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EFFECT OF IRON ON THE GROWTH AND SIDEROPHORE PRODUCTION OF MYCOBACTERIA

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SUMMARY :

To gain a better understanding of the role of iron in the pathogenesis of tuberculosis, the growth and production of siderophores were studied in the presence of different concentrations of free iron *in vitro* with *M. smegmatis* and virulent, avirulent and low virulent strains of *M. tuberculosis.* Increase in the concentrations of iron caused an appreciable increase in the growth (as assessed by cell dry-weight and log viable counts) of all 4 strains. This was, however accompanied by a significant decrease in the production of both exochelins and mycobactins, suggesting that these siderophores are necessary only under iron-deficient conditions. The growth and production of siderophores were significantly higher with the virulent strain of *M.tuberculosis* than with the avirulent (or) the low virulent strains.

INTRODUCTION :

With the onset of infection and an elevation in the body temperature, the host limits the availability of essential nutrients by a process called nutritional immunity (1). Of these nutrients, iron is recognised to be vital for the survival and proliferation of microorganisms within the host.

The total concentration of iron in mammalian sera is about 10^{-5}M and the iron requirement of most aerobic bacteria is about 10% (2,3). However, the concentration of free iron in mammalian serum is about 10^{-18}M (4) only, as most of it is bound to transferrin. This concentration of free iron is too low to support the growth of microorganisms. To overcome this limitation of iron, microorganisms produce low molecular weight compounds known as siderophores, which have high affinity constants for iron (5).

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To meet the demand for iron, mycobacteria also synthesize and utilize specific high affinity iron-binding compounds which help them grow in the iron-restricted conditions of the host (1.6) and also participate in the uptake of iron across the thick lipid cell wall (7). Two types of iron-chelating compounds are produced by mycobacteria : exochelin, occurs extracellularly to act as scavenger and mycobactin, occurs on the cell wall to act as transporter and as a store for iron.

Our present knowledge of several host-defence mechanisms in tuberculosis is incomplete, particularly with respect to nutritional immunity. Thus for instance, very little is known about the-iron seqestration mechanisms adopted by mycobacteria during infections like tuberculosis. In this report we have examined the influence of iron on the growth of mycobacteria and the production of exochelins and mycobactins and also the differences in the iron-chelating mechanisms between the virulent, avirulent and low virulent strains of *M. tuberculosis* in addition to that of a saprophyte. *M. smegmatis in vitro.*

MATERIALS AND METHODS :

Organisms used : Four different strains of mycobacteria namely, a saprophyte *M. smegmatis* (TMC 1515), an avirulent strain of *M. tuberculosis* $H_{37}R_a$ (TMC 201), a standard virulent strain of hf. *tuberculosis* $H_{37}R_v$ (TMC 102) and a South Indian variant of *M. tuberculosis* low virulence in the guinea-pig (SILV) were employed in these investigations. All organisms were kindly supplied by the Bacteriology Department of the Tuberculosis Research Centre, Madras.

Growth of Organisms in vitro : The four strains were grown on Lowenstein-Jensen (LJ) slopes to their log phase and then subcultured into a synthetic medium (8). This medium was made metal iron-free' by autoclaving with alumina (chromatography grade). Immediately before inoculation, the medium was supplemented with magnesium sulphate (4 µg/ml), manganous sulphate (0.1 µg/ml) and zinc sulphate (0.5 µg/ml) ; in addition ferrous sulphate was added in concentrations of 0, 0.02, 0.1, 0.5, 2 and 4 µg/ml (ferrous iron is converted to ferric iron under aerobic conditions). The media was inoculated with different strains and incubated at 37°C. The organisms were harvested on the 8th day after inoculation for *M. smegmatis* and on the 35th day for the three *M. tuberculosis* strains.

Determination of cell dry-weight : Cell dry-weights were determined using pre-weighed filters with during to constant weight at 100°C.

Extraction and estimation of exochelins : A saturated solution of ferric chloride (in distilled water) was added to the cell-free culture filtrates until it just formed a precipitate. The precipitate was removed by filtration through Whatman No. 541 paper and exochelins were extracted from the filtrate with chloroform (9). Chloroform extracts were evaporated and the exochelins were estimated gravimetrically.

Extraction and estimation of Mycobactins : Mycobactin extraction was carried out by a modification of the method of White and Snow (10). Washed cells which were freshly harvested, were extracted overnight with cold ethanol at room temperture. A saturated solution of ferric chloride (in ethanol) was added to extract the mycobactin in the ferri form. Cells were removed by filtration and given a final wash with ethanol. The combined ethanol extracts were then mixed with an equal volume of chloroform in a separating funnel and distilled water added to provide two phases. The aquoes phase containing the polar materials was discarded. The organic phase was dried over anhydrous sodium sulphate and evaporated to dryness. The resulting mycobactin reside was estimated gravimetrically.

Determination of viable counts : In another experiment, the effect of increasing concentration of iron on the multiplication as assessed by the log viable counts of the 4 strains of mycobacteria, was examined. The organisms were initially grown (4 days for *M. smegmatis* and 2 weeks for *M. tuberculosis* strains) in synthetic liquid media containing iron in concentrations ranging from 0 to 4 µg/ml. Serial ten-fold dilutions (ranging from 1/10 to 1/10000) were prepared from these cultures (neat) and 10 µl from each of the dilutions and neat cultures were inoculated on to duplicate slopes of LJ. The bacterial growth (log viable counts) was measured by counting the colonies following incubation at 37°C for 4 days for *M. smegmatis* and 4 weeks for the *M. tuberculosis* strains. All the estimations were set up in hexaplicate, and the estimations were undertaken after coding the flasks; those inoculated with *M. smegmatis* were coded separately due to the differences in the period of incubation. The results were statistically analysed employing ANOVA (analysis of variance) and t-tests (paired and unpaired).

RESULTS :

Cell dry-weight : There was an appreciable increase in the growth of all 4 strains of mycobacteria (Pig. 1) with increasing concentrations of iron in the medium ($P < 0.001$). the increase in the growth of the saprophytic *M smegmatis* being substantially higher than with any of the *M. tuberculosis* strains.

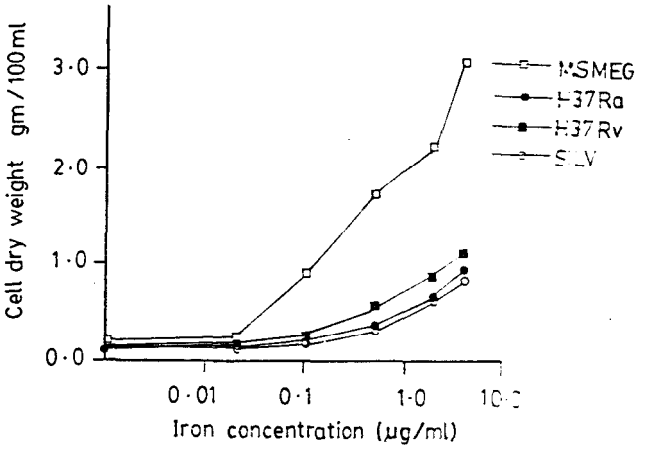

Fig 1 Effect of various concentrations (0, 0.02, 0.1, 0.5, 2.0 and 4.0 µg/ml of iron in the medium on the mean cell dry weight (g/100 ml of medium) of *M. smegmatis* and $H_{37}R_a$ and $H_{37}R_v$ and the SILV strains of *M. tuberculosis*

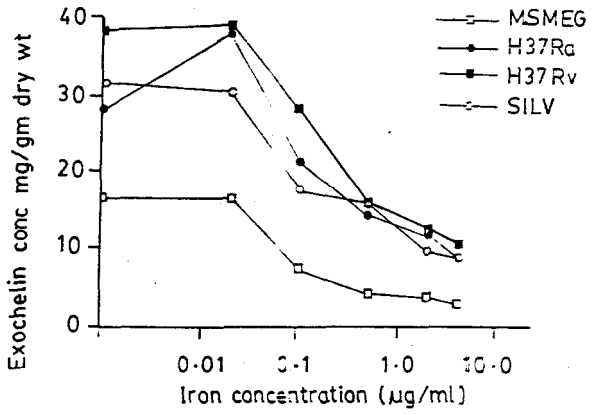

Fig 2 Effect of various concentrations (0, 0.02, 0.1, 0.5, 2.0 and 4.0 µg/ml) of iron in the medium on the mean exochelin concentrations (mg/g dry weight of cells) of *M. smegmatis* and $H_{37}R_a$, $H_{37}R_v$ and SILV strains of *M. tuberculosis*

The 2-way analysis of variance for comparison between the 3 *M. tuberculosis* strains indicates that the differences in the growth is significant ($P < 0.001$), with the growth of the virulent $H_{37}R_v$ being higher than that with the avirulent or the low virulent strains.

Exochelins and mycobactins : A marked decrease was observed in the production of exochelins and mycobactins with increasing concentrations of iron in the medium (Figs. 2 and 3). The concentration of both these siderophores, particularly at the higher concentrations of iron, was appreciably lower with *M. smegmatis* than with any of the *M. tuberculosis* strains. The 2-way analysis of variance indicates that the differences between the strains in the contents of the 2 siderophores are highly significant ($P < 0.001$). with the production of both exochelins and mycobactins being greater with $H_{37}R_v$ than that with the avirulent or the low virulent strains.

The correlation coefficients for the variousassociations observed are presented in the Table 1. All the correlation coefficients were statistically significant ($P < 0.01$). There was a strong linear association between the growth of the bacilli and increasing concentrations of iron in the medium with the correlation coefficients exceeding 0.90. The release of exochelins and the production of mycobactins were negatively correlated with increase of iron in the medium but the association was not as strong as that observed with cell dry-weight. The release of exochelins seemed to parallel the production of

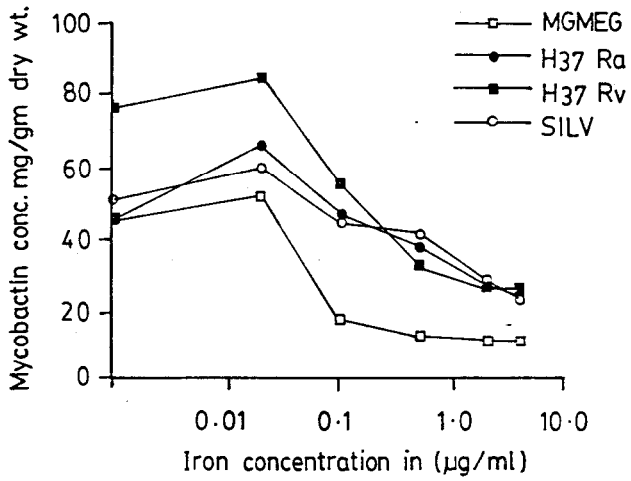

Fig 3 Effect of various concentrations (0, 0.02. 0.1, 0.5, 2.0 and 4.0 µg/ml) of iron in the medium on the mean mycobactin concentrations (mg/g dry weight of cells) of *M. smegmatis* ad $H_{37}R_a$, $H_{37}R_v$ and SILV strains of *M. tuberculosis*

mycobactins, suggest that both exochelins and mycobactins are probably necessary for the sequestration of iron by mycobacteria under conditions of iron-deficiency and that there is probably no role for these compounds in the presence of high concentration of iron as this element is probably taken up passively under these conditions.

Log viable counts : As seen with the cell dry-weight in the previous experiment, there was a significant increase ($P < 0.001$) in the log VC of all the 4 strains following prior incubation with increasing concentrations of iron in the synthetic medium (Fig. 4), the growth of the saprophytic *M. smegmatis* being substantially higher with any of the *M. tuberculosis* strains. The 2-way analysis of variance shows that the differences between the three *M. tuberculosis* strains are highly significant ($P < 0.001$) with the growth (log VC) of the virulent strain being higher than that of the avirulent or the SILV strains.

DISCUSSION :

Pathogenic microorganisms use one of two mechanisms to invade a host. The first is to utilize or produce compounds such as toxins and hydrolytic enzymes that may exploit and weaken the host and

Table 1. The correlation co-efficients for the various associations

Association between	M. smegmatis	$H_{37}R_a$	$H_{37}R_v$	SILV
Iron concentrations and cell dry-weight	+ 0.91	+ 0.98	+ 0.993	+ 0.98
Iron concentrations and release of exochelin	- 0.62	- 0.67	- 0.75	- 0.69
Iron concentrations and production of mycobactins	- 0.52	- 0.81	- 0.70	- 0.83
Release of exochelins and production of mycobactins	**+ 0.97**	**+ 0.94**	**+ 0.98**	**+ 0.95**

the second is to exploit an already weakened, and hence more susceptible host. The acquisition of iron by the invading microorganisms is important in both cases. The growth of all bacterial pathogens can be enhanced by the presence of available iron in the host tissues (11). In the case of *E. coli* 0111, iron compounds can enhance the virulence 10000 - fold (12). Further, *in vitro* experiments with avirulent and virulent strains of *E. coli* showed that bacteria deprived of iron in human sera stopped multiplying and died in a very short time. In contrast to virulent bacteria, which were effectively inhibited in mammalian serum, virulent bacteria were able to obtain iron and multiply (13).

In the present study, increasing concentration of iron caused an appreciable increase in the growth of all the four strains of mycobacteria. The growth of the virulent strain is greater than that of the avirulent of the low virulent strains of *M. tuberculosis.* The viability of the four strains of mycobacteria is also enhanced by increasing concentrations of iron in the medium, and the viability is greater with the pathogenic strains of *M. tuberculosis* ($H_{37}R_v$ and SILV) than with the avirulent ($H_{37}R_a$) strain. This observation supports the idea that iron is a critical nutrient for bacterial growth and that it probably enhances the virulence of the pathogenic strain.

Siderophore synthesis is repressed when iron is present, and induced when iron is depleted from the culture medium (14). The growth of virulent bacteria in animals has been associated with the

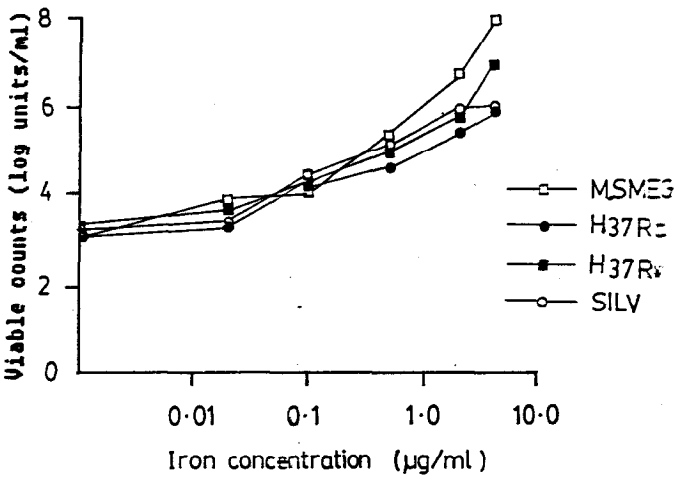

Fig 4 Effect of different concentrations (0, 0.02, 0.1, 0.5, 2.0 and 4.0 µg/ml) of iron in the medium on the mean log viable counts/ml observed at the end of the incubation period on the LJ slopes of *M. smegmatis* and $H_{37}R_a$, $H_{37}R_v$ and SILV strains of *M. tuberculosis*

prolific production of siderophores that supply bacteria with iron from complexes of transferrin and iron (15). Findings presented here show that the concentrations of both exochelins and mycobactins are highest under iron-deficient conditions (0 and 0.02 µg/ml). suggesting a role in the sequestration of iron for these compounds. The decrease in the concentrations of these compounds with increasing concentrations of iron demonstrates that iron is taken up passively under iron-rich conditions. The release of exochelins and the production of mycobactins is greater with the virulent strain than with either avirulent or the low virulent strains of *M. tuberculosis.* Thus, the virulent strain appeared to be more capable of sequestering iron and thereby registers a higher growth due to its ability to synthesize greater amounts of both siderphores. Interestingly, the behaviour of the low virulent strain appeared to be similar to that of the avirulent strain with respect to growth and the production of siderophores.

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