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Virulence and the Role of Iron in *Pseudomonas aeruginosa* Infection

J. J. BULLEN, C. G. WARD,[1] AND S. N. WALLIS
National Institute for Medical Research, Mill Hill, London, NW7 1AA, England

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The virulence of *Pseudomonas aeruginosa* can be enhanced by passage in mice or rabbits. Enhanced virulence has some specificity for the host in which the passage is done. Experimental infection in the peritoneal cavity of cannulated rabbits has shown that the injection of iron compounds can lead to a rapid and fatal growth of an otherwise nonlethal dose of bacteria. In vitro the unsaturated iron-binding proteins present in the peritoneal fluid can halve the growth rate of *P. aeruginosa*. The restricted rate of growth is restored to normal if the iron-binding proteins are saturated with iron. Exactly the same results are achieved with purified transferrin. Both fatal and nonfatal infections with *P. aeruginosa* cause a sharp fall in the percentage of saturation with Fe of the plasma and peritoneal fluid. In both normal and infected animals the peritoneal fluid is invariably less saturated than the plasma. Specific antiserum not only protects against death but also against the fall in iron saturation of the plasma and peritoneal fluid. In both fatal and nonfatal infections a high proportion of viable bacteria are unphagocytized in the peritoneal cavity.

The mechanisms controlling resistance to *Pseudomonas aeruginosa* are by no means yet understood. The frequent association of the organism with dibilitating conditions such as burns (24) or immunosuppressive treatment (21) strongly suggests that it is uniquely equipped to take advantage of a decline in natural resistance. In this paper we have examined the effect of passage on virulence, as well as the influence of iron compounds on resistance. Both these factors have a significant effect on the outcome of infection.

One of the technical problems of work of this kind is to be able to infect animals and to remove samples repeatedly without causing injury, which, in itself, can enhance infection. With intraperitoneal infections this difficulty can be overcome by the use of permanent cannulae inserted 4 to 10 days beforehand.

MATERIALS AND METHODS

P. aeruginosa strain 2171. The organism was isolated from a hospital patient and was obtained from the Cross Infection Laboratory, Central Public Health Laboratories, Colindale, London. Cultures 4- to 6-h old were used for all the experiments.

Method of passage and storage. The methods used for passage in mice have been described previously (12). For passage in rabbits about 10^9 bacteria were injected intraperitoneally and the peritoneal exudate was harvested 7 h later.

P. aeruginosa antiserum. Antiserum against *P. aeruginosa* 2171 was prepared in a pony by twice weekly intravenous injections of formalized or live cultures over a period of 3 months.

Human transferrin. A sample of purified human transferrin was obtained from Henry J. Rogers (25). Its iron binding capacity was as follows: saturated iron-binding capacity (SIBC) 1.4×10^{-4} M; unsaturated iron-binding capacity (UIBC) 1.67×10^{-4} M (45% saturated with Fe).

Hematin hydrochloride. Sterile hematin powder was prepared as described previously (3).

Ferric ammonium citrate. This contained 20% (wt/wt) iron estimated colorimetrically (10). The citrate was dissolved in 0.85% saline and sterilized by autoclaving.

Human red cells. Human O group cells were suspended in sterile saline and washed 6× by centrifugation. They were then resuspended in saline to give a 50% suspension (vol/vol).

Polymorphonuclear leukocyte lysosome extract. The extract from the cells was prepared by the method of Cohn and Hirsch (9). It contained 0.5 mg of protein per ml and the pH value was approximately 7.0.

Measurement of LD_{50} in mice. Groups of 10 TO mice (NIMR) weighing 23 to 25 g each were injected intraperitoneally with 10-fold dilutions of culture. Analysis of the results was done by converting the proportion of deaths at each dose to a probit and fitting a probit regression line (11). The mean lethal dose (LD_{50}) and 95% fiducial limits were calculated from this line.

[1] Present address: Department of Surgery, University of Miami School of Medicine, Jackson Memorial Hospital, Miami, Fla. 33136.

Measurement of LD_{100} in rabbits. The LD_{100} was measured by injecting groups of two animals intraperitoneally with dilutions of the organism in saline. The LD_{100} was taken as the smallest dose which killed both animals in 3 days.

Peritoneal cannula. The cannula was made of Delrin (Du Pont de Nemours, Del.) and had a removable perspex plug (Fig. 1 and 2). The barrel was 17 mm long with a 16-mm flange at the bottom. The internal diameter was 5 mm. The perspex plug (21 mm long) projected 3 mm beyond the end of the cannula. The two hexagonal locking nuts when screwed tightly together prevented any movement of the barrel. The cannula was sterilized with 0.05% chlorhexidine gluconate (Hibitane, ICI Pharmaceuticals, Cheshire, England). It was then washed with sterile saline.

The cannulae were well tolerated. Within a few days the peritoneal endothelium had grown over the flange. Complete occlusion of the cannula opening was prevented by the projecting perspex plug. The internal aspect of the abdominal wound was covered by peritoneal endothelium in a day or two. The optimum time for experimentation was 4 to 14 days. After this time there was an increasing tendency for the peritoneal endothelium to grow over the projecting plug.

Samples of peritoneal exudate. Under anaesthesia 30 ml of warmed heparinized saline (0.15 mg of heparin per ml) was introduced via the cannula. The abdomen was gently kneaded for 1 min. A perforated tube was then introduced and 10 ml of exudate was removed.

Viable counts of P. aeruginosa in exudate. A 1-ml amount of exudate was homogenized at 50,000 rpm for 30 s in a Sorvall micro homogenizer (5 ml) (Ivan Sorvall, Norwalk, Conn.) cooled with ice. This disrupted at least 90% of the tissue cells present. This material was used for the total viable count. A 6-ml amount of the original exudate in a 15-ml siliconized conical centrifuge tube was centrifuged at $100 \times g$ for 10 min. This deposited all the tissue cells. A 1-ml amount of supernatant from the top of the tube was taken for the 'extracellular' count. Viable counts were done by the methods described previously (6).

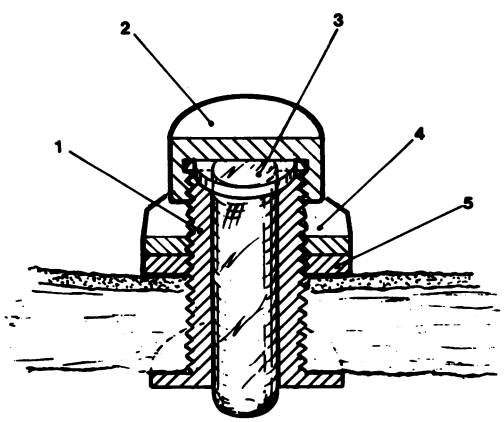

FIG. 1. *Diagram of peritoneal cannula inserted in abdominal wall. (1) Barrel, (2) cap, (3) perspex plug, (4, 5) locknuts.*

Growth of P. aeruginosa in peritoneal fluid in vitro. Peritoneal fluid was obtained by washing out the peritoneal cavity with 200 ml of heparinized Hanks solution. Cells were removed by centrifugation and the supernatant was concentrated by ultrafiltration under nitrogen by using a Sartorius high pressure filter no. 16208 with membrane no. 12136 (Sartorius, Göttingen, West Germany) to give a protein content of 10 to 15 mg per ml. The concentrated material was sterilized by membrane filtration. Before use Na HCO_3 was added to a final concentration of 0.2%. The experiments were done by the methods described previously (5). With 5% CO_2 in the gas phase the pH value of the fluid was 7.4 at 37 C.

Measurement of iron-binding capacity of plasma and peritoneal fluid. The concentrated peritoneal fluids and plasma samples were centrifuged at 1,500 $\times$ *g* or more for 30 min before measurement of the UIBC. The UIBC and the SIBC were measured by the methods described previously (5) except that in the case of the SIBC 4:7 diphenyl-1:10-phenanthoroline in amyl alcohol was used to estimate the ferrous iron (23).

Measurement of protein. Protein in plasma and peritoneal fluid was measured by the method of Warburg and Christian (29).

RESULTS

Effects of passage. Previous observations by Forsberg and Bullen (12) showed that repeated passage of the original strain in mice led to a fall in LD_{50} from 1.26×10^6 to 4.1×10^4 (MP4). Continued passage of MP4 reduced the LD_{50} to the remarkably low figure of 10^1. However, this figure cannot be considered for comparison since it was subsequently shown that the 'conventional' mice which were used for the test were more susceptible to infection than the 'specific pathogen free' (SPF) mice used later. All the results given below were obtained with SPF mice. (The 95% fiducial limits are given in brackets.) The original strain had an LD_{50} for SPF mice of 9.35×10^5 (3.9×10^5 to 2.3×10^6) and an LD_{100} for rabbits of 10^9. After 20 passages of MP4 in rabbits the culture (E59/20) had a mouse LD_{50} of 8.8×10^2 (3.7×10^2 to 2.14×10^3) and for the rabbit a LD_{100} of 10^7. After a further 20 passages in rabbits the culture (E59/40) had a mouse LD_{50} of 1.5×10^5 (6.15×10^4 to 3.57×10^5) but the rabbit LD_{100} remained at 10^7. Thus, continued passage in rabbits led to a loss of virulence for mice but the enhanced virulence for rabbits was maintained. These results confirm those of Forsberg and Bullen (12) and show that the virulence of *P. aeruginosa* can be greatly influenced by passage. They also suggest that repeated passage in

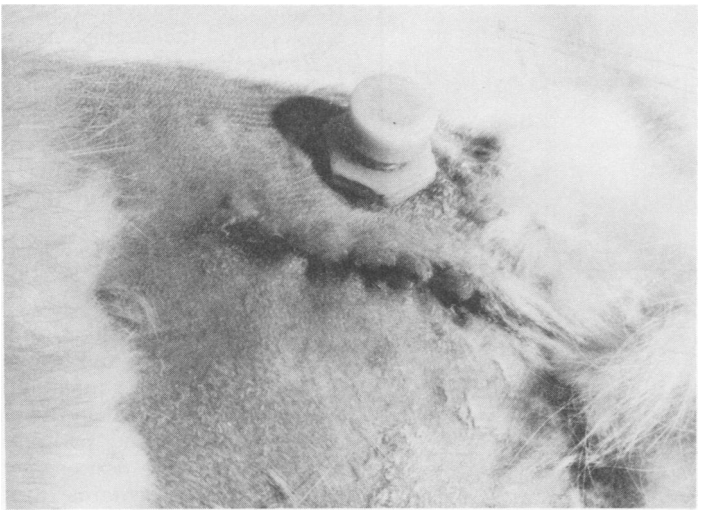

FIG. 2. *Peritoneal cannula in rabbit 2 days after insertion.*

one species can lead to loss of virulence for another.

Cellular response to peritoneal infection. Intraperitoneal infection with *P. aeruginosa* is accompanied by a massive exudation of polymorphonuclear leukocytes. Figure 3 shows the polymorph response in nonlethal, lethal, and a lethal infection induced with a normally sublethal dose plus hematin. There was no significant difference in the response from the three different types of infection. In all cases there was comparatively little change in the mononuclear cell population.

Effect of iron compounds on cultures of different virulence. Immediately after injection of 2.3×10^8 of the original strain there was a rapid fall in the viable count (Fig. 4). Initially, the total count was slightly higher than the extracellular count, but both were similar by 3.5 h and remained so for the rest of the experiment. After the count had fallen to approximately 10^4 per ml it persisted for a long time (Fig. 4).

In contrast to the original strain the passaged culture (E59/40) grew rapidly when slightly less organisms (1.5×10^8) were injected. The animal died with a massive infection (Fig. 4). In the majority of the samples the extracellular count was similar to the total count.

Injection of a sublethal dose (2.7×10^6) of the original strain with enough ferric ammonium citrate to give 5 mg of iron per kg (live weight) had no untoward effect although the organisms persisted as usual (Fig. 5). The same dose (2.7×10^6) of the passaged strain (E59/40) with the same amount of ferric ammonium citrate led to rapid bacterial growth and death (Fig. 5). A similar dose of the passaged strain without added iron failed to cause death (Fig. 5).

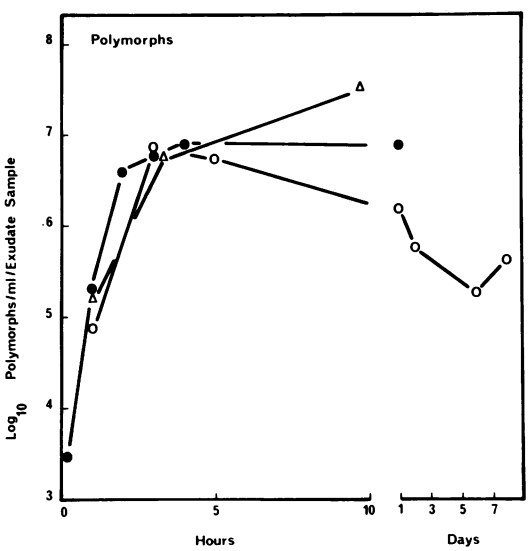

FIG. 3. *Polymorphonuclear leukocytes in peritoneal exudate after P. aeruginosa infection. Symbols:* ●, *lethal infection; original strain;* ○, *nonlethal infection, strain E59/20;* △, *lethal infection E59/40 (with hematin).*

Heme compounds were equally effective in stimulating the growth of the passaged strain, but failed to stimulate the growth of the original strain. In the experiments shown in Fig. 6 the animals received 10 ml of a 50% suspension of washed human red cells. The hemoglobin released by the slowly lysing red cells was 3.4 mg per ml in the sample taken at 1 h. This fell to 2 mg per ml at 6 h but there was still 0.8 mg

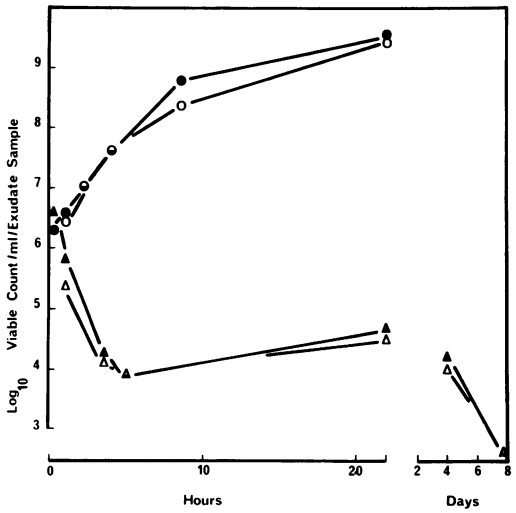

FIG. 4. *P. aeruginosa in peritoneal cavity. Symbols: ▲, original strain total count; △, extracellular count; ●, E59/40 total count; ○, extracellular count.*

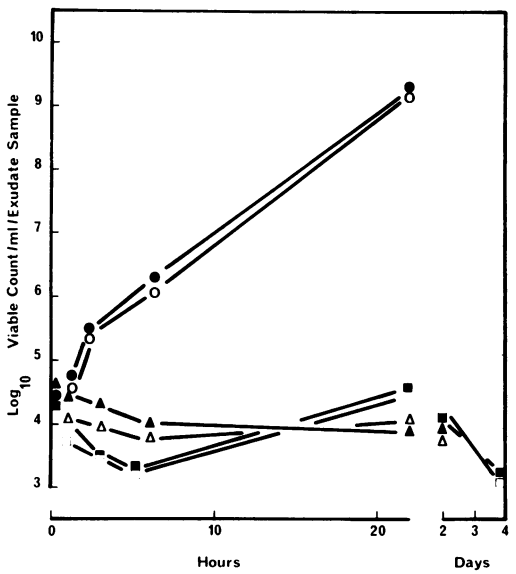

FIG. 5. *P. aeruginosa in peritoneal cavity plus ferric ammonium citrate (5 mg of Fe per kg of live weight). Symbols: ▲, original strain, total count; △, extracellular count; ●, E59/40 total count; ○, extracellular count; ■, E59/40 (control) without added Fe; □, extracellular count.*

per ml at 24 h. Simultaneous injection of 1.4×10^6 of the passaged strain with the red cells resulted in death at 24 h (Fig. 6). In similar circumstances the injection of 9×10^6 of the original strain had no effect (Fig. 6). Very similar results were obtained with animals receiving 1 g of hematin with the culture intraperitoneally. Those given 1.7×10^6 organisms of the passaged strain died; those given 2.5×10^6 organisms of the original strain survived.

Protective effect of specific antibody. A 2-ml amount of antiserum 2171 given intravenously 18 h previously completely protected against 7×10^9 of the passaged strain E59/40. In the experiments shown in Fig. 7 the unprotected animal received 1.5×10^8 bacteria and the protected animal 2.4×10^8 bacteria. In the protected animal there was a large uptake of bacteria by phagocytic cells immediately after injection but after 6 h there was a high proportion of extracellular organisms which persisted for a long time. In another experiment it was shown that 2 h after infection 99% of the total viable bacterial count in the body (lungs, liver, spleen, kidneys, and peritoneal exudate) was still present in the peritoneal cavity (Table 1).

Iron-binding proteins in plasma and peritoneal fluid during infection. In the normal rabbit before infection or administration of antiserum it was found that the peritoneal fluid was invariably less saturated than the plasma (Table 2). If the plasma and peritoneal fluid were compared on the basis of the same concentration of protein it was found that the total iron-binding capacity (TIBC) of the peritoneal fluid was invariably greater than the plasma.

The mean total protein concentration in the plasma of four of our normal rabbits was 57.7 mg per ml. The mean total protein concentration in the peritoneal fluid of five normal rabbits given by Maurer et al. (19) was 18.4 mg per ml. This means that the plasma contains

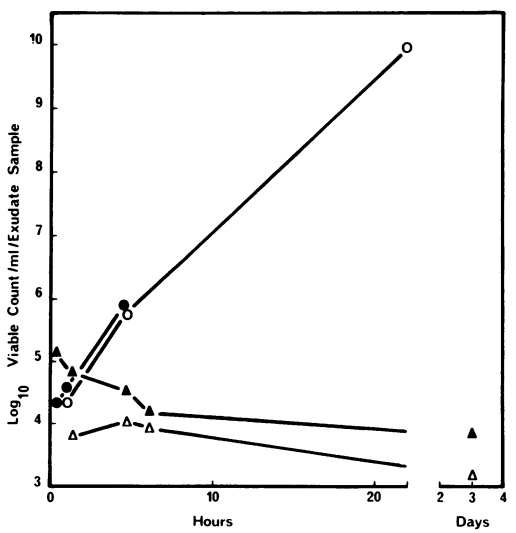

FIG. 6. *P. aeruginosa in peritoneal cavity plus human red cells. Symbols: ▲, original strain, total count; △, extracellular count; ●, E59/40 total count; ○, extracellular count.*

about three times the amount of protein as the peritoneal fluid. If the data in Table 2 are taken into account the peritoneal fluid in vivo has a TIBC about the same or slightly less than plasma. Both lethal and nonlethal infections produced a rapid and persistent fall in the percentage of saturation of the plasma and peritoneal fluid, but the peritoneal fluid was invariably less saturated than the plasma (Table 2). Specific antibody had a striking protective effect since neither the percentage saturation with iron nor the total iron binding capacity of the plasma and peritoneal fluid was altered during infection (Table 2).

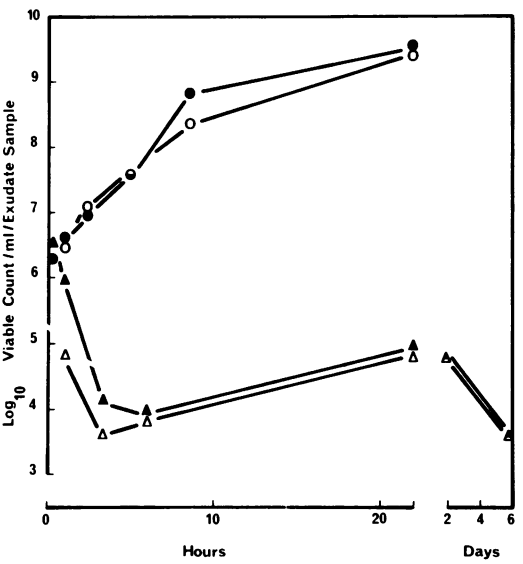

FIG. 7. *P. aeruginosa in peritoneal cavity. The effect of protective antibody. Strain E59/40. Symbols: ●, total count without antibody; ○, extracellular count; ▲, total count with antibody; △, extracellular count.*

Growth of P. aeruginosa in peritoneal fluid, transferrin, or broth in vitro. The peritoneal fluid (Table 3) was obtained from a rabbit infected with *P. aeruginosa* 6 days before. After concentration it contained 16 mg of protein per ml and was 34% saturated with iron. The organisms grew relatively slowly in this material but just over twice as fast when it was saturated with iron. Peritoneal fluids from normal or passively immunized animals gave similar results. The human transferrin (Table 3) was 46% saturated with iron and was added to ¼ strength 199 medium containing 0.2% Na HCO_3 (5). The rate of growth in the transferrin solution was identical to that obtained with the peritoneal fluid. Saturating the transferrin with

TABLE 1. *Distribution of P. aeruginosa in protected animals 2 h after infection*[a]

Material from rabbit	*P. aeruginosa* viable count (×10⁶)		Percentage of total body count
	Count/g	Total	
Liver	0.1	7.0	0.33
Spleen	7.0	9.5	0.45
Lungs	0.005	0.06	0.003
Kidneys	0.008	0.15	0.007
Peritoneal exudate	42	2100	99.2

[a] Intraperitoneal dose, 6.4 × 10⁹.

TABLE 2. *Iron-binding capacities of plasma and peritoneal fluids (PF) of infected and protected animals*[a]

Infection	Sample	Hours after infection	Plasma and peritoneal fluid (10 mg of protein/ml)			Saturation with Fe (%)	TIBC peritoneal fluid/plasma ratio
			SIBC 10^{-6} M Fe	UIBC 10^{-6} M Fe	TIBC 10^{-6} M Fe		
Sublethal	Plasma	0	5.0	2.0	7.0	71	3.4
	PF		11.6	12.5	24.1	48	
	Plasma	7	5.9	4.9	10.8	55	3.0
	PF		9.1	23.7	32.8	28	
Lethal	Plasma	0	13.1	2.9	16.0	82	1.7
	PF		19.3	8.6	27.9	69	
	Plasma	7	3.9	6.0	9.9	39	1.6
	PF		5.2	11.0	16.2	32	
Protected with antibody	Plasma	0	9.7	2.4	12.1	80	2.3
	PF		10.3	17.3	27.6	37	
	Plasma	7	9.7	2.7	12.4	78	1.9
	PF		8.7	14.4	23.1	38	

[a] Data calculated for samples adjusted to same protein concentration, 10 mg/ml.

TABLE 3. *Growth of P. aeruginosa in peritoneal fluid, transferrin, or broth in vitro*

Culture fluid	*P. aerguinosa* generation time (min)
Peritoneal fluid	72
Peritoneal fluid + Fe	34
Transferrin + 199 medium	72
Transferrin + 199 medium + Fe	34
Broth	29

iron gave a rate of growth identical to that obtained with the peritoneal fluid saturated with iron. Organisms cultured in broth under the same conditions grew slightly faster than those in peritoneal fluid plus iron, or transferrin plus iron (Table 3).

Experiments with leukocyte lysosomal extract. At pH 7.4 and with 5% CO_2 in the gas phase (5) the passaged strain of *P. aeruginosa* was rapidly destroyed by undiluted lysosomal extract at 37 C. However, the lysosomal extract was largely ineffective in the presence of peritoneal exudate which had been added to give a final concentration of 2.2 mg of protein per ml. It was clear, therefore, that although the lysosomal extract by itself was bactericidal against *P. aeruginosa* it was rapidly inactivated in the presence of peritoneal fluid.

DISCUSSION

It is well recognized that the virulence of *P. aeruginosa* for experimental animals can vary considerably (8, 12, 15, 16, 18). However, apart from the experiments of Forsberg and Bullen (12), little or nothing has been done about the effect of passage on the virulence of this organism. This could be of more than academic interest since there are many reports to show that *P. aeruginosa* can be passed from patient to patient within hospitals (17, 26, 28). If this should occur frequently it seems quite possible that the virulence for man could be enhanced.

The work reported here has confirmed the initial observations of Forsberg and Bullen (12) that the virulence of *P. aeruginosa* can be unusually labile when compared with some other gram-negative bacteria. With *Escherichia coli*, for example, repeated passage for several years entirely failed to enhance the virulence of these organisms, although their initial virulence was preserved (3). One rather suprising result was that the *P. aeruginosa* virulent for mice became far less so after repeated passage in rabbits. This tendency towards host specificity suggests that tests for virulence in experimental animals may have little relevance to the pathogenicity of these organisms for man.

Previous studies with many different species of bacteria had shown that saturating the iron-binding capacity of the serum transferrin or the injection of heme compounds could greatly enhance virulence (3) or abolish passive immunity (2, 7). There is also good evidence that iron-binding proteins play an important role in resistance (4). Nevertheless, the effect of iron varies greatly with different organisms and different hosts. With *E. coli*, for example, the injection of iron compounds can enhance virulence by a factor of 10,000- to 100,000-fold (3). In the case of *P. aeruginosa*, the degree of enhancement by iron is about 1,000-fold in mice injected with a passaged strain (12), but only 10- to 50-fold in rabbits in the present work. Nevertheless, a combination of passage and iron gave an enhancement of virulence for rabbits of at least 1,000-fold.

Infection produced well-marked changes in the iron metabolism of the host. Falls in the plasma iron after infection have been reported before (13, 22). This also occurred in rabbits (Table 2), but it was surprising to find that the peritoneal fluid was invariably less saturated than the plasma (Table 2). According to Morgan (20), the percentage of saturation of the lymph and plasma is approximately the same. Why the peritoneal fluid should be different is not yet known. The total iron-binding capacity of the peritoneal fluid was greater than that of the plasma when calculated on the basis of the same protein concentration (Table 2) but similar to that of plasma when calculated on the basis of the actual concentration of protein in vivo.

Specific antibody not only protects against death but also prevents any fall in the percentage of saturation of the plasma or peritoneal fluid (Table 2). Since bacterial endotoxin can cause a fall in the serum iron (1), which is mediated by a substance liberated from leukocytes (14), it appears that antibody also protects against some or all of the effects of endotoxin. Protection against the lethal effects of endotoxin by antiserum has been reported before (27).

One striking feature of the experiments in vivo was the large percentage of bacteria that was unphagocytized. What is the mechanism that inhibits their growth? Iron-binding proteins appear to play some part. Cell-free peritoneal exudates reduce the normal growth rate of *P. aeruginosa* by a half. This is almost certainly due to unsaturated transferrin in the exudate since transferrin itself gave identical results

(Table 3). In both cases normal growth is restored by saturating the iron-binding protein with iron. However, this mechanism does not explain survival of the host, since even the slowly growing organisms would produce an overwhelming infection eventually. One possibility is that the rate of phagocytosis is just adequate to remove enough of the slowly growing bacteria to produce the observed effects and that those organisms that are phagocytized are killed so quickly that they can make no contribution to the total count. This possibility needs further investigation.

In the early stages of sublethal infections (0 to 3 h) there was evidence that a high proportion of bacteria was phagocytized and this particularly applied to the less virulent strain (Fig. 6). During this period, however, the bulk of phagocytosis was due to mononuclear cells whose numbers did not greatly alter throughout the infection. It was also clear that the presence of protective antibody did not increase the number of viable bacteria within the leukocytes in the latter stages of infection (Fig. 7). In addition, it is worth pointing out that although it is obvious that bacteria do escape from the peritoneal cavity, their numbers in terms of the percentage of the total are small, at least during the first few hours (Table 1). Thus, the viable counts from the peritoneal cavity probably do represent a fairly accurate picture of the infection as a whole.

As far as cationic proteins from the polymorphonuclear leukocytes are concerned, it is clear that these can be lethal to *P. aeruginosa* in the right environment, but it was shown that these proteins are rapidly inactivated by peritoneal exudate and could therefore make no contribution to controlling infection if they were liberated into the peritoneal cavity.

In conclusion, it appears that virulence in *P. aeruginosa* can be altered relatively easily and that this can have a profound influence on the outcome of infection. Virulence can be enhanced by iron compounds. Unsaturated iron-binding proteins appear to play a significant role in resistance by slowing down bacterial growth. Specific antibody not only prevents death but also protects against at least one aspect of the effect of endotoxin. Lastly, in rabbits at least, very large numbers of viable bacteria remain extracellular during fatal or nonfatal infections and exactly how their growth is inhibited in resistant animals has yet to be discovered.

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