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Correlation Between Phagocytic Activity and Metabolic Response of Polymorphonuclear Leukocytes Toward Different Strains of *Escherichia coli*

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The bactericidal activity, the phagocytic capacity, and the metabolic stimulation of polymorphonuclear leukocytes challenged with different strains of *Escherichia coli* were studied. It was found that only two strains out of 10 tested stimulated the oxygen consumption and carbohydrate metabolism of leukocytes and were readily killed by the phagocytes. The lack of killing of the other eight strains was shown to be due to absent or poor phagocytosis rather than to resistance to intracellular killing. Evidence was presented that the surface K antigen plays an important role in conferring antiphagocytic properties to some strains of *E. coli*. It was suggested that K antigen acts by interfering with the early step of the phagocytic process, that is, the attachment step.

It has been shown that the rate of clearance of *Escherichia coli* from the blood stream of mice varies with different strains of the microorganism (6). This finding has been interpreted in terms of differential susceptibility of *E. coli* strains to phagocytosis, and the supposed antiphagocytic property of a given strain of *E. coli* has been attributed to its content in K antigen (6, 7). The surface K antigen has been already shown to make *E. coli* O inagglutinable and resistant to complement lysis (4) and, due to these properties, the name of "impedin" has been proposed for it (2). In this paper the interaction in vitro between a class of professional phagocytes, that is, polymorphonuclear leukocytes (PMN), and different strains of *E. coli* has been examined. It is shown that some strains of *E. coli* are not susceptible to phagocytosis by PMN, and this is reflected in their inability to mount a number of metabolic responses, known to occur during challenge of phagocytes with particulate material. Data are also presented that support the role of K antigen as an antiphagocytic factor.

MATERIALS AND METHODS

Leukocytes. Both guinea pig and human PMN were used. Guinea pig PMN were obtained from peritoneal exudates produced by intraperitoneal injection of 1% sterile casein in isotonic saline. The exudates were collected 12 to 14 h after injecting casein (50 ml), and cells were harvested by centrifugation at 250 × *g* for 7 min, washed in Krebs-Ringer phosphate (KRP) without Ca^{2+}, and finally suspended in KRP. Suspensions containing less than 95% PMN were discarded. Human leukocytes were obtained from heparinized venous blood by dextran sedimentation, 1 ml of 15% dextran (molecular weight, 150,000 to 200,000) solution in isotonic saline being added to 6 ml of blood. After sedimentation of erythrocytes, leukocytes were harvested by centrifugation at 250 × *g* for 7 min, washed, and resuspended in KRP without Ca^{2+}.

Percentage of PMN in the preparations used ranged from 65 to 80%.

E. coli. The following strains of *E. coli* have been employed in this study: (i) two strains widely used for studies of bacterial genetics, K-12λ- and K-12 Hfr R, obtained from the stock collection of the Institute of Microbiology of the University of Genova; (ii) five strains belonging to the group of enteropathogenic *E. coli* (O78:B-, O111:B4, O55:B5, O26:B6, O126:B16) from the stock collection of the Institute of Microbiology of the University of Trieste; (iii) two strains isolated from feces of two children with acute diarrhea. One of these two strains (E88) was classified by the International Escherichia Center (World Health Organization), Statens Seruminstitut of Copenhagen, as O6:H1 and the other (E71) was classified as H9, but it could not be classified with available 159 O group sera; (iv) one strain (ECR) isolated from the feces of a healthy 3-month-old baby and classified by the same Center in Copenhagen as O18 ac, O23:H-.

All the strains used were grown overnight in nutrient broth (Difco) at 37 C. After washing three times with saline, suspensions in KRP were prepared and were then adjusted to the desired concentration with a Leitz model M photrometer equipped with an A filter.

Assessment of bactericidal and phagocytic activities. Essentially the method described by McRipley and Sbarra was followed, with a few modifications (10). For each experimental system two tubes were prepared containing 0.1 ml of *E. coli* suspension (10^9/ml) and 0.1 ml of PMN suspension (2×10^8/ml). The ratio of bacteria to phagocyte was 5:1. Control tubes contained 0.1 ml of *E. coli* suspension and 0.1

ml of KRP. The tubes were incubated at 37 C in a shaking water bath.

The bactericidal activity was assayed by removing the tubes at selected time intervals (0, 30, and 60 min), and 0.8 ml of 5% sterile saponin was added to lyse phagocytes and release intracellular bacteria. The concentration of saponin employed was not toxic for the test bacteria. However, when *E. coli* grown at 45 C, instead of 37 C, were used as test bacteria, it was found necessary to lower the concentration of saponin to 0.5%. The saponin-containing tubes were allowed to stand for 15 min at room temperature, after which appropriate dilutions were made and plated on nutrient agar. Viable counts from these tubes gave total viable bacteria at the particular time interval in the absence and presence of leukocytes.

For the assessment of the phagocytic activity (engulfment) two experimental tubes and two control tubes, prepared as described above, were allowed to incubate for 30 min. At this time, 9.8 ml of KRP was added, and the tubes were centrifuged at 250 × *g* for 10 min to sediment leukocytes but not extracellular bacteria. From the supernatant 0.2 ml was removed and added to 0.8 ml of saponin. After 15 min this mixture was appropriately diluted and plated. The pellet was resuspended with KRP to 10 ml, and the mixture was centrifuged again at 250 × *g* for 10 min. This was done to liberate bacteria trapped between leukocytes. From the second supernatant 0.2 ml was removed and added to 0.8 ml of 5% saponin, and, after standing for 15 min, the mixture was diluted and plated. The viable counts obtained from supernatants 1 plus 2 gave the extracellular viable bacteria. The pellet was resuspended to 10 ml with KRP, and 0.2 ml of this suspension was added to 0.8 ml of 5% saponin and, after 15 min, diluted and plated. This gave the number of live bacteria associated with phagocytes (intracellular or adherent bacteria).

Preparation of E. coli extracts. The technique described by Glynn and Howard was followed (4). According to this technique, acetone-dried bacteria were homogenized, and polysaccharide was extracted by cold ethanol. The extracts were tested for agglutination inhibiting activity.

Estimation of agglutination inhibiting activity. The ability of extracts to inhibit the agglutination of sheep erythrocytes by rabbit antibody was measured by the method described by Glynn and Howard (4). The anti-sheep erythrocyte antibody used was bought from Behringwerke, Marburg/Lahn (agglutination titer, 1:2,048).

Biochemical assays. Oxygen uptake by intact cells was determined polarographically with a Clark oxygen electrode. Oxidation of glucose via the hexose monophosphate shunt by intact cells was determined using glucose labeled in carbon 1 ([1-^{14}C]glucose). Cell suspensions were incubated with [1-^{14}C]glucose in capped flasks equipped with a central well containing 0.25 ml of 20% KOH. The $^{14}CO_2$ produced was trapped by KOH. The content of the central well was transferred into vials for liquid scintillation counting with a Beckman LS-100 spectrometer. These methods have been described in greater detail in previous papers (12, 16, 19).

Electron microscopy. Suspensions of leukocytes, incubated with bacteria, were fixed by addition of an equal volume of a 3% solution of purified glutaraldehyde in 0.2 M cacodilate buffer, pH 7.2, containing 2% sucrose. After 1 h the suspension was centrifuged, and the pellet was resuspended in 1% osmium tetroxide solution in 0.1 M cacodilate buffer, pH 7.2. After dehydration in graded ethanols, the cell pellet was embedded in Dow epoxy resin 332 (9). Ultrathin sections, prepared by an ultrotome III (LKB), were doubly stained with an aqueous solution of 2% uranyl acetate for 30 min and lead citrate (25) and examined in a Philips EM 300 electron microscope.

RESULTS

Bactericidal activity. Figure 1 shows that only two strains of *E. coli* (K-12λ- and K-12 Hfr R) out of the 10 tested were extensively killed by guinea pig PMN. Two other strains (O55 B5 and O26 B6) were moderately killed, and the other strains were virtually insensitive to phagocytic killing over 60 min of incubation with PMN. The question arose whether the lack of killing reflected an impaired or impeded phagocytosis or was due to a true resistance to intracellular killing. This was assessed with the experiments reported in the next paragraph.

Phagocytic activity. Figure 2 shows that live bacteria present in the mixture at the end of incubation were largely recovered in the supernatant and only a small percentage was present in the pellet, i.e., was associated with cells. The percentage of cell-associated bacteria probably reflected the trapped or adherent bacteria, since it was not appreciably different among the easily killed strains and the "not-killed" strains. It was concluded that the lack of killing of certain *E. coli* strains reflected lack of phagocytosis. The above conclusion was confirmed directly by electron microscopy of PMN exposed to one of the strains insensitive to the phagocytic killing (*E. coli* O111 B4), to one strain partially sensitive to phagocytosis (O26 B6), and to an easily phagocytosable strain (K-12λ-). Figure 3 shows that the number of PMN containing bacteria was very low when the strain used was O111 B4 and progressively increased when *E. coli* O26 B6 or *E. coli* K-12λ- were used.

Biochemical response of PMN challenged with E. coli. It is known that phagocytosis is accompanied by a series of changes in the metabolism of leukocytes, including an increased oxygen uptake and an increased oxidation of glucose, via the hexose monophosphate shunt (1, 16, 17, 20, 21).

Table 1 shows that both $^{14}CO_2$ production and the KCN-insensitive oxygen uptake were markedly stimulated in PMN exposed to the phagocytosable strains, whereas the metabolic response was poor or absent in PMN exposed to

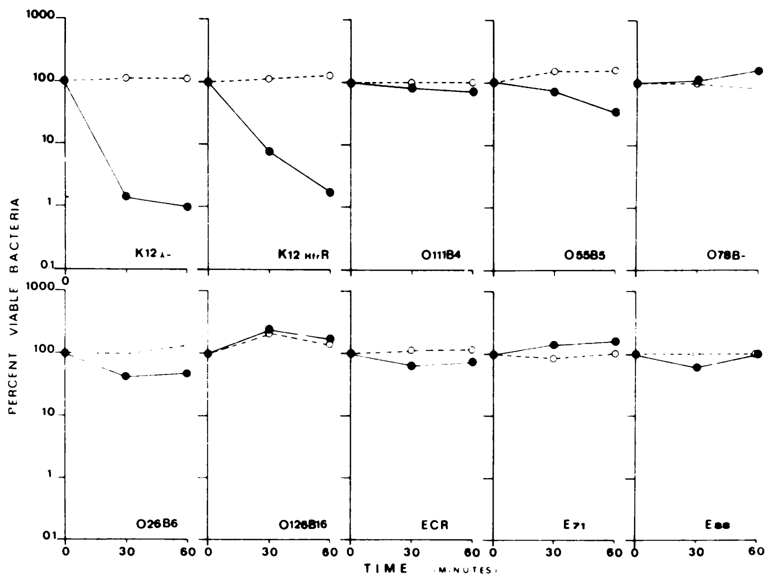

FIG. 1. *Viability of 10 strains of E. coli incubated with (●) or without (○) guinea pig PMN. Individual points represent the mean of five experiments for the two K-12 strains, of four experiments for O111 B4, O55 B6, O26 B6, O78 B-, O126 B16, and ECR, and of two experiments for E71 and E88.*

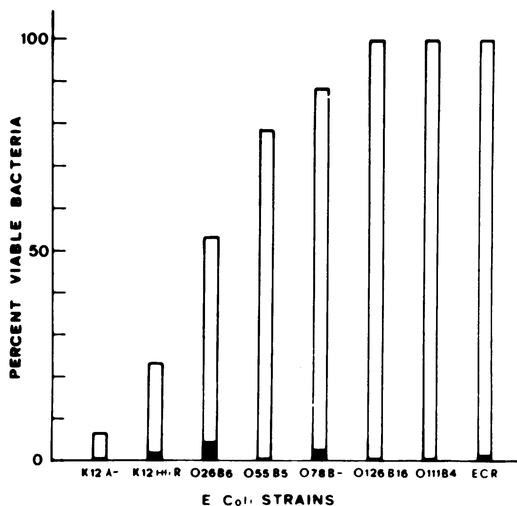

FIG. 2. *Phagocytic capacity of guinea pig PMN exposed to different strains of E. coli. Each column represents the percentage of live bacteria recovered in the supernatant (open part of the column) or in the cell pellet (solid part of the column) after centrifugation of the incubation mixture. Bacteria were incubated with PMN for 30 min. Values are the mean of duplicate viable counts.*

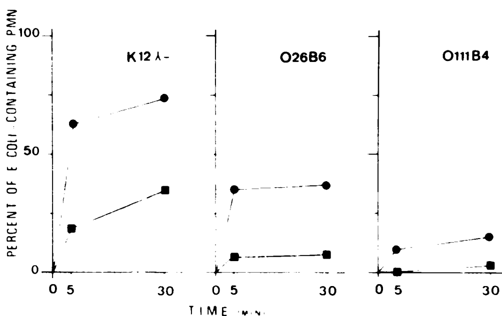

FIG. 3. *Evaluation by electron microscopy of the engulfment of three strains of E. coli by guinea pig PMN. Leukocytes containing one (●) or more (■) bacteria were enumerated on electron micrographs.*

the strains resistant to phagocytosis, and, in general, it paralleled the degree of phagocytability. It is likely that this lack of response was not due to some kind of "poisoning" of PMN, as suggested by the example reported in Fig. 4, where PMN are shown to be able to increase their oxygen uptake when exposed to K-12λ- after previous exposure to O111 B4.

Experiments with human leukocytes. Electron microscopy was performed, and bactericidal activity and oxygen uptake of human PMN exposed to *E. coli* K-12λ- or O111 B4 were determined. The results paralleled those obtained with guinea pig PMN (Rottini and Tedesco, unpublished data).

Relationship between K antigen and resistance of phagocytosis. (i) Inhibition of erythrocyte agglutination. The ability of K antigen to inhibit the agglutination of sheep erythrocytes by rabbit antibody has been taken as a measure of its "impedin" properties (3). Table 2 shows that extracts from strains resistant to phagocytosis inhibited agglutination of sheep

TABLE 1. *Metabolic response of guinea pig PMN exposed to phagocytosable and non-phagocytosable strains of E. coli*[a]

Strain	Oxygen uptake[b] (natoms of O/min)	$^{14}CO_2$ production[c] (counts/min)
K-12λ-	61	8,176
O 26 B 6	31	4,047
O 55 B 5	24	3,390
O 78 B-		0
O 111 B 4	9	834
O 126 B 16	10	1,471
E 71	9	793
ECR	5	

[a] The values are given as oxygen consumption or as $^{14}CO_2$ production of PMN exposed to bacteria minus oxygen consumption or $^{14}CO_2$ production of resting PMN and bacteria alone.

[b] Assay medium: KRP, 2 mM KCN, 1.5×10^7 PMN, and 3×10^8 *E. coli*. Volume, 2 ml. Temperature, 37 C.

[c] Assay medium was as for oxygen uptake plus 4 μmol of glucose. The initial specific activity of glucose was 1.7×10^5 counts/min per μmol. Incubation, 15 min. Temperature, 37 C.

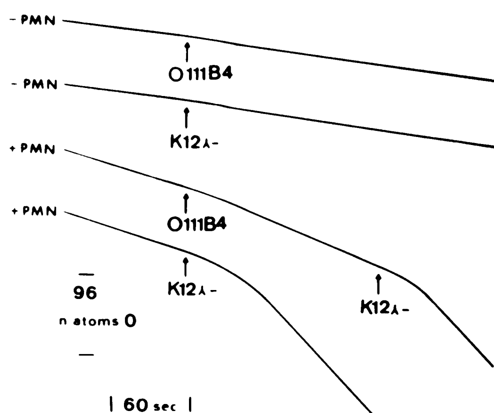

FIG. 4. *Oxygen uptake of PMN exposed to phagocytosable and non-phagocytosable E. coli. Assay medium was as described in footnote (b) to Table 1.*

erythrocytes, whereas no inhibition was detected with the extracts of phagocytosable strains.

(ii) Effect of growth temperature on E. coli phagocytability. Growth at unusual temperatures (18 or 45 C) has been reported to lower the amount of K antigen in some strains of *E. coli* (4). In our experiments *E. coli* O111 B4, after

TABLE 2. *Agglutination inhibiting activity of crude extracts of E. coli*

Strain	Agglutination inhibition titer
K-12 λ-	0
K-12 Hfr R	0
O 26 B 6	1:16
O 55 B 5	1:4
O 78 B-	1:4
O 111 B 4	1:8
E 71	1:32
E 88	1:4
ECR	1:32

overnight growth at 45 C, became agglutinable by homologous anti-O serum (Table 3), susceptible to phagocytic killing (Fig. 5), and able to induce the respiratory burst in PMN (Fig. 6).

(iii) Effect of K antigen-rich extracts on E. coli K-12λ- phagocytosis. Figure 7 shows that *E. coli* K-12λ- incubated with PMN in the presence of the extract of a non-phagocytosable strain (*E. coli* O111 B4) are less susceptible to

TABLE 3. *Effect of growing E. coli O111 B4 at 45 C on its O agglutinability*

Strain	Agglutination titer[a]	
	Anti-OK serum	Anti-O serum
O111 B4, grown at 37 C	1:512	1:8
O111 B4, grown at 45 C	1:512	1:1, 024
O111 B4, grown at 37 C and heated at 100 C for 60 min	1:1, 024	1:1, 024

[a] Coli sera from Behringwerke, Marburg/Lahn, were used.

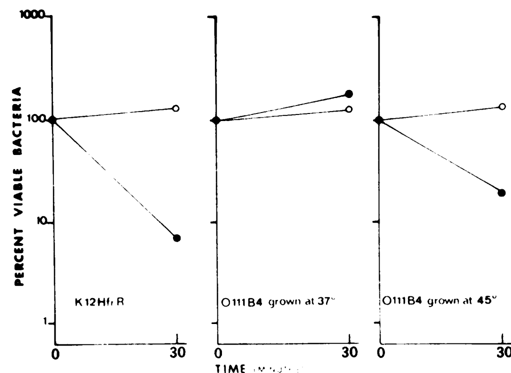

FIG. 5. *Effect of growth temperature on susceptibility of E. coli O111 B4 to phagocytic killing. Symbols: ●, bacteria plus PMN; ○, bacteria alone. Values are the mean of duplicate experiments.*

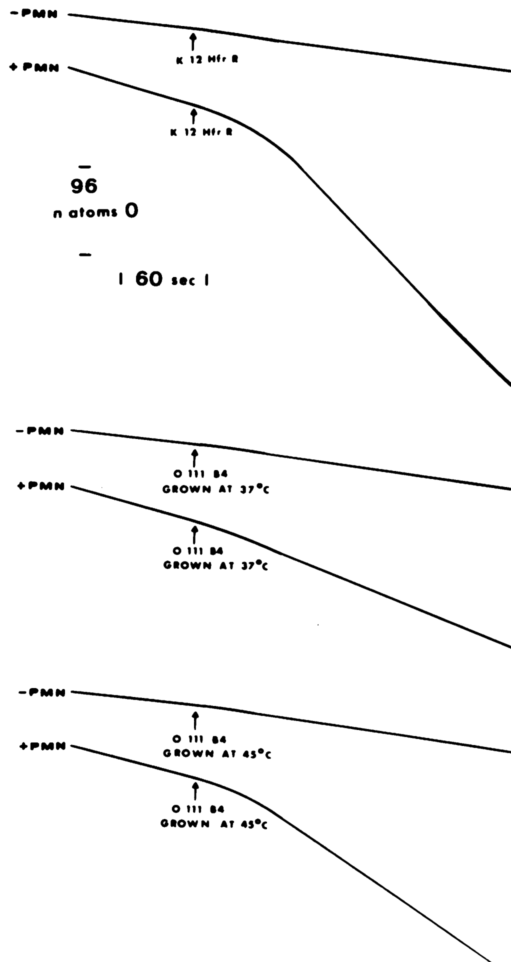

FIG. 6. *Effect of growth temperature on the ability of E. coli O111 B4 to stimulate oxygen uptake by PMN. Assay medium was as described in footnote (b) to Table 1.*

phagocytosis than in the absence of the extract. Also the extract of *E. coli* O111 B4 inhibited oxygen consumption by PMN exposed to heat-killed *E. coli* O111 B4 without affecting the rate of oxygen uptake by resting PMN (not shown).

DISCUSSION

The results reported in this paper emphasize the role of the bacterium in phagocytosis by showing that several strains of *E. coli* are resistant to phagocytosis by PMN leukocytes and do not induce in these cells the regular metabolic burst that accompanies phagocytosis. What is the peculiarity of these strains with respect to those which are susceptible to the phagocytic killing? Our data support the role of K antigen, as it has been already suggested by Glynn and Howard (2, 4, 6). In fact, (i) the ability of crude extracts of *E. coli* to inhibit the agglutination of sheep erythrocytes, which is taken as a measure of the amount of K antigen in the extract, was found only in the extracts obtained from the strains resistant to phagocytosis; (ii) it was possible to render a strain susceptible to phagocytosis and able to stimulate the metabolism of PMN by growing it under conditions where no or little synthesis of K antigen takes place; (iii) the K antigen-rich extract of a non-phagocytosable strain inhibited phagocytosis of a strain that was readily ingested by PMN in the absence of the extract.

It is generally believed that the endocytic act involves two stages, i.e., attachment of the particle to the phagocyte surface and engulfment. The attachment is dependent on the ability of the cell surface to recognize and bind the particle. During this stage, rearrangements of the molecular architecture of the plasma membrane occur which make it possible for metabolic stimulation and engulfment to take place (15). The two events, however, are independent of each other, as indicated by the occurrence of engulfment without metabolic burst (11) and of metabolic burst without en-

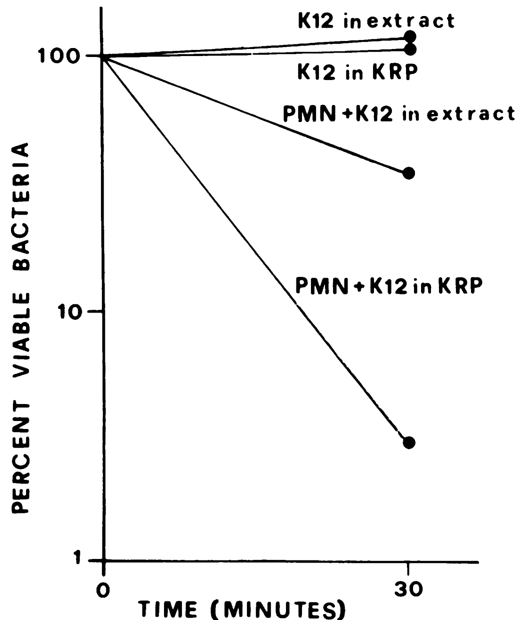

FIG. 7. *Effect of a K antigen-containing extract from E. coli O111 B4 on the phagocytic killing of E. coli K-12λ- by PMN leukocytes. Before use the extract was dialyzed overnight against KRP buffer. The content of K antigen in the extract was assayed by passive hemagglutination (titer, 1:512). E. coli K-12λ- (10^8) suspended in 0.1 ml of the extract or in 0.1 ml of KRP were used in each assay.*

gulfment. The latter case has been shown to hold for PMN challenged with mycoplasma (22) or with substrates that are not phagocytosable because of their large size coated with molecules for which PMN possess a surface receptor, such as concanavalin A (14), C3b (F. Tedesco, S. Trani, M. R. Soranzo, and P. Patriarca, FEBS Lett., in press), and immune complexes (5). It appears, therefore, that the mechanism that triggers the metabolic stimulation lies in some still unknown events occurring at the level of plasma membrane. This view is also supported by the possibility of inducing a metabolic burst in leukocytes with several chemical agents that only interact with plasma membrane (8, 13, 18).

Therefore, the absence of the metabolic burst in PMN challenged with K antigen-rich *E. coli* suggests an early impairment of the attachment stage. One could further speculate that such impairment may recognize one of the following two mechanisms. (i) The microorganisms become attached to the phagocytic surface, but the presence of the K antigen inhibits the conformational changes occurring within the plasma membrane following the attachment; and (ii) the K antigen acts as a real impedin of the attachment. This second hypothesis seems to be supported by the electron micrographs of PMN exposed to non-phagocytosable *E. coli*, showing no bacteria attached to the phagocyte surface.

The mechanism of the regulation of bacterial phagocytosis by PMN has been discussed by Van Oss and Gillman (23, 24) in terms of relative hydrophobicity of bacterial surface with respect to phagocyte surface. Bacteria which are more hydrophobic than granulocytes get readily phagocytosed by them, whereas the more hydrophilic bacteria resist phagocytosis. The list, given by the authors, of hydrophilic bacteria included *E. coli* O111 and O55, which have been shown in our study to resist phagocytosis. Therefore, it is tempting to speculate that the property whereby K antigen makes *E. coli* non-phagocytosable is an increase in the hydrophilicity of the bacterial surface. However, other properties of K antigen such as the degree of polymerization (7) and its steric distribution on the bacterial surface should be taken into account.

Therefore, if it is reasonable to admit that K antigen inhibits the attachment stage of phagocytosis, the properties of the molecule that are crucial for such an impairment are still to be clarified.

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