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Influence of Anti-Slime Glycolipoprotein Serum on the Interaction Between *Pseudomonas aeruginosa* and Macrophages

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Glycolipoprotein, a purified fraction of the exopolysaccharide slime of *Pseudomonas aeruginosa*, was identified as responsible for a number of the biological activities of viable cells, including toxicity and immunogenicity capable of stimulating protective antibody against the lethal effects of viable cells. Antiserum against glycolipoprotein also mediated the phagocytosis and subsequent killing of viable *P. aeruginosa* by unstimulated mouse peritoneal macrophages. In the absence of anti-glycolipoprotein serum, macrophages did not significantly reduce the number of bacteria. The presence of complement in the experimental mixture did not affect the reduction of bacteria by the macrophage in the presence of anti-glycolipoprotein serum. The limiting effect of antiserum concentration on macrophage activity was studied, and maximal activity was found at 2%, with no further increase in activity at 5%. Preopsonization of the bacteria with anti-glycolipoprotein serum had little effect on the course of phagocytosis within the experimental conditions. Variations in bacterium-to-macrophage input ratios, ranging from 30:1 to 1:30, did not affect the capacity of the macrophages for phagocytosis.

The biological characteristics of the extracellular slime glycolipoprotein (GLP) of *Pseudomonas aeruginosa* suggest that it is an important cellular component to consider in the pathogenesis of this organism (1). The isolated GLP has been purified and analyzed chemically, and its homogeneity has been demonstrated physically and immunologically (14). Experimentally, GLP production has been detected in vivo (8), and injection of highly purified preparations of GLP has been shown to elicit the same toxic, leukopenic, and lethal manifestations that have been observed after lethal infection of mice with viable bacteria (14). Furthermore, in appropriate concentrations, GLP has been shown to act as a protective antigen in active and passive immunization by preventing the toxic, leukopenic, and lethal effects in mice challenged with viable cells (14). A functional relationship between indirect hemagglutinating and passive protection activities was observed in vivo after passive immunization of mice (15).

The macrophage is known to occupy a central position in the immune defense mechanism, and its role in the resistance of mice after experimental infection with *P. aeruginosa* may be decisive (5). Evidence of its importance in natural infection has come from a recent report suggesting that the susceptibility of cystic fibrosis patients to pulmonary colonization by *P. aeruginosa* may stem from a defect in macrophage phagocytic activity (6).

This report attempts to clarify the protective activity of antiserum produced against the purified GLP of *P. aeruginosa* by focusing on its effects on macrophage activity. Experiments were designed to examine the influence of anti-GLP serum on the interaction between *P. aeruginosa* and mouse peritoneal macrophages, especially the early, most critical event of the phagocytic process, i.e., attachment or association of the bacterial cells to macrophages.

MATERIALS AND METHODS

Organism. The bacterium used in this study, *P. aeruginosa* BI, was originally isolated from a clinical specimen and has been previously described (2).

Antigen. The GLP fraction was purified from the extracellular slime layer of strain BI and used as antigen. As previously described (1, 14), slime was extracted from 18-h bacterial cultures, precipitated with ethanol, and dialyzed against distilled water. The dialysate was centrifuged at 105,000 × *g* for 3 h, and the supernatant fluid, which contained the GLP fraction, was lyophilized. Purity and homogeneity were demonstrated by chromatography, sedimentation pattern, and immunodiffusion (14).

Animals. White male rabbits weighing 3 to 4 kg were housed individually. White male Swiss mice

weighing 18 to 20 g were housed 10 per cage. All animals were supplied with water and Purina chow ad libitum.

Anti-GLP serum. On day zero, rabbits received subcutaneous injections in four separate sites for a total of 2 mg of GLP in equal volumes of 0.1 M phosphate-buffered saline, pH 7 (PBS), and Freund incomplete adjuvant. Groups of three rabbits were bled before immunization and 18 to 20 days after immunization. Pooled sera were stored in 1-ml portions at −20°C. The rabbit anti-GLP serum used in this study had a titer of 1:640 by indirect hemagglutination, and by passive immunization it protected 80% of the mice challenged with viable *P. aeruginosa*.

Macrophages. Macrophages were collected from the peritoneal cavity of mice as described by Bjornson and Michael (4). Three milliliters of Hanks balanced salt solution with sodium bicarbonate, pH 7.4 (HBSS; Difco Laboratories, Detroit, Mich.), containing heparin (100 U/ml) was injected intraperitoneally into mice. Peritoneal fluids were aspirated with siliconized Pasteur pipettes, pooled in siliconized tubes, and centrifuged at 200 × *g* for 10 min at 5 to 8°C. Sedimented cells were washed and resuspended in HBSS containing 1% gelatin to a final concentration of 6×10^6 macrophages/ml. Differential counts of these suspensions confirmed that 80 to 90% of the cells were macrophages, and at least 95% were viable as determined in a hemocytometer by negative staining with trypan blue following the manufacturer's directions (GIBCO Laboratories, Grand Island, N.Y.).

Reaction mixtures. Bacteria were harvested from 18-h Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants, washed twice in PBS, and suspended finally in HBSS to a turbidity equivalent to approximately 10^6 viable cells/ml. Colony counts were made on Trypticase soy agar (BBL) for each experimental suspension to determine the precise numbers of viable bacteria. The experimental reaction mixture consisted of 0.2 ml of the bacterial suspension, 0.2 ml of serum (usually prediluted to 20%), 1.0 ml of the macrophage suspension, and HBSS to a final volume of 2.0 ml, with appropriate substitutions wherever called for. Except in the preopsonization experiments, serum was added to the bacterial suspension with gentle mixing, and the macrophages were then added without delay. The mixtures were made in siliconized tubes and rotated end-over-end (9 rpm) on a multipurpose rotater (model 150V, Scientific Instruments, Inc., Springfield, Mass.) at 37°C. Based on experiments presented in Results, a bacterium-to-macrophage ratio of 1:30 was chosen for its convenience and was used in all experiments except where otherwise indicated.

Bacterial opsonization. For some experiments, serum-bacteria mixtures were incubated at 37°C for 30 or 60 min with gentle mixing before being added to the macrophages. Certain mixtures were further incubated at 4°C for an additional 18 h and then centrifuged at 1,650 × *g* for 10 min; the sediment was washed twice in PBS and resuspended finally in the original volume of HBSS. Macrophages were then added, and the reaction mixture was incubated as described above.

The role of heat-labile serum components in the opsonization of strain BI was examined by adding 0.2 ml of 50% normal mouse or rabbit serum to 0.2 ml of a suspension of strain BI (10^6 viable bacteria/ml) before the addition of macrophages (6×10^6 viable cells/ml) to the reaction mixture. Similarly, the opsonic activity of heated (56°C for 30 min) anti-GLP serum was compared with that of the unheated serum. The influence of guinea pig complement (Flow Laboratories, McLean, Va.; final concentration in reaction mixtures, 50 50% hemolytic complement units [CH_{50}] /ml) on opsonization and killing of strain BI was examined. In these experiments, the concentration of complement was determined spectrophotometrically by the hemolytic assay of Kabat and Mayer (10).

Assay of bacteria-macrophage interaction. Immediately after reaction mixtures were constituted, 0.1-ml samples were placed in 0.9 ml of cold HBSS containing saponin (2.5%, wt/vol); viable bacterial counts were then made to establish the total number of viable bacteria in the reaction mixture at zero time. At subsequent time intervals, 0.1 ml of the reaction mixture was placed in 0.9 ml of cold HBSS to stop phagocytosis. After centrifugation at 200 × *g* for 5 min to sediment macrophages and macrophage-associated bacteria, the number of free, viable bacteria remaining in the supernatant fluid was determined by agar plate counts (17). The number of surviving bacteria was determined by viable count after disruption of macrophages with saponin 2.5% (wt/vol) (9). Experiments were repeated at least five times with murine peritoneal macrophages obtained from different donors. Viable counts were performed in duplicate, and the results are presented as average values.

RESULTS

Effect of anti-GLP serum on phagocytosis. The presence of anti-GLP serum resulted in a significant decrease in the numbers of viable *P. aeruginosa* in the supernatant fluids of the reaction mixture (Fig. 1). The results indicate that great numbers of bacteria (84%) quickly became macrophage associated during the first 60 min of interaction in the presence of anti-GLP serum.

In the absence of anti-GLP serum, bacterial association with macrophages was consistently observed, but at a significantly reduced level. The presence of normal serum, mouse or rabbit, did not significantly enhance the activity of the macrophage suspension. In the absence of macrophages, anti-GLP serum failed to significantly reduce the numbers of viable bacteria in the supernatant fluids of the reaction mixture. In these experiments, control bacterial suspensions in the absence of macrophages showed no decrease in the number of viable bacteria but rather some evidence of an increase, detected usually after 120 min of incubation.

Role of complement. When the opsonizing activity of heat-treated anti-GLP serum (56°C for 30 min) was compared with that of the un-

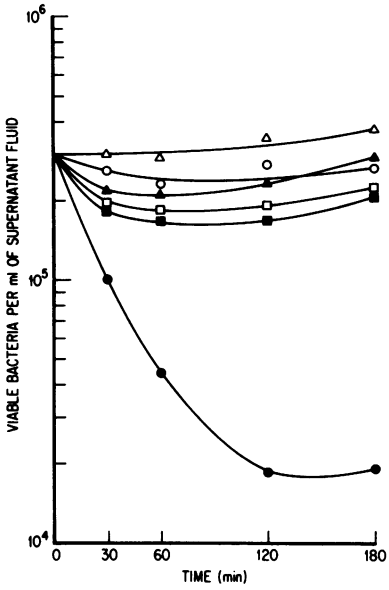

FIG. 1. *Influence of anti-GLP serum on the phagocytosis of P. aeruginosa by mouse peritoneal macrophages. Reaction mixtures were varied as follows: 2% anti-GLP serum with macrophages (●), 2% anti-GLP serum without macrophages (○), 5% normal mouse serum with macrophages (■), 5% normal rabbit serum with macrophages (□), macrophages without serum (▲), and P. aeruginosa alone (△). Other experimental details were as described in the text.*

treated anti-GLP serum, it was found to be essentially unchanged (Fig. 2). The concentration of complement contained in the untreated serum was assayed and found to be approximately 60 CH_{50} per ml in the rabbit serum and undetectable in the mouse serum. As in the previous experiments (Fig. 1), macrophages, in the absence of anti-GLP serum, demonstrated a consistent but reduced level of interaction with the bacterial cells, and heated, like unheated, anti-GLP serum alone failed to reduce the number of bacteria significantly.

In other experiments, guinea pig complement in known quantity (50 CH_{50}/ml, final concentration) was added to certain reaction mixtures to determine whether the presence of complement enhanced the opsonic activity of anti-GLP serum. The results (Fig. 3) indicated that opsonization of strain BI by anti-GLP serum did not require the presence of complement. Further, complement in the absence of anti-GLP serum failed to act as an opsonin, triggering macrophage uptake of bacteria, nor did complement together with anti-GLP serum reduce the number of viable bacteria in the absence of macrophages.

Bactericidal activity of the macrophages was also investigated under the conditions of these experiments. Killing was observed only in those macrophage reaction mixtures that contained anti-GLP serum (Fig. 4). In the presence of anti-GLP serum, the viable bacterial count was reduced by approximately 74% during the 2-h incubation period. The presence of complement did not appear to be a necessary component of this activity. It should also be noted that in the absence of macrophages, no bactericidal activity was observed in reaction mixtures containing anti-GLP serum and complement.

Effect of serum concentration on phagocytosis. Incubation of *P. aeruginosa* strain BI with macrophages, in the presence of various concentrations of anti-GLP serum at 37°C, showed that the bacterium-macrophage interaction was dependent on the serum concentration (Fig. 5). The experiment showed that maximal activity resulted with serum concentrations of 2 to 5%. A further dilution of serum to 0.4% resulted in a significantly decreased activity.

Effect of preopsonization. To determine

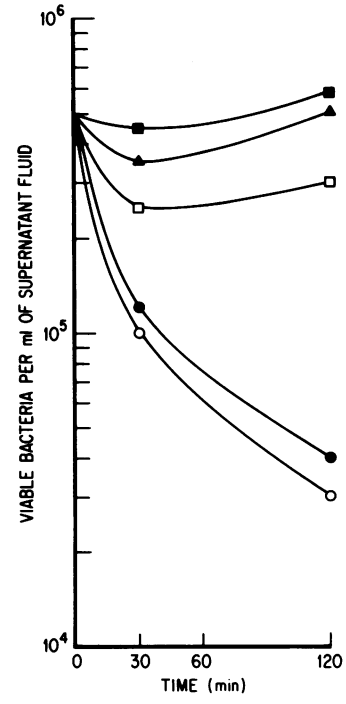

FIG. 2. *Effect of heat on the activity of anti-GLP serum in the phagocytosis of P. aeruginosa by mouse peritoneal macrophages. Reaction mixtures were varied as follows: 2% heated (56°C, 30 min) anti-GLP serum with macrophages (●), 2% anti-GLP serum with macrophages (○), 2% anti-GLP serum without macrophages (▲), macrophages without serum (□), and P. aeruginosa alone (■). Other experimental details were as described in the text.*

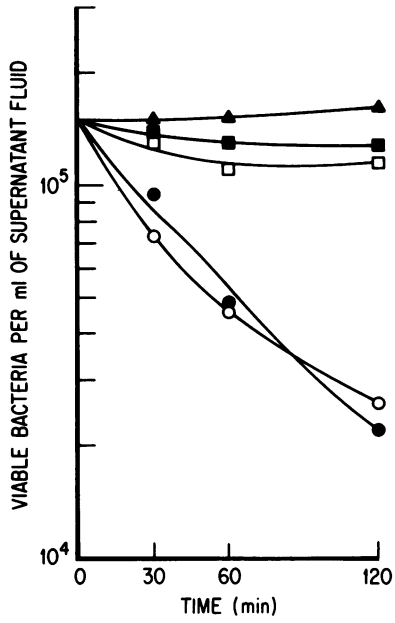

FIG. 3. *Influence of complement on the activity of anti-GLP serum in the phagocytosis of P. aeruginosa by mouse peritoneal macrophages. Serum was heated at 56°C for 30 min before it was added to the reaction tubes. Reaction mixtures were varied as follows: 2% anti-GLP serum and macrophages (●), 2% anti-GLP serum with macrophages and 50 CH_{50} of complement per ml (○), 2% anti-GLP serum with 50 CH_{50} of complement per ml and no macrophages (▲), 50 CH_{50} of complement per ml and macrophages without serum (□), and P. aeruginosa alone (■). Other experimental details were as described in the text.*

the effect of various conditions of opsonization on the phagocytic process, experiments were performed with bacteria preopsonized with anti-GLP serum before their addition to the macrophages in the reaction mixture (Fig. 6). As a control, anti-GLP serum and bacteria were added to reaction tubes without preopsonization. Preopsonization of strain BI with anti-GLP serum (2%) at 37°C for 30 min, or at 37°C for 1 h followed by 4°C overnight, did not enhance phagocytic activity over that observed without the preopsonization of the bacteria. However, experiments using the mixtures containing bacteria opsonized for 30 min only suggest that the initial rate of association may be faster under certain conditions of preopsonization. Washing of the preopsonized bacteria before their addition to the macrophages made little, perhaps no, difference. The results showed virtually no effect relating to time conditions of preopsonization, and further indicated that the immediate addition of anti-GLP serum to reaction mixtures was fully effective in promoting phagocytic activity.

Effect of bacterium-to-cell ratio. Incubation of 3×10^6 macrophages/ml with various numbers of viable bacteria in the presence of anti-GLP serum (2%) showed that a wide range of bacterium-to-macrophage ratios were effective in promoting bacterial interaction with macrophages (Fig. 7). At bacterium-to-macrophage ratios of 30:1, 10:1, and 1:30, 85 to 94% of the bacteria became macrophage associated within 2 h. Bacterial excess apparently did not affect adversely the capacity of the macrophages for phagocytosis.

DISCUSSION

Although macrophages might reasonably be expected to participate in the host response, few studies have attempted to clarify the characteristics of macrophage participation in *P. aeruginosa* experimental infections. The phagocytic

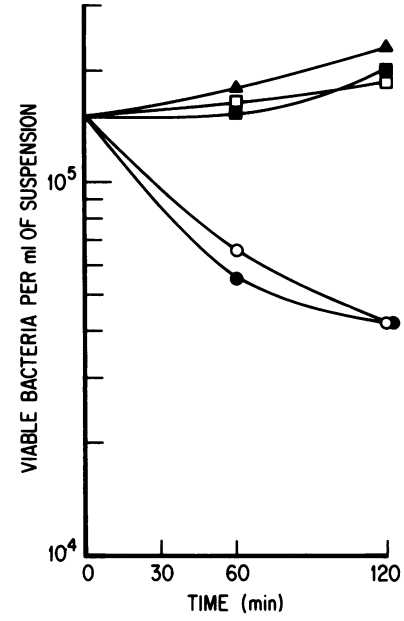

FIG. 4. *Influence of complement on the activity of anti-GLP serum in killing of P. aeruginosa by mouse peritoneal macrophages. Serum was heated at 56°C for 30 min. The number of viable bacteria was determined from the reaction suspension after disruption of macrophages with 2.5% saponin. Reaction mixtures were varied as follows: 2% anti-GLP serum and macrophages (●), 2% anti-GLP serum with 50 CH_{50} of complement per ml and macrophages (○), 2% anti-GLP serum and 50 CH_{50} of complement per ml (▲), 50 CH_{50} of complement per ml and macrophages without serum (□), and P. aeruginosa alone (■). Other experimental details were as described in the text.*

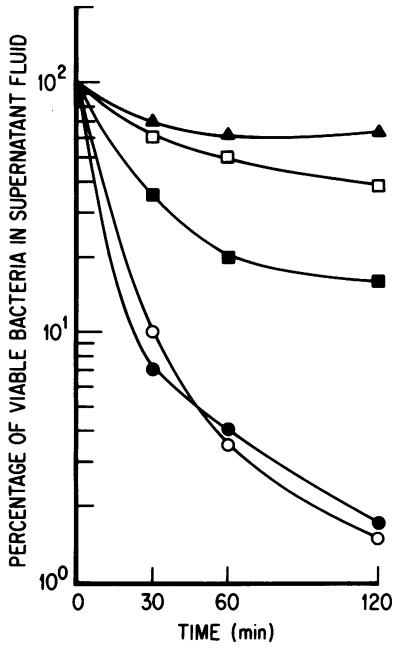

FIG. 5. *Effect of concentration of anti-GLP serum on phagocytosis of P. aeruginosa by mouse peritoneal macrophages. The concentration of anti-GLP serum was varied as follows: 5% (●), 2% (○), 0.4% (■), 0.08% (□), and 0.016% (▲). Other experimental details were as described in the text.*

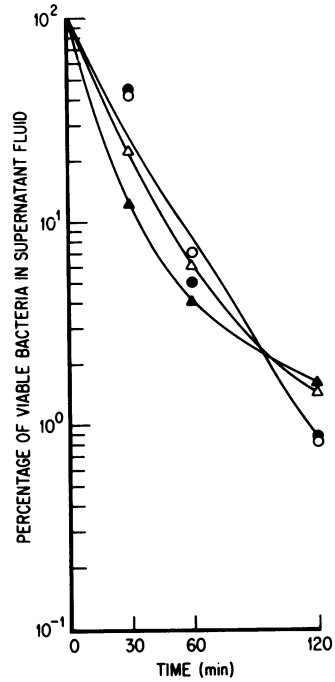

FIG. 6. *Effect of preopsonization on phagocytosis of P. aeruginosa by mouse peritoneal macrophages. Before their addition to macrophages, bacteria were opsonized under the following conditions: incubation at 37°C for 1 h and then 18 h at 4°C (▲), incubation at 37°C for 1 h and then 18 h at 4°C and two washings in PBS (●), and incubation at 37°C for 30 min (△). As a control, bacteria and anti-GLP serum were held separately at 37°C for 1 h and then 18 h at 4°C before being added separately to the macrophages (○). Other experimental details were as described in the text.*

studies that have been reported (4, 13, 16) have had to rely on heterogeneous antigen mixtures for the preparation of antibody in examining the role of cellular and humoral factors in resistance to experimental *Pseudomonas* infection. Although one can explore general aspects of the host response by using of crude antigenic preparations, the factors in *P. aeruginosa* invasiveness and host resistance can be fully understood only by studying purified components of the interaction.

GLP extracted and purified from the exopolysaccharide slime of *P. aeruginosa* has been identified as the cellular faction responsible for a number of biological activities of whole cells (14). Among these activities is the stimulation of antibody capable of protecting against the toxic, leukopenic, and lethal effects of viable cells in experimental infection. The results presented in this report confirm still further the efficacy of GLP in mediating biologically active responses to whole viable cells, and they suggest a mode of action that may determine successful host resistance within the experimental conditions.

Our results have indicated that although few viable *P. aeruginosa* interacted and became associated with macrophages in the absence of specific antibody, significantly more viable cells became macrophage associated in the presence of anti-GLP serum. Similar results were obtained by Bjornson and Michael (4), who used murine peritoneal macrophages and human antisera produced with a polyvalent vaccine prepared from lipopolysaccharides of *P. aeruginosa*, and by Reynolds and Thompson (13), who used rabbit alveolar macrophages and rabbit antiserum produced against a *P. aeruginosa* whole-cell vaccine. The present findings represent the first demonstration that macrophages can interact with *P. aeruginosa* in the presence of antiserum raised to a purified antigen, namely, GLP.

In our system, complement did not appear to be necessary for the association of the macrophages with *P. aeruginosa*, nor for the subsequent bactericidal activity exhibited by the macrophages, nor for the enhancement of these ac-

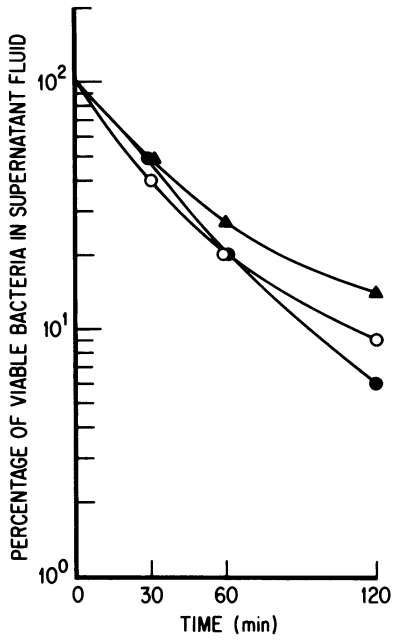

FIG. 7. *Effect of the bacterium-to-macrophage ratio on the phagocytosis of P. aeruginosa by mouse peritoneal macrophages. Concentrations of bacteria were varied to establish bacterium-to-cell ratios of 1: 30 (●), 10:1 (▲), and 30:1 (○). Other experimental details were as described in the text.*

tivities. Little difference in macrophage association was noted when heated (56°C, 30 min) serum was compared with unheated serum or when known amounts of complement were added to the reaction mixture. Complement, in the absence of anti-GLP serum, did not promote phagocytosis, as other investigators have observed (4, 18). In the absence of macrophages, anti-GLP serum and complement exhibited no bactericidal activity. This was expected, since the resistance of most strains of *P. aeruginosa* to specific antiserum has been well documented (18). However, macrophages in the presence of heated anti-GLP serum effectively ingested and killed *P. aeruginosa*. Similarly, a study by Reynolds and Thompson (13) showed that the addition of complement to the assay system did not enhance phagocytosis by alveolar macrophages. Bjornson and Michael (4) also found that immunoglobulin opsonins were required for bactericidal activity of mouse macrophages but that heat-labile serum factors were not.

Some investigators have found that macrophages ingest bacteria in the absence of specific antibody. Thomas et al. (16), using heat-killed *P. aeruginosa*, reported a high rate of phagocytosis of radiolabeled organisms by monolayers of mouse peritoneal macrophages in the presence of 10% heated (60°C, 2 h) calf serum. Cohn (7), using normal unheated serum, observed phagocytosis and killing of bacteria by macrophages; however, he observed little or no phagocytosis in the presence of heated serum (56°C, 30 min). No attempt was made in either study to examine the possibility that natural antibodies might be present in the serum used. Bjornson and Michael (5) found that phagocytic activity associated with normal sera could be removed by absorption with the test bacterium at low temperatures. This indicated the presence of natural antibodies to *P. aeruginosa* in these sera, and specific agglutination reactions confirmed that anti-*P. aeruginosa* immunoglobulins were indeed present.

The influence of preopsonization on the kinetics of phagocytosis by granulocytes has been studied from some microorganisms (11). Although a similar study for *P. aeruginosa* has yet to be done, this investigation has touched on several related factors. We have shown that prolonged periods of preopsonization were not required for maximal association of *P. aeruginosa* with macrophages. In other words, at the serum concentration (2%) used, the experimental procedure of mixing bacteria, serum, and macrophages at zero time was not rate limiting for phagocytosis by 60 min. We did not exclude the possibility that the initial rate of bacterial cell-macrophage interaction is faster when the bacterial cell is preopsonized. In fact, our results indicate, although inconclusively, that such may be the case. Other experiments indicated that the concentration of serum was a limiting factor for the attachment of *P. aeruginosa* to the macrophages; dilution beyond a minimal concentration of 2% resulted in sharply decreased association. However, in the wide range tested, the bacterium-to-macrophage ratio did not appear to be a critical factor for association. At ranges from 10:1 to 1:30 bacterium to macrophage, only an 8% difference separated the highest from the lowest number of macrophage-associated bacteria. These results were similar to those of other investigators (3). Given the presence of a minimum level of anti-GLP serum, the insensitivity of this system to potentially limiting factors suggests that it might contain the elements of a defensive response under the more diffuse conditions of in vivo infection. Anti-slime serum has a demonstrated effectiveness in protecting mice against experimental *P. aeruginosa* infection (1). The key to this protection may reside in a vigorous macrophage defense mediated by serum opsonins directed against GLP.

The experiments presented here focused on the factors related to the early critical events of phagocytic process, i.e., attachment or associa-

tion of the bacterial cells to macrophages, while only touching on the bactericidal aspects of this in vitro system. More fundamentally, these experiments provide supporting data for the hypothesis that the GLP of *P. aeruginosa* is the cellular component of central importance in establishing the experimental infection and invoking an effective defense against it.

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