R-plasmid RP1 increases sensitivity of *Proteus mirabilis* to normal body defences

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Summary. The bactericidal action of non-immune whole blood on *Proteus mirabilis* was increased when the bacteria contained the R-plasmid RP1. This effect was due mainly to increased phagocytosis. Iron-depleted stationary-phase cells were more sensitive than carbon-depleted cells. The contribution of serum was usually negligible but was increased during a minor non-specific infection. Most plasmid-containing phenotypes were more sensitive than were those without plasmids but there were considerable differences between stationary and exponentially-growing cells. The R-plasmid-mediated increase in sensitivity to phagocytosis may be due in part to the presence of additional glycosylated proteins in the outer membrane.

Introduction

To survive in an animal host a microbe must resist the normal humoral and cellular defences (Smith, 1984), and the outer surface, which interacts initially with these defences, may play a large part in determining the final outcome of an infection.

Plasmid RP1, which was originally described in organisms isolated from nosocomial antibiotic-resistant infections (Lowbury *et al.*, 1969), determines various cell-envelope changes as well as carrying genes for resistance to β -lactam antibiotics, aminoglycosides and tetracycline (Onaolapo and Klemperer, 1986; Onaolapo *et al.*, 1987). The effects of RP1 on the resistance of a clinical isolate of *Proteus mirabilis* to killing by whole blood and serum and on phagocytosis have, therefore, been investigated. Since the properties of the cell envelope may depend on the environment (Brown and Williams, 1985), we studied various phenotypes and, in an attempt to characterise the plasmid-mediated features, the composition of the outer membrane of the cells was investigated.

Materials and methods

Bacterial cultures

Proteus mirabilis PB13, with (R⁺) and without (R⁻) plasmid RP1, and its routine maintenance and the media

for carbon and iron-limited chemostat cultures have been described previously (Onaolapo *et al.*, 1987). In the case of batch cultures, for carbon depletion (C- cells) 4 mM glucose was used, and for iron depletion (Fe- cells) the same medium was used as for iron-limited chemostat cultures. A batch culture medium with all ingredients in excess (Fe+ cells) was the same medium as for Fe- cells plus excess iron (0.005 mM FeSO₄). Batch cultures were aerated by shaking in a water bath at 120 times/min. The dilution rate for chemostat cultures was 0.1 h⁻¹.

Bactericidal action of blood

Fresh heparinised human or pooled guinea-pig blood was incubated in a shaking water bath at 37°C with an equal volume of a suspension of bacteria in saline (*E*₄₇₀ 0.2), which had previously been washed twice in saline. The bacteria:human polymorphonuclear leukocyte (PMN) ratio was approximately 50:1 as determined microscopically after staining with Giemsa and assuming that approximately 66% of white cells isolated are PMNs (Diem and Lentner, 1970). Guinea-pig leukocytes were tested at the same concentration. Samples (0.1 ml) were removed at timed intervals and added to 0.9 ml of sterile water at 0°C for 15 min to lyse the red cells, before diluting in saline and counting bacteria on nutrient agar.

Bactericidal action of serum

Serum was prepared from clotted blood, and its action was tested as for whole blood except that the addition to sterile water was omitted. Serum was either tested at 50%, with equal volumes of serum and bacterial suspension in saline, or at 30%, when the proportions were adjusted by adding saline. Heat-inactivated serum was held at 50°C for 30 min. To inactivate the alternative

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complement pathway, 10 mM ethylene glycol tetraacetate (EGTA) was added.

Phagocytosis

Human and guinea-pig leukocytes were prepared by mixing 17 ml of blood with 3 ml of acid-citrate-dextrose (trisodium citrate H_2O 22 g, citric acid H_2O 8 g, D-glucose 25 g, water to 1 L) and adding this mixture to 10 ml of dextran 75 (Fisons) 6% w/v in saline. After standing for 2 h at 4°C the buffy coat was centrifuged at 900 rpm for 12 min at 4°C , the pellet was resuspended in 0.6 ml of cold distilled water and 0.2 ml of 0.6 M saline was added. The cells were centrifuged again and the procedure for lysing red cells repeated until pure leukocytes were obtained. These were washed twice in phosphate-buffered saline and resuspended to a concentration of 2.5×10^6 cells/ml.

Rat leukocytes were obtained by intraperitoneal lavage with 25 ml of ice-cold Hanks's balanced salt solution containing heparin 10 $\mu\text{g/ml}$ (HBSS) 4 h after injection of 20 ml of mussel glycogen 2% w/v in saline. After washing with HBSS, the leukocytes were resuspended in Eagle's Minimum Essential Medium plus 2 mM glutamine (Williams *et al.*, 1986) to a concentration of 2.5×10^6 cells/ml. Phagocytosis was assayed by luminol-enhanced chemiluminescence (Easmon *et al.*, 1980), with a lumi-

nometer (LKB 1250) and 0.2 mM luminol. Before testing, bacteria were opsonised by incubation for 15 min at 37°C with serum (10% v/v in phosphate-buffered saline) from the same source as the leukocytes.

Chemical properties of outer membranes

Outer membranes were prepared by the sarkosyl method of Filip *et al.* (1973) and the proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Lugtenberg *et al.*, 1975). After separation, proteins were transferred to nitrocellulose paper with a transblot cell (Towbin *et al.*, 1979). Non-specific binding sites were blocked by saline containing 10 mM Tris HCl, pH 7.4, and Tween 0.3% w/v (TBS-Tween) (Batteiger *et al.*, 1982) and the paper strips incubated with various (see fig. 6) peroxidase-labelled lectins (Sigma) (30 $\mu\text{g/ml}$ TBS-Tween) (Tsang *et al.*, 1983).

Results

Bactericidal action of whole blood on *P. mirabilis* R^+ and R^- cells

Stationary-phase R^+ bacteria were more sensitive to the bactericidal action of whole blood than

Fig. 1. Effect of guinea-pig blood on stationary-phase *P. mirabilis* R^+ (●, ■) and R^- (○, □) when depleted of carbon (■, □) or iron (●, ○). Results are the average of two experiments.

were R^- cells and both cultures were more sensitive when depleted of carbon (C-) than when depleted of iron (Fe-) (fig. 1). Blood was tested at a concentration of 50% and even when as many as 99% of bacteria disappeared rapidly there were always a few survivors that started to regrow. Results were similar with human and guinea-pig blood, but the different types of bacteria had to be compared with the same blood sample, because individual samples varied widely. Fig. 2 demonstrates the problem; the donor reported a trivial non-specific respiratory infection after the tests shown in fig. 2B had been completed.

Phagocytosis of R^+ and R^- *P. mirabilis* cells

With a ratio of 50:1 bacteria:leukocytes, results were similar to those for whole blood. Stationary phase R^+ cells were more sensitive than R^- cells and C- cells more sensitive than Fe- cells. Fig. 3 shows the action of rat leukocytes. Similar results were obtained with human and guinea-pig cells. When the ratio was increased to 500:1, the difference between R^+ and R^- cells was striking. R^+ cells depressed the human leukocyte response so much that R^+ cells now appeared to be the most resistant. The change with R^- cells was much smaller and the activity of human leukocytes was

Fig. 2. Effect of human blood (●, ○) and serum (▲, △) on iron-depleted stationary-phase *P. mirabilis* R^+ (●, ▲) and R^- (○, △). Samples of blood were from the same donor separated by a 2-month interval; (●---●) and (○---○) show the response after a minor non-specific infection.

Fig. 3. Chemiluminescence response of rat leukocytes to stationary-phase *P. mirabilis* R⁺ (●, ■) and R⁻ (○, □), when depleted of carbon (■, □) or iron (●, ○). Leukocyte response in the absence of bacteria shown as (▲) and bacterial response in the absence of leukocytes as (▼). Graphs are the average of two experiments with four readings/point/experiment.

Fig. 4. Chemiluminescence response of human leukocytes to stationary phase iron-depleted *P. mirabilis* R⁺ (●) and R⁻ (○). Bacteria: leukocyte ratio 50:1 (●—●, ○—○) and 500:1 (●---●, ○---○). Leukocyte response is shown as (▲) and bacterial response as (▼). Graphs are the average of two experiments, with four readings/point/experiment.

slightly depressed (fig. 4). R^+ cells also depressed the guinea-pig leukocytes response but R^- cells increased it slightly, though not in proportion to the number of bacteria.

*Bactericidal action of serum on R^+ and R^- *P. mirabilis* cells*

A serum effect could not normally be detected when it was tested at its concentration in blood (approximately 30%) (fig. 2A), but at a concentra-

tion of 50% some bactericidal activity could be demonstrated with guinea-pig or human serum and differences between cultures were detected. As with whole blood, stationary phase cells grown in Fe— conditions that were R^+ were more sensitive than equivalent R^- cells (fig. 5A). However, when grown in C— conditions, there was little difference between R^+ and R^- cells (fig. 5B). Chemostat-grown bacteria were also tested. The relative sensitivities of R^+ and R^- bacteria remained the same, but the Fe— cells were even more sensitive (fig. 5C) and the C— cells were the most resistant

Fig. 5. Effect of guinea-pig serum on stationary-phase *P. mirabilis* R^+ (●, ■, ▲, ▼) and R^- (○, □, △, ▽), when depleted of iron (●, ○, ▲, △) or carbon (■, □, ▼, ▽). Stationary-phase cells are shown as (●, ○, ■, □) and chemostat-grown cells as (▲, △, ▼, ▽). Serum treated with EGTA is shown as (●---●, ○---○) and when heat inactivated as (▲---▲, △---△). Graphs are the average of two experiments.

of all (fig. 5D). Serum was inactivated by EGTA (fig. 5A) and by heat (fig. 5C).

Outer-membrane composition of R^+ and R^- bacteria

No significant difference could be detected in Fe— and Fe+ cells between the outer-membrane-protein profiles of stationary phase R^+ and R^- cells by SDS-PAGE. However, when the proteins of Fe— outer membranes were treated with lectins, two glycosylated proteins were detected in the outer membrane of R^+ cells but not in R^- cells. The one with a mol. wt of 28×10^3 reacted with concanavalin A (con A); the other with a mol. wt of 31×10^3 reacted with con A, soybean and pea lectins (fig. 6). It was noted that the protein of mol. wt 31×10^3 could be detected in whole-cell preparations of R^- as well as of R^+ cultures (not shown). Other glycosylated proteins of mol. wt (10^3) 36 and 39 were recognised by con A as a single band in outer membranes of both strains. Fe+ and C— cells were not investigated.

Fig. 6. Outer-membrane proteins of *P. mirabilis* separated by SDS-PAGE, transferred to nitrocellulose paper and incubated with peroxidase-labelled lectins. R^+ : tracks 2, 4, 6, 8; R^- : tracks 1, 3, 5, 7. Lectins: tracks 1, 2 soybean; 3, 4 wheat germ agglutinin; 5, 6 concanavalin A; 7, 8 pea lectin. Mol. wts are shown.

Discussion

The effect of plasmid RP1 on the virulence of its host bacterium appears to depend on the location of the infection. Although *Pseudomonas aeruginosa* with RP1 was originally described as fully virulent (Lowbury *et al.*, 1969), the plasmid decreased mortality in *Ps. aeruginosa* infections in experimental burns in rodents (McManus *et al.*, 1981; Wretling *et al.*, 1985) and when injected intravenously, but not when injected intraperitoneally (Wright *et al.*, 1977). The increased sensitivity to normal body defences associated with the R-plasmid reported here is likely to be associated with a loss of virulence, whereas plasmid-mediated effects on adhesion might be expected to enhance virulence (Onaolapo *et al.*, 1987). It is noteworthy that in the absence of iron the increased sensitivity of R^+ cells is small. Unbound iron, which would be readily available to bacteria, is virtually absent from normal body fluids (Griffiths, 1983). When R^+ and R^- bacteria were grown in normal urine, the presence of iron-regulated proteins in their outer membranes confirmed the absence of iron (Onaolapo, 1986).

The action of whole blood correlated well with that of its PMN content, when both were tested with a bacteria:PMN ratio of 50:1. However, when this ratio was increased to 500:1, as might occur in a leukopenic patient, the depression of the response to R^+ cells was striking. Easmon *et al.* (1980) found that phagocytic activity reached a plateau when with *Staphylococcus aureus* or *Ps. aeruginosa*, the bacteria: PMN ratio was raised above 100. *P. mirabilis* R^- appeared to have a similar effect. However, R^+ cells, particularly when iron-depleted, have very different outer membranes from R^- cells, particularly with respect to the nature of their constituent fatty acids (Onaolapo, 1986), which might affect the phagocytic process.

Outer-membrane differences, particularly with respect to lipid constitution, may also explain differences in serum sensitivity, as the complement membrane attack complex is thought to insert into a hydrophobic membrane site and disrupt the outer membrane completely (Taylor, 1983). The inactivation of serum by heat and EGTA suggested that its action was mediated by the classical complement pathway, presumably due to normal antibody present in the serum.

An RP1-mediated increase in sensitivity to serum was reported by Wretling *et al.* (1985) but their bacteria were grown in a nutrient-rich complex medium. Results reported here demonstrate the importance of testing a variety of environmentally-

determined phenotypes. It seems likely that early in an infection cells will be growing exponentially but that in many in-vivo situations cells will be in stationary phase and have different properties. Iron-limitation is likely to be the usual nutrient-determined phenotype (Shand *et al.*, 1985) but some plasmid-containing cells may be carbon-limited in view of the additional synthetic burden (Levin, 1980).

Phagocytosis is a multistage process beginning with adhesion and it is of interest that RP1 promotes adhesion to inert surfaces, possibly because of an increase in the electronegativity of the bacterial surface (Onaolapo *et al.*, 1987). Furthermore, RP1 is associated with an increase in hydrophobicity of iron- and carbon-depleted stationary-phase cells (Onaolapo and Klemperer, 1986) and increased hydrophobicity has been correlated with increased

susceptibility to phagocytosis of *Salmonella* (Xiu *et al.*, 1983). However, interaction with living cells usually also involves specific receptors on the target cell recognised by specific molecules on the bacteria (Stendahl, 1983) and it has been suggested that lectin-like sites on macrophages might recognise carbohydrate residues on glycoproteins (Stahl *et al.*, 1978). While R⁻ and R⁺ cells had apparently identical glycoproteins of mol. wt (10³) 36 and 39 when iron-depleted, the R⁺ ones had two additional glycoproteins detectable in the outer membrane, one of mol. wt 28 × 10³ containing glucose and mannose residues and one of 31 mol. wt 31 × 10³ containing fructose and galactosamine residues as well.

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REFERENCES

- Batteiger B, Newhall W J, Jones R B 1982 The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. *Journal of Immunological Methods* **55**: 297–307.
- Brown M R W, Williams P 1985 The influence of environment on envelope properties affecting survival of bacteria in infections. *Annual Review of Microbiology* **39**: 527–556.
- Diem K, Lentner C 1970 Total leucocyte count and distribution at various ages. In: Documenta Geigy 7th edn. Ciba-Geigy, Basle, p 619.
- Easmon C S F, Cole P J, Williams A J, Hastings M 1980 The measurement of opsonic and phagocytic function by luminol-dependent chemiluminescence. *Immunology* **41**: 67–74.
- Filip C, Fletcher A, Wilf J, Earhart C F 1973 Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. *Journal of Bacteriology* **115**: 717–722.
- Griffiths E 1983 Availability of iron and survival of bacteria in infection. In: Easmon C S F *et al.* (eds) Role of the envelope in the survival of bacteria in infection. Medical Microbiology 3, Academic Press, London, pp 153–177.
- Levin B R 1980 Conditions for the existence of R-plasmids in bacterial populations. In: Mitsuhashi S *et al.* (eds) Antibiotic resistance: transposition and other mechanisms. Springer Verlag, Berlin, p. 197.
- Lowbury E J L, Kidson A, Lilly H A, Ayliffe G A J, Jones R J 1969 Sensitivity of *Pseudomonas aeruginosa* to antibiotics: emergence of strains highly resistant to carbenicillin. *Lancet* **2**: 448–452.
- Lugtenberg B, Meijers J, Peters R, van der Hoek P, van Alphen L 1975 Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K12 into four bands. *FEBS Letters* **58**: 254–258.
- MacManus A T, Northam W J, Moody E E M 1981 Suppression of *Pseudomonas aeruginosa* burn surface infection by plasmid RP1. *Abstracts of the Annual Meeting, American Society for Microbiology*, p 17.
- Onaolapo J A 1986 Effect of R-plasmid RP1 on the properties of *Proteus mirabilis*. PhD thesis, Aston University.
- Onaolapo J A, El-Haffar M I A, Townley D, Klemperer R M M 1987 R-plasmid RP1 promotes adhesion of Gram-negative bacteria to medical prostheses and glass. *Journal of Medical Microbiology* **24**: 227–232.
- Onaolapo J A, Klemperer R M M 1986 Effect of R-plasmid on surface hydrophobicity of *Proteus mirabilis*. *Journal of General Microbiology* **132**: 3303–3307.
- Shand G. H, Anwar H, Kadurugamuwa J, Brown M R W, Silverman S H, Melling J 1985. In vivo evidence that bacteria in urinary tract infection grow under iron-restricted conditions. *Infection and Immunity* **48**: 35–39.
- Smith H 1984 The biochemical challenge of microbial pathogenicity. *Journal of Applied Bacteriology* **57**: 395–404.
- Stahl P D, Rodman J S, Miller M J, Schlesinger P H 1978 Evidence for receptor-mediated binding of glycoproteins, glycoconjugates and lysosomal glycosidases by alveolar macrophages. *Proceedings of the National Academy of Sciences of the USA* **75**: 1399–1403.
- Stendahl O 1983 The physicochemical basis of surface interaction between bacteria and phagocytic cells. In: Easmon C S F *et al.* (eds) Role of the envelope in the survival of bacteria in infection. Medical Microbiology 3, Academic Press, London, pp 137–152.
- Taylor P W 1983 Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. *Microbiological Reviews* **47**: 46–83.
- Towbin H, Staehelin T, Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the USA* **76**: 4350–4354.
- Tsang V C W, Peralta J M, Simons A R 1983 Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. *Methods in Enzymology* **92**: 377–391.
- Williams P, Lambert P A, Haigh C G, Brown M R W 1986 The influence of the O and K antigens of *Klebsiella aerogenes* on

- surface hydrophobicity and susceptibility to phagocytosis and antimicrobial agents. *Journal of Medical Microbiology* **21**: 125-132.
- Wretling B, Becker K, Haas D 1985 Inc P-1 R-plasmids decrease the serum resistance and the virulence of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **131**: 2701-2704.
- Wright B L, Carrick L, Jollick J D 1977 The effect of an R-factor on the virulence of *Pseudomonas aeruginosa* in the mouse. *Abstracts of the Annual Meeting, American Society for Microbiology*, p 35.
- Xiu J H, Magnusson K E, Stendahl O, Edebo L 1983 Physiochemical surface properties and phagocytosis by polymorphonuclear leucocytes of different serogroups of *Salmonella*. *Journal of General Microbiology* **129**: 3075-3084.