Effect of pH and Haem Compounds on the Killing of *Pasteurella septica* by Specific Antiserum

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SUMMARY

The killing of *Pasteurella septica* by horse antiserum has features not previously associated with serum bactericidal reactions. The present work showed that lowering the pH from 7.4 to 6.8 abolished the action of antiserum. The bactericidal effect and the degradation of RNA seen when antiserum is added to *P. septica* growing in horse serum, were abolished at pH 6.8 in much the same way as when haem compounds were added to the system. Addition of chloramphenicol, rifampicin or puromycin to *P. septica* growing apparently normally in antiserum at pH 6.8 or in antiserum containing haem compounds led to rapid killing of the bacteria and to degradation of their RNA. Addition of these antibiotics to *P. septica* growing in normal serum produced only bacteriostasis and did not induce RNA breakdown. In contrast, nalidixic acid, although inhibiting growth, did not induce rapid killing and RNA breakdown under the same conditions. These findings were unexpected and led to a reassessment of ideas concerning the mechanism of action of specific antiserum on *P. septica*. Although iron compounds clearly abolish the bactericidal effects of antiserum, an explanation of the antibacterial action of such antiserum based simply on an interference with bacterial iron supply is no longer sufficient. The process is more complex and must involve other factors.

INTRODUCTION

Certain clinical conditions involving abnormalities in iron metabolism, such as haemolysis and saturation of serum transferrin with iron, make patients more susceptible to bacterial infections (Bullen, Rogers & Griffiths, 1972*a*, 1974; Sussman, 1974; Weinberg, 1974). Similarly, in experimental animals, injection of iron compounds can greatly reduce host resistance to certain bacteria and abolish passive immunity to others. A working hypothesis to account for such observations, involving interactions between bacteria, iron-binding proteins and antibodies, was proposed by Bullen, Rogers & Lewin (1971). It is suggested that specific antibody acting in concert with transferrin, or lactoferrin, interferes directly with normal bacterial iron metabolism, making iron unavailable to the cell and leading to inhibition of bacterial growth. Saturating the iron-binding capacity of the iron-binding protein or the addition of haem compounds overcomes this inhibition.

Specific antibody, transferrin and a heat-labile factor in fresh serum, which appears to be complement, have been implicated in the bacteriostatic and bactericidal action of sera on *Pasteurella septica* (Bullen & Rogers, 1969; Bullen *et al.* 1971; Griffiths, 1974*b*). Both of these antibacterial effects can be abolished by the addition of iron compounds. Iron compounds also abolish passive immunity to *P. septica* in mice. Passively immunized animals given iron compounds fail to control the growth of *P. septica* in the normal way, the growth

of the organisms in iron treated animals being identical to that seen in unimmunized controls (Bullen *et al.* 1968).

The bacteriostatic action of antiserum on *P. septica* is sensitive to small changes in pH. In rabbit serum, the bacteriostatic effect seen at pH 7.4 is lost at pH 6.8 (Bullen *et al.* 1971). Similar effects have been observed with serum and *Clostridium welchii* (Bullen, Dobson & Wilson, 1964) and with bovine colostrum and *Escherichia coli* (Bullen, Rogers & Leigh, 1972*b*). This effect of pH has been interpreted as evidence for the importance of iron-binding proteins in these antibacterial reactions. In the case of rabbit serum and *P. septica*, Bullen *et al.* (1971) argue that if antibody and complement alone were involved, it would be difficult to see why adjusting the pH to 6.8 should abolish bacteriostasis, since this pH is well within the range for the optimum reaction of antibody and complement (Boyd, 1966). The iron-binding property of transferrin, however, is known to be pH dependent, the association constant between transferrin and Fe^{3+} being reduced by lowering the pH. This, it was proposed, would make Fe^{3+} normally bound by transferrin more readily available to the bacteria and would overcome the antibacterial effects of the serum components in much the same way as would added iron.

The present paper reports the effect of a small change in pH on the killing of *P. septica* by horse antiserum. The results show that the bactericidal reaction seen in horse serum, like the bacteriostatic effect in rabbit serum, is abolished by lowering the pH from 7.4 to 6.8. However, the idea that the inhibition is abolished by an increased availability of transferrin-bound iron is not substantiated. The work further shows that an explanation of the antibacterial action of *P. septica* antiserum based simply on an interference with bacterial iron supply (Bullen *et al.* 1971) is no longer sufficient. The process is more complex and must involve other factors. A preliminary report of some of these results has been published (Griffiths, 1974*c*).

METHODS

Organisms and media. *Pasteurella septica* (strain CN3449, Wellcome Research Laboratories) was grown, harvested and counted as described by Griffiths (1971).

Sera. Whole normal horse serum was obtained from the Wellcome Research Laboratories (horse serum No. 3) and kept at -70°C . Specific *P. septica* horse-antiserum was as used by Griffiths (1971). Sera were sterilized by membrane filtration.

Bacterial growth in serum. *Pasteurella septica* was grown in serum at 37°C in jacketed culture vessels, the contents of which were stirred with the aid of magnetic followers and maintained at the required pH by the gas mixture passing through the vessels (Griffiths, 1971). A pH of 7.4 was established with a mixture of equal volumes of (95 % air + 5 % CO_2) and (95 % N_2 + 5 % CO_2). For pH 6.8, the gas mixture was changed to equal volumes of (75 % air + 25 % CO_2) and (75 % N_2 + 25 % CO_2). In some experiments 100 % CO_2 was passed through the vessel for a minute or two, to lower the pH rapidly from 7.4 to 6.8. When the required pH was attained, the 25 % CO_2 gas mixture was switched to pass through the vessel, to maintain the pH at 6.8.

In other experiments, the pH of the serum was controlled by the automatic addition of 1 M-HCl using a Radiometer (Copenhagen) titrator. When the pH-stat was used, the vessels were under normal atmospheric conditions.

The temperature in the vessels was lowered from 37°C to 1 to 2°C by circulating ice-cold water as described by Griffiths (1974*a*).

Loss of radioactive label from RNA of P. septica in antiserum. The bacteria were labelled by growing them in serum (4 ml) containing [^{32}P]orthophosphate (8 $\mu\text{Ci/ml}$ serum) or

[¹⁴C]-uracil (1 to 2 μ Ci/ml serum) (Griffiths, 1974a). Labelled bacteria were collected by centrifugation at room temperature, resuspended in a 10 % (v/v) mixture of papain digest broth in 0.15 M-NaCl (10 % broth-saline), centrifuged, and finally resuspended in 1 ml 10 % broth-saline. They were used immediately.

Labelled bacteria in 10 % broth-saline (0.2 ml) were added to fresh, normal, unlabelled horse serum and incubated under controlled conditions for 1½ h. Specific antiserum, sufficient to give a 15 % mixture, was then added and a similar volume of normal serum was added to control vessels. Procedures for assaying the loss of radioactive label from *P. septica* during the inhibitory reactions were described by Griffiths (1974a). Any contribution to the radioactive count from ¹⁴C-label in DNA was ignored in the present experiments.

Chemicals. Haemoglobin, chloramphenicol, nalidixic acid, puromycin dihydrochloride and rifampicin were obtained from Sigma, potassium cyanide and 2,4-dinitrophenol were from BDH, and *o*-[³²P]phosphate (50 to 100 Ci/mg P) and [2-¹⁴C]uracil (62 Ci/mol) were from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Abolition of the bactericidal effect of antiserum at pH 6.8

Recent work on the mechanism of action of antiserum on *P. septica* showed that the process operates by interfering with the biochemistry of the bacterial cell (Griffiths, 1971, 1974a, b). Addition of antiserum to *P. septica* growing rapidly in fresh horse serum at pH 7.4 leads, after a delay of about 40 min, to a rapid degradation of ribosomal RNA, loss of ribosomes and cell death (Griffiths, 1974a). In the present work the effect of pH on both RNA breakdown and cell death was examined. It should be emphasized that possible complications introduced by variations in the metabolic state of the organisms as they adjust to growth in serum are eliminated by ensuring that antiserum is always added to *P. septica* already fully adapted to growth in serum (Griffiths, 1971).

Fig. 1 shows the effect of adding antiserum to *P. septica* growing in fresh horse serum under 5 % CO₂ (pH 7.4) and under 25 % CO₂ (pH 6.8). The antiserum was ineffective when added to *P. septica* growing under 25 % CO₂ and the bacteria grew normally (Fig. 1a). The presence of 25 % CO₂ in the gas phase also prevented the degradation of RNA which occurs after the 40th minute in antiserum under 5 % CO₂ (Fig. 1b). That this effect was due to a lowering of the pH and not to an increased concentration of CO₂ was shown by using an automatic titrator to control the pH. The bactericidal action of antiserum and the degradation of RNA was abolished by adjusting the pH of the sera to 6.8 with 1 M-HCl.

Effect of pH changes during growth on the action of antiserum

A possible explanation for the abolition of the killing action of antiserum was that *P. septica* had been changed somehow by growth in serum at pH 6.8. Therefore, labelled bacteria were grown first in horse serum at pH 7.4 and the pH was then lowered to 6.8 either 5 min before or 5 min after the addition of the antiserum. The pH was lowered and maintained at 6.8 either by addition of 1 M-HCl by the automatic titrator or by using 100 % CO₂ for a few minutes, followed by 25 % CO₂. In each case, lowering the pH abolished the antibacterial action of the antiserum and allowed the bacteria to grow normally.

Antibiotic-induced RNA breakdown and cell death in antiserum at pH 6.8

If the antiserum is operating by interfering with the organism's iron supply and the abolition of killing at pH 6.8 is due to the increased availability of iron (Bullen *et al.* 1971),

Fig. 1. Effect of CO₂ concentration on the action of *P. septica* antiserum. Specific antiserum (15 %, v/v) was added at zero time. (a) Viability; (b) breakdown of RNA. ○, Serum under 5 % CO₂ (pH 7.4); ●, serum under 25 % CO₂ (pH 6.8). Bacterial RNA was prelabelled with [¹⁴C]uracil and the results are expressed as percentages of label in RNA at time zero.

Fig. 2

Fig. 3

Fig. 2. Action of chloramphenicol on *P. septica* growing in normal serum and in antiserum at pH 6.8. ●, Bacteria in antiserum, no chloramphenicol; ○, bacteria in antiserum with chloramphenicol added at 5th minute; ▲, bacteria in normal serum with chloramphenicol added at 5th minute. Antiserum (15 %, v/v) was added at zero time; an equal volume of normal serum was added simultaneously to the normal serum control.

Fig. 3. Fate of stable RNA in *P. septica* on addition of chloramphenicol. ●, Bacteria growing in antiserum at pH 6.8; ○, bacteria in antiserum at pH 6.8 with chloramphenicol added at 5th minute; □, bacteria in normal serum at pH 6.8 with chloramphenicol added at 5th minute. Antiserum was added at zero time. Bacterial RNA was prelabelled with [¹⁴C]uracil and the results are expressed as percentages of label in RNA at time zero.

then it would be expected that *P. septica* growing in antiserum at pH 6.8 would behave like *P. septica* growing in normal serum. Addition of chloramphenicol (200 µg/ml) to *P. septica* growing in normal serum at pH 6.8 produced bacteriostasis. There was no rapid killing of the organisms, nor any loss of label from the RNA (Figs. 2 and 3). However, the addition of chloramphenicol to *P. septica* growing, apparently normally, in antiserum at pH 6.8 resulted in the breakdown of RNA (Fig. 3) and in a large loss of viability (Fig. 2). Using ^{32}P as label and fractionating for label in RNA and DNA (Griffiths, 1971) showed that there was no loss of DNA from *P. septica* under these conditions, although 80 to 90 % of the ^{32}P was lost from the RNA fraction. As in the normal antibacterial system at pH 7.4, there was a delay of about 40 to 50 min after the addition of chloramphenicol before the onset of RNA breakdown and cell death.

One possible interpretation of the results with chloramphenicol was that the degradation of RNA and cell death resulted from the action of antiserum on chloramphenicol-inhibited bacteria. Any agent capable of stopping bacterial multiplication might therefore be expected to give a similar result. To see if this was the case, selected antibiotics were tested for their ability to induce RNA breakdown and cell death in *P. septica* growing in antiserum at pH 6.8. Nalidixic acid was chosen as an inhibitor of DNA synthesis, rifampicin as an inhibitor of RNA synthesis and puromycin as another inhibitor of protein synthesis. None of these antibiotics interfered with the normal bactericidal system at pH 7.4.

Addition of nalidixic acid (50 µg/ml) to *P. septica* growing in antiserum at pH 6.8 and to *P. septica* in normal serum at pH 6.8 produced the same inhibitory effect and some loss of viability. There was no loss of radioactive label from the RNA fraction in either case. In contrast, rifampicin (10 µg/ml) and puromycin (100 µg/ml) behaved like chloramphenicol; both induced RNA breakdown and extremely rapid killing of bacteria in antiserum at pH 6.8 but not in bacteria in normal serum at pH 6.8. Like chloramphenicol, there was a delay of about 40 min between the time of addition of the antibiotics and the onset of killing and loss of label from the RNA. Figs. 4 and 5 compare the effects of puromycin and nalidixic acid.

Effect of low pH on the binding of antibody to P. septica

To see if the lowered pH prevented the binding of antibody to *P. septica*, labelled bacteria were incubated with antiserum (15 %, v/v) for 20 min at pH 6.6 at 1 to 2 °C. A control sample of bacteria was incubated under identical conditions in normal serum. The bacteria were then collected by centrifugation and transferred into normal serum at 37 °C and pH 7.4. Chloramphenicol was added to each and the incubation continued. Antiserum-treated organisms were killed (Fig. 6) and their RNA degraded in normal serum at 37 °C in the presence of chloramphenicol, showing that the low pH had not interfered with the binding of the antibody (or antibodies) to the bacteria. Normal bacteria remained in stasis. In this system, the usual delay of about 40 min between the time of addition of chloramphenicol and the onset of killing and RNA breakdown was reduced to about 20 min.

Properties of the antibiotic-induced killing process in antiserum at pH 6.8

Uncouplers and inhibitors of oxidative phosphorylation protect *P. septica* against the antibacterial action of antiserum (Griffiths, 1974*b*). The present work showed that the antibiotic-induced killing of *P. septica* in antiserum at pH 6.8 could also be prevented by such agents. The addition of KCN (5×10^{-3} M) or 2,4-dinitrophenol (4×10^{-3} M) to the bacteria in serum at pH 6.8 immediately following the antiserum and chloramphenicol, abolished the bactericidal effect.

The antibiotic-induced killing in the presence of antiserum at pH 6.8 also resembled the

Fig. 4

Fig. 4. Effect of nalidixic acid and puromycin on *P. septica* growing in normal serum and in antiserum at pH 6.8. □, Bacteria in normal serum plus nalidixic acid; ■, bacteria in antiserum plus nalidixic acid; ○, bacteria in normal serum plus puromycin; ●, bacteria in antiserum plus puromycin. Antiserum (15 %, v/v) or an equal volume of normal serum was added at zero time and the antibiotics 5 min later.

Fig. 5

Fig. 5. Effect of nalidixic acid and puromycin on the stability of *P. septica* RNA. ■, Bacteria in antiserum at pH 6.8 with nalidixic acid added at 5th minute; ○, bacteria in normal serum at pH 6.8 with puromycin added at 5th minute; ●, bacteria in antiserum at pH 6.8 with puromycin added at 5th minute. Bacterial RNA was prelabelled with [¹⁴C]uracil and the results expressed as percentages of label in RNA at time zero.

normal (pH 7.4) antiserum-mediated killing system with regard to temperature sensitivity. Lowering the temperature from 37 °C to 1 to 2 °C before the onset of RNA breakdown and cell death prevented loss of label and rapid killing. On returning the temperature to 37 °C, rapid breakdown of RNA and killing of *P. septica* took place as before.

Antibiotic-induced killing of P. septica in antiserum in the presence of haem compounds

The antibacterial action of antiserum on *P. septica* can be abolished by the addition of haemin or haemoglobin (Bullen *et al.* 1971; Griffiths, 1971, 1974a; and unpublished observations). In the presence of haem compounds, the bacteria multiply apparently normally in antiserum, just as when the pH is lowered from 7.4 to 6.8. It has been proposed that haem compounds abolish the action of antiserum by the fact that they are not bound to transferrin and can thus provide the inhibited bacteria with iron or haem (Bullen *et al.* 1971, 1972a, 1974). If depletion of iron or haem from a critical cellular role was the only factor involved in the inhibitory process, then it would be expected that bacteria growing in antiserum plus haem would behave like bacteria growing in normal serum. That this is not the case is shown in Figs. 7 and 8. The addition of chloramphenicol to *P. septica* growing in antiserum containing haemoglobin (2×10^{-4} M) resulted in a large loss of viability and in the

Fig. 6. Lethal action of chloramphenicol on *P. septica* pretreated with specific antibody. ●, Bacteria pre-treated with antiserum and resuspended in fresh serum without antibody; ○, control bacteria pre-treated in the same way with normal serum and resuspended in fresh serum. Chloramphenicol was added to both at 5th minute.

degradation of RNA. There was a delay of about 40 min between addition of chloramphenicol and the onset of RNA breakdown and cell death. As before, the addition of chloramphenicol to bacteria growing in normal serum, at pH 7.4, produced only bacteriostasis and no breakdown of RNA. Puromycin and rifampicin behaved like chloramphenicol, inducing a rapid bactericidal effect in the presence of antiserum plus haemoglobin but not in normal serum. Nalidixic acid behaved differently, having the same effect on *P. septica* growing in antiserum plus haemoglobin as on the bacteria growing in normal serum. There was some killing in both instances but no loss of label from the RNA.

DISCUSSION

This work shows that a small change in pH will abolish the bactericidal effect of horse antiserum on *P. septica*. Clearly, though, the results do not support the idea that the abolition of the antibacterial action of *P. septica* antiserum, by lowering the pH to 6.8, is due to the increased availability of transferrin-bound iron. That the inhibition was abolished by changing the pH to 6.8 after adding antiserum contrasts with work using added ferric iron (Griffiths, 1971, 1974*a*). The addition of sufficient Fe^{3+} to oversaturate the serum transferrin did not prevent the antiserum from initiating the inhibitory process, but it did reverse these effects after a delay of 2 to 3 h. The pH-effect resembles the action of haem compounds, which also abolish the killing immediately (Griffiths, 1971, 1974*a*). If the antiserum-induced lesion involves a blockage in bacterial iron supply and the abolition of the killing at pH 6.8 is due simply to the increased availability of iron, then it would be expected that *P. septica* in antiserum at pH 6.8 would behave like *P. septica* in normal serum. Since the two systems behaved quite differently on treatment with antibiotics, doubt is cast on the original explanation.

Fig. 7

Fig. 8

Fig. 7. Effect of chloramphenicol on *P. septica* growing in antiserum containing haemoglobin. ○, Bacteria in antiserum plus haemoglobin; ●, bacteria in antiserum plus haemoglobin plus chloramphenicol. Antiserum (15 %, v/v) was added at time zero and followed after 2 min by haemoglobin (2×10^{-4} M). Chloramphenicol was added at 5th minute.

Fig. 8. Action of chloramphenicol on *P. septica* growing in antiserum containing haemoglobin - fate of stable RNA. ○, Bacteria growing in antiserum containing haemoglobin; ●, bacteria in antiserum plus haemoglobin with chloramphenicol added at 5th minute. Antiserum was added at zero time and haemoglobin (2×10^{-4} M) 2 min later. Bacteria were prelabelled with [14 C]uracil and the results expressed as percentages of label in RNA at time zero.

The abolition of the bactericidal action of antiserum at pH 6.8 is not, therefore, due to an increased availability of free ferric iron in the serum. Indeed, it is difficult to see how such a small change in pH could have had an effect on the availability of transferrin-bound iron sufficient to abolish completely the antibacterial action of the antiserum. Experimental studies suggest that only below pH 6.5, where the configuration of the metal binding sites of transferrin appear to undergo a transformation, would any significant release of Fe^{3+} occur (Aasa & Aisen, 1968; Aisen & Liebman, 1968*a, b*). The effect of pH on the bactericidal action of antiserum on *P. septica* must therefore be distinguished from the effect of pH on the growth of organisms in serum. Elin & Wolff (1973) have shown that the growth of *Candida albicans* in human serum is inversely proportional to the pH and directly related to the percentage iron saturation of serum transferrin.

The discovery that the addition of certain antibiotics to *P. septica* growing, apparently normally, in antiserum at pH 6.8 or in antiserum containing haem compounds, restored the bactericidal action of antiserum was unexpected and led to a reassessment of ideas concerning the mechanism of action of specific antiserum on *P. septica*. Since this antibiotic-induced killing was dependent on bacterial oxidative energy metabolism and temperature sensitive, the mechanism responsible is the same as, or part of, the normal antiserum-mediated killing process seen at pH 7.4 (Griffiths, 1974*a, b*). That the antibiotic-induced killing and degradation of RNA could be achieved only with antiserum plus chloramphenicol, puromycin or rifampicin, and not with antiserum and nalidixic acid, suggests that RNA breakdown and cell death is not due simply to the action of antiserum on *P. septica* inhibited by any growth-stopping antibiotic. It remains to be seen whether the cessation of protein synthesis *per se* is involved in the inhibitory process.

The present findings, together with previous work (Griffiths, 1971, 1974*a, b*), show that, although iron compounds can interfere with the bactericidal action of antiserum on *P. septica*, an explanation of the antibacterial action of antiserum based on an interference by antibody with bacterial iron supply is no longer sufficient. If the depletion of iron or haem from a critical cellular role was the only factor in the inhibitory process, then the addition of haem would be expected to restore the system to normal. Bacteria growing in both normal serum and in antiserum plus haem should therefore have responded in the same way to the antibiotics. Since they did not, the process is more complex and must involve other factors. The present results show that antibody, possibly in conjunction with complement, has some effect on the bacteria which is not reversed by haem compounds or by low pH. *Pasteurella septica* with antibody attached, growing apparently normally in antiserum at pH 6.8 or in the presence of haem compounds, is susceptible to a remarkably rapid bactericidal reaction in the presence of certain antibiotics which in normal circumstances would only have produced bacteriostasis.

The mechanism whereby surface-bound antibody or antibodies, plus complement factors, direct changes leading to RNA breakdown and cell death in *P. septica*, and the way in which iron compounds counteract them, thus remains unclear. This is in contrast to the situation in *Escherichia coli*, where the antibacterial action of some sera can be explained by an interference with bacterial iron supply (Bullen *et al.* 1972*b*; Griffiths, 1972; Rogers, 1973). Perhaps a distinction should be made between the role of iron compounds in providing an essential growth factor and their ability to abolish the bactericidal effects of certain antisera.

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