THE ANTAGONISM OF TETRACYCLINE AND FERRIC IRON IN VIVO

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SUMMARY. To test the hypothesis that the in-vivo antibiotic action of tetracycline might be affected by ferric iron and the enhancement of infection by ferric iron by tetracycline, the actions of intraperitoneal antibiotic and local ferric ammonium citrate, given separately and together, were measured in the dorsal skin of guinea-pigs bearing lesions due to staphylococci, streptococci, a Proteus sp., an Erysipelothrix sp., Clostridium perfringens, Pseudomonas aeruginosa, Aeromonas hydrophila and Klebsiella pneumoniae.

Tetracycline, given in two intraperitoneal doses of 25 mg/kg at 0 and 2 h after intracutaneous challenge, maintained plasma concentrations of 4–6 μg/ml for more than the first 4 h of infection, after which the local lesions had become largely insusceptible to the antibiotic. The intracutaneous injection of Fe+++ 10 μg in a volume of 0.1 ml containing the bacteria was sufficient to enhance infection by those strains susceptible to this effect.

The in-vivo efficacy of tetracycline was not always related to low MIC; a low MIC was sometimes associated with little action and a high MIC with moderate action.

Sixteen organisms were tested. The iron diminished the tetracycline effect only feebly with one staphylococcal strain and the strain of E. rhusiopathiae. In only one case, with a strain of Proteus sp., was the tetracycline action grossly diminished. On the other hand, tetracycline diminished the enhancement effect of iron moderately with three strains of staphylococci and one strain each of K. pneumoniae, P. aeruginosa and C. perfringens, and strongly with two strains of staphylococci, a group-C streptococcus and one strain each of K. pneumoniae, E. rhusiopathiae and A. hydrophila.

It is evident that the diminution of tetracycline action by moderate excess of readily available Fe+++, whether endogenous or administered, is an unlikely event (three instances among the 16 tested) whereas the diminution of the infection-enhancing effect of iron by tetracycline is much more likely (12 instances among the 16). Insofar as a decrease in iron available for enhancement of infection is valid evidence of a

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diminution of the iron available for necessary physiological processes of the subject treated, our results suggest that these processes might be affected by tetracycline.

INTRODUCTION

The work of Weinberg (1957 and 1971) showed that tetracycline forms complexes with metallic cations and suggested that the high affinity of tetracycline for iron might affect the availability of iron in the tissues during treatment with the antibiotic. We have made a small number of tests of the effect of iron on various local infections in guinea-pig skin and their modification by systemic tetracycline. Average concentrations of tetracycline in plasma after a dose of 25 mg/kg given intraperitoneally were of the order of 5 µg/ml. This concentration parallels that achieved in clinical situations and had a substantial action on several of the infections tested. It was, therefore, considered suitable for the measurement of the effect that excess iron might have on the antibacterial action of tetracycline on the infection-enhancing effect of Fe++, used at the local concentration found by Miles et al. (1978) to enhance various infections.

MATERIALS AND METHODS

Bacteria. The test strains and (in brackets) their sources were: Staphylococcus aureus CN57H, BI and M1 (Professor Wilson-Smith), PS80, 16201 and 16334; Streptococcus group C, D181 (Wellcome Research Laboratories); Proteus sp. Graves (Professor A. C. Wardlaw); Klebsiella pneumoniae KGP, A3 and K701 (Miles et al., 1976); Pseudomonas aeruginosa 5515 and 5525 (Dr M. T. Parker); Erysipelothrix rhusiopathiae EW2 (Dr A. W. Gledhill); Clostridium perfringens SR9; Aeromonas hydrophila Kulp, NCTC 7810.

Culture media. With the exception of C. perfringens, which was cultivated in Robertson's cooked meat medium (RCM), all strains were grown in Todd-Hewitt Broth (Oxoid) and suspended for injection in saline 0.85% containing Todd-Hewitt Broth 2.5%.

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 the intracutaneous injection of bacteria and depilated next morning for measurement of the lesions.

Iron. Ferric iron, as ferric ammonium citrate, was given intracutaneously. The 0.1-ml injection volumes contained Fe+++ 10 µg.

Tetracycline hydrochloride buffered with ascorbic acid (tetracycline 500 mg and ascorbic acid 1250 mg) was administered intraperitoneally (ip) or in some cases intravenously (iv). Usually, 25 mg/kg was given at 0 and 2 h after the local injections of bacteria. In one instance, only the 0-h dose was given; in four instances a single dose was given 4 h before infection (table II).

Plasma concentrations of tetracycline were estimated by a well-diffusion assay. At hourly intervals after tetracycline injections, blood samples were taken and heparinised to prevent clotting. The plasma, diluted 1 in 4 in phosphate buffer (pH 4.5), was placed in 4-mm wells in an assay plate containing Diagnostic Sensitivity Test Agar (DST; Oxoid) seeded with a suspension of Bacillus cereus spores (NCIB 8849). A standard series of dilutions of tetracycline in phosphate buffer (pH 4.5) containing horse serum 25% v/v was included in each assay. Incubation was for 18 h at 30°C.

In-vitro susceptibility tests of strains to tetracycline. All strains were grown in Todd Hewitt Broth for 18 h, except C. perfringens which was grown in RCM. An inoculum of c. 10^6 cfu was applied with a multi-point inoculator (Denley Instruments Ltd) to DST agar, containing saponin-lysed horse blood 5% and appropriate concentrations of tetracycline. Minimum inhibitory concentrations (MICs) of tetracycline were estimated after incubation for 24 h at 37°C. The MIC was defined as the lowest concentration completely suppressing visible growth as compared with a control plate without antibiotic.

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 two graded doses of washed bacterial suspension in the dorsal skin of the guinea-pig. The method was fully described by Miles et al. (1976). Unless otherwise stated, two concentrations of each test bacterial suspension, undiluted (1 in 1) and diluted (1 in 10), were tested in batches of three guinea-pigs, the 1 in 1 concentration being selected to produce a mature lesion of 8-12 mm diameter. Up to six strains per animal were tested. The mean lesion diameter was usually linear with respect to log 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 log dose, in terms of the horizontal logarithmic distance between the lines at a given level of response. Thus, at the level of diameter of 9.5 mm, if the horizontal distance is log_{10} 1.4 between the response lines for control and for the Fe^{++} series, 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 0.0.3 as a possible depression; the corresponding values of 3.0 or more and 0.33 or less are considered significant.

Occasionally the slope of the plot of lesion size with iron and tetracycline was not parallel with the slope for control lesions. Usually the slopes where the lower doses produced small lesions were shallower than the control slopes. For an approximate estimate of distances in such cases, a slope was drawn parallel to the control slope through the mid-points of the deviant lines (figs. 3 and 4). The horizontal distance was then measured between these compromise lines. As in our previous work, no attempt was made to fit plots statistically or to determine the error of observed distances. The basis for arriving at limits of significance were fully discussed by Miles et al. (1976). The iron enhancement of S. aureus strain MI was great but the response lines were so shallow and far apart that a numerical estimate of enhancement was impossible. As explained fully by Maskell and Miles (1984), this type of enhancement is indicated by + symbols; the ++ results in table I indicate a very strong enhancement.

**RESULTS**

*Plasma concentrations of tetracycline*

Fig. 1 shows typical examples of concentrations attained in guinea-pigs on either the one-dose or the two-dose regimen. A two-dose regimen enabled slightly higher concentrations to be maintained for a longer period. A concentration of tetracycline of about 5 μg/ml in the plasma for more than 4 h after ip injection may be confidently assumed.

*Declining efficacy of tetracycline with age of local lesions*

In spite of the above findings the efficacy of the tetracycline falls sharply within 4 h, either because the lesions are less permeable to the circulating antibiotic or the bacteria are intraleukocytic and, therefore, relatively inaccessible to the drug.

Bacteria were injected at −4, −2 and 0 h in six guinea-pigs, and at 0 h a single ip dose of tetracycline was given to three guinea-pigs. Fig. 2 shows the effect on S. aureus PS80. The E factor changed from 0.01 with bacteria injected at the same time as the tetracycline to 0.6 with bacterial lesions that were 4 h old when tetracycline was given. The results with four organisms tested in this way are summarised in table I, inhibition at 0 h being rated 100. The results are compatible with those found with several organisms inhibited by penicillin and streptomycin by Miles et al. (1957) and extended by others (e.g., Burke, 1961; Alexander and Altemeier, 1965; Noble, 1965).
Fig. 1.—Tetracycline concentrations in plasma of guinea-pig after ip injections of 25 mg/kg (see Methods): ○—○ one-dose regimen (mean values for three animals); •—• two-dose regimen (mean values for two animals).

Fig. 2.—Diminishing efficacy of intravenous tetracycline on the size of local skin lesions due to S. aureus PS80 with increasing age of the lesions: T = tetracycline-treated animals; C = control animals.
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Table I
Declining effect of tetracycline with increase in the age of local infection

<table>
<thead>
<tr>
<th>Organism</th>
<th>Effect on local lesion* when intravenous tetracycline was given at different times after injection of stated organism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Streptococcus D181</td>
<td>100</td>
</tr>
<tr>
<td>A. hydrophila Kulp</td>
<td>100</td>
</tr>
<tr>
<td>S. aureus B1</td>
<td>100</td>
</tr>
<tr>
<td>S. aureus PS80</td>
<td>100</td>
</tr>
</tbody>
</table>

* The effect is expressed as a percentage of the effect observed with the antibiotic given at time 0 h (i.e., at the time of injection of the bacteria).

Inhibition of tetracycline by guinea-pig serum

It is noteworthy that guinea-pig serum added to the in-vitro mixtures for assay of serum concentrations of tetracycline in concentrations of 20% or 50% decreased measurable concentrations by between 14 and 30%. Accordingly the tetracycline samples were all maintained and assayed in 25% serum and the results reflect the plasma concentrations of effective antibiotic in vivo.

The separate and combined effects of iron and tetracycline on infection in the skin

Table II summarises the results obtained in studies with 16 test strains. Six guinea-pigs were used for each test. Three were controls in which organisms alone and organisms with local Fe+++ were injected into the skin. The other group was similarly treated but also received the tetracycline. It was thus possible to estimate the effect of tetracycline alone, of Fe+++ alone, and of combinations of systemic tetracycline and local Fe+++.

Many of the tests with the two substances were repeated with similar results. In addition, most of the strains were first tested with iv tetracycline only, with results very similar to those recorded as E factors for tetracycline alone; however, with S. aureus C57H the tetracycline effect was equivalent to an E factor of 0·09. A positive effect of this degree suggests that in this case tetracycline was antagonised by Fe+++.

Low MIC and high anti-infective action are not necessarily correlated. Conversely, organisms with a high MIC, e.g., Proteus sp. Graves, may be substantially affected by tetracycline in vivo. Such results emphasise that estimates of MIC are inadequate as indicators of in-vivo efficacy. The minimum bactericidal concentration might have been a better guide.

The raw data from which interaction of the two compounds can be deduced are summarised in the E values. With Proteus sp. Graves, for example, it may be deduced that tetracycline is antagonised by the Fe+++; an E value of 0·02 with tetracycline alone was raised to 5·6 when iron was added. On the other hand, an enhancement by Fe+++ alone of 2·24 was increased only a little, to 5·6, by the tetracycline. In another example, the tetracycline effect on Streptococcus D181 diminished quite substantially from 0·008 to 0·002 with added iron, and the iron effect was strongly diminished by tetracycline.
TABLE II
Action of local ferric ammonium citrate (Fe+++ and intraperitoneal tetracycline (Tet), separately and in combination, on various local infections of guinea-pig skin

<table>
<thead>
<tr>
<th>Infected strain</th>
<th>MIC (µg/ml)</th>
<th>Tetracycline given at (h)</th>
<th>Tet alone</th>
<th>Fe+++ alone</th>
<th>Tet and Fe+++ combined</th>
<th>Tet by Fe+++</th>
<th>Fe+++ by Tet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aureus C57H</td>
<td>1</td>
<td>0 and 2</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MI</td>
<td>2</td>
<td>0 and 2</td>
<td>0.4</td>
<td>++</td>
<td>0.2</td>
<td>0.5</td>
<td>+</td>
</tr>
<tr>
<td>BI</td>
<td>0.5</td>
<td>0 and 2</td>
<td>0.008</td>
<td>+</td>
<td>0.008</td>
<td>1.0</td>
<td>150</td>
</tr>
<tr>
<td>PS80</td>
<td>0.5</td>
<td>0 and 2</td>
<td>0.17</td>
<td>+</td>
<td>0.55</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>16201</td>
<td>64.0</td>
<td>0 and 2</td>
<td>0.6</td>
<td>3.2</td>
<td>0.8</td>
<td>1.3</td>
<td>4</td>
</tr>
<tr>
<td>16334</td>
<td>0.5</td>
<td>0 and 2</td>
<td>0.19</td>
<td>1.9</td>
<td>0.24</td>
<td>1.3</td>
<td>8</td>
</tr>
<tr>
<td>Proteus sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Graves</td>
<td>64.0</td>
<td>0 and 2</td>
<td>0.008</td>
<td>5.6</td>
<td>0.002</td>
<td>2.0</td>
<td>2800</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KGP</td>
<td>4.0</td>
<td>-4†</td>
<td>1.0</td>
<td>10.6</td>
<td>10.5</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>A3</td>
<td>2.0</td>
<td>-4†</td>
<td>0.09</td>
<td>4.4</td>
<td>0.14</td>
<td>1.6</td>
<td>32</td>
</tr>
<tr>
<td>K701</td>
<td>1.0</td>
<td>-4†</td>
<td>0.5</td>
<td>2.5</td>
<td>1.0</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5515</td>
<td>32.0</td>
<td>-4</td>
<td>0.25</td>
<td>1.0</td>
<td>0.4</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>5525</td>
<td>2.0</td>
<td>0 and 2</td>
<td>3.0</td>
<td>1.26</td>
<td>2.4</td>
<td>-§</td>
<td>-§</td>
</tr>
<tr>
<td>Erysipelothrix sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EW2</td>
<td>2.0</td>
<td>0 and 2</td>
<td>0.015</td>
<td>0.60</td>
<td>0.04</td>
<td>2.7</td>
<td>150</td>
</tr>
<tr>
<td>C. perfringens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S9R</td>
<td>&lt;0.06</td>
<td>0 and 2</td>
<td>0.40</td>
<td>1.60</td>
<td>0.20</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kulp</td>
<td>&lt;0.5</td>
<td>0 and 2</td>
<td>0.37</td>
<td>32.0</td>
<td>0.56</td>
<td>1.5</td>
<td>57</td>
</tr>
</tbody>
</table>

* Significant factors of decrease in enhancement of infection by Fe+++ or in decrease of the antibiotic action of tetracycline indicated by bold type.
† Enhancement great but response lines so shallow and far apart that numerical estimates of enhancement impossible.
§ Tetracycline enhances infection; no estimate of antagonism possible.

Figs. 3 and 4 exemplify two typical results where Fe+++ and tetracycline affected each other. With A. hydrophila Kulp, both the Fe+++ and the tetracycline antagonisms were substantial, though whereas the tetracycline was strongly antagonistic, the Fe+++ was less so. The same kind of result is featured in fig. 4; with S. aureus 16334, the differences in the E factors were much smaller.

The factors of apparent antagonism (columns 4 and 5 in table II) were obtained as follows: for antagonism of tetracycline by Fe+++ by dividing the E factor for tetracycline (column 1) by the combined E factor (column 3); for antagonism of Fe+++ by tetracycline the E factor in column 2 was divided by that in column 3. The figures show clearly that Fe+++ only rarely antagonised tetracycline. If a factor of 2.5 or more is deemed significant, feeble antagonism occurred with only two of the 16 strains (S. aureus PS80, and the E. rhusiopathiae strain) and strong antagonisms only with Proteus sp. Graves. In sharp contrast, tetracycline affected the Fe+++ feebly with S. aureus PS80, K. pneumoniae 701 and the Ps. aeruginosa 5515, moderately with S. aureus strains 16201 and 16334, and the C. perfringens strain, and strongly or very strongly with S. aureus strains M1 and B1, Streptococcus D181, K. pneumoniae A3, the
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FIG. 3.—Change in response in lesion size due to two doses of A. hydrophila Kulp induced by Fe$^{+++}$, tetracycline and the two combined. For the calculation of E factors from response lines not parallel to the control line (broken line) parallel lines were drawn through the midpoints of the original lines: Fe$^{+++}$ = ferric ammonium citrate; T = tetracycline; C = control.

FIG. 4.—Responses with S. aureus 16334. Explanation and symbols as in fig. 3.
Erysipelothrix strain and the A. hydrophila strain. That is, in tests with a combination of tetracycline and ferric iron, tetracycline either moderately or strongly antagonised the effect of iron on 12 of the 16 strains investigated.

The dose of local Fe+++ was of the order of size that has proved useful in distinguishing readily enhanced from feebly enhanced organisms. As larger doses of Fe+++ often enhance more effectively (Miles et al., 1976), we tested the effect of increasing the dose of Fe+++ from 10 µg to 90 µg with S. aureus B1. There was only a small rise in E value for iron alone (1·2 to 1·6), but the E value for tetracycline, unaffected by 10 µg of Fe+++, was decreased from 0·008 to 0·06 by 90 µg. Because this represents an enormous local concentration of Fe+++, that is likely to occur only in extreme experimental conditions, we did not test other organisms in this way.

DISCUSSION

Miles, Maskell and Evans (unpublished data) observed that, as measured in vitro, the molar ratio of Fe+++ to tetracycline at neutralisation point changed with the concentration of tetracycline used. Thus tetracycline at a concentration of 1 µg/ml was antagonised by iron (Fe+++ 4·25 µg/ml and the molar ratio at this point is 34·5. The corresponding figures for tetracycline 5 µg/ml was 28 µg of Fe+++ with a molar ratio of 45·4. This ratio increased so that for tetracycline 125 µg/ml, it was 85·7.

A tetracycline concentration of 5 µg/ml was maintained in the plasma for a period of 4 h or more when a total of 50 mg was given ip during the first 2 h. Clearly we were dealing with a depot effect.

In several studies in rabbits and mice it appears that plasma concentrations are exceeded after intravenous injection in actively metabolising tissues only, such as liver, kidneys, lungs, spleen and intestines; no such concentration is recorded for tissues like the skin. Because plasma tetracycline is rapidly excreted and destroyed, the local concentration is clearly the result of a dynamic state of continuous replenishment (see review by Barber, 1964). We demonstrated that, despite the maintenance of the plasma tetracycline during the first 4 h, there was a sustained and rapid diminution of local efficacy of the drug. A similar diminution of efficacy of Fe+++ occurs in the first 4 h (Miles et al., 1976). During this important period the concentration of tetracycline at the site of infection will certainly rise above that in healthy tissues because of an increased blood volume in the vessels of the inflamed tissue and the exudation of tetracycline-containing plasma, though the tetracycline concentration in the tissue is unlikely in these circumstances to approach that in the plasma.

From the studies of Miles et al. (1976), the total volume of exudate in the first 4 h in iron-treated lesions is about 135 µl and at any one time during that period is about 14 µl. The 10 µg of Fe+++, on the other hand (Miles et al., 1976), is not replenished and declines from 9 µg at 0 h to 4 µg at 4 h. The average concentration of iron during this period is (9 + 4)/2 = 6·5 µg in 14 µl, which is 0·46 µg/ml. According to Miles, Maskell and Evans (unpublished data), this amount neutralises tetracycline 5 µg/ml. As there is renewal of the tetracycline in the lesion while the iron is either disappearing from the lesion or is being absorbed to the tissues beyond the reach of the tetracycline, tetracycline dominates the lesion in spite of the initial high concentration of iron. Since the local effect of both agents declines substantially within 4 h, only in the early period of infection will their interaction be crucial.
Our data do not permit a definite conclusion that true antagonism occurs. For example, a diminution of the effect of tetracycline by iron may be due wholly to the infection-enhancing power of iron, which would mask the action of the antibiotic. Alternatively, it may be that the direct complexing of some of the tetracycline with ferric iron leaves only some tetracycline to act as an antibiotic. Many other speculations are possible about the point in the chain of reactions at which the action of the antibiotic and the enhancement of infection by iron (see Weinberg, 1978) may be critically affected, but none can be identified from our data. Accordingly, "apparent" antagonism is the only suitable designation of the observed effects. However the effect is accomplished, the two reagents can react \textit{in vivo}, sometimes to the detriment of their primary effects.

We conclude that the efficacy of tetracycline treatment will only rarely be diminished by an excess of readily available iron; on the other hand, therapeutically effective tetracycline in the circulation can substantially affect enhancement of infection by ferric iron, as predicted by Weinberg (1971). As to implications for clinical medicine, there are a number of circumstances in which this antagonism might affect the issue. First, the available endogenous iron required by bacteria for growth in the body might be diminished by tetracycline therapy. This would presumably result in a lessening of a particular infection attributable not to the direct action of the antibiotic but to denial of an essential nutrient for the invader. Second, there may be circumstances of clinical iron-deficiency whereby endogenous iron is decreased to a significant degree by the tetracycline or where the effect of exogenous iron necessary for clinical improvement might be prejudiced by the antibiotic. The evidence for the assumption that diminution of iron available for physiological processes can be equated with diminution of the exogenous iron available for enhancement of infection is clearly very indirect but is justified on the grounds that tetracycline, having an unusually high avidity for iron (Albert, 1973), could not fail to compete for any of the endogenous cation in the tissues available for either pathological or physiological processes. These conclusions would also apply to related antibiotics such as doxycycline, methacycline and oxytetracycline, which are strongly neutralised by \(\text{Fe}^{+++}\) \textit{in vitro} (Miles, Maskell and Evans, unpublished data).

Whether the magnitude of the competition is such as to affect the availability of iron to a pathological degree is a moot point, but at least the possibility should be borne in mind.

\textbf{REFERENCES}