Hayes W (eds) (1968) Experiments in microbial genetics. Oxford: Blackwell Scientific Publication, pp 184-185
5. Griffin FM (1982) Mononuclear cell phagocytic mechanisms and host defense. In: Gallin JI, Fauci AJ (eds) Host Defense Mechanisms. Vol 1. New York: Raven Press, pp 31-53
6. Guyer MS, Reed RR, Steitz JA, Low KB (1980) Identification of a sex-factor-affinity site in *E. coli* gd. Cold Spring Harbor Symp Quant Biol 45:135-140
7. Horwitz MA (1982) Phagocytosis of microorganisms. Rev Infect Dis 4:104-123
8. Mangan DF, Snyder JS (1979) Mannose-sensitive interaction of *Escherichia coli* with human peripheral leukocytes in vitro. Infect Immun 26:520-527
9. Ofek I, Rest RF, Sharon N (1992) Nonopsonic phagocytosis of microorganisms. ASM News 58:429-435
10. Ofek I, Sharon N (1988) Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. Infect Immun 56: 539-547
11. Ohman L, Hed J, Stendahl O (1982) Interaction between human polymorphonuclear leukocytes and two different strains of type 1 fimbriae-bearing *Escherichia coli*. J Infect Dis 146:751-757
12. Ohman L, Maluszynska G, Magusson E, Stendahl, O (1988) Surface interaction between bacteria and phagocytic cells. Prog Drug Res 32:131-147
13. Pruzzo C, Debbia E, Satta G (1982) Mannose inhibitable adhesins and T3-T7 receptors of *Klebsiella pneumoniae* inhibit phagocytosis and intracellular killing by human polymorphonuclear leukocytes. Infect Immun 36:949-957
14. Pruzzo C, Guzmàn CA, Calegari L, Satta G (1989) Impairment of phagocytosis by the *Klebsiella pneumoniae* mannose inhibitable adhesin/T7 receptor. Infect Immun 57:975-982
15. Pruzzo C, Debbia E, Satta G (1980) Identification of the major adherence ligand of *Klebsiella pneumoniae* in the receptor for coliphage T7 and alteration of *Klebsiella* adherence properties by lysogenic conversion. Infect Immun 30:562-570
16. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A laboratory manual (2nd ed). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
17. Stendahl O (1983) The physicochemical basis of surface interaction between bacteria and phagocytic cells. In: Easmon CSF, Brawn MRW, Jeljaszewicz J, Lambert PA (eds) Medical Microbiology. Vol 3. New York: Academic Press, pp 137-151
18. Svanborg Eden C, Bjursten LM, Hull R, Magnusson KE, Moldavano Z, Leppler H (1984) Influence of adhesins on the interaction of *Escherichia coli* with human phagocytes. Infect Immun 44:672-680
19. Van Oss CJ (1978) Phagocytosis as a surface phenomenon. Annu Rev Microbiol 32:19-39