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The Effect of Iron and Transferrin on the Killing of *Escherichia coli* in Fresh Serum

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Summary. Iron in excess of the binding capacity of transferrin allows *Escherichia coli* to grow in fresh serum. This appears to be due to two mechanisms. Firstly iron stimulates growth as it is an essential metabolite which is not available to *E. coli* in serum. Secondly, it interferes with bacterial killing, a process normally mediated by natural antibody and complement.

INTRODUCTION

Iron is an essential growth factor for micro-organisms (Stephenson, 1949). Their ability to compete successfully with the host for iron appears to be a feature of pathogenicity (Weinberg, 1966). Conversely the ability of the host to limit the availability of iron to the pathogen may be associated with resistance to infection. For example, Jackson and Burrows (1956) showed that non-pigmented mutants of *Pasteurella pestis* are relatively avirulent for mice but their virulence is restored when the animals are also injected with iron compounds. This *in vivo* effect is paralled *in vitro* as iron greatly enhances the growth of *P. pestis* and many other bacteria in human serum (Jackson and Morris, 1961; Schade, 1963). Even the protective effect of specific antisera against *Clostridium welchii* type A (Bullen, Cushnie and Rogers, 1967) and *Pasteurella septica* (Bullen, Wilson, Cushnie and Rogers, 1968) can be completely overcome by injection of iron compounds. It seems unlikely that this could be due to iron acting solely as a growth requirement and suggests that iron might also interfere with immune mechanisms. Consequently experiments have been carried out *in vitro* to elucidate the effect of iron and the iron binding plasma protein, transferrin, on anti-bacterial mechanisms. This paper reports their effects on the killing of *Escherichia coli* by fresh serum.

The bactericidal action of fresh serum depends upon 'natural' antibody and all nine components of complement (Inoue, Yonemasu, Takamiyawa and Amano, 1968; Goldman, Ruddy, Austin and Feingold, 1969). Lysozyme is not essential but can, at times, markedly potentiate bacterial killing (Glynn and Milne, 1967). Consequently, as well as the effect of iron on bacterial growth in fresh serum, its effects on other antibody and complement mediated functions and on lysozyme have been studied.

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METHODS AND MATERIALS

Iron solutions were added to aliquots of fresh serum in iron free tubes. 0·1 ml of iron solution was added to 1 ml of serum and after mixing was left at room temperature for 30 minutes to allow binding of iron by transferrin. Next 0·1 ml of washed *E. coli* in iron free saline was added to each tube. The tubes were gassed with 5 per cent CO_2 in air, stoppered, and incubated in a water bath at 37° with shaking. Tubes were removed at intervals and the bacteria counted by serial dilution and pour plates using tryptic soy agar (Difco).

Serum

Fresh human serum was obtained from venous blood allowed to clot at room temperature in iron free tubes. When necessary, complement was inactivated by heating at 56° for 30 minutes.

Bacteria

E. coli, serotype O 141, K 85, H 49, was originally isolated from the urine of a patient with pyelonephritis. This organism has been used previously in experiments showing the effect of parenteral iron injections on pyelonephritis due to *E. coli* (Fletcher and Goldstein, 1970). The bacteria were grown overnight in trypticase soy broth and stored in 1 ml aliquots at −20°. When required an aliquot was incubated in 100 ml of broth for 4 hours. The bacteria were washed in iron free saline and then diluted in saline so the 0·1 ml of the suspension contained a suitable number of organisms.

Metals

Ferrous ammonium sulphate 20 mg/100 ml (Caraway, 1963) was used as a stock solution. Ferric ammonium sulphate was prepared in the same way. Other metal salts (Fisher Pure Chemicals) were dissolved in iron-free water immediately before use.

Transferrin

Human iron free transferrin (Behringewerke) was dissolved in serum or other media as required.

Haemoglobin

Pure haemoglobin was prepared by chromatography on Sephadex CM 50 (Huisman and Dozy, 1965).

Iron free water and glassware were used throughout.

The effect of iron and transferrin was also investigated in the following systems.

(a) Haemolytic antibody, complement and complement fixation were titrated by the method of Campbell, Garvey, Cremer and Sussdorf (1963) using sheep red cells and rabbit anti sheep haemolysin (Baltimore Biological Laboratories). Fresh human or guinea-pig serum was used as complement. Complement fixation was also carried out using dilutions of an *E. coli* suspension in barbital buffer as antigen and fresh human serum as the source of both antibody and complement.

(b) The acid lysis test on red cells of a patient with paroxysmal nocturnal haemoglobinuria (PNH) was performed as described by Dacie and Lewis (1968).

(c) Egg white lysozyme (Pentex) was titrated in tris buffer at pH 8·0 by the method of Repaske (1958) using a stock culture of *Bacillus megaterium* as the indicator organism.

(d) Phagocytosis and killing of *E. coli* by cells from guinea-pig peritoneal exudate was measured by the method of McRipley and Sbarra (1967).

RESULTS

Fig. 1 shows the effect of iron on the growth of *E. coli* in both fresh and complement inactivated serum. The inoculum of *E. coli* multiplied only slowly in complement inactivated serum. When iron had been added there was a lag phase and then rapid growth with a

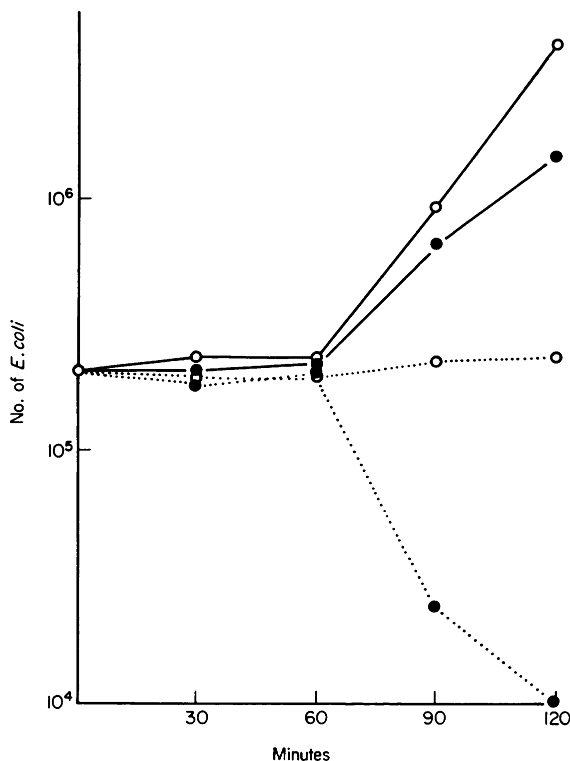

FIG. 1. The effect of iron, 2×10^{-4} molar, on the growth or killing of *E. coli* in fresh (●) and complement inactivated (○) serum. —, +Fe; ...., −Fe.

generation time of about 18 minutes. In fresh serum there was the same lag period before rapid killing, the count of viable bacteria being halved in about 10 minutes. Addition of iron to fresh serum completely reversed killing and allowed the bacteria to grow although a little more slowly than in complement inactivated serum. If iron were acting only as a growth factor the net effect of growth stimulation as seen in complement inactivated serum together with the rate of killing seen in fresh serum should result in some bacterial killing. Instead the bacteria grew, suggesting that iron protects *E. coli* from the bactericidal action of fresh serum.

This suggestion was confirmed by supplying iron to the bacteria as haemoglobin. Fig. 2 shows that added haemoglobin stimulated bacterial growth in complement inactivated serum but did not interfere with killing of *E. coli* in fresh serum.

The protection of *E. coli* from bactericidal activity in fresh serum required the presence of free iron. Table 1 shows the effect of adding increasing quantities of iron to fresh and heated serum. Both growth stimulation and prevention of killing did not occur until the iron binding capacity of transferrin in the serum was exceeded. When the serum was enriched with purified transferrin neither effect occurred until this transferrin was also saturated.

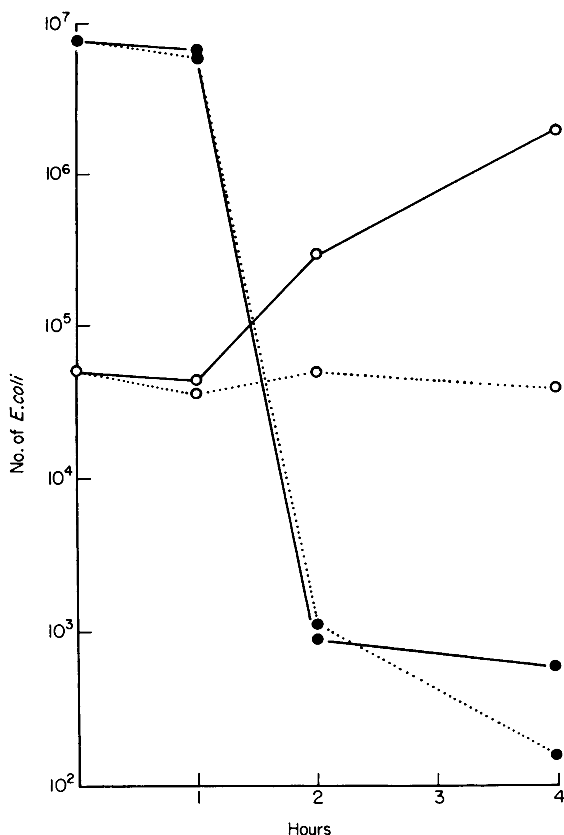

FIG. 2. The effect of haemoglobin, 5 mg/ml of serum, on the growth or killing of *E. coli* in fresh (●) and complement inactivated (○) serum. —, +Hb; ...., −Hb.

Exposure of *E. coli* to iron then washing in iron free saline before mixing with fresh serum did not affect killing. Adding iron after mixing the bacteria with fresh serum reversed killing after the usual lag period. Adding iron after bacterial killing had started also reversed killing but only after the same lag period.

Ferrous and ferric ions were equally effective in blocking killing. Other transitional metal ions, Cr^{+++}, Cu^{++}, Zn^{++}, Mn^{++}, Co^{++} and Ni^{++}, which are known to react with transferrin (Tan and Woodworth, 1969), had no effect.

HAEMOLYTIC COMPLEMENT AND ANTIBODY

In paired experiments, the presence of iron in the same concentration as promotes

bacterial growth (2×10^{-4} molar), did not affect the titre of haemolytic complement or antibody in a system using sheep red cells and rabbit antibody in barbital buffer pH 7·4. This was true whether the iron was added to the fresh serum, i.e. complement, to the antibody, or to the red cells or whether the antibody and red cell dilutions were made in buffer containing transferrin. In a separate experiment heated human serum was used as a source of natural anti-sheep red cell antibodies when again the presence of iron made no difference to the titre of these antibodies.

TABLE 1

THE EFFECT OF IRON ON THE FATE OF *Escherichia coli*, 5×10^4 BACTERIA/ML, IN FRESH AND COMPLEMENT INACTIVATED SERUM

	Fate of *Escherichia coli* in:			
Amount of iron added (μg/ml of serum)	Fresh serum	Fresh serum with added transferrin, 5 mg/ml of serum	Complement inactivated serum	Complement inactivated serum with added transferrin 5 mg/ml of serum
0	K	K	I	I
1	K	K	I	I
2	K	K	I	I
3	G	K	G	I
4	G	K	G	I
5	G	K	G	I
10	G	G	G	G

K = killing; I = growth inhibited; G = rapid growth (1 mg of transferrin binds 1·3 μg of iron).

TABLE 2

ACID LYSIS OF RED CELLS FROM A PATIENT WITH PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA (PNH) WITH AND WITHOUT ADDED IRON

	Per cent lysis of red cells with:	
	No added iron	Added iron 2×10^{-4} molar
PNH cells+fresh serum	1·4	2·8
PNH cells+fresh serum+HCl	16·2	31·7
PNH cells+heat inactivated serum+HCl	0	1·2

ACID LYSIS OF PNH CELLS

The presence of iron (2×10^{-4} molar) increased the sensitivity to lysis of cells from a patient with paroxymal nocturnal haemoglobinuria when incubated in compatible serum (Table 2). There was certainly no protection from this complement dependent action.

PHAGOCYTOSIS AND KILLING BY GUINEA-PIG PERITONEAL LEUCOCYTES

A suspension of *E. coli* was opsonized by incubating with fresh guinea-pig serum for 10 minutes at 37°, and then washed before being incubated with a suspension of leuco-

cytes. In this experiment the concentration of leucocytes was 4×10^7/ml and the *E. coli* 5×10^7/ml. The presence of iron (2×10^{-4} molar) during opsonization or during phagocytosis made no difference to the killing of the bacteria (Fig. 3).

COMPLEMENT FIXATION

The presence of iron (2×10^{-4} molar) made no difference to complement fixation using *E. coli* as the antigen and fresh serum as the source of both antibody and complement.

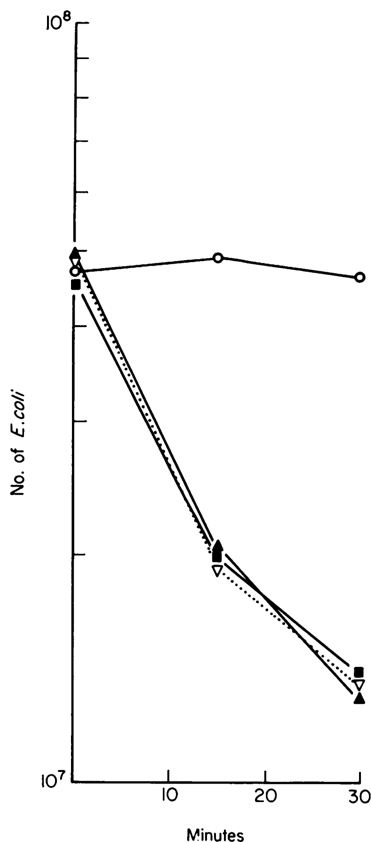

FIG. 3. The effect of iron, 2×10^{-4} molar, on the killing of *E. coli* by guinea-pig peritoneal leucocytes. ○, Opsonized in the presence of iron but no cells added; ■, opsonized in the presence of iron; ▽, opsonized in the absence of iron; ▲, opsonized in the absence of iron, iron added to cell suspension.

EGG WHITE LYSOZYME ACTIVITY

Titration of lysis of a suspension of *B. megaterium* by lysozyme in the presence and absence of iron showed no difference (Fig. 4). The killing of this organism by fresh serum is not affected by iron although its rate of growth in complement inactivated serum was greatly increased.

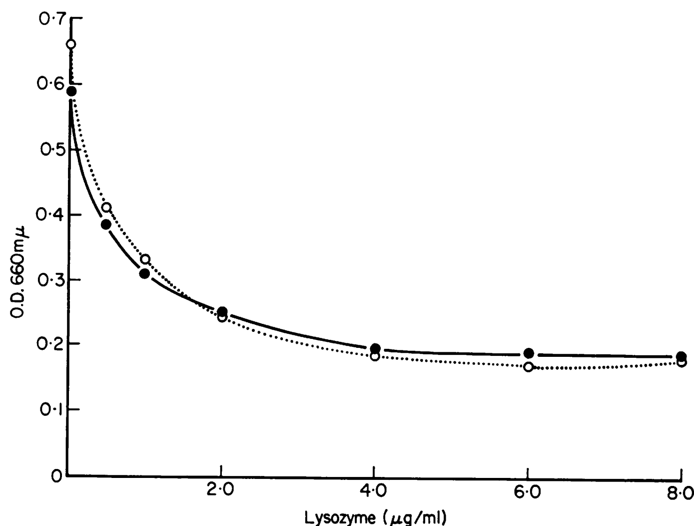

FIG. 4. The effect of iron, 25 μg/ml, on the lysis of *Bacillus megaterium*. ●, +Fe; ○, −Fe. Increasing quantities of lysozyme were added to a suspension of *B. megaterium* in Tris buffer containing transferrin 10 mg/ml. 1 mg of transferrin can bind 1·3 μg of iron.

DISCUSSION

Free iron, that is not bound by transferrin, allows *E. coli* to grow rapidly in fresh serum. Measurements of the rates of growth and killing suggest that unbound iron is not only acting as a required growth factor for the bacteria but also blocks the serum's bactericidal action. This suggestion was confirmed by the effect of haemoglobin which stimulates bacterial growth in complement inactivated serum but does not affect killing in fresh serum.

The killing of *E. coli* by fresh serum is mediated by natural antibody and all nine components of haemolytic complement (Inoue *et al.*, 1968). Lysozyme, although not essential, may increase the rate of killing. In the present experiments iron had no effect on the lysis of *B. metagerium* by lysozyme nor on other complement and antibody dependent functions which it was possible to examine *in vitro*. Hadding and Müller-Eberhard (1967) have reported that ferrous but not ferric ions block the action of the ninth component of haemolytic complement when purified isolated components are assayed. Complement fixation and phagocytosis of bacteria do not require C9 and so no effect of iron would be expected. However, there was also no demonstrable effect upon complement lysis of sheep red cells or of PNH red cells which requires C9. Furthermore, there was no difference in the influence of added ferrous and ferric ions. These experiments did not show that iron is anticomplementary nor that it interferes with the binding of antibody to cells.

Bullen and Rogers (1969) have suggested that antibody may interfere with the iron chelating ability of bacteria. However, it is difficult to see how this would result in rapid bacterial killing which is reversible by iron particularly since the organisms were previously cultured in iron rich broth. It seems more likely that iron affects the surface of bacteria. Jandl and Simmons (1957) showed that several metallic cations, particularly ferric ions, can bind serum proteins to the surface of red cells. This does not interfere with complement lysis of red cells but the same effect may protect bacteria. It might make them similar to

the strains of *E. coli* that are resistant to lysis in fresh serum because of their thick coat of 'O' antigen (Feingold, 1969).

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