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The effect of antipolymorphonuclear leucocyte serum on *Pseudomonas aeruginosa* infection in rabbits

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Summary. Studies were made on the rate of phagocytosis and killing of *Pseudomonas aeruginosa* by phagocytic cells in the peritoneal cavity of rabbits. In sublethal and lethal infections the phagocytosed bacteria were killed very quickly. In antibody-protected animals, the polymorphs became loaded with living bacteria, but this had little effect on the decline in infection. In sublethal infections and in protected animals the proportion of intracellular bacteria labelled with ^{32}P or [^{14}C]uracil was high and antibody greatly enhanced phagocytosis. In lethal infections the rate of phagocytosis was insufficient to prevent the development of a fatal septicaemia.

Antipolymorphonuclear leucocyte serum (APS) completely suppressed the normal polymorph response to infection and greatly reduced resistance. The macrophages in the peritoneum, which were not affected by APS, delayed bacterial growth for several hours but were eventually unable to control bacterial multiplication. The outcome of infection appeared to depend almost entirely on the ratio of bacteria to phagocytes and the presence of antibody. Iron-binding proteins probably make a significant contribution to resistance by reducing the rate of multiplication of extracellular bacteria.

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INTRODUCTION

In a previous paper (Bullen, Ward and Wallis, 1974) we reported that the virulence of *Pseudomonas aeruginosa* could be enhanced by passage and by the injection of iron compounds. We also suggested that the presence of unsaturated iron binding proteins in the blood and tissue fluids could make a significant contribution to resistance by slowing down the rate of bacterial growth.

Nevertheless, it is clear that many other factors also contribute to resistance. In particular, there is a massive influx of polymorphonuclear leucocytes into the peritoneal cavity following infection. To explore the role of these cells we have studied the rate of phagocytosis and the rate of killing of *P. aeruginosa in vivo*, as well as the effect of completely suppressing the polymorph response with antipolymorphonuclear leucocyte serum (APS).

MATERIALS AND METHODS

P. aeruginosa

The strain of *P. aeruginosa* was the same as the one used previously (Bullen *et al.*, 1974). It was stored in liquid nitrogen.

P. aeurginosa *antiserum*

This was also the one used previously (Bullen *et al.*, 1974).

Cannulated rabbits
The technique used for cannulating rabbits and the methods used for taking samples and making viable counts of bacteria were those described by Bullen *et al.* (1974).

Ammonium chloride–Tris solution
Nine volumes of 0·83 per cent ammonium chloride (w/v) + 1 volume Tris buffer (pH 7·5, 0·17 M).

Measurement of the total and extracellular viable bacteria in peritoneal exudates
The methods previously described (Bullen *et al.*, 1974) were used. The extracellular count was made on the supernatant fluid after centrifugation of the exudate at 100 *g* for 10 min.

Labelling of P. aeruginosa *with* ^{32}P *or* [^{14}C]*uracil*
The labelling procedure was usually carried out by growing the bacteria in rabbit plasma containing the radioactive label. In some experiments, the plasma was replaced by papain digest broth or peritoneal fluid (Bullen *et al.*, 1974). Normal rabbit plasma (3 ml) containing [^{32}P]orthophosphate (neutralized, 200 μCi) or [^{14}C]uracil (10 μCi) (Radiochemical Centre, Amersham, Bucks) was inoculated with sufficient bacteria to give approximately 10^6 or 10^7 cells/ml, as required, and incubated at 37° for 2½ h. The labelled bacteria were collected by centrifugation at room temperature for 30 min, resuspended in unlabelled rabbit plasma (5 ml) at 37° and grown for a further 45 min to chase the radioactive label into high molecular weight material. The bacterial suspension was again centrifuged and the bacteria resuspended in saline (5 ml) at 37°. They were used immediately.

Measurement of total and extracellular labelled bacteria
The same samples were used for counting the labelled bacteria and making the viable counts. Samples (1 ml) were mixed with 4 ml of cold Na_2HPO_4–NaH_2PO_4 buffer (0·2 M, pH 7·3) in ^{32}P experiments, or with 4 ml of a solution of unlabelled uracil (1 mg/ml) in 0·15 M NaCl in [^{14}C]uracil experiments. These were filtered immediately through 0·45 μm Millipore filters. The filters were washed with 2–4 ml of ice-cold 5 per cent (w/v) trichloracetic acid and then dried for 30 min at 60–65°. All filters were counted in a Beckman LS133 liquid scintillation counter using a scintillation solution containing 4 g of 2,5-diphenyloxazole in 1 l of toluene. Results are expressed as counts per minute (c.p.m.) above background.

Antipolymorphonuclear leucocyte serum (APS)
The antiserum was prepared in a pony. Cells for immunization were obtained from cannulated rabbits (Bullen *et al.*, 1974). The rabbits were injected with 20 ml of 15 per cent nutrient gelatin, and after 18 h a further 50 ml of gelatin was given. Four hours later 200 ml of heparinized Hanks's solution (0·15 mg heparin/ml) was introduced into the peritoneum. The abdomen was kneaded gently for 1 min and the cells removed. After centrifugation the supernatant fluid was discarded and any red cells lysed by suspending the cells in ammonium chloride–Tris buffer. The leucocytes were washed twice in Hanks's solution without heparin.

1–2 × 10^9 Cells were obtained from each rabbit, of which approximately 85 per cent were polymorphonuclear leucocytes. The polymorphs were separated from macrophages and lymphocytes by the technique of Bøyum (1974). 73·5 ml of 32·8 per cent Hypaque (Winthrop Laboratories, Surrey) was mixed with 176·5 ml of 8 per cent Ficoll (Pharmacia, Uppsala, Sweden). Twenty millilitres of this mixture was placed in 50-ml centrifuge tubes and 20 ml of cell suspension containing 5–6 × 10^7 cells per ml was carefully layered on top. The tubes were centrifuged at 400 *g* for 40 min. The supernatant was discarded and the polymorphs resuspended in Hanks's solution to contain 1 × 10^9 cells/ml (*circa* 95 per cent polymorphs). The vaccine consisted of equal parts of this cell suspension and Freund's complete or incomplete adjuvant (Difco).

At weekly intervals the pony received two intramuscular doses in the neck of 8 ml of vaccine made with complete adjuvant and 1 dose of 8 ml of vaccine made with incomplete adjuvant. Five weeks later the animal was given 4 × 10^9 polymorphs intravenously. This caused a severe reaction. The animal was bled 7 days later.

RESULTS

The uptake and destruction of *P. aeruginosa* by exudate cells

Sublethal infections
The animals were given approximately 10^6 bacteria and after 5–20 h when the viable count in exudate

samples was usually about 10^3/ml the labelled organisms (*circa* 10^4) were given via the cannula. Samples were taken 10 and 60 min later and the total and extracellular counts of the viable and labelled bacteria measured.

In three separate experiments the proportion of the total viable count which was intracellular was low (mean 12 per cent), whereas the proportion of intracellular labelled bacteria was high (mean 78 per cent) (Table 1, Fig. 1).

The presence of only small numbers of viable bacteria within the phagocytes, combined with the fact that the living labelled organisms were taken up extremely rapidly suggested that once the bacteria were phagocytosed they were killed very quickly, and that the rate of phagocytosis was relatively high.

Lethal infections

In lethal infections with approximately a hundred lethal doses of bacteria (*circa* 2×10^9) the total number of organisms in the peritoneum was very large, yet the rate of killing of phagocytosed organisms was still good at 1 h after infection. This was shown by the low proportion of viable intracellular organisms (mean 5 per cent) (Table 1). However, the relatively low proportion of labelled intracellular organisms (mean 39 per cent) (Table 1, Fig. 1) showed an apparent inefficiency of the phagocytic process as a whole which was probably related to the relatively high ratio of bacteria to phagocytic cells.

Passively immunized animals

Passively immunized animals received 2·0 ml of specific *P. aeruginosa* antibody (Bullen *et al.*, 1974) intravenously 18 h before infection with a normally lethal dose of $2{\cdot}5–3{\cdot}0 \times 10^9$ bacteria. All the animals survived. One hour after infection the total viable count was only a tenth of that observed in lethally infected animals (Table 1). The uptake of the labelled bacteria (mean 73 per cent) was almost as good as that seen in sublethal infections (Table 1, Fig. 1). This showed that phagocytosis in the presence of specific antibody was extremely efficient. The infection declined fairly rapidly (Fig. 2) but the polymorphs became loaded with living bacteria. This was shown by the relatively high proportion of viable intracellular bacteria (mean 72 per cent) (Table 1).

Table 1. *P. aeruginosa* infection in the peritoneal cavity. Viable counts and counts of ^{32}P- and ^{14}C-labelled bacteria

Infection	Time after infection (h)	Time after labelled bacteria (mins)	Viable bacteria: Total (count/ml)	Viable bacteria: Supernatant (count/ml)	Labelled bacteria: Total count (c.p.m.)	Labelled bacteria: Supernatant count (c.p.m.)	Intracellular bacteria: Viable percent	Intracellular bacteria: Labelled percent
Sublethal	20	10	$5{\cdot}0 \times 10^5$	$5{\cdot}0 \times 10^5$	148,050(^{32}P)	66,780	0	55
		60	$7{\cdot}0 \times 10^5$	$5{\cdot}4 \times 10^5$	27,682	6159	23	78
Sublethal	20	10	$8{\cdot}1 \times 10^2$	$8{\cdot}6 \times 10^2$	1502(^{32}P)	192	0	87
		60	$4{\cdot}6 \times 10^2$	$4{\cdot}6 \times 10^2$	359	57	0	84
Sublethal	5	10	$5{\cdot}7 \times 10^4$	$5{\cdot}3 \times 10^4$	2275(^{14}C)	444	7	80
		60	$8{\cdot}3 \times 10^3$	$5{\cdot}0 \times 10^3$	682	120	40	82
Passively immune	1	10	$7{\cdot}0 \times 10^6$	$3{\cdot}7 \times 10^6$	52,901(^{14}C)	22,744	48	57
		60	$1{\cdot}5 \times 10^6$	$6{\cdot}6 \times 10^5$	17,409	2095	56	88
Passively immune	1·5	10	$1{\cdot}2 \times 10^6$	$1{\cdot}4 \times 10^5$	2252(^{14}C)	765	88	66
		60	$1{\cdot}2 \times 10^6$	$4{\cdot}3 \times 10^4$	837	166	96	80
Lethal	1	10	$5{\cdot}5 \times 10^7$	$4{\cdot}9 \times 10^7$	21,151(^{14}C)	13,237	10	37
		60	$6{\cdot}8 \times 10^7$	$6{\cdot}0 \times 10^7$	10,950	6573	11	38
Lethal	1	10	$2{\cdot}5 \times 10^7$	$3{\cdot}7 \times 10^7$	50,070(^{14}C)	33,629	0	33
		60	$4{\cdot}8 \times 10^7$	$4{\cdot}2 \times 10^7$	21,685	11,763	12	46
Lethal (+APS)	4·7	10	$4{\cdot}5 \times 10^6$	$4{\cdot}8 \times 10^6$	31,149(^{32}P)	20,819	0	33
		60	$2{\cdot}6 \times 10^6$	$4{\cdot}0 \times 10^6$	8471	6305	0	26
Lethal (+APS)	4·5	10	$1{\cdot}0 \times 10^6$	$1{\cdot}0 \times 10^6$	62,925(^{32}P)	44,083	0	30
		60	$1{\cdot}0 \times 10^6$	$9{\cdot}5 \times 10^5$	18,859	11,821	5	37

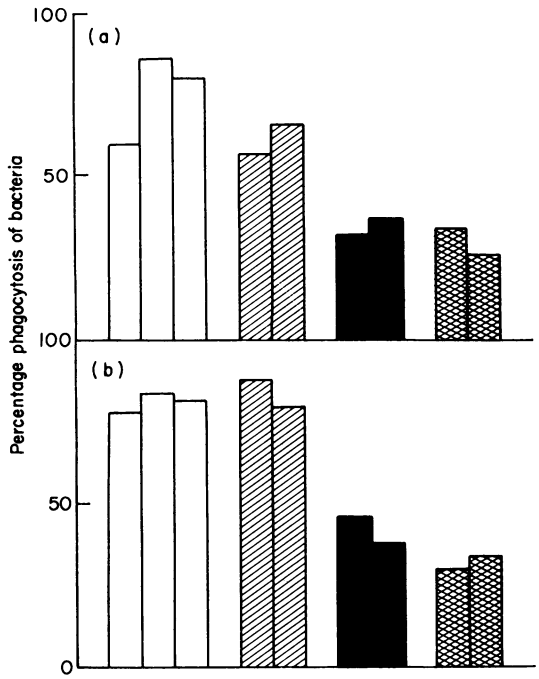

Figure 1. Percentage phagocytosis of ^{32}P- or ^{14}C-labelled bacteria (a) 10 and (b) 60 min after introduction into peritoneal cavity. Open columns, sublethal infection; hatched columns, passively immune animals; solid columns, lethal infection; cross-hatched columns, lethal infection in APS-treated animals.

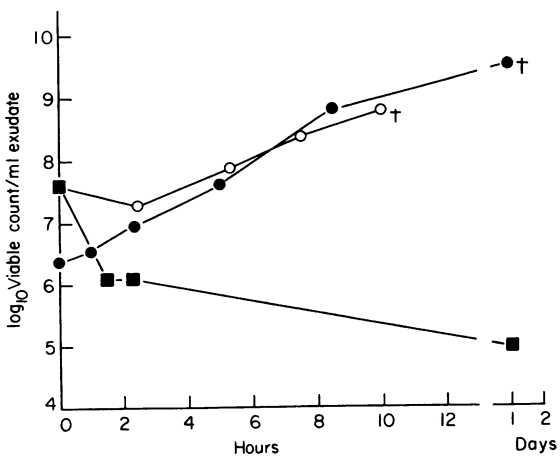

Figure 2. Effect of APS on passive immunity to large doses of *P. aeruginosa*. Viable counts of bacteria in peritoneal cavity. (●) Normal animal, no antibody, fatal infection; (■) passively immune animal, survived; (○) passively immune animal treated with APS, fatal infection; (†) death.

Experiments with antipolymorphonuclear serum

The effect of APS on normal animals

Crude APS contained some anti-red cell antibodies, and although intravenous injection of non-toxic doses produced no obvious lysis of red cells it did result in the appearance of considerable numbers of normoblasts in the circulating blood.

The APS was heated to 56° for 30 min and absorbed three times at room temperature with washed packed rabbit red cells (three volumes of serum to one of packed cells). The absorbed APS did not cause the appearance of normoblasts.

Intravenous injection of 1·0 ml of absorbed APS/kg live weight produced severe shock which was usually fatal. This toxic effect could be avoided if small doses of APS were given the previous day. The final routine consisted of three preliminary intravenous doses of absorbed APS on the first day (0·125 ml/kg at 9.30 h, 0·25 ml/kg at 14.00 h, and 0·25 ml/kg at 17.30 h). Next day 2·0 ml of APS/kg was given intravenously. Provided it was injected slowly no shock occurred. Further doses of 2·0 ml/kg were given every 4–5 h up to a total of four.

Intravenous injection of absorbed APS produced local thrombophlebitis in the ear veins. These lesions eventually healed. APS (2 ml/kg) produced a profound polymorphonuclear leukopenia which persisted for at least 6 h after the last injection. There was often a temporary fall in the number of mononuclear cells in the blood but in the peritoneum there was little or no observable change in the mononuclear cell population. In all other respects the animal appeared quite healthy.

The polymorph response in normal rabbits

In the normal rabbit the polymorph response to infection is extremely rapid (Bullen *et al.*, 1974) (Table 2, Fig. 3). Two to 3 h after infection the total white cell population in the peritoneum rose from about 1×10^5 cells/ml/sample to $6{\cdot}5 \times 10^6$–1×10^7 cells/ml/sample of which 85–95 per cent were polymorphs. If the infection was a fatal one the polymorph count continued to rise, but in non-fatal infections the proportion of polymorphs declined in step with the fall in the viable bacterial count (Bullen *et al.*, 1974) (Fig. 3).

The effect of APS on infected rabbits

The lethal dose of *P. aeruginosa* for a normal rabbit was 10^7 bacteria. Treatment with APS completely abolished the initial polymorph response to infection

Table 2. The effect of APS and *P. aeruginosa* antiserum on infection in the peritoneal cavity

Anti-polymorph serum	*P. aeruginosa* protective anti-serum	Log_{10} dose *P. aeruginosa*	Log_{10} total white cells per ml exudate (h)				Percentage polymorphs in exudate (h)				Log_{10} bacterial count per ml exudate (h)				Fate of rabbit
			1	3–6	6–10	24–28	1	3–6	6–10	24–28	1	3–6	6–10	24–28	
No	No	6·7	5·9	6·8	6·8	6·7	11	94	88	30	4·1	3·5	3·3	4·5	Survived
Yes	No	6·3	5·4	5·4	5·5	—	0·5	13	16	—	4·5	4·5	4·7	8·5	Died
Yes	No	6·1	5·2	5·3	5·3	5·7	1·3	2·3	2·8	10	4·4	4·0	3·9	9·1	Died
Yes	No	5·9	5·1	—	5·1	6·0	0	—	10	5	4·0	—	3·5	8·9	Died
Yes	No	6·1	5·2	5·7	5·1	6·1	0	0	1·2	10	4·5	4·1	4·1	9·8	Died
Yes	Yes	6·2	4·9	5·0	5·6	—	1·0	2·3	0·5	—	2·9	3·4	3·3	—	Survived
Yes	Yes	6·4	5·0	5·2	—	—	5·0	2·0	—	—	3·7	2·6	—	—	Survived
Yes	Yes	9·1	5·0	5·1	4·5	—	3·0	2·0	3·0	—	7·1	7·1	7·3	—	Died
Yes	Yes	9·5	4·7	5·1	5·2	—	0	< 10	< 10	< 10	7·3	7·9	8·8	—	Died
No	Yes	9·5	6·9	6·9	—	7·5	97	95	—	84	6·1	6·1	—	5·0	Survived

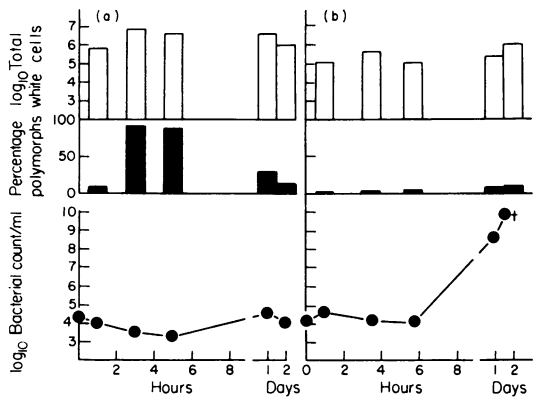

Figure 3. Total white cells, percentage of polymorphs, and viable counts of *P. aeruginosa* in peritoneal cavity of (a) normal and (b) APS-treated animals.

(Table 2, Fig. 3). There was very little effect on the mononuclear cell population. The result of infection in APS-treated rabbits was greatly influenced by the presence or absence of specific antibody. In animals not receiving antibody a thousandth of a normal lethal dose (*circa* 10^4 bacteria) was fatal. In the experiments shown in Table 2 and Fig. 3 a tenth of a normal lethal dose (*circa* 10^6 bacteria) was given. The most striking feature of the experiments was that the bacteria did not grow immediately (Table 2, Fig. 3). In the early stage of infection the ingested bacteria were killed quickly with a mean intracellular viable count of about 1 per cent (Table 1) but the uptake of labelled organisms was relatively poor (mean 32 per cent) (Table 1, Fig. 1).

If the same dose of bacteria (*circa* 10^6) was given to APS-treated animals which had received specific antibody (2 ml) the animals survived (Table 2). During the early hours of infection the viable bacterial counts were far less than in animals not given antibody (Table 2) which suggested that the antibody greatly enhanced the phagocytic power of the mononuclear cells.

Normal rabbits passively immunized with 2·0 ml of *P. aeruginosa* antiserum were protected against very large doses of bacteria (3×10^9) (Fig. 2, Table 2), and there was a rapid decline in the viable count in the peritoneal cavity (Fig. 2). Microscopical examination of the peritoneal exudate 2–3 h after infection showed large numbers of polymorphs. No bacteria were seen (Fig. 4). *P. aeruginosa* antiserum (2·0 ml) did not protect animals treated with APS against the same large doses of bacteria (Fig. 2, Table 2). The infection was held in check for about 2 h but thereafter the rate of bacterial growth was the same as that in an unprotected animal (Fig. 2) and death occurred in about 12 h. At 2–3 h after infection very few polymorphs could be seen in the peritoneal exudate and

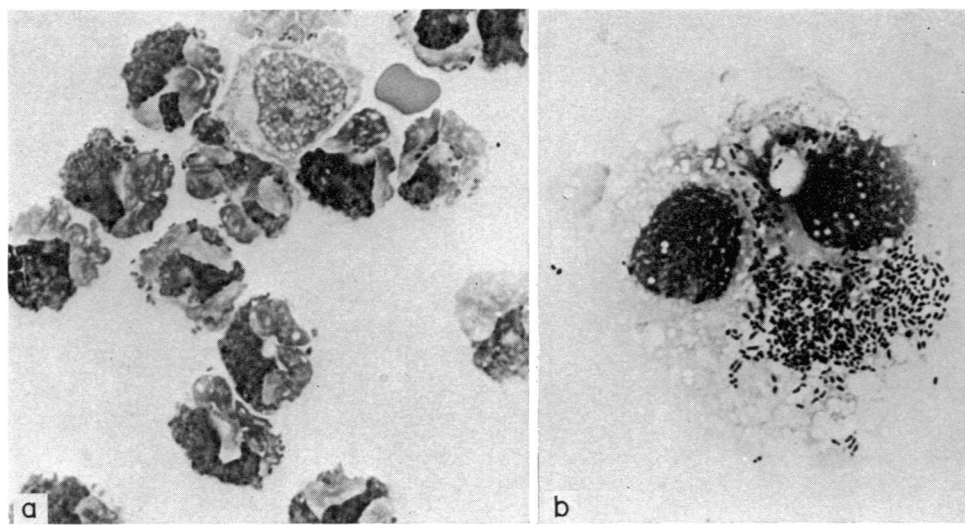

Figure 4. Passively immunized rabbits given $2{\cdot}5$–$3{\cdot}0 \times 10^9$ *P. aeruginosa*. Films of peritoneal exudate 2–3 h after infection. (a) Normal animal. Cells largely polymorphs. No bacteria seen. (Objects on periphery of polymorphs are granules not bacteria.) Animal survived. (b) Animal treated with APS. Mononuclear cells. Bacteria easily seen. Animal died. (Leishman stain; magnification × 980.)

large numbers of bacteria were seen within the macrophages. Some extracellular bacteria were also visible (Fig. 4).

DISCUSSION

The massive exudation of polymorphs that occurs in *P. aeruginosa* infections in the rabbit peritoneal cavity (Bullen *et al.*, 1974) suggests that these cells probably play an important role in resistance. Past experience, however, showed that the majority of living bacteria were extracellular (Bullen *et al.*, 1974) and the way in which bacterial growth was controlled was uncertain.

One possibility was that ingested bacteria were killed so quickly that viable intracellular organisms were rarely found, with the result that the phagocytic role of the polymorph was not immediately apparent. In the present experiments the radioactive label was not destroyed by phagocytosis. We could, therefore, compare the phagocytic efficiency of the cell with its bactericidal power.

In sublethal infections the proportion of viable intracellular bacteria compared with the total viable count was low, whereas the proportion of labelled intracellular bacteria was high with a mean value of 73 per cent (Table 1, Fig. 1). This suggested that the phagocytosed bacteria were killed extremely quickly and that phagocytosis was highly efficient. However, the total viable count declined only slowly over many hours (Bullen *et al.*, 1974) (Fig. 3), which implied that the bacteria were growing extracellularly at a rate almost fast enough to replace those killed inside the polymorphs. This agrees with previous results where it was shown that *P. aeruginosa* could grow in cell free peritoneal exudate with a generation time of 72 min, which is about half its normal rate of growth (Bullen *et al.*, 1974). The fall in the percentage of polymorphs in the exudate which coincides with the fall in the total bacterial count (Fig. 3) may also help to explain why it took so long before this infection finally disappeared (Bullen *et al.*, 1974).

In lethal infections the total viable count rose steadily (Bullen *et al.*, 1974) (Table 1). The failure to control the infection was not due to inefficient killing of ingested bacteria since the number of viable intracellular bacteria was low (Table 1). In this situation the rate of growth of the extracellular bacteria is crucial. Since it is known that *P. aeruginosa* can grow in extracellular fluid (Bullen *et al.*, 1974) with a generation time of 72 min, and since the uptake of labelled bacteria was low (mean 39 per cent) (Table 1, Fig. 1) it appeared that the polymorphs were overwhelmed by the excess of organisms. This would also explain why the injection of iron compounds enhances virulence since the saturation of the iron binding protein in the exudate with Fe, or the presence of haem compounds would allow the bacteria to grow twice as quickly with a generation time of 34 min (Bullen *et al.*, 1974).

In passively immunized animals the situation was quite different. Here the uptake of the labelled organisms (mean 73 per cent) was almost as good as that seen in non-lethal infections (Table 1, Fig. 1). Since the number of bacteria introduced into the peritoneal cavity was similar to or greater than that given in lethal infections the increased efficiency of phagocytosis must be attributed to the presence of antibody. The increased efficiency of phagocytosis did lead to a fall in the bactericidal power of the phagocyte since between 48 and 96 per cent of the total viable count was intracellular (Table 1). This fall in killing efficiency, which may perhaps have been only temporary, appeared to be of little importance since the total viable count fell steadily (Fig. 2).

The use of antipolymorphonuclear leucocyte serum confirmed the importance of the polymorph in resistance. Sera of this kind have been prepared by others (Chew, Stephens and Lawrence, 1936; Humphrey, 1955; Moeschlin, Meyer, Israels and Tarr-Gloor, 1954; Simpson and Ross, 1971), but have been little used to explore the role of the polymorph in infection, although Humphrey (1955) noticed that APS-treated guinea-pigs were unusually susceptible to local infection, and Chew *et al.* (1936) showed that APS treated animals were more susceptible to staphylococcal infection. In our experience suppression of the polymorph exudate led to a profound loss of resistance (Figs 1, 2 and 3 and Table 2).

The great advantage of APS over other means of immunosuppression is its selective power. In APS-treated animals there was little alteration in the number of mononuclear cells in the peritoneal exudate. APS was also non-toxic if given carefully. However, it was interesting that the mononuclear cells in APS-treated animals could delay bacterial growth for several hours (Fig. 3, Table 2). The mononuclear cells had a rapid bactericidal effect on ingested organisms but their phagocytic efficiency was low with an average uptake of labelled organisms of only 32 per cent. This is no better than that seen in lethal infections in

normal animals with far larger doses of bacteria (Table 1, Fig. 1) and presumably is one of the factors in the situation that leads to the eventual death from septicaemia.

The results of the present work may also clarify a technical point. It is sometimes argued that bacteria associated with phagocytic cells are not really intracellular at all but are merely attached to the outside of the cell. This seems unlikely in our experiments since in several instances there were no viable bacteria associated with the cells at all whereas the average number of labelled organisms within the cells was over 80 per cent (Table 1). Since the labelled cells were alive when introduced into the peritoneum, these figures suggest that the bacteria were in fact phagocytosed, unless one postulates that mere attachment of the bacteria to the outside of the cell could cause death, which seems unlikely.

In conclusion, it appears that the phagocytic mononuclear cells (macrophages) in the peritoneum provide some degree of resistance to *P. aeruginosa* but their importance is far outweighed by the massive exudation of polymorphs that normally occurs. The importance of polymorphs in *P. aeruginosa* infections have been mentioned before (Fox and Lowbury, 1953; Jones and Dyster, 1973) but the observations were made *in vitro* which does not necessarily match all the conditions *in vivo*.

Specific antibody can enhance the phagocytic power of both mononuclear cells and polymorphs but the resistance of the animal is very greatly impaired if the normal polymorph response is suppressed. The outcome of infection appears to depend almost entirely on the ratio of bacteria to phagocytes and the presence or absence of specific antibody. Since *P. aeruginosa* can grow in cell-free peritoneal fluid the rate of extracellular growth is also important. In this respect the unsaturated iron binding proteins in the peritoneal fluid probably make a significant contribution to resistance since their presence halves the normal rate of bacterial multiplication (Bullen *et al.*, 1974).

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