THE VARIABLE RESPONSE OF BACTERIA TO FREE HAEMOGLOBIN IN THE TISSUES

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SUMMARY. The local enhancement of infection by exogenous ferric iron, as ferric ammonium citrate, and by ferrous iron as guinea-pig haemoglobin, was assessed in studies with 55 strains of bacteria injected into the skin of guinea-pigs. The test organisms included Staphylococcus aureus, Streptococcus spp., Klebsiella spp., Escherichia coli and Pseudomonas aeruginosa. Four strains of Bacteroides spp. were tested with haemoglobin only. As previously reported with other strains, enhancement of infection by members of a given species by ferric iron was variable; in this study infection with only 11 of 59 strains was enhanced. Haemoglobin either of equal or lesser iron content was a more potent enhancer, affecting 27 of the 59 strains. The enhancement ranged from two-fold to 80-fold, the higher figures on the whole being characteristic of haemoglobin enhancement. Some few instances of depression by both haemoglobin and ferric ammonium citrate were noted. A few tests were made with systemic haemoglobin but the concentrations attainable were largely ineffective. Enhancement of infection did not appear to be related to the capacity of a strain to lyse or digest host red blood cells.

In so far as guinea-pigs, whose antibacterial defences are lowered by ferric or ferrous iron, represent human subjects at risk of infection because of clinical circumstances characterised by excess of available iron—either exogenous or as a result of haemolysis—our results with organisms of a kind commonly associated with infection in hospitals suggest that only a small proportion of environmental bacteria can take advantage of any decreased resistance associated with iron excess.

INTRODUCTION

We have shown (Miles, Khimji and Maskell, 1979) that an increase in the availability of iron in the tissues of many host animal species studied by a number of investigators diminishes resistance to infection with only a minority of strains of a given pathogenic bacterial species. This variability in the response of members of a species was evident with test species of the genera Mycobacterium, Bacillus, Clostridium, Corynebacterium, Listeria, Erysipelothrix, Klebsiella, Proteus, Streptococcus, Staphylococcus, Bordetella, Haemophilus, Aeromonas and Mycoplasma. Our
detailed observations (Miles, Pillow and Khimji, 1976) of a number of Klebsiella strains suggested that absence of response to excess body iron is related to virulence of the test organism for guinea-pigs; the less virulent forms are unable to multiply in the presence of the host's defences to the point where a supply of iron is necessary for their further growth in vivo; and neither free iron nor the microbial iron chelator, enterochelin (see Miles and Khimji, 1975), would enhance the infection in these circumstances.

In 1968, Bornside and Cohn deduced that free haemoglobin enhances bacterial virulence when present in peritoneal exudates of dogs after strangulating intestinal obstruction, and concluded that the presence of lysed red cells decreased tissue resistance to infection. In the same year, Bullen and Rogers (1968) confirmed the infection-enhancing power of haemoglobin. We have accordingly compared the effect in the skin of guinea-pigs of local ferric iron and guinea-pig haemoglobin on infections by strains of bacteria, mainly Staphylococcus aureus, Proteus spp., Klebsiella spp., Escherichia coli, Pseudomonas aeruginosa and streptococci. Some were laboratory strains, and others were recently isolated by the clinical microbiology staff of The London Hospital. Our results with ferric ammonium citrate are similar to those of our survey with local and systemic iron.

**MATERIALS AND METHODS**

**Bacteria.** The designations and, in brackets, sources of the strains tested were as follows: *Proteus vulgaris* and *mirabilis* PR1, PR4, PR10, PR28, PR30 (A. A. Miles), Graves (Professor A. C. Wardlaw) and two recent clinical isolates 66 and 524; *Klebsiella aerogenes* K60, K72, K225, K701, A4 and KGP (Miles et al., 1976) and ten recent clinical isolates, 59, 61, 140, 475, 539, 574, 712, 946, 'Turner' and 'Rankin'; *Escherichia coli* laboratory strain 2882 and eight isolates from bacteraemic patients, 442, 491, 513, 621, 674, 816, 913 and 914; one strain of *Aeromonas hydrophila* (NCTC 7810); *Pseudomonas aeruginosa*, six recent clinical isolates, 276, 277, 279, 289, 724 and 941; *Streptococcus pyogenes* group A R80/6720, R80/6721, R80/6722, R80/6728 and R80/6733 (Dr M. T. Parker) and one clinical isolate 819; *Str. zooepidemicus*, group C, CN771 and CN/D181 (Wellcome Research Laboratories); two clinical isolates of *Bacteroides fragilis*, one of *B. distasonis* and one of *B. thetaiotaomicron*.

**Culture media.** The bacteria were generally grown in Todd-Hewitt broth, but the *Bacteroides* spp. were grown in Robertson's cooked meat medium. For tests of haemolysis, layered nutrient-agar (Oxoid) plates containing 5% blood were used.

**Animals.** Albino female guinea-pigs of the Hartley strain weighing 300–500 g were used. They were clipped over the dorsal part of the trunk for intracutaneous injection and depilated next morning for measurement of lesions.

**Iron.** The injection was 0.1 ml in volume and contained 3–10 μg of Fe+++ as ferric ammonium citrate, or 2.4–3.5 μg of Fe++ as guinea-pig haemoglobin.

**Guinea-pig haemoglobin.** Blood was collected by cardiac puncture and heparinised. The cells were washed three times in phosphate-buffered saline (pH 7.3) and centrifuged at 3000 rpm for 15 min. The washed cell deposit was resuspended in distilled water to the original volume of blood and stored overnight at −20°C. The suspension was thawed and centrifuged at 45000 g for 20 min; the supernate was removed and centrifuged again at 45000 g for a further 20 min to give a final supernate. Sodium chloride to a final concentration of 0.85% was added, and the solution was sterilised by membrane filtration (Millipore GS 0.22 μm pore size). The concentration of haemoglobin in each batch was determined spectrophotometrically (Coulter Counter Model S plus IV); concentrations varied between 14 and 33 mg/ml. Immediately before injection, a bacterial suspension was mixed with an equal volume of haemoglobin, ferric ammonium citrate or saline solution.

**Measurement of infectivity.** The infectivity of the strains was as a rule estimated from the
diameter of the 18-h lesions induced by the injection of graded doses of washed bacterial suspension in the dorsal skin of the guinea-pig as described by Miles et al. (1976). Bacterial suspensions for injection were made in saline containing Todd-Hewitt broth 2.5%. Unless otherwise stated, two concentrations of bacteria were tested in batches of three guinea-pigs; the initial concentration was selected to produce a mature lesion of 8–12 mm diameter. According to the virulence of the strain, this varied between a three-fold concentration and a ten-fold dilution of the original 18-h broth culture. A dilution of this, usually ten-fold but occasionally five-fold was also tested. Up to six strains per animal were tested. The mean lesion diameter is usually linear with respect to log₁₀ dose, for diameters from 6 to 20 mm and with a slope varying from 2.5 to 6.0 according to the strain tested. Because the response lines were linear and in most tests were approximately parallel, differences between two sets of lesions could be estimated with sufficient accuracy from the plots of mean diameter on log₁₀ dose, in terms of the horizontal logarithmic distance between the lines at a given level of response. Thus, at the level of a diameter of 9.5 mm, if the horizontal distance is log₁₀ 1.4 between the response lines for control and for Fe⁺⁺⁺, the ratio of the infective potencies is antilog 1.4 = 25. Differences are expressed as E, the enhancement factor. As regards E factors of < 1, 0.3 for example represents a 3.3-fold depression of lesion size. E values of 2.0–2.9 are considered as a possible enhancement and 0.5–0.3 as a possible depression; the corresponding values of 3.0 or more and 0.33 or less are considered significant.

RESULTS

Strain variation in relation to enhancement of virulence by local haemoglobin or ferric iron salt

The effect was examined of local ferric iron and haemoglobin on the development of intracutaneous lesions produced by 55 strains, representing seven species of bacteria chosen mainly because they are common infecting agents in hospital wards. The table records the dosage of ferric and ferrous iron, the incidence of enhancement among the strains of each species tested and the degree of enhancement (E) observed. The range of positive E values for each species is cited. A number of strains were tested several times. Sometimes the E values were exceptionally high. In each case we have given the average for the lower set of figures and have indicated in footnotes where much higher E values were recorded.

The general conclusion is clear; ferric iron enhanced only 11 of the 55 strains, the most readily enhanced being E. coli and Klebsiella spp., whereas enhancement by haemoglobin was evident with 27 of the 55 strains. In most cases haemoglobin enhanced those that were enhanced by Fe⁺⁺⁺. Members of all species except Bacteroides were enhanced, in some cases substantially and even more than the cited figures suggest. As indicated in the footnotes, one strain of S. aureus was enhanced 18-fold, one of Str. zooepidemicus 20-fold; one strain of E. coli was enhanced 13-fold with Fe⁺⁺⁺ and 16-fold with haemoglobin.

Between 3 and 10 μg of Fe⁺⁺⁺ were used at each injection site and 2.4–3.5 μg of Fe⁺⁺ as haemoglobin. On the reasonable assumption that the haemoglobin owed its enhancing effect to the contained iron, weight for weight the ferrous iron in the haemoglobin was in general more effective than ionic Fe⁺⁺⁺.

As regards depression by either of the iron preparations, one strain of Ps. aeruginosa (not enhanced by haemoglobin) was depressed by Fe⁺⁺⁺, one strain of Str. pyogenes was depressed by both and one strain of S. aureus was depressed by Fe⁺⁺⁺ but not affected by haemoglobin.
TABLE
Incidence and factors (E) of enhancement of skin lesion size produced by bacterial strains tested with local ferric iron (ferric ammonium citrate) and local ferrous iron (haemoglobin).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Tests with Fe+++ (F.A.C.)</th>
<th>Tests with Fe+++ (Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose per site (µg)</td>
<td>Number enhanced/ Number tested</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>10</td>
<td>1/8</td>
</tr>
<tr>
<td><em>Str. pyogenes</em> (group A)</td>
<td>3</td>
<td>1/6</td>
</tr>
<tr>
<td><em>Str. zooepidemicus</em> (group A)</td>
<td>3</td>
<td>0/2</td>
</tr>
<tr>
<td><em>E. coli</em> (group C)</td>
<td>10</td>
<td>4/7</td>
</tr>
<tr>
<td><em>Proteus sp.</em></td>
<td>3-3.5</td>
<td>1/8</td>
</tr>
<tr>
<td><em>K. aerogenes</em></td>
<td>10**</td>
<td>5/16</td>
</tr>
<tr>
<td><em>Aeromonas sp.</em></td>
<td>2.4</td>
<td>1/1</td>
</tr>
<tr>
<td><em>Ps. aeruginosa</em></td>
<td>3.5</td>
<td>0/6</td>
</tr>
</tbody>
</table>

F.A.C. = ferric ammonium citrate; E = factor of enhancement (see Methods).
Highest values recorded: * 18.2, † 20.8, § 12.6, ‡ 15.8.
** Four tests with 3 µg only.
$ This strain was not enhanced in two other tests.
+, ++, +++ = moderate, strong and very strong enhancement, not measurable as a numerical factor.

The slopes of the dose-response plots (lesion size in relation to bacterial dose) in control and iron-treated sites were reasonably parallel. In some cases a compromise was necessary. When there was a substantial deviation from parallelism of iron-treated site diameters with the control because the larger doses were disproportionately more enhanced, a line was drawn parallel to the control through the mid-point of the steeper response line (fig. 1a; cf. fig. 2, Miles et al., 1976). In a few other instances (fig. 1b) the slopes, though reasonably parallel, were very shallow and far apart indicating a very large difference not measurable as a ratio of potencies. In such cases the enhancement is cited as +, ++ and ++++. Slopes were particularly shallow with *Proteus* spp. and some *E. coli* strains; with most other species they ranged from 2.4-6.5 with a median of about 3.5.

We claim neither great accuracy nor precision in our estimates of enhancement. Nevertheless it is very evident that weight for weight of iron content, haemoglobin is a more effective enhancer of infection than ferric ammonium citrate. But even so, haemoglobin enhancement is by no means a general phenomenon; only 27 of 55 strains tested were enhanced by haemoglobin and with nine of them the enhancement was only between 2.1 and 2.7.

*Bacteraemic strains of E. coli.* The eight *E. coli* strains were from bacteraemic patients but no deduction can be made about their having a relatively high virulence because most of them came from grossly compromised patients; e.g., those under treatment with immunosuppressive drugs, and some with diabetes or terminal cancer. As with other species, they were more susceptible to enhancement by haemoglobin than by ferric ammonium citrate.

In our previous comparison (Miles et al., 1979) we found little local enhancement of
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*Fig. 1.* (a) Enhancement by haemoglobin of skin lesions produced by a *Klebsiella* strain. (b) Enhancement by haemoglobin or ferric iron salts of skin lesions produced by a strain of *E. coli*. The very shallow response lines make numerical estimation of enhancement impossible. C = control with no haemoglobin or iron salts added.

**E. coli** by ferric ammonium citrate; with the present strains there was enhancement by ferric ammonium citrate and even more so by haemoglobin. One strain was violently infective for guinea-pig skin; the usual moderate doses employed produced great spreading lesions invading a large part of one flank. Only when tested in low doses was it possible to get measurable lesions with this strain. Even then the slope of the dose response was so shallow (fig. 1b) that a numerical estimate of enhancement was impossible.

**Bacteroides spp.** The reactions in guinea-pig skin of two strains of *B. fragilis*, one of *B. distasonis* and one of *B. thetaiotaomicron* were tested with haemoglobin but not ferric ammonium citrate; they were not enhanced.

**Some miscellaneous observations**

**Systemic iron.** A few comparisons were made of local and systemic haemoglobin. In one experiment the maximum dose of intravenous haemoglobin (150 mg/kg, corresponding to Fe+++ 0.5 mg) was evidently too small to enhance infection by one strain each of *S. aureus*, *Str. zooepidemicus*, *K. aerogenes*, *Aero. hydrophila* and *E. coli*. Indeed the lesions due to the *Klebsiella* strain were depressed some 30-fold. However, in another experiment with the *Klebsiella* strain, the aeromonad and two bacteraemic *E. coli* strains, the equivalent of 1.5 mg of systemic Fe+++ did not affect the klebsiella, the two coliforms were enhanced about 3-fold and the aeromonad, which in nearly all tests has been well enhanced by local Fe+++ and Fe+++, was enhanced 11-fold by systemic Fe++. 

![Diagram](image-url)
Haemolysis. The pathogenicity of strains enhanced by free haemoglobin may have in part depended on their capacity to lyse host red blood cells, thus providing a readily available source of iron. Accordingly, five strains that were well enhanced by local iron, namely *E. coli* 28B2, *K. aerogenes* KGP, *S. aureus* 12, the aeromonad and *Str. zooepidemicus* C (D181) (Miles et al., 1979) were tested on nutrient-agar plates containing 5% horse or guinea-pig blood. The coliform and the klebsiella affected neither blood, the staphylococcus was feebly β-haemolytic on horse and strongly so on guinea-pig blood. The aeromonad was strongly β-haemolytic on both media, and the *Str. zooepidemicus* strain was haemolytic on horse blood alone. Thus the staphylococcus and the aeromonad may have benefited by the release of available iron by their haemolytic powers. Another test was made with the eight bacteraemic strains of *E. coli*. None was β-haemolytic on guinea-pig blood; one had no effect on blood and the other seven produced only varying degrees of haemodigestion around their colonies. In this group there was no suggestion that even haemodigestion aided their infectivity.

Availability of Fe³⁺ in free haemoglobin. This was tested indirectly by measuring the capacity of ferric ammonium citrate (Fe³⁺⁺⁺) and guinea-pig haemoglobin to reverse the inhibition by ethylene diamine di-ortho-hydroxyphenyl acetic acid (EDDA) of *K. aerogenes* K72 in plates of chemically defined medium (Miles and Khimji, 1975). Plates (9 cm) contained 16 ml of defined medium (DMSCP) with EDDA 15 μg/ml. Dilutions of ferric ammonium citrate and guinea-pig haemoglobin were applied to the surface of plates in fish spines containing 27-μl volumes (Lightbown and Sulitzeanu, 1977).

![Fig. 2.—Reversal of EDDA-inhibition of *Proteus* 054 by iron as ferric ammonium citrate (Fe³⁺⁺⁺) and guinea-pig haemoglobin (Hb) (Fe⁺⁺⁺).]
Weight for weight of iron, the Fe\(^{++}\) in haemoglobin was slightly more effective than Fe\(^{+++}\) in ferric ammonium citrate. A direct comparison is not possible because the slopes of diameters of exhibition zones produced were not parallel (fig. 2) but it is clear that both are highly effective in reversing iron chelation by the synthetic compound.

**DISCUSSION**

The work with haemoglobin was confined to the simple observation of the degree to which lesions were increased in size. None of the more elaborate tests that we applied to detailed studies on ferric ammonium citrate, such as establishing the living bacterial content of the lesions, was used. We have assumed that enhanced lesion size is equivalent to enhancement of infection. The main object of our investigation was to see how far the variable response of bacteria to excess ferric iron in host tissues (Miles *et al.*, 1979) is applicable to Fe\(^{++}\) presented as guinea-pigs’ own haemoglobin, administering as far as possible comparable weights of iron in the preparations used. In our previous comparison of systemic and local Fe\(^{+++}\) we used 10 \(\mu\)g of local Fe\(^{+++}\). The number of positive results with *S. aureus* and *K. aerogenes* and the degree of enhancement is about the same as recorded previously, but at the lower dose of Fe\(^{+++}\) (3\(\mu\)g or less) the streptococci and the *Proteus* spp. are much less enhanced. For the other species the results are much the same as in the previous paper. It is clear first that the response in a given species is highly variable, ranging from enhancements of 80-fold to no enhancement; and second that weight for weight the Fe\(^{++}\) in haemoglobin is a more effective enhancer than Fe\(^{+++}\) as ferric ammonium citrate.

The contention based on a number of investigations in the USA, that haemoglobin in the exudates of the inflamed peritoneum enhances infection by *E. coli* in dogs, rabbits, guinea-pigs and rats was admirably summarised and extended by Bornside and Cohn (1968). Bornside and Cohn (1968) also tested four strains of *Acinetobacter*, only one of which was enhanced, whereas a strain each of a species of *Proteus*, *Providencia* and *Pseudomonas* was fully enhanced, *Serratia marcescens* and *Bordetella bronchiseptica* mildly enhanced, and *Bacteroides* spp. not affected. Bullen and Rogers (1968) established that homologous lysed red cells and heterologous crystalline haemoglobin abolished the protective effect of antibody in *Pasteurella septica* infection of mice and extended their observations to intraperitoneal infection of guinea-pigs by *E. coli* with the same result. It should be noted however that in most of the investigations cited by Bornside and Cohn (1968) fairly large intraperitoneal doses of haemoglobin-stained exudates were tested. Relatively large weights of haemoglobin, from 7–28 mg in the mouse peritoneum and 50–236 mg in the guinea-pig peritoneum were used by Bullen and Rogers (1968). It is therefore possible that massive haemoglobin-containing exudates in the peritoneum will enhance a larger proportion of common hospital infecting organisms than is indicated by our relatively low doses. But our results stress the fact that a general assumption of the dangers of enhancement of moderately pathogenic organisms by free iron in the tissues is not so prevalent a hazard as appears from the extensive literature on the experimental enhancement of infection by iron.

We are indebted to the Clinical Microbiology Department of The London Hospital for many of the strains recently isolated from clinical infection.
REFERENCES


