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Effect of Hemoglobin on the Growth of Mycobacteria and the Production of Siderophores

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ABSTRACT

Hemoglobin is known to support the growth of several bacterial species. The growth and the production of siderophores by 4 strains of mycobacteria in the presence of hemoglobin was studied *in vitro.* The findings were compared with those obtained in the presence of equivalent concentrations of iron in the medium. Increase in the concentrations of hemoglobin caused an appreciable increase in the growth of all 4 strains. This was however, accompanied by a significant decrease in the production of both exochelins and mycobactins. It was also observed that hemoglobin supported the growth of all strains as well as that with free iron and the concentrations of both siderophores was significantly higher in the presence of hemoglobin than in that of free iron.

Key words: Hemoglobin; *Mycobacterium.*

INTRODUCTION

A bacterium must rat to proliferate in host tissues: the major nutritional limitation in that environment appears to be iron*. To meet the demand for iron, mycobacteria synthesize and utilize specific high affinity iron-binding compounds (Siderophores) which help them grow in the iron-restricted environment of the host[1]. Two types of siderophores are produced by mycobacteria[3]: exochelin, occurs extracellularly and the mycobactin, which is cell-associated. Recent work[4] has demonstrated that the growth of *Mycobacteriurn tuberculosis* strains was increased with increasing concentrations of iron in the medium and that the concentrations of exochelins and mycobactins, which are highest under iron-deficient conditions, register a marked decrease.

Hemoglobin is known to support the growth of several bacterial species[5].

TABLE I

Effect of haemoglobin on the growth of mycobacteria

Strain	Source of iron	Mean cell dry-weight ± standard deviation (g/100 ml) in the presence of the following concentrations of iron (ug/ml				
		0.02	**0.1**	**0.5**	**2.0**	**4.0**
M. smegmatis	Free iron	0.332±0,021	0.822±0.060	1.590±0.060	2.300±0.220	3.780±0.200
	Haemoglobin	0.242±0.020	0.946±1.040	1.772±0.040	2.054±0.220	3.610±0.320
$H_{37}Ra$	Free iron	0.102±0.008	0.164±0.010	0.316±0.012	0.618±0.016	0.904±0.050
	Haemoglobin	0.106±0.008	0.192±0.012	0.398±0.006	0.716±0.034	1.056±0.060
$H_{37}Rv$	Free iron	0.116±0.010	0.220±0.016	0.522±0.036	0.822±0.020	1.052±0.016
	Haemoglobin	0.162±0.010	0.256±0.024	0.636±0.034	1.020±0.036	1.272±0.080
SILV	Free iron	0.104±0.016	0.194±0.010	0.282±0.010	0.538±0.046	0.822±0.030
	Haemoglobin	0,126±0.028	0.230±0.016	0.340±0.024	0.684±0.038	0.880±0.048

No information is available about the effect of hemoglobin on the growth of 4 strains and mycobacteria and the production of exochelins and mycobactins *in vitro.* The findings were compared with those obtained in the presence of equivalent concentrations of iron in the medium.

MATERIALS AND METHODS

The four strains of Mycobacteria investigated were a saprophyte, *Mycobacterium smegmatis* (TMC 1515), and avirulant $H_{37}Ra$ (TMC 201), virulent $H_{37}Rv$ (TMC 102) and South Indian Low Virulent (SILV) strains of *M. tuberculosis.* These strains were inoculated into a synthetic medium (100 ml.) which was prepared in iron-free glassware[6].

Two sets of flasks were set up for each strain; Fe^{++} (in the form of $FeSo_4$) was added in concentrations of 0.02, 0.1, 0.5, 2 & 4 µg/ml to one set of flasks and hemoglobin was added in concentrations of 5.8, 29, 145, 580 and 1160 µg/ml (corresponding to the free iron concentrations from 0.02 to 4 µg/ml) to another set. The concentration of hemoglobin to be added to the medium in lieu of iron was calculated on the basis of its molecular weight and its capacity to bind 4 atoms of iron per mole. Incubation was at 37°C and the experiment was set up in quadruplicate. The estimations were performed after coding the flasks at 8th day for *M. smegmatis.* and at 35th day for 3 *M. tuberculosis* strains.

Cell dry weights were determined using pre-weighed filters with drying to constant weight at 100°C. Exochelins were extracted from the cell-free culture filtrates with chloroform[7] and mycobactins were extracted from the cells with ethanol[8] and both the siderophores were estimated gravimetrically.

Hemoglobin (human) was purchased from Sigma Chemical Company,USA. The results were analysed employing Student's t-test (paired and unpaired).

RESULTS

As with free iron, there was an appreciable increase ($P<0.01$) in the growth of all strains of Mycobacteria in the presence of increasing concentrations of hemoglobin as the source of iron (Table I). With M. *smegmatis,* the growth in the presence of hemoglobin was higher than in that of free iron only at concentrations of 0.1 and 0.5 µg/ml ($P<0.01$). However, the growth in the presence of hemoglobin was higher than with free iron at all concentration levels in the three *M. tuberculosis* strains ($P<0.01$ for all).

The release of exochelins (Table II) and the production of mycobactins (Table III) was also significantly higher in the presence of hemoglobin than in that of free iron with all four strains ($P<0.001$), and as with free iron, there was. a decrease with increasing concentrations of hemoglobin in the medium. The growth and the production of exochelins and mycobactins of the virulent $H_{37}Rv$ strain being higher ($P<0.01$) than that of the

TABLE II

Effect of haemoglobin on exochelin concentrations

Strain	Source of iron	Mean exochelin concentrations (mg/g dry weight of cells) ± standard deviation in the presence of the following concentrations of iron (ug/ml)				
		0.02	0.1	0.5	2.0	4.0
M. smegmatis	Free iron	18.15±1.78	5.45±0.83	3.38±0.50	2.50±0.47	2.37±0.20
	Hemoglobin	20.01±3.08	9.27±0.98	7.24±0.62	3.23±0.49	2.75±0.35
$H_{37}Ra$	Free iron	39.31±2.22	24.48±l.64	15.81X3.21	11.29±1.60	9.40±0.71
	Hemoglobin	43.65±7.00	28.58±3.98	17.58±2.68	14.00±0.69	10.30±0.63
$H_{37}Rv$	Free iron	34.82±3.07	16.28±1.30	12.76±1.05	l2.76±1.05	10.94±0.82
	Hemoglobin	53.03±9.35	35.05±2.65	21.27±l.07	15.68±1.08	13.10±0.13
SILV	Free iron	32.90±5.37	20.66±1.06	14.22±0.54	10.01±1.56	9.11±1.04
	Hemoglobin	44.21±6.36	26.14±1.93	20.52±2.00	13.11±1.00	10.80±0.95

TABLE III

Effect of haemoglobin on mycobactin concentrations

Strain	Source of iron	Mean mycobactin concentrations (mg/g dry weight of cells) ± standard deviation in the presence of the following concentrations of iron (ug/ml)				
		0.02	**0.1**	**0.5**	**2.0**	**4.0**
M. smegmatis	Free iron	47.28±5.28	17.02±1.95	14.61±3.63	10.42±0.49	9.52±0.48
	Hemoglobin	65.91±8.20	25.94±1.66	18.08±0.48	15.17±1.99	11.49±0.84
$H_{37}Ra$	Free iron	63.51±6.33	42.49±4.44	34.87±2.76	24.24±1.34	20.50±0.68
	Hemoglobin	75.79±5.97	49.52±3.01	38.97±2.08	29.32±0.59	25.06±0.48
$H_{37}Rv$	Free iron	82.16±3.36	54.24±3.71	30.62±1.69	24.94±0.97	22.36±0.67
	Hemoglobin	89.82±2.97	62.34±1.41	37.80±0.61	28.97±0.70	26.26±1.54
SILV	Free iron	60.30±15.46	41.42±2.04	35.56±1.36	27.06±1.93	20.67±0.76
	Hemoglobin	64.65±2.30	49.89±2.58	41.03±2.50	30.68±0.41	23.89±0.13

avirulent or the low virulent strains of *M. tuberculosis* in the presence of either free iron (or) hemoglobin.

DISCUSSION

Hemoglobin is a conjugated protein, composed of a protein, globin, and an iron containing prosthetic group, haem. Eaton et al[5] showed that the combination of bacteria and blood in a wound can have lethal consequences, probably because hemoglobin iron supports prolific bacterial growth. Further, rats inoculated intraperitoneally with pathogenic *Escherichia coli* and small amounts of hemoglobin died, whereas the animals injected with *E. coli* alone survived. The effective component of hemoglobin is iron. In the case of *E. coli* 0111, iron compounds can enhance the virulence 10000-fold[9].

In the present study, increase in the concentrations of iron (or) those of hemoglobin as the source of iron caused an appreciable increase in the growth of all 4 strains of Mycobacteria. The increase in the growth in the presence of hemoglobin is greater than that with free iron. The growth of the virulent strain of *M. tuberculosis* is greater than those of the avirulent (or) the low virulent strains in the presence of free iron (or) hemoglobin. 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. The decrease of the concentrations of exochelins and mycobactins demonstrates that these compounds are necessary only under iron-deficient conditions. The production of both these siderophores is greater with the virulent strain than. with either avirulent (or) low virulent strains of *M. tuberculosis.* Thus, the virulent strain appeared to be more capable of sequestering iron and thereby registering a higher growth due to its ability to synthesize greater amounts of both siderophores.

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REFERENCES

1. Weinberg ED: Iron and infection. *Microbiol Rev* **42:** 45-66, 1978.

2. Barclay R. Ratledge C: Iron binding compounds of *Mycobacterium avium, Mycobacterium intracellulare* and mycobactin dependent *Mycobacterium paratuberculosis. J Bacterial* **153:** 1138-1146, 1983.

3. Katledge C: Iron and Nutrition. III: Ratledge C, Stanford JL, eds. The Biology of mycobacteria. London: Academic Press. 185-221, 1982.

4. Barclay K, Ratledge C: Mycobactins and exochelins of *M. tuberculosis* and other related species. *J Gen Microbiol* **134:** 771-776, 1988.

5. Eaton JW, Brandt P, Mahoney JR: Haptoglobin: a natural bacteriostat. *Science* **215:** 691-693, 1982.

6. Ratledge C, Hall MJ: Influence of

metal ions on the formation of mycobactins in *M. smegmatis. J bacterial* **108:** 314-319, 1971.

7. Macham LP, Ratledge C, Nocton JC: Extracellular iron acquisition by mycobacteria. *Infect Immun* **12:** 1242-1251, 1975.

8. Snow GA: Mycobactins: iron-chelating growth factors from mycobacteria. *Bacteriol Rev* **34:** 99-125, 1970.

9. Bullen JJ, Leigh LC, Roger HJ: The effect of iron compounds on the virulence of *E. coli* for guinea pigs. *Immunology* **15:** 581-588, 1963.

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