Failure of iron to promote attachment of gonococci to human spermatozoa under physiological conditions

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SUMMARY The effect of iron on the attachment of gonococci to human spermatozoa was investigated using the three iron salts, ferric chloride, ferric nitrate, and ammonium ferric citrate (AFC). Ferric chloride and ferric nitrate were found to be unsuitable for such studies because they were insoluble at physiological pH values, produced a marked decrease in the pH of unbuffered medium (Ringer’s solution), and agglutinated spermatozoa. AFC, in contrast, was soluble at physiological pH, did not affect the pH value of Ringer’s solution, and did not agglutinate spermatozoa. When gonococci and spermatozoa were mixed together in media with and without AFC, the proportion of spermatozoa with adherent gonococci was approximately the same in each case. Thus, in contrast to a previous report, we have found that the addition of iron does not increase the attachment of gonococci to human spermatozoa.

Introduction

The ability of bacteria to acquire iron from host tissues is an essential feature of the pathogenesis of many bacterial infections (Weinberg, 1978), and recent studies with Neisseria gonorrhoeae suggest that this phenomenon may prove to be important in gonorrhoea. Addition of exogenous iron has been shown to promote the growth of gonococci in vitro (Kellogg et al., 1968), to induce reversion of type 4 colonies which are unassociated with virulence to colonies with virulence-associated type 1 morphology (Hafiz et al., 1977), to enhance the survival of serum-susceptible strains of N. gonorrhoeae in the presence of serum (Johnson et al., 1978; Norrod and Williams, 1978), and to enhance the virulence of gonococci for chicken embryos (Payne and Finkelstein, 1975). Furthermore, strains of gonococci isolated from patients with disseminated disease exhibit an enhanced ability to acquire iron in experimental infections of chicken embryos (Payne, et al., 1978), and one such strain produces an iron-chelating factor which promotes growth of gonococci in low-iron medium (Payne and Finkelstein, 1978).

It has also been reported that addition of iron salts promotes the attachment of gonococci to human spermatozoa (James et al., 1976). Such an effect could be important because factors which enhance attachment of gonococci to host tissues would tend to increase the likelihood of the organisms successfully colonising host epithelial surfaces. This study was therefore undertaken in an attempt to confirm and extend the observation that iron salts promote attachment of gonococci to spermatozoa.

Materials and methods

BACTERIA
Type 1 gonococci (Kellogg et al., 1963) of strain 192A (McGee et al., 1976) were used in all studies.

REAGENTS
Stock solutions of ferric chloride and ferric nitrate (100 mmol) were prepared, sterilised, and stored as previously described (James et al., 1976). Ammonium ferric citrate (AFC) containing 20.5-22.5% (w/w) iron (Hopkin and Williams) and tri-ammonium citrate (Hopkin and Williams) were each dissolved in distilled water and sterilised by filtration through a 0.22µm Millipore filter. As the exact amount of iron in the AFC was unknown, stock solutions were prepared on the basis that there was 20.5% (w/w) iron present. However, the actual iron concentration may have been up to 10% more than the values quoted in the results. Ringer’s
solution (RS) was prepared as described previously (James et al., 1976), filter-sterilised and stored in plastic containers. Eagle’s minimal essential medium (MEM) was prepared by diluting a 10× concentrated solution (GIBCO) to single strength with sterile pyrogen-free water for injection. The MEM was supplemented with HEPES buffer (50 mmol) and the pH was adjusted to a value of 7.2 with sterile 1 mol NaOH.

**PREPARATION OF SPERMATOZOA**

Samples of human semen from a single donor were used throughout these studies. The semen samples were diluted to 20 ml in either RS or MEM within five minutes of collection and centrifuged at 1000×g for five minutes at room temperature. The pelleted spermatozoa were resuspended in fresh medium and washed twice. The spermatozoa were finally resuspended in 5 ml of medium, counted in a haemocytometer, and diluted so that the final concentration was in the range 10⁶-10⁷ cells/ml.

**PREPARATION OF ERYTHROCYTES**

Five millilitres of blood from each of three donors (blood groups A, AB, and O) were collected separately into heparinised tubes (Sigma). Each sample was diluted to a volume of 25 ml in RS, and the erythrocytes were sedimented by centrifugation at 1000×g for five minutes. The erythrocytes were resuspended in RS, washed twice, and then resuspended in 25 ml of RS for use in agglutination studies.

**MIXTURE OF SPERMATOZOA AND BACTERIA**

Gonococci were grown for 18-22 hours at 37°C on GC agar base (Difco) supplemented with 2% Isovitalex (Baltimore Biological Laboratories) in an atmosphere of 5% CO₂ in air. The bacteria were scraped from the surface of the agar, suspended in 5-10 ml of either RS or MEM, and agitated on a Rotamix Deluxe at maximal amplitude for 30-60 seconds. The suspension was then centrifuged at 70×g for three minutes to remove clumped bacteria. The upper portion of the suspension was removed for use in experiments, care being taken not to disturb the sediment, and the number of viable gonococci was determined by plating out serial 10-fold dilutions. The number of gonococci used in different experiments varied in the range 10⁶-10⁷ colony-forming units/ml.

The test mixture used in attachment experiments consisted of 0.5 ml of gonococcal suspension, 0.1 ml of either an iron solution or control fluid (that is distilled water or a solution of ammonium citrate), and 0.4 ml of sperm suspension. In any given experiment, the spermatozoa and gonococci were both suspended in either RS or MEM but never in a mixture of the two. The iron solutions were routinely preincubated with the gonococci for 20-30 minutes and then, following the addition of spermatozoa, the test mixtures were incubated in a water bath at 37°C for 15 minutes, during which time the suspensions were agitated at intervals by gentle manual shaking.

**EVALUATION OF ATTACHMENT OF GONOCOCCI TO SPERMATOZOA**

At the end of the incubation period, two separate drops from each suspension were placed on a glass slide using a Pasteur pipette and allowed to dry in air. The slides were fixed overnight in methanol, then Gram stained. A minimum of 200 spermatozoa for each drop was counted at ×650 magnification, and the percentage with one or more diplococci attached was determined. All slides were coded before microscopical examination to reduce observer bias in determining the degree of attachment.

**Results**

**SOLUBILITY OF IRON SALTS**

In initial experiments, attempts were made to supplement HEPES-buffered MEM, pH 7.2, with iron in the form of either ferric chloride or ferric nitrate. Both these salts however reacted upon mixing

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of iron compounds and pH value on attachment of gonococci to spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suspension containing</td>
<td>Suspending medium</td>
</tr>
<tr>
<td>Gonococci + spermatozoa</td>
<td>RS</td>
</tr>
<tr>
<td></td>
<td>RS + 0.1 mmol FeCl₃·6H₂O</td>
</tr>
<tr>
<td></td>
<td>RS + 0.1 mmol Fe(NO₃)₃·9H₂O</td>
</tr>
<tr>
<td></td>
<td>RS</td>
</tr>
<tr>
<td></td>
<td>RS + AFC</td>
</tr>
<tr>
<td></td>
<td>RS + AC</td>
</tr>
<tr>
<td></td>
<td>RS</td>
</tr>
<tr>
<td></td>
<td>RS + 0.1 mmol FeCl₃·6H₂O</td>
</tr>
</tbody>
</table>

+ Positive — negative  
* Counts performed on unclumped spermatozoa only  
AFC = ammonium ferric citrate (final concentration of iron approximately 10 µg/ml); AC = ammonium citrate (final concentration 40 µg/ml); RS = Ringer’s solution
Failure of iron to promote attachment of gonococci to human spermatozoa

with MEM to produce insoluble orange precipitates. In subsequent experiments therefore RS was used as the diluent, since ferric chloride and ferric nitrate are soluble in this fluid (James et al., 1976). RS is a simple salt solution with negligible buffering capacity, and several batches tested during the course of these studies had pH values within the range 6.2-6.8. With all batches of RS studied, addition of either ferric chloride or ferric nitrate at a final concentration of 0.1 mmol resulted in a dramatic drop in the pH value to within the range 3.0-3.5 (Table 1). Attempts to adjust the pH of the solution to its original value by addition of sodium hydroxide resulted in the formation of a precipitate. To study the effect of iron on gonococcal attachment to cells at physiological pH values, a further group of experiments was therefore conducted in which the soluble iron-complex AFC was used. When added to RS, AFC (final concentration of iron approximately 10 μg/ml) lowered the pH value only marginally (Table 1) and had no effect at all on the pH of MEM buffered with HEPES (Table 2).

**Table 2 Effect of ammonium ferric citrate on attachment of gonococci to spermatozoa**

<table>
<thead>
<tr>
<th>Suspending medium</th>
<th>pH value</th>
<th>% of spermatozoa (mean ± SD) with adherent gonococci</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM + AFC</td>
<td>7.1</td>
<td>33.1 ± 2.8</td>
</tr>
<tr>
<td>MEM + AC</td>
<td>7.2</td>
<td>32.1 ± 0.6</td>
</tr>
</tbody>
</table>

AFC = ammonium ferric citrate (final concentration of iron approximately 10 μg/ml; AC = ammonium citrate (final concentration 40 μg/ml)

To assess the effect of iron on the interaction of gonococci and spermatozoa, attachment studies were performed using gonococci and spermatozoa suspended in either RS or RS supplemented with ferric chloride, ferric nitrate, or AFC. In addition, gonococci and spermatozoa were suspended in RS, the pH of which had been adjusted (by addition of HCl) to a value comparable to that of RS supplemented with ferric chloride or ferric nitrate (Table 1). When a sperm suspension was added to RS supplemented with either ferric chloride or ferric nitrate (final pH 3.1) a white precipitate was produced which was seen on microscopical examination to consist of agglutinated spermatozoa (Fig. 1). Gonococci were not necessary for agglutination since it readily occurred in suspensions containing only

**Fig. 1 Agglutination of human spermatozoa suspended in Ringer's solution containing 0.1 mmol ferric chloride. (Gram stain; × 180 magnification)**
spermatozoa (Table 1). Agglutination was not detected however when spermatozoa were added to RS not supplemented with iron (Fig. 2), to RS at a low pH value, or to RS supplemented with AFC (Table 1). Similarly in experiments in which AFC or ammonium citrate was added to MEM buffered with HEPES (pH 7.2) no agglutination of spermatozoa occurred. The agglutination of cells in the presence of ferric chloride or ferric nitrate was not specific for spermatozoa, since an identical pattern of agglutination resulted when erythrocytes rather than sperm were added to the various media described above.

EFFECT OF IRON SALTS ON ATTACHMENT OF GONOCOCCI TO SPERMATOZOA

The proportion of spermatozoa with gonococci attached to them did not vary markedly when cells suspended in RS pH 6.8 were compared with cells suspended in RS containing either AFC or ammonium citrate (Table 1). A slight increase in the proportion with adherent gonococci was however observed with spermatozoa suspended in RS pH 3.1 (Table 1). When spermatozoa were suspended in RS supplemented with either ferric chloride or ferric nitrate, accurate determination of the proportion with attached gonococci was not possible owing to the tendency of the spermatozoa to agglutinate. In such samples however counts performed on free spermatozoa indicated that about 75% of the cells had adherent gonococci (Table 1).

In experiments in which HEPES-buffered MEM (pH 7.2) was used as the suspending medium, addition of iron (at a final concentration of 10 μg/ml) in the form of AFC did not have any effect on the proportion of spermatozoa to which gonococci attached (Table 2).

Discussion

Previous work on the interaction of gonococci with spermatozoa in vitro indicated that adherence of the bacteria is enhanced in the presence of iron salts (James et al., 1976). The findings presented here however suggest that the circumstances of their study may have been rather adverse and that the degree of attachment may have been raised for a number of reasons. In our study, addition of the iron salts, ferric chloride or ferric nitrate, produced immediate agglutination of the sperm cells. Some cells entangled in the clumps would be less accessible to gonococci and consequently the ratio of gonococci to accessible

Fig. 2 Human spermatozoa suspended in Ringer's solution not supplemented with iron salts. (Gram stain; × 180 magnification)
sperrmatozoa (that is, spermatozoa which have an opportunity to come into contact with a gonococcus) would be increased. This would be expected to result in an apparent increase in the degree of attachment of gonococci if counts were performed only on free spermatozoa, since the degree of attachment has been shown to be directly related to the ratio of gonococci to spermatozoa in the reaction mixture (James-Holmquest et al., 1974). In our study, the agglutination of spermatozoa by the iron salts appeared to be non-specific with regard to cell type, since erythrocytes of three different blood groups were also agglutinated. James et al. (1976), in contrast, made no comment about agglutination of spermatozoa; however they used pooled semen samples which had been frozen in glycerol and incubated their mixtures on a continually shaking water bath. These experimental conditions were slightly different from ours and may possibly have led to less agglutination of spermatozoa than seen by us.

Attachment of gonococci to spermatozoa in the presence of ferric chloride or ferric nitrate was also apparently enhanced because these iron salts produced a marked decrease in the pH value of the unbuffered suspending medium (RS). When the attachment of gonococci to spermatozoa suspended in RS pH 6.8 was compared to the attachment observed with gonococci and spermatozoa suspended in RS pH 3.1 (the pH value of RS supplemented with 0.1 mmol ferric chloride or ferric nitrate) enhanced attachment occurred at the lower pH value (Table 1). Thus, some of the enhanced attachment apparently produced by ferric chloride or ferric nitrate is due, at least in part, to the effect these salts have on the pH of the medium. This is further borne out by the observation that iron in the form of AFC, which failed to lower the pH value of RS to any marked degree, failed to enhance attachment of the bacteria to the spermatozoa.

The observation that the pH value of RS supplemented with ferric chloride or ferric nitrate at a final concentration of 0.1 mmol is in the range 3.0-3.5 has important implications in that this pH range is several orders of magnitude lower than that found in vivo. Thus, observations made on the interaction of gonococci with spermatozoa under these conditions may not be relevant to interactions that occur under conditions of physiological pH. Since ferric chloride and ferric nitrate were insoluble at pH values close to neutrality, subsequent studies were performed using the soluble iron complex AFC. This compound was soluble in both RS pH 6.8 and in MEM buffered with HEPES at pH 7.2, the normal pH value of spermatozoa (Diem and Lentner, 1970). The presence of AFC did not enhance attachment of gonococci to spermatozoa with either RS or MEM as the suspending medium. Thus, no evidence was obtained that iron enhanced attachment of gonococci to sperm cells under conditions of physiological pH.

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References

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