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The Effect of Iron Compounds on the Virulence of *Escherichia coli* for Guinea-Pigs

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Summary. Ferric ammonium citrate, haematin hydrochloride, lysed guinea-pig red cells and crystalline human haemoglobin greatly enhanced the virulence of *Escherichia coli* 0111/B4/H2 when injected intraperitoneally into normal guinea-pigs. The viable counts of *E. coli* in the peritoneal fluid of normal guinea-pigs given a sub-lethal infection were very variable and the bacteria eventually disappeared. In lethal infections in animals treated with iron compounds the bacteria grew extremely rapidly and death occurred when the counts reached 10^9–10^{10}/ml of peritoneal fluid. It is suggested that the mechanisms underlying non-specific immunity of guinea-pigs to *E. coli* may not be dissimilar to those involved in passive immunity to *Clostridium welchii* Type A in guinea-pigs, and in passive immunity to *Pasteurella septica* in mice.

INTRODUCTION

Resistance to bacterial infection takes many forms, and it is convenient to classify the different manifestations of resistance under various headings, such as natural or non-specific immunity, active immunity and passive immunity. While examples of each of these different classes can be demonstrated experimentally, it does not mean necessarily that each separate type of resistance is governed by entirely different mechanisms. In this paper we show that a variety of iron compounds greatly enhance the virulence of *Escherichia coli* for normal guinea-pigs, and the results suggest that non-specific resistance to *E. coli* involves mechanisms which have a close link to those operating in passive immunity to *Clostridium welchii* Type A in guinea-pigs, and *Pasteurella septica* in mice, where iron compounds are equally effective in causing overwhelming infections in otherwise resistant animals (Bullen, Cushnie and Rogers, 1967; Bullen, Wilson, Cushnie and Rogers, 1968).

MATERIALS AND METHODS

Guinea-pigs

Normal guinea-pigs (230–300 g) of either sex were obtained from the Rowett Research Institute Colony.

Escherichia coli

E. coli strain 0111/B4/H2 was obtained from Dr Joan Taylor, Central Public Health Laboratory, London. During a period of 2 years the strain was passaged forty-five times in

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adult guinea-pigs by intraperitoneal injection of a dilute homogenate of infected spleen obtained from dying animals. This procedure did not appear to increase its virulence for guinea-pigs; the lethal dose remaining in the region of 10^8 organisms. Between passages the organism was stored in frozen spleens at −20°. Stock cultures for experiments were kept at −20° in liver brain medium. Before use the organism was subcultured once or twice in papain digest broth, pH 7·4; the last subculture being grown for 5–18 hours. The culture was centrifuged, the supernatant fluid removed, and the organisms resuspended in 0·85 per cent NaCl at a concentration of approximately $1{\cdot}5 \times 10^8$ organisms/ml. Suitable dilutions of the culture were prepared in 0·85 per cent NaCl and a viable count done on plates of MacConkey medium by the method of Bullen, Wilson and Cordiner (1961). The serological identity of the culture was checked at frequent intervals by slide agglutination tests with an *E. coli* 0111/B4 agglutinating antiserum (Burroughs Wellcome & Co.).

Ferric ammonium citrate

This contained 20 per cent w/w iron estimated colorimetrically by the method of Collins, Diehl and Smith (1959): 0·625 g was dissolved in 100 ml of 0·85 per cent NaCl, and sterilized by autoclaving at 15 lb for 15 minutes. The solution was mixed with the bacteria and injected intraperitoneally in amounts sufficient to give 5 mg Fe/kg live weight.

Haematin hydrochloride

Haematin hydrochloride (British Drug Houses Ltd) (2 g) was ground in a ball mill in water (25 ml) for 3 hours. After centrifugation the water was removed and the haematin placed in ethyl alcohol for 30 minutes. The mixture was again centrifuged, the alcohol discarded, and the haematin dried in a dessicator over $CaCl_2$.

Lysed guinea-pig red cells

Normal adult guinea-pigs were anaesthetized with ether and the heart blood removed into a syringe containing a trace of heparin. The cells were washed three times in 0·85 per cent NaCl and lysed by the addition of approximately 5 volumes of distilled water. The red cell stromata were removed by centrifugation at 30,000 ***g*** for 20 minutes at 0° and the supernatant fluid sterilized by filtration through a Seitz EK pad. The haemoglobin content was estimated by the carboxyhaemoglobin (Haldane) method (Wintrobe, 1951).

Crystalline human haemoglobin

Crystalline human haemoglobin was prepared by the method of Drabkin (1949). The crystals were dissolved in 0·85 per cent NaCl and sterilized by filtration through a Seitz EK pad.

Viable counts of E. coli *in the peritoneal cavity*

Normal guinea-pigs were infected intraperitoneally with *E. coli* in 0·85 per cent NaCl, or with *E. coli* mixed with various iron compounds. The animals were killed at intervals by a blow on the head. The peritoneal fluid was removed with a pipette and viable counts done in the usual way.

RESULTS

THE VIRULENCE OF *E. coli* 0111/B4/H2 FOR NORMAL GUINEA-PIGS

Intraperitoneal injection of less than 10^5 *E. coli* in 0·85 per cent NaCl failed to kill normal guinea-pigs, while doses of 10^8 *E. coli* or more were invariably fatal within 48 hours. Doses of 10^5–10^7 *E. coli* gave very variable results (Table 1).

TABLE 1

TITRATION OF *E. coli* IN GUINEA-PIGS

E. coli intraperitoneally	Death rate (2 days)
$2{\cdot}5 \times 10^4$	0/12
$2{\cdot}6 \times 10^5$	2/6
$5{\cdot}2 \times 10^5$	1/6
$2{\cdot}6 \times 10^6$	2/6
$5{\cdot}2 \times 10^6$	5/6
$1{\cdot}0 \times 10^7$	3/6
$1{\cdot}1$–$2{\cdot}2 \times 10^8$	7/7

THE EFFECT OF IRON COMPOUNDS ON THE VIRULENCE OF *E. coli* 0111/B4/H2

The iron compounds were mixed with the organisms immediately before intraperitoneal injection. The volume injected was 1·0 ml except in the experiments with lysed red cells where it varied from 1·0 to 5·0 ml. In every experiment a control group received identical numbers of organisms in the same volume of 0·85 per cent NaCl. Frequent checks were made on the identity of the *E. coli* causing death by performing slide agglutination tests on 50 colonies selected at random from MacConkey agar or blood agar plate cultures of peritoneal exudate or spleen. In every case all the colonies were agglutinated immediately by the 0111/B4 antiserum.

TABLE 2

THE EFFECT OF INTRAPERITONEAL INJECTION OF FERRIC AMMONIUM CITRATE ON THE DEATH RATE OF GUINEA-PIGS INFECTED WITH *E. coli*

E. coli intraperitoneally	Fe intraperitoneally (mg/kg)	Death rate (2 days)
Nil	5	0/5
$3{\cdot}4$–$3{\cdot}7 \times 10^4$	Nil	0/10
$3{\cdot}4$–$3{\cdot}7 \times 10^4$	5	25/25
$3{\cdot}4 \times 10^3$	5	10/10
$3{\cdot}4 \times 10^2$	5	8/10

FERRIC AMMONIUM CITRATE

Intraperitoneal injection of ferric ammonium citrate equivalent to 5 mg Fe/kg was not toxic to normal guinea-pigs. The iron greatly enhanced the virulence of *E. coli*. All the animals receiving a few thousand organisms died within 2 days, and even a few hundred organisms gave rise to a high percentage of fatal infections (Table 2).

HAEMATIN HYDROCHLORIDE

A dose of 100 mg of haematin hydrochloride was as effective as ferric ammonium citrate in enhancing the lethal effect of *E. coli*. Reducing the dose to 50 mg caused less deaths, while 10 mg had no effect (Table 3).

TABLE 3

THE EFFECT OF INTRAPERITONEAL INJECTION OF HAEMATIN HYDROCHLORIDE ON THE DEATH RATE OF GUINEA-PIGS INFECTED WITH *E. coli*

E. coli intraperitoneally	Haematin intraperitoneally (mg)	Death rate (2 days)
Nil	100	0/6
$1{\cdot}8$–$3{\cdot}2 \times 10^4$	Nil	0/16
$1{\cdot}8$–$3{\cdot}2 \times 10^4$	100	21/21
$1{\cdot}8$–$8{\cdot}7 \times 10^3$	100	14/24
$1{\cdot}5$–$8{\cdot}4 \times 10^2$	100	12/20
$2{\cdot}8 \times 10^4$	50	6/10
$2{\cdot}8 \times 10^4$	10	0/10

LYSED GUINEA-PIG RED CELLS

Doses of lysed guinea-pig red cells containing 100–192 mg haemoglobin were as effective as ferric ammonium citrate in enhancing the lethal effect of *E. coli*. Doses containing *ca.* 50 mg haemoglobin were slightly less effective, while 5 mg haemoglobin had no effect (Table 4).

TABLE 4

THE EFFECT OF INTRAPERITONEAL INJECTION OF LYSED GUINEA-PIG RED CELLS ON THE DEATH RATE OF GUINEA-PIGS INFECTED WITH *E. coli*

E. coli intraperitoneally	Lysed red cells (mg haemoglobin)	Death rate (2 days)
Nil	192	0/10
$2{\cdot}5$–$6{\cdot}6 \times 10^4$	Nil	0/30
$5{\cdot}5$–$6{\cdot}6 \times 10^4$	166–192	24/25
$3{\cdot}7 \times 10^4$	108	8/8
$3{\cdot}6 \times 10^3$	100	9/10
$3{\cdot}6 \times 10^2$	100	6/10
$2{\cdot}5$–$4{\cdot}0 \times 10^4$	47–54	26/33
$2{\cdot}5 \times 10^4$	5	0/5

CRYSTALLINE HUMAN HAEMOGLOBIN

Crystalline haemoglobin (135 mg) appeared to be as effective as the other iron compounds in enhancing the lethal effect of *E. coli* (Table 5).

TABLE 5

THE EFFECT OF INTRAPERITONEAL INJECTION OF CRYSTALLINE HUMAN HAEMOGLOBULIN ON THE DEATH RATE OF GUINEA-PIGS INFECTED WITH *E. coli*

E. coli intraperitoneally	Crystalline haemoglobin (mg)	Death rate (2 days)
Nil	135	0/3
$4{\cdot}3 \times 10^4$	Nil	0/3
$4{\cdot}3 \times 10^4$	135	9/10

THE EFFECT OF IRON COMPOUNDS ON THE GROWTH OF *E. coli* IN NORMAL GUINEA-PIGS

Intraperitoneal injection of $1{\cdot}8$–$6{\cdot}6 \times 10^4$ *E. coli* in 0·85 per cent NaCl had no apparent effect on normal guinea-pigs (Tables 1–5). Viable counts on the peritoneal fluid were very variable, but there was some evidence that the bacteria grew slowly for a few hours in the majority of animals. Thereafter the counts declined steadily, although it was surprising to find that a few animals contained small numbers of *E. coli* in the peritoneal cavity for as long as 60 hours after infection (Fig. 1). When ferric ammonium citrate (equivalent

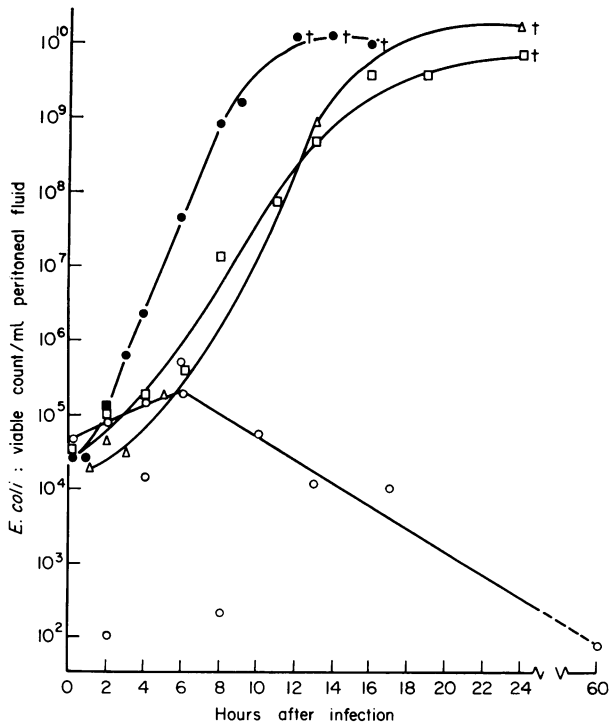

FIG. 1. The effect of iron compounds on the growth of *E. coli* in normal guinea-pigs. *E. coli* injected intraperitoneally with: ○, Normal saline; ●, lysed guinea-pig red cells = 170 mg haemoglobin; □, haematin hydrochloride (100 mg); △, ferric ammonium citrate = 5 Fe/Kg. †, Moribund animals.

to 5 mg Fe/kg), haematin (100 mg) or lysed guinea-pig red cells (equivalent to 170 mg haemoglobin) were injected with the bacteria the organisms grew rapidly in the peritoneal cavity. The rate of growth was remarkably consistent in individual animals, in contrast to the wide variations shown when the bacteria were injected in 0·85 per cent NaCl (Fig. 1). The animals were severely ill or moribund by the time the viable count of *E. coli* had reached 10^9–10^{10}/ml of peritoneal fluid.

DISCUSSION

There is good evidence to suggest that the iron binding protein, transferrin, may play an important role in the mechanism of resistance to a number of bacterial infections. Transferrin, together with the β_2- and γ-globulins, is an essential component of the bacteriostatic system in horse serum, which has a powerful inhibitory effect on *Cl. welchii* Type A *in vitro*. (Rogers, 1967). This system has two important features: it is dependent on the maintenance of a relatively high Eh and pH of the medium (Bullen, Dobson and Wilson, 1964), and the inhibitory effect on bacterial growth is abolished if sufficient iron is added to the medium to saturate the transferrin (Rogers, 1967). Recent work (unpublished) has also shown that iron compounds can abolish the inhibitory effect of serum against *P. septica*.

In passively immunized animals treated with iron compounds and injected with *Cl. welchii* or *P. septica*, bacterial growth is extremely rapid, and closely follows that observed in fatal infections in unimmunized animals. This may be contrasted to the situation in passively immunized animals not treated with iron where the bacteria either grow for a short period and then decline rapidly, as is the case with *Cl. welchii* (Bullen, *et al.*, 1967) or, like *P. septica*, show a rapid decline in numbers immediately after injection (Bullen *et al.*, 1968). The fact that iron compounds abolish both the bacteriostatic effect of serum *in vitro* and passive immunity *in vivo* strongly suggests that the bacteriostatic effect of serum proteins may play a decisive role in resistance to infection. At the moment the mechanism of inhibition of bacterial growth is not fully understood, although for obvious reasons it appears that the iron metabolism of the organisms must be closely involved.

In the present paper we have shown that iron compounds can greatly enhance the virulence of *E. coli* for guinea-pigs. Not only are the iron treated animals killed by very small numbers of *E. coli* but growth curves *in vivo* show that the bacteria multiply at a remarkably consistent rate in individual animals, whereas in animals not given iron the bacterial counts are extremely irregular before the organisms eventually disappear (Fig. 1).

In the experiments with *Cl. welchii* and *P. septica*, already mentioned, the virulence of the bacteria was kept as constant as possible by repeated unselected passage through susceptible animals. Repeated passage of *E. coli* 0111/B4/H2 in guinea-pigs seemed to have little effect on its virulence, and a characteristic feature of the titration in guinea-pigs was the wide range of bacterial numbers that killed some but not all of the animals (Table 1). This closely resembles the result obtained in mice with a non-pigmented mutant strain of *Pasteurella pestis* (Jackson and Burrows, 1956). This organism is of some interest. Its virulence for mice was normally low but was greatly enhanced when the animals were treated with iron compounds. This is exactly comparable to the results obtained with *E. coli* in guinea-pigs. Jackson and Burrows (1956) suggested that the non-pigmented mutant was unable to metabolize some iron compound that was utilized by the virulent parent strain. If one assumes that both the *E. coli* and the mutant strain of

P. pestis possess all the factors necessary for rapid growth *in vivo* except the ability to overcome a defence mechanism associated with transferrin, which normally prevents these organisms acquiring iron, then the basis for their virulence when the animals were injected with iron compounds is largely explained. This suggestion is supported by the observation that the virulence of non-pigmented mutants of *P. pestis* derived from avirulent strains is not enhanced by iron. These bacteria presumably lack additional virulence factors which are not associated with iron metabolism.

There is also some evidence that resistance to *P. pestis* may be more closely associated with iron metabolism than with phagocytosis by tissue cells. Burrows (1955) states that the *P. pestis* mutant strain M7 which is normally of low virulence for mice has the ability to develop resistance to phagocytosis and to inhibit polymorphonuclear leucocytes *in vivo* in a way similar to that shown by virulent stains; yet this strain is one of the non-pigmented mutants whose virulence is greatly enhanced by iron compounds (Jackson and Burrows, 1956). Burrows (1962) also points out that injected iron compounds had no effect on the mobilization of polymorphs, nor on their ingestive capacity, nor on the formation of antibodies, and did not enhance the toxicity of killed *P. pestis*. Iron did not appear to inhibit the phagocytic power of polymorphonuclear leucocytes in *Cl. welchii* infections (Bullen *et al.*, 1967).

Apart from the work with *P. pestis*, *P. septica* and *Cl. welchii*, already mentioned, several authors have shown that iron compounds can have a significant effect on the growth of other bacteria *in vivo*. Sword (1966) showed that iron enhanced the virulence of *Listeria monocytogenes* for mice. Normal mice treated with iron compounds had far more viable organisms in the liver and spleen than untreated animals, although iron did not appear to enhance infection in actively immunized animals. Martin, Jandle and Finland (1963) found that iron compounds markedly enhanced the virulence of *Klebsiella pneumoniae* for rats and mice, but had no significant effect with *Pseudomonas aeruginosa*. Chandlee and Fukui (1965) found that iron compounds greatly enhanced the virulence of *Klebsiella pneumoniae* and *Salmonella typhimurium* in mice and guinea-pigs. No enhancement was found with *Salmonella typhi*, *Pseudomonas aeruginosa*, or two species of Streptococcus. It is clear that further work is required on the effect of iron compounds on experimental infection with a large variety of organisms. Nevertheless the results obtained with *E. coli* suggest that the mechanisms of resistance in normal guinea-pigs to *E. coli* are not entirely dissimilar to those involved in guinea-pigs passively immune to *Cl. welchii* Type A, and those in mice passively immune to *P. septica*. In all three instances the injection of iron compounds with the bacteria leads to loss of resistance and death accompanied by overwhelming bacterial infection. While specific antibody obviously plays an essential role in the passively immune animals it seems highly probable that transferrin, and probably other serum proteins, are closely involved in all three cases, in a defence mechanism which operates by interfering with the iron metabolism of the bacteria. It also seems probable that this defence mechanism consists essentially of a bacteriostatic mechanism, which effectively limits bacterical growth *in vivo*.

REFERENCES

BULLEN, J. J., CUSHNIE, G. H. and ROGERS, H. J. (1967). 'The abolition of the protective effect of *Clostridium welchii* Type A antiserum by ferric iron.' *Immunology*, **12**, 303.

BULLEN, J. J., DOBSON, A. and WILSON, A. B. (1964). 'Bacteriostatic effects of specific antiserum on *Clostridium welchii* Type A. The role of Eh and pH of the medium.' *J. gen. Microbiol.*, **35**, 175.

BULLEN, J. J., WILSON, A. B. and CORDINER, K. (1961). 'The effect of normal and immune sera on the

growth of *Clostridium welchii* Type A in the allantoic cavity of embryonated hen eggs.' *J. path. Bact.*, **82**, 383.

BULLEN, J. J., WILSON, A. B., CUSHNIE, G. H. and ROGERS, H. H. (1968). 'The abolition of the protective effect of *Pasteurella septica* antiserum by iron compounds.' *Immunology*, **14**, 889.

BURROWS, T. W. (1955). 'The basis of virulence for mice of *Pasteurella pestis*.' *Mechanisms of Microbial Pathogenicity. 5th Symposium. Soc. Gen. Microbiol.*, pp. 152. Cambridge University Press.

BURROWS, T. W. (1962). 'Genetics of virulence in bacteria.' *Brit. med. Bull.*, **18**, 69.

CHANDLEE, G. C. and FUKUI, G. M. (1965). 'The role of iron in endotoxin induced non-specific protection.' *Bact. Proc.*, M. **33**, 45.

COLLINS, P. F., DIEHL, H. and SMITH, G. F. (1959) '2, 4, 6-Tripyridyl-S-triazine as a reagent for iron.' *Analyt. Chem.*, **31**, 1862.

DRABKIN, D. L. (1949). 'A simplified technique for a large scale crystallization of human oxyhemoglobin. Isomorphous transformations of hemoglobin and myoglobin in the crystalline state.' *Arch. Biochem.*, **21**, 224.

JACKSON, S. and BURROWS, T. W. (1956). 'The virulence-enhancing effect of iron on non-pigmented mutants of virulent strains of *Pasteurella pestis*.' *Brit. J. exp. Path.*, **37**, 577.

MARTIN, C. J., JANDLE, J. H. and FINLAND, M. (1963). 'Enhancement of acute bacterial infections in rats and mice by iron and their inhibition by human transferrin.' *J. infect. Dis.*, **112**, 158.

ROGERS, H. J. (1967). 'Bacteriostatic effects of horse sera and serum fractions on *Clostridium welchii* Type A, and the abolition of bacteriostasis by iron salts.' *Immunology*, **12**, 285.

SWORD, C. P. (1966). 'Mechanisms of pathogenesis in *Listeria moncytogenes* infection. I. Influence of iron.' *J. Bact.*, **92**, 536.

WINTROBE, M. M. (1951). *Clinical Haematology*, 3rd edn, p. 320. Kimpton, London.