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Impairment of Phagocytosis by the *Klebsiella pneumoniae* Mannose-Inhibitable Adhesin-T7 Receptor

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It has been previously shown that *Klebsiella pneumoniae* K59 carrying the mannose-inhibitable adhesin-T7 receptor (MIAT) efficiently binds to polymorphonuclear leukocytes (PMNs) incubated at 4°C but is not efficiently bound and internalized by phagocytes incubated at 37°C. Pretreatment of K59 with compounds that bind the MIAT ligand (D-mannose, UV-inactivated T7 phages, and pepsin-digested anti-MIAT antibodies) enables PMNs to phagocytize and kill these bacteria. In this article, we show that the incubation temperature has no direct effect on expression of either the MIAT or the PMN receptors. These receptors were always expressed at 37°C when PMNs were treated with substances that impaired their ability to rearrange their surfaces (glutaraldehyde and cytochalasins B and D). Pretreatment of inert PMNs with concanavalin A or succinyl concanavalin A drastically reduced binding of K59 to phagocytes at both 4 and 37°C. The same pretreatment carried out with metabolically active PMNs enabled them to efficiently phagocytize the MIAT-positive strain. When phagocytes were treated with K59 bacteria, they became unable to ingest and kill a K59 mutant not expressing the MIAT which was sensitive to phagocytosis. If this pretreatment was performed in the presence of D-mannose, UV-inactivated T7 phages, and pepsin-digested anti-MIAT antibodies, PMNs maintained their phagocytic activity against the MIAT-negative strain. In the presence of K59 bacteria, a very low chemiluminescence response was generated; in contrast, a significant response was observed when bacteria were previously absorbed with UV-inactivated T7 phages and pepsin-digested anti-MIAT antibodies. These results support our previous suggestion that the MIAT adhesin triggers changes in the cell surface, inhibiting further binding and phagocytosis.

Bacterial adhesins are surface compounds that mediate the specific binding of bacteria to surfaces (4, 17, 18, 21). Several adhesins involved in adherence to epithelial cells have also been shown to mediate efficient binding to human polymorphonuclear leukocytes (PMNs) and have been shown to be necessary for the phagocytosis of bacteria carrying them (1–3, 5, 11, 13, 22–24, 26, 31, 34–38). On the other hand, other adhesins were shown not to mediate binding to PMNs and not to be involved in the phagocytosis of bacteria (5, 6, 22, 36).

We have previously described (27–30) a *Klebsiella pneumoniae* adhesin (mannose-inhibitable adhesin-T7 receptor [MIAT]) which also acts as the receptor for coliphages T3 and T7 and has unique properties with respect to the adhesins mentioned above. In fact, in the absence of serum, the *K. pneumoniae* strains carrying the MIAT (MIAT positive) efficiently bind to PMNs incubated at 4°C but are not efficiently bound and phagocytized by PMNs incubated at 37°C. In contrast, the *Klebsiella* strains not carrying the MIAT adhesin (MIAT negative) are efficiently phagocytized at 37°C by these cells. We have also shown that pretreatment of MIAT-positive strains with compounds that bind the MIAT ligand (such as D-mannose, UV-inactivated T7 bacteriophages, or pepsin-digested anti-MIAT antibodies) enables PMNs to phagocytize and kill *Klebsiella* strains carrying such an adhesin.

These findings have raised the problem of understanding why bacteria that efficiently bind to inert PMNs are not ingested and killed by metabolically active phagocytes. In this article, we study the mechanism by which the MIAT ligand works. We show that the interaction of the MIAT-positive strains with their phagocyte receptor prevents PMNs from binding, ingesting, and killing *Klebsiella* strains both via the MIAT ligand and via other systems.

MATERIALS AND METHODS

Strains, media, and buffer. All strains used are listed in Table 1. Luria-Bertani (LB) broth and LB agar were used throughout this work. For radioactively labeling bacteria, the minimal medium described by Clowes and Hayes was used (8). Phosphate-buffered saline (0.1 M KH_2PO_4–0.1 M Na_2HPO_4–0.15 M NaCl [pH 7.2 to 7.4]) (PBS) and Hanks balanced salt solution were used for the preparation of leukocytes and for phagocytosis experiments.

Radioactive labeling of bacteria. ^{3}H-labeled bacteria were prepared by inoculating 0.2 ml of an overnight bacterial culture into 20 ml of minimal medium containing (per milliliter): 20 μg of cold thymidine, 1 μg of cold uridine and 1 μCi of [^{3}H]thymidine. When cultures reached the optical density of 1.2 optical units, bacteria were washed three times with PBS and were diluted to a final concentration of 5×10^8 CFU/ml by a spectrophotometric method confirmed by pour plate colony counts.

PMN preparation. Human PMNs were obtained from healthy adult donors as described by Boyum (7). PMN counts were performed by standard methods, and the final leukocyte pellet was suspended at a concentration of about 5×10^6 PMNs per ml of PBS.

***Klebsiella*-leukocyte association on cover slips.** Bacterial association with the PMN monolayer was examined as described by Mangan and Snyder (16). Briefly, a drop (0.05 ml) of a suspension of PMNs (10^6 PMNs per ml) was pipetted onto glass cover slips (20 by 22 mm). The cover slips were incubated in high humidity at 37°C for 1 h. Nonadherent

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TABLE 1. Bacterial and viral strains

Strain(s)	Source, reference, and characteristics
K. pneumoniae	
K59	MIAT positive; belongs to Genoa Microbiology Institute collection; is unencapsulated and presents receptors for coliphages T3 and T7 (27).
KRTT1 and KRTT2	MIAT-negative unencapsulated spontaneous mutants of strain K59, selected as resistant to coliphages T3 and T7 (27).
KRTT1 Nal^r and KRTT2 Nal^r	Mutants of strains KRTT1 and KRTT2 resistant to nalidixic acid (this work).
Coliphages T3 and T7	From Genoa Microbiology Institute collection (27, 33).

PMNs were removed by dipping the cover slips into PBS. The cover slips were then placed in plastic culture dishes containing 10 ml of *Klebsiella* suspension (10^7 bacteria per ml). The dishes were incubated at either 37 or 4°C for various periods after which each cover slip was rinsed in cold PBS and allowed to air dry. The dried cover slips were Giemsa stained and examined under oil immersion (×1,000). Each PMN was scored as positive if two or more bacteria could be seen attached to the cells at 4°C or associated (attached plus internalized) with the cells at 37°C. The same experiments were also performed by treating PMNs, at 4 or 37°C, with either cytochalasin B (CB) or cytochalasin D (CD) at a final concentration of 5 μg/ml. In other experiments, PMNs were fixed in glutaraldehyde (GA; 0.25%) containing saline at 4 and 37°C and were washed twice with 0.1 M glycine and twice with saline.

***Klebsiella* binding and internalization into PMNs.** To differentiate ingested bacteria from those simply bound, the fluorescence-quenching method of Ohman et al. was used (24). Briefly, fluorescein isothiocyanate-labeled bacteria were added to PMN monolayers. At different incubation times, monolayers were rinsed with buffer and were immediately examined with an incident-light fluorescence microscope. The total number of bacteria associated with 100 PMNs was determined. Then three drops of crystal violet (0.8 mg/ml in 0.15 M NaCl) were added to the spot for a few seconds. The attached bacteria lost their fluorescence, whereas the ingested bacteria remained fluorescent and were counted. Results are expressed as the number of internalized bacteria per 100 PMNs and the number of bound bacteria per 100 PMNs according to the formula: number of bound bacteria per 100 PMNs = number of associated bacteria per 100 PMNs − number of internalized bacteria per 100 PMNs.

Effect of temperature shift on phagocytosis. Bacteria were added to PMNs suspended in Hanks balanced salt solution at either 4 or 37°C. After 10, 30, and 60 min of incubation, samples were taken from the phagocytosis mixtures and were centrifuged twice for 5 min at 160 × *g*. The pellets containing PMN-associated bacteria were washed twice with cold PBS. Slides of the pellets were prepared and Giemsa stained, and bacterial association with PMNs was evaluated as the percentage of 200 PMNs examined having two or more bacteria attached to or within the cytoplasmic periphery. Meanwhile, after 60 min of incubation, the original 4°C (or 37°C) phagocytosis mixture was subdivided; one sample was mantained at the same temperature and the other was shifted to 37°C (or 4°C). At timed intervals, samples were taken, centrifuged, and washed as described above. Slides of the pellets were prepared and Giemsa stained, and bacterial association with PMNs was evaluated.

Experiments in which the effect of temperature shift on binding and internalization was studied were performed as described above, with the difference that fluorescein isothiocyanate-labeled bacteria were used. The number of bacteria bound and internalized per 100 PMNs was evaluated as described above.

Phagocytosis of radioactively labeled bacteria. The rate of association with PMNs of ^{3}H-labeled bacteria was determined without serum by the procedure described by Verhoef et al. (40). Unless otherwise specified, the bacteria:PMN ratio was 100:1. In mixed phagocytosis, PMNs were pretreated with unlabeled K59 (or KRTT1) bacteria (bacteria: PMN ratio, 5:1). ^{3}H-labeled bacteria were then added, and the radioactivity associated with the PMNs was evaluated as above.

***Klebsiella* survival assay.** The survival of *K. pneumoniae* during incubation with PMNs was assayed without serum as previously described (16). The bacteria:PMN ratio was approximately 100:1. In mixed phagocytosis of bacteria having different antibiotic resistance markers, PMNs were pretreated with K59 nalidixic acid-sensitive (or KRTT1 nalidixic acid-sensitive) bacteria (bacteria:PMN ratio, 5:1). Nalidixic acid-resistant bacteria were then added (bacteria: PMN ratio, 100:1), and the rate of killing was evaluated by the duplicate pour plate method on LB agar containing nalidixic acid (final concentration, 100 μg/ml).

Lysosomal enzyme release. Bacteria and PMN mixtures were prepared as previously described (16). Lysozyme and lactate dehydrogenase were assayed in the supernatants of the centrifuged phagocytosis mixtures as reported by Mangan and Snyder (16). Lactoferrin concentrations were measured by the Mancini radial immunodiffusion technique as described by Leffell and Spitznagel (14). β-Glucuronidase activity was determined by the method of Fishman (12), using standard reagents (Sigma Chemical Co.).

Chemiluminescence assay. Luminol-induced chemiluminescence (CL) responses were evaluated as previously described (36).

Phagocytosis experiments in the presence of different substrates. UV-inactivated T7 phages and pepsin-digested antibodies were prepared and added to the phagocytosis mixtures as already described (28). D-Mannose and lectins were added to the phagocytosis mixtures at final concentrations of 2 mg/ml and 50 μg/ml, respectively.

Bacterial opsonization. Bacteria were preopsonized by 30-min incubation at 37°C with fresh pooled human serum (10%) and were then washed twice.

Statistical analyses. Statistical analyses were performed by the Student's *t* test. The difference between two samples was considered statistically significant for $P < 0.05$.

RESULTS

Effect of blocking PMNs by various means on the capability of MIAT-positive *K. pneumoniae* to bind them. The finding mentioned above that the MIAT ligand mediates binding to PMNs at 4 but not at 37°C (28) could be explained in two ways. (i) Temperature could interfere with the MIAT ligand

in such a way that at 37°C it becomes unable to bind PMNs; alternatively, the incubation temperature could change the phagocyte receptors for the MIAT, making PMNs able to interact with bacterial cells at 4 but not at 37°C. (ii) The MIAT ligand, after binding its specific receptor in the PMN membrane, could trigger a rearrangement in the phagocyte surface such that bacterial binding via the MIAT is impaired. Since rearrangement requires a metabolically active cell, this event occurs at 37°C but cannot occur at 4°C.

The first hypothesis would predict that under no circumstances should binding of *Klebsiella* strain K59 to PMNs occur when the phagocytosis mixture is incubated at 37°C. In contrast, the second hypothesis would predict that PMNs fixed with GA or treated with drugs (such as CB and CD) that specifically impair their capability to rearrange the cell surface should be bound by K59 at 37°C, exactly as untreated phagocytes incubated at 4°C are bound.

It is evident (Table 2) that phagocytes incubated for 20 min at 37°C before being fixed with GA bound the MIAT-positive cells also at 37°C with the same efficiency as untreated PMNs incubated at 4°C. In addition, PMNs treated with GA after incubation at 4°C bound K59 cells both at 4 and 37°C equally well. The results also show that MIAT-positive cells, at both 4 and 37°C, bound PMNs treated either at 4 or 37°C with either CD or CB with the same efficiency as untreated PMNs incubated at 4°C. It is interesting that binding of K59 to PMNs fixed with GA or treated with CB or CD under all temperature conditions was prevented by D-mannose, UV-inactivated T7 phages, and pepsin-digested anti-MIAT antibodies. This indicates that in all cases bacterial binding was mediated by the MIAT ligand.

These findings show that the incubation temperature has no direct effect on the expression and activity of the MIAT or the PMN receptors, since in phagocytes blocked by various means such binding efficiently occurred at 37°C.

Effect of temperature shift on the capability of MIAT-positive *K. pneumoniae* to associate with human PMNs and evaluation of binding and internalization. If the suggestion that binding of the MIAT ligand to its specific receptor triggers changes in the PMN membrane such that the phagocyte receptor becomes no longer accessible to the MIAT ligand is correct, *Klebsiella* strain K59 bound to phagocytes at 4°C should gradually detach from them after being shifted to 37°C, while a gradual increase in the number of bacteria bound to PMNs should be observed when phagocytosis mixtures are shifted from 37 to 4°C. It was found that at 37°C most of the few bacteria interacting with PMNs were simply externally bound and occurred in a very low number throughout the experiment (Fig. 1). As expected, at 4°C almost no internalization occurred, while the number of adhering bacteria was high, reaching values higher than 450 bacteria per 100 PMNs.

When the incubation temperature was shifted from 37 to 4°C, it was found that the number of adhered bacteria rapidly increased, changing, after 60 min of incubation at 4°C, from 195 to 430 bacteria per 100 PMNs. In contrast, the number of internalized bacteria was very low.

In experiments in which the effect of the temperature shift from 4 to 37°C was studied, we found no change in the number of internalized bacteria, which remained very low. In contrast, a rapid decrease in the number of attached bacteria was observed (from 450 to 190 bacteria per 100 PMNs).

Effect of concanavalin A (ConA) and succinyl-ConA on binding, phagocytosis, and killing of the MIAT-positive *Klebsiella* strains. The results described above show that human

TABLE 2. Effect of PMN pretreatment with GA, CB, or CD on *Klebsiella* strain K59 association with human PMNs at 37 and 4°C

Substrate added	% Attachment to PMNs[a]											
	Untreated		Pretreated at 37°C with:						Pretreated at 4°C with:			
			GA		CB		CD		GA		CB	
	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C	4°C	37°C
None	87 (78–95)	21 (18–29)	72 (64–89)	62 (54–70)	77 (68–83)	81 (70–98)	85 (80–91)	84 (72–90)	80 (67–93)	74 (61–82)	84 (78–91)	88 (79–95)	82 (74–89)	89 (85–93)
D-Mannose	10 (5–19)	88 (81–99)	9 (6–18)	12 (8–12)	14 (9–19)	12 (4–19)	11 (8–17)	16 (14–19)	14 (8–24)	20 (8–33)	16 (12–20)	20 (17–23)	12 (10–14)	13 (11–17)
UV-killed T7	13 (6–19)	89 (80–96)	13 (6–24)	16 (10–22)	8 (2–11)	9 (3–15)	17 (14–20)	17 (11–21)	8 (1–19)	9 (2–17)	13 (10–16)	13 (9–16)	15 (14–17)	19 (15–21)
Pepsin-digested anti-MIAT antibodies	14 (7–20)	84 (78–99)	10 (7–15)	13 (10–17)	13 (8–17)	6 (2–10)	15 (13–16)	17 (13–22)	12 (5–20)	11 (4–33)	8 (5–11)	12 (8–15)	9 (8–10)	13 (11–16)

[a] Bacterial attachment to PMNs was assayed on cover slips. PMN monolayers were incubated at 4°C (or 37°C) and treated with GA, CB, or CD as described in Materials and Methods. Each sample was then moved to both 4 and 37°C, and bacteria were added at a final bacteria:PMN ratio of 100:1. The results are expressed as the percentage of PMNs with two or more bacteria adherent at 60 min and represent an average of three experiments. The numbers in parentheses indicate extreme values. The differences between association values of samples to which substrates were added and those of the respective control to which no substrate was added were significant ($P < 0.01$).

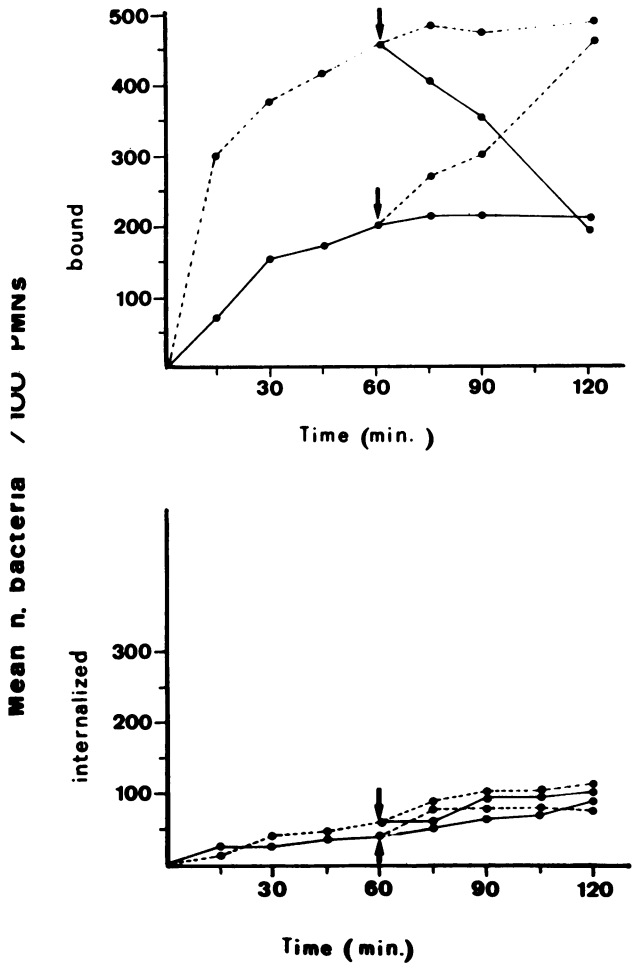

FIG. 1. K59 binding to and internalization by PMNs at 4 and 37°C and after temperature shifts. The experiment was performed as described in Materials and Methods. Bacteria were incubated at 4°C (-----) or 37°C (——). Shifts from 4 to 37°C (----——) and from 37 to 4°C (————) are shown. Arrows indicate shift times. Data are mean values of two experiments.

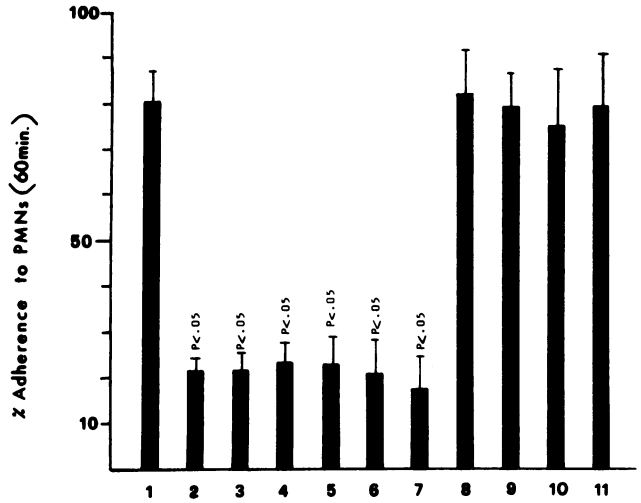

FIG. 2. Effect of lectins on adherence to PMNs of the MIAT-positive strain K59. The bacteria:PMN ratio was 100:1. Experiments were performed on cover slips as described in the text. Results represent an average of three experiments and are expressed as the percentage of PMNs with two or more bacteria bound. Bars at the top indicate standard deviation. P was calculated by comparing mean values of the treated samples with those of untreated controls; differences were considered significant for $P < 0.05$. Where P is not indicated, the differences were not significant. Bars: 1, PMNs plus K59 (4°C; control); 2, PMNs pretreated with ConA plus K59 (4° C); 3, PMNs fixed at 37°C with GA and then pretreated with ConA plus K59; 4, PMNs fixed at 4°C with GA and then pretreated with ConA plus K59; 5, PMNs pretreated with succinyl-ConA plus K59 (4°C); 6, PMNs fixed at 37°C with GA and then pretreated with succinyl-ConA plus K59; 7, PMNs fixed at 4°C with GA and then pretreated with succinyl-ConA plus K59; 8, PMNs pretreated with *A. hypogea* lectin plus K59 (4°C); 9, PMNs pretreated with soybean agglutinin plus K59 (4°C); 10, PMNs plus K59 bacteria pretreated with ConA for 20 min and then washed (4°C); 11, PMNs plus K59 bacteria pretreated with succinyl-ConA for 20 min and then washed (4°C).

PMNs must be metabolically active to be able to reject binding by *Klebsiella* strains carrying the MIAT ligand. However, they do not yet demonstrate that it is the interaction of the MIAT with its specific receptor in the phagocyte membrane that causes inhibition of both bacterial binding to PMNs and phagocytosis.

To evaluate this possibility, we have taken advantage of the fact that ConA binds D-mannose like MIAT and could mask the receptors for MIAT. If this were really the case, PMNs incubated at 4°C as well as PMNs treated with GA, CB, or CD and incubated either at 4 or 37°C should not be able to bind MIAT-positive bacteria when pretreated with ConA.

Pretreatment with ConA of PMNs blocked by various means drastically reduced binding of MIAT-positive *K. pneumoniae* to them both at 4 and 37°C (Fig. 2, bars 1 to 4).

It is interesting that under these conditions *Klebsiella* strains bound to PMNs with efficiencies identical to those with which they bound to blocked phagocytes in the presence of either D-mannose- or UV-inactivated T7 coliphages or anti-MIAT antibodies (Table 2). This indicates that ConA masks PMN receptors for the MIAT and, in addition, excludes the possibility that ConA cross-links bacteria with phagocytes.

Therefore, if binding of its specific receptor by the MIAT ligand was required for preventing bacterial association with metabolically active PMNs and phagocytosis, metabolically active PMNs pretreated with ConA should become able to associate with and kill MIAT-positive *Klebsiella* strains. ConA-treated PMNs associated with and killed the MIAT-positive bacteria with a much greater efficiency than the untreated PMNs did (Fig. 3 and 4, bars 1 and 2), with rates of association and killing of *Klebsiella* strain K59 virtually identical to the rates at which nonpretreated PMNs associated with and killed MIAT-positive bacteria in the presence of mannose, UV-killed coliphage T7, or anti-MIAT antibodies (28). It is also interesting that ConA pretreatment did not influence K59 strain association with and killing by human PMNs in the presence of D-mannose, UV-inactivated T7 phages, or anti-MIAT antibodies (Fig. 3 and 4, bars 3 to 5). Finally, when MIAT-positive *Klebsiella* cells were treated with ConA and washed, they remained unable to bind metabolically active PMNs and resistant to phagocytosis (Fig. 3 and 4, bars 12).

These findings indicated that the MIAT ligand impairs phagocytosis of *Klebsiella* strain K59 through binding to its receptors in the PMN membrane. To confirm this, we analyzed the effect of three other lectins, *Arachis hypogea*

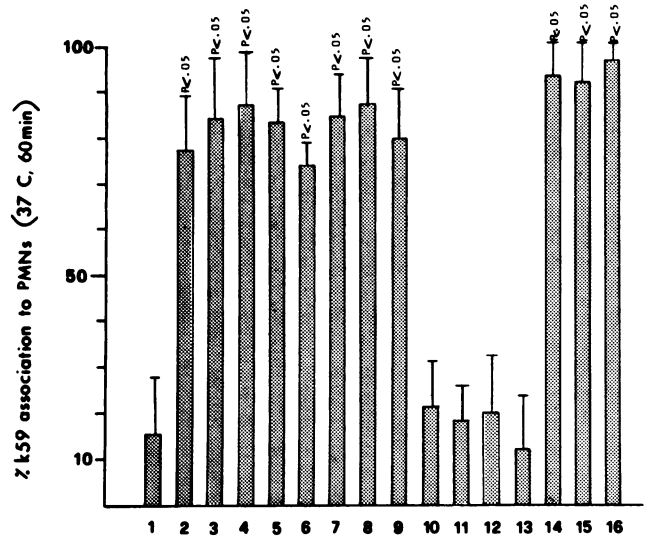

FIG. 3. Effect of lectins on K59 association with PMNs. Experiments were performed on cover slips at 37°C. Results are expressed as the percentage of PMNs with two or more bacteria associated (attached plus ingested) and represent an average of three experiments. Bars at the top indicate standard deviation. *P* was calculated as for Fig. 2. Bars: 1, PMNs plus K59 (37°C; control); 2, PMNs pretreated with ConA plus K59; 3, PMNs pretreated with ConA plus K59 pretreated with D-mannose; 4, PMNs pretreated with ConA plus K59 pretreated with UV-inactivated T7 phages; 5, PMNs pretreated with ConA plus K59 pretreated with pepsin-digested anti-MIAT antibodies; 6, PMNs pretreated with succinyl-ConA plus K59; 7, PMNs pretreated with succinyl-ConA plus K59 pretreated with D-mannose; 8, PMNs pretreated with succinyl-ConA plus K59 pretreated with UV-inactivated T7 phages; 9, PMNs pretreated with succinyl-ConA plus K59 pretreated with pepsin-digested anti-MIAT antibodies; 10, PMNs pretreated with *A. hypogea* lectin plus K59; 11, PMNs pretreated with soybean agglutinin plus K59; 12, PMNs plus K59 bacteria pretreated with ConA for 20 min and then washed; 13, PMNs plus K59 bacteria pretreated with succinyl-ConA for 20 min and then washed; 14, PMNs plus opsonized K59; 15, PMNs pretreated with ConA plus opsonized K59; 16, PMNs pretreated with succinyl-ConA plus opsonized K59.

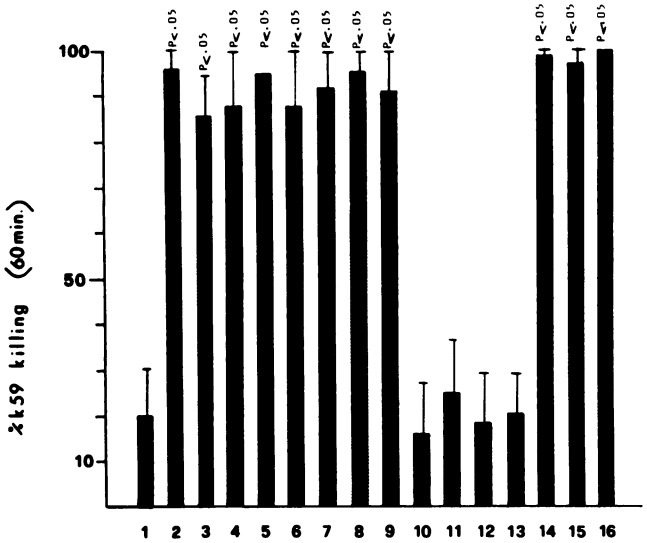

FIG. 4. Effect of lectins on K59 killing by PMNs. Results represent an average of three experiments. Bars at the top represent standard deviation. *P* was calculated as for Fig. 2. Bars 1 to 16 are as described in the legend to Fig. 3.

and soybean agglutinins, which bind sugars other than mannose, and succinyl-ConA, which recognizes this sugar, on the capability of human PMNs to associate with and kill the K59 strain. PMNs pretreated with either *A. hypogaea* or soybean agglutinin remained able to bind K59 bacteria and unable to associate with and kill the K59 strain (Fig. 2, bars 8 and 9, and Fig. 3 and 4, bars 10 and 11).

In contrast, the other mannose-binding lectin, succinyl-ConA, which compared with ConA has a greatly reduced cross-linking activity, demonstrated exactly the same effects as ConA. PMNs blocked by different means and pretreated with succinyl-ConA were not bound by the MIAT-positive *Klebsiella* strain (Fig. 2, bars 5, 6, and 7) while metabolically active PMNs pretreated with this lectin associated with and killed K59 bacteria with an efficiency virtually identical to that with which untreated PMNs associated with and killed these bacteria in the presence of mannose, UV-inactivated T7 phage, or anti-MIAT antibodies (Fig. 3 and 4, bars 6 to 9).

Pretreatment (followed by washing) of the MIAT-positive *Klebsiella* cells with succinyl-ConA did not modify their inability to bind metabolically active PMNs or their resistance to phagocytosis (Fig. 3 and 4, bars 13).

To further confirm that binding of the MIAT-specific receptors was necessary for strain K59 to prevent phagocytosis, we have studied association with metabolically active PMNs and killing of opsonized K59 cells. Opsonized *Klebsiella* cells should in fact associate with PMNs via the receptors for the C3b component of the complement, thus avoiding interaction of the MIAT ligand with its receptor. Such bacteria should therefore bind to metabolically active PMNs and also be sensitive to phagocytosis. In addition, their capability to associate with PMNs and sensitivity to killing should not be increased by pretreating phagocytes either with ConA or with succinyl-ConA.

Opsonized *Klebsiella* strain K59 associated very efficiently with metabolically active PMNs and were very rapidly killed by them, and pretreatment of phagocytes with the two mannose-binding lectins did not influence either binding or killing of the MIAT-positive strains (Fig. 3 and 4, bars 14, 15, and 16).

Effect of PMN pretreatment with K59 on MIAT-negative strain phagocytosis. The results described above suggest the possibility that the binding of the MIAT to its PMN specific receptor triggers changes in the PMN membrane, preventing it from binding and ingesting other bacteria. Thus, PMNs pretreated with MIAT-positive *Klebsiella* cells should be unable to ingest and kill the MIAT-negative mutants. It was found that this pretreatment caused a greater than sixfold reduction in the number of engulfed bacteria (Fig. 5) and in the sensitivity to intracellular killing (Fig. 6). If PMN treatment was performed in the presence of D-mannose, UV-inactivated T7 phages, or pepsin-digested anti-MIAT antibodies, no effect on the MIAT-negative strain phagocytosis was observed.

CL induction and degranulation during phagocytosis of MIAT-positive *Klebsiella* cells under different conditions. In another series of experiments, we have studied the effect of the MIAT ligand interaction with its receptor in the PMN membrane on both CL response and degranulation. Results are reported (Fig. 7) for experiments in which we evaluated PMN luminol-enhanced CL responses induced by K59 bacteria under conditions in which the adhesin could bind its receptor and under other conditions in which the binding was prevented by various means. It was found that in the

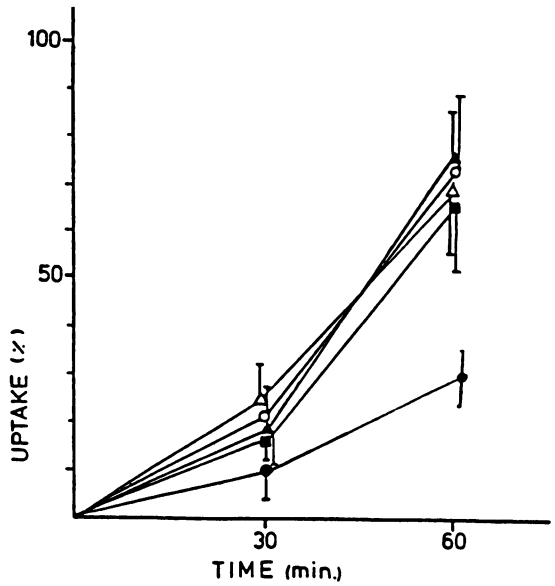

FIG. 5. Effect of phagocyte pretreatment with K59 bacteria on uptake by PMNs of MIAT-negative strains. ^{3}H-labeled strain KRTT1 was added to PMNs (bacteria:PMN ratio, 100:1) pretreated with K59 (●), KRTT1 (▲), K59 in the presence of D-mannose (○), K59 adsorbed with UV-inactivated T7 phages (■), or K59 pretreated with pepsin-digested anti-MIAT antibodies (△) (bacteria: PMN ratio, 10:1). At timed intervals, radioactivity associated with phagocytes was evaluated. Results represent an average of three experiments. Bars indicate standard deviation. Similar results were obtained with ^{3}H-labeled strain KRTT2.

presence of K59 bacteria, a very low CL response was generated. In contrast, a high response was observed when bacteria were previously adsorbed with UV-inactivated T7 phages, D-mannose, and pepsin-digested anti-MIAT antibodies.

Finally, the extent of degranulation by PMNs after ingestion of the MIAT-positive strain was studied by measuring the amounts of granule enzymes released into supernatants by exocytosis. Supernatants were assayed for lysozyme, β-glucuronidase, and lactoferrin. The amounts of enzymes released after 30 and 60 min of incubation were the same when the MIAT could interact with its PMN receptor and when the interaction was inhibited by T7 phages and pepsin-digested anti-MIAT antibodies (Table 3). Lactate dehydrogenase, a cytoplasmic enzyme, was not released from PMNs infected with strain K59; therefore, the release of granule proteins was not due to leukocyte lysis.

DISCUSSION

The major aim of this work was to investigate the apparently paradoxical behavior of MIAT-positive *K. pneumoniae* strains which, although able to bind inert PMNs, are not ingested and killed by metabolically active phagocytes. The results presented in this article support our previous suggestion (28) that the MIAT adhesin binds to a specific receptor in the membrane of PMNs which, when bound by MIAT but not by other ligands, triggers changes in the cell surface receptors which inhibit further MIAT-mediated binding and phagocytosis.

The fact that PMNs made unable to ingest bacteria by various means (low temperature or GA, CB, and CD treatment) always bound MIAT-positive cells demonstrates that metabolically normal phagocytes carry receptors to which the MIAT ligand binds. However, these receptors are no longer functional after active phagocytes have been bound by the MIAT ligand.

In phagocytosis mixtures incubated at 4°C and then shifted to 37°C, the degree of association of MIAT-positive bacteria with PMNs rapidly decreased with time after the shift, while in mixtures incubated first at 37°C and then shifted to 4°C the degree of K59 association was very low before the shift and then increased with time. These findings suggest that binding of the MIAT adhesin to its receptor in the PMNs triggers a change in the phagocyte surface which results in modification or removal of the MIAT receptors.

The experiments performed with CB (which impairs microfilament function and energy metabolism [19, 25]) and CD (which more specifically alters microfilament function [20]), which showed that PMNs treated with these compounds are able to bind MIAT-positive bacteria at 4 and 37°C in a number which increases with time, suggest that the impairment by MIAT of the ability of PMNs to bind further MIAT-positive bacteria may require microfilament function.

The role of MIAT in triggering receptor modification is also strongly supported by the observation that when PMNs were pretreated with the mannose-binding lectins ConA or succinyl-ConA (but not with other lectins) they bound at 4°C fewer MIAT-positive bacteria than did the untreated controls. With the same pretreatment at 37°C, they bound and internalized the MIAT-positive strain with an efficiency similar to that at which untreated PMNs phagocytize them in presence of D-mannose, UV-killed T7 phages, or anti-MIAT antibodies. This shows that when the receptors for the MIAT ligand are masked by ConA the interactions of

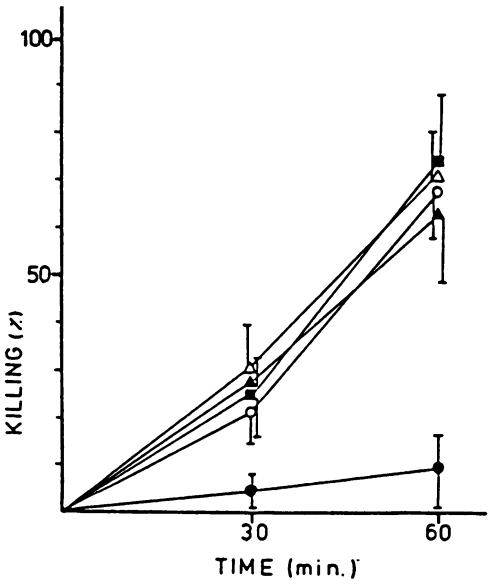

FIG. 6. Effect of phagocyte pretreatment with K59 bacteria on killing of MIAT-negative strains by PMNs. Strain KRTT1 Nal^r was added to PMNs (bacteria:PMN ratio, 100:1) pretreated with K59 (●), KRTT1 (▲), K59 in the presence of D-mannose (○), K59 adsorbed with UV-inactivated T7 phages (■), or K59 pretreated with anti-MIAT antibodies (△) (bacteria:PMN ratio, 10:1). At timed intervals, KRTT1 Nal^r killing was evaluated by using a duplicate pour plate method on LB agar containing nalidixic acid (final concentration, 100 μg/ml). Results represent an average of three experiments. Bars indicate standard deviation. Similar results were obtained with strain KRTT2 Nal^r.

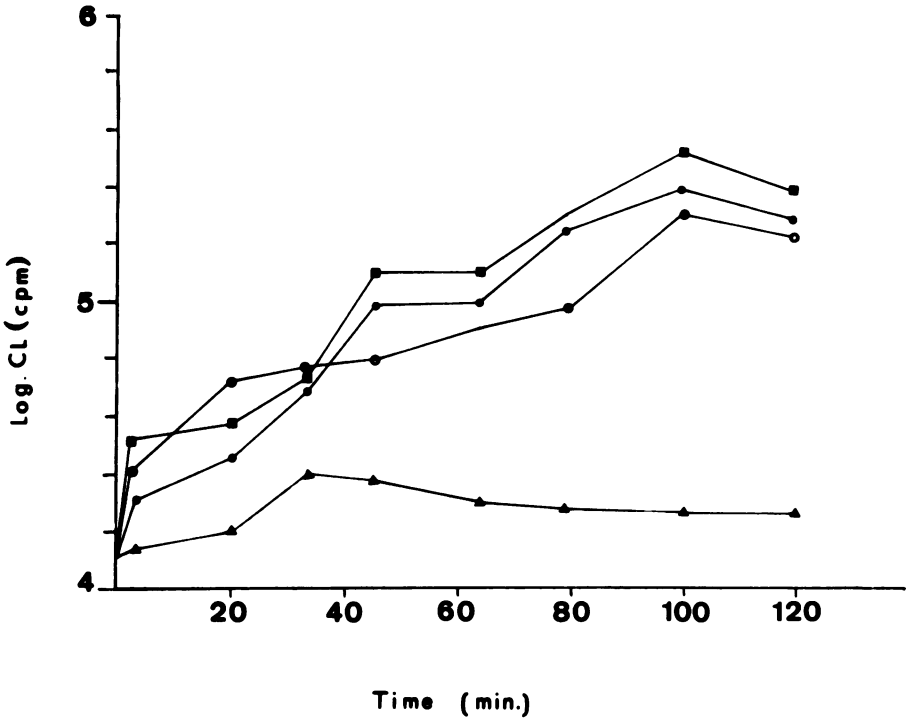

FIG. 7. Luminol-induced CL response by PMNs in the presence of the MIAT-positive strain K59 and the effect of MIAT masking on such induction. Results (an average of two experiments) are shown for K59 (▲), K59 plus UV-inactivated T7 phages (■), K59 plus D-mannose (●), and K59 plus pepsin-digested anti-MIAT antibodies (○).

MIAT-positive bacteria with PMNs are equal to those of MIAT-negative cells. It is not the presence of the MIAT ligand in the bacterial cells per se that makes bacteria unable to bind PMNs and resistant to phagocytosis, but, rather, the interaction of the MIAT ligand with a mannose-containing receptor in the phagocyte membrane.

These results are confirmed by the experiments in which luminol-induced CL responses to K59 bacteria were evaluated in the presence or absence of substances that inhibit MIAT interactions with its receptors. In fact, in the presence of D-mannose, UV-inactivated T7 phages, and anti-MIAT antibodies, the CL response to K59 was significantly higher than in conditions under which MIAT could interact with its receptor.

The capability of MIAT to block phagocytosis is also supported by the observation that PMNs pretreated with K59 are unable to engulf and kill the strains that do not carry the MIAT.

TABLE 3. Exocytosis of lysozyme, lactoferrin, and β-glucuronidase by human PMNs incubated with strain K59

Treatment	Enzyme release (% of total cell content)[a]					
	Lysozyme		Lactoferrin		β-Glucuronidase	
	30 min	60 min	30 min	60 min	30 min	60 min
K59	10	20	12	25	4	7
K59 + UV-inactivated T7 phages	17	19	19	24	5	6
K59 + anti-MIAT antibodies	16	19	18	21	5	8

[a] The amount of lysosomal enzyme release was evaluated as described in Materials and Methods.

In conclusion, this work has clearly shown that, besides the previously described bacterial adhesins that mediate binding to PMNs and stimulate phagocytosis, there also exist adhesins that mediate binding to PMNs but prevent phagocytosis and killing. Although other bacterial cell components that protect bacteria from phagocytosis were previously described (9, 10, 13, 15, 32, 35, 39, 41), to our knowledge this is the first demonstration that binding of a bacterial adhesin to its specific receptor in the PMN surface prevents bacterial killing and phagocytosis. It also shows that a bacterial strain resistant to phagocytosis can drastically impair phagocytosis of another strain of the same species which is prone to engulfment and killing by PMNs. Similar new findings are important because they show that interactions between bacteria and phagocytes are very complex and that bacteria have an additional and novel mechanism by which they protect themselves from host defenses. The study of this mechanism can help to further clarify the complex interactions between bacteria and PMNs.

The finding that bacterial strains resistant to phagocytosis can also impair phagocytosis of strains of the same species which are sensitive to phagocytosis could also have practical importance in naturally occurring infections. It is possible that a relatively small portion of an infecting bacterial population, by becoming resistant to phagocytosis (by mutation or by recombination), might render the entire population resistant to phagocytosis and killing.

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