

## Colonial Dissociation and Susceptibility to Phagocytosis of *Pseudomonas aeruginosa* Grown in a Chamber Implant Model in Mice

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*Pseudomonas aeruginosa* strains were grown in 1-cm plastic chambers sealed at both ends with porous Millipore filters and implanted in the peritonea of mice. Mucoïd and nonmucoïd strains of *P. aeruginosa* isolated from a patient with cystic fibrosis largely retained their phenotypes when grown for up to 1 year in this in vivo system, although colonial dissociation occurred, as observed in chronic lung infections of patients with cystic fibrosis. In the absence of added opsonins, *P. aeruginosa* M2 cells taken directly from the in vivo system were significantly more susceptible to phagocytosis than were the same *P. aeruginosa* cells after being washed in buffer. Phagocytosis of in vivo-grown *P. aeruginosa* cells could be further enhanced by using a porin protein F-specific monoclonal antibody.

Although the properties of pathogenic bacteria are often studied after in vitro growth, there is evidence to suggest that an organism may differ in terms of its growth rate (4), envelope composition (14), and serum sensitivity (7) when grown in vivo. Day et al. (4) developed a simple model for the in vivo growth of organisms whereby bacteria, contained within 1-cm-long plastic chambers sealed at both ends with porous membrane filters (Millipore Corp.), could be grown in the peritonea of laboratory animals and easily recovered for subsequent examination. This chamber implant model, originally developed for the in vivo growth of *Staphylococcus aureus* (4), has been used for the in vivo growth of *Escherichia coli* (7, 9), *Streptococcus* spp. (6), and *Bordetella pertussis* (3). We have now used this chamber implant model for the growth of *Pseudomonas aeruginosa* in vivo.

Chambers (1 cm by 7 mm), constructed as described by Day et al. (4), were inoculated with 500 to 5,000 CFU of *P. aeruginosa* and sealed at both ends with 0.22- $\mu$ m Millipore filters. Two chambers per mouse were inserted into the peritonea (4) of 3-month-old male or female BALB/c or B6D2 (F<sub>1</sub>) mice. The *P. aeruginosa* strains used for growth experiments included strains 3F.9M (mucoïd) and 3F.9NM (nonmucoïd), obtained from the sputum of a patient with cystic fibrosis; M2, a strain traditionally used for mouse pathogenicity studies (16); and our laboratory wild-type strain H103 (10).

For growth experiments, mice were killed by cervical dislocation at regular intervals throughout the experimental period and the chambers were removed. The chamber contents were extracted with a Pasteur pipette after removal of one of the filter paper ends. Viable counts were performed on the chamber contents by using tenfold serial dilutions and plating 100- $\mu$ l quantities on duplicate nutrient agar plates. Colonial morphology was assessed by the method of Philips (13) after overnight incubation at 37°C followed by overnight incubation at room temperature on nutrient agar and cetrimide agar plates.

*P. aeruginosa* 3F.9M (Fig. 1) and 3F.9NM (data not shown) displayed a typical bacterial growth curve when grown in chambers implanted in the mouse peritoneum, with recognizable lag, logarithmic, and stationary phases. Doubling times of 51 and 47 min were recorded for strains 3F.9M and 3F.9NM, respectively, during logarithmic-phase growth. Maximum viable counts of  $\geq 10^9$  CFU/ml were reached after 24 h of growth in vivo, and viable cells could still be recovered at a concentration of  $\geq 10^7$  CFU/ml after 9 to 12 months in the mouse peritonea. Growth curves similar to those shown in Fig. 1 were demonstrated for *P. aeruginosa* strains H103 and M2 during the first 2 weeks of in vivo growth (data not shown). The extension of the growth curve beyond 6 months relied on very few experimental points (Fig. 1). This was caused by a breakdown in the integrity of some chambers after about 6 months in vivo such that the filters came unstuck from the chamber barrel, allowing *P. aeruginosa* to leak into the mouse peritonea and cause peritonitis. Thus, after 6 months, 7 of the 40 mice prepared at the beginning of these experiments developed peritonitis associated with leaky chambers, whereas prior to 6 months, only 1 mouse developed peritonitis. These leaky chambers were not considered when the growth curve was drawn. An additional three mice died of unknown causes during these experiments. Only one of these deaths occurred during the first 6 months after chamber implantation.

Although the mucoïd colony type was largely retained in the *P. aeruginosa* 3F.9M culture growing in vivo, nonmucoïd dissociants emerged after 1 week (Fig. 1). The mucoïd and nonmucoïd colony types continued to be maintained alongside one another for the full 1-year period of the experiment. Nonmucoïd dissociants were represented by both typical and coliformlike colony types. Similar dissociation from the typical into the coliformlike colony type occurred for the *P. aeruginosa* 3F.9NM culture growing in vivo, with dissociants from typical to coliformlike bacteria first observed after 6 weeks of growth and thereafter constituting between 10 and 100% of the cells present in chambers.

The properties of retention of mucoïd phenotype by strain 3F.9M growing in the mouse peritoneum and of colonial

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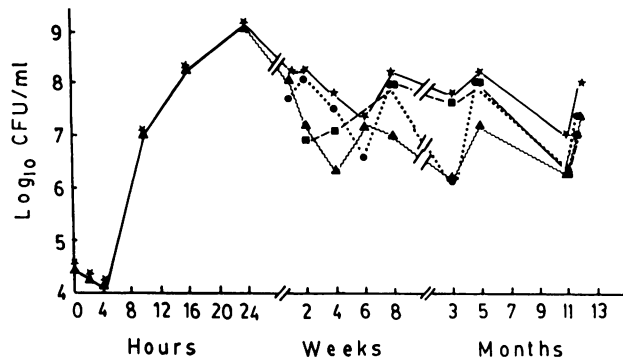


FIG. 1. Emergence and growth of mucoid (▲), typical (●), and coliformlike (■) colony types in *P. aeruginosa* 3F.9M culture growing in vivo and total bacteria recovered (★) at indicated intervals.

dissociation by both mucoid and nonmucoid strains have clinical parallels in patients with cystic fibrosis who have chronic lung infections. The mucoid phenotype of *P. aeruginosa* is exceedingly rare in clinical isolates, except in the case of patients with cystic fibrosis, among whom it is frequently the major isolate (5). Mucoid *P. aeruginosa*, when isolated from the sputum of patients with cystic fibrosis, is usually accompanied by the simultaneous isolation of a nonmucoid variant having the same serotype and pyocin type (2, 17, 19). The suggestion is that colony-type dissociation is occurring in the lower respiratory tract of these patients (19). Zierdt and Schmidt (18) demonstrated the ability of a single strain of *P. aeruginosa* to dissociate into different colony types when grown on laboratory media. Reports have also been made on colonial dissociation in primary cultures of *P. aeruginosa* isolated from general clinical specimens (12, 18). The results presented in the present paper demonstrate that colonial dissociation is a property of pure cultures of both mucoid and nonmucoid *P. aeruginosa* growing in vivo. This ability to dissociate into different colony types, a phenotypic variation which presumably represents changes occurring at the level of the cell envelope, may be an important factor in the ability of *P. aeruginosa* to survive in different, and often adverse, environments.

One of the advantages of the chamber implant system is that in vivo-grown bacteria are easily recovered for further studies. Thus, we were able to study in vivo-grown *P. aeruginosa* cells by using an in vitro phagocytosis assay. Phagocytosis of strain M2 grown in vitro in BM2 glucose minimal medium (11) or in vivo in the chamber implant model was performed by a modification of the visual inspection assay described by Speert et al. (15), with the mouse macrophage cell line P388<sub>D1</sub>. We have previously shown, with protein F-specific monoclonal antibodies as opsonins, that this cell line gave results in phagocytosis experiments that were almost identical to those obtained with unelicited mouse peritoneal macrophages (1). Our earlier studies, however, utilized in vitro-grown bacteria and thus did not precisely reflect the situation expected during a *P. aeruginosa* infection. Therefore, in this study we compared in vitro- and in vivo-grown isolates of strain M2 with respect to their susceptibility to both nonopsonic and opsonic phagocytosis by the macrophage cell line P388<sub>D1</sub>. As stated above, the mode of growth of strain M2 in the chamber implant model was similar to that of strains 3F.9M and 3F.9NM (data not shown). For the experiments described below, strain M2

was grown in chambers for 3 days on three separate occasions.

Bacteria were washed twice in phagocytosis buffer by centrifugation and suspended in phagocytosis buffer. Bacteria were then added to a P388<sub>D1</sub> monolayer to give a bacteria/macrophage ratio of 20:1. Monoclonal antibody MA5-8, specific for protein F (titer of 10<sup>8</sup>) (10), was added at 10% of the assay volume. The suspensions were then incubated at 37°C for 90 min in a CO<sub>2</sub> atmosphere. Following incubation, cells were scraped from the dish with a rubber policeman and suspended by gentle pipetting. Aliquots of this suspension were cytocentrifuged onto glass slides and stained with Diff-quick (Canlab, Vancouver, British Columbia, Canada) for viewing under oil at a magnification of ×1,000. The number of bacteria in each of 60 cells was counted in each of three independent experiments, and statistical analyses were performed by using Student's *t* test.

Unwashed in vivo-grown bacteria showed significantly higher phagocytosis ( $P < 0.01$  by Student's *t* test) than unwashed in vitro-grown bacteria (Fig. 2). This increased phagocytosis seemed to be due to loosely bound, naturally occurring opsonins picked up by strain M2 during growth in the peritoneal chambers, since washing the in vivo-grown cells twice in phagocytosis buffer caused a significant ( $P < 0.01$ ) threefold decrease in nonopsonic phagocytosis (Fig. 2). Washing the in vitro-grown bacteria twice with phagocytosis buffer resulted in a small, insignificant alteration ( $P > 0.5$ ) in phagocytosis. A single experiment with strain H103 resulted in similar data (not shown).

Experiments performed with the outer membrane protein F-specific monoclonal antibody MA5-8 resulted in a significant increase ( $P < 0.01$ ) in phagocytosis of both in vitro-grown and in vivo-grown strain M2 cells compared with unopsonized cells (Fig. 2), thus demonstrating that in vivo-grown strain M2 contained surface-accessible protein F epitopes. It was interesting to note that the average increase in bacteria per macrophage resulting from opsonization appeared lower for in vivo-grown (1.6 to 2.4 bacteria per macrophage) than for in vitro-grown (2.4 to 4.0 bacteria per

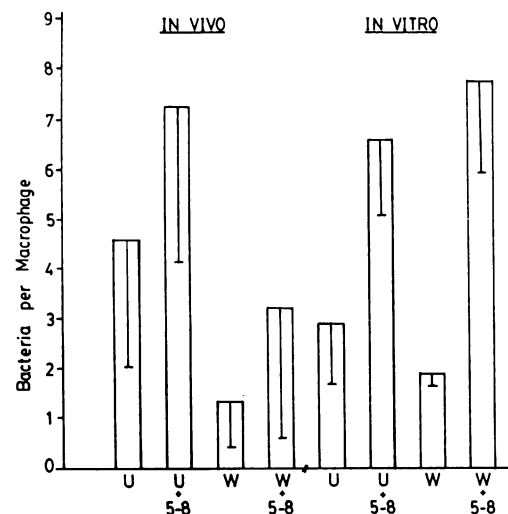


FIG. 2. Phagocytosis by mouse macrophage cells P388<sub>D1</sub> of in vivo-grown and in vitro-grown *P. aeruginosa* M2 cells, washed (W) and unwashed (U), with (+5-8) and without the addition of anti-protein-F monoclonal antibody MA5-8. Each column represents the arithmetic mean (with standard deviation shown by the descending error bar) of the uptake observed in three independent experiments.

macrophage) cells, regardless of whether they were unwashed or washed. This could be explained if the degree of surface exposure of protein F was somewhat reduced in the *in vivo*-grown cells or, alternatively, if there was incomplete masking of protein F sites by *in vivo*-acquired material.

Previously published work from this laboratory demonstrated the ability of a monoclonal antibody raised against *P. aeruginosa* outer membrane protein F to protect mice against lethal infections with *P. aeruginosa* M2 (8). The results presented in the present paper agree with this earlier work, suggesting that this protection may be related to an increase in phagocytosis of anti-F-coated *P. aeruginosa* cells. Furthermore, anti-F monoclonal antibody appears to enhance phagocytosis of *in vivo*-grown *P. aeruginosa* by acting synergistically with naturally occurring opsonin(s). Further studies in our laboratory are aimed at the identification of this naturally occurring opsonin(s).

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