Exochelin-mediated Iron Uptake into *Mycobacterium leprae*[1]

Richard Malcolm Hall, Paul Robert Wheeler, and Colin Ratledge[2]

Iron uptake into mycobacteria is mediated by the exochelins which are low molecular weight siderophores of, as yet, chemically undefined structures. More than one exochelin is produced by each species of mycobacterium and they occur as two types: a) those from saprophytic mycobacteria cannot be extracted into any organic solvent and are termed water-soluble exochelins; b) those from pathogenic species which, when complexed with iron, can be extracted into chloroform and are termed the chloroform-soluble exochelins ([9]).

We have shown that exochelins can remove iron from ferritin, ferric phosphate and can reverse the bacteriostatic effect of serum on the growth of *Mycobacterium smegmatis* and *M. bovis* BCG, which would infer that they can also acquire iron from transferrin ([6]). The exochelins are therefore ideally suited for both *in vitro* and *in vivo* functions. Their production by *M. paratuberculosis* enables this organism to grow *in vivo* ([1]) even though it lacks mycobactin, which is involved in another aspect of iron metabolism—possibly that of iron storage ([9]).

Although little is known of the metabolic incompetences of *M. leprae* ([14]), the presence of a cytochrome system has been reported ([3]), thus inferring the presence of iron within the organism. It would in any case be difficult to imagine, even with an organism such as *M. leprae*, that it would be completely devoid of iron. If we then deduce that *M. leprae* does contain iron, the next question to ask is how might the organism acquire this metal from its host tissue. Since *M. leprae* cannot be grown in a laboratory medium we cannot examine its culture filtrates for the presence of an exochelin as with other mycobacteria. We therefore have resorted to examining the role of exochelins from other mycobacteria as possible donors of iron for *M. leprae*, knowing that there is a certain amount of cross specificity between the exochelins of each type ([13]). However, since the water-soluble exochelins are taken up by an active transport process ([12]) and the others by a passive or facilitated diffusion mechanism, there is apparently no means whereby the pathogenic mycobacteria can transport the exochelins from the saprophytic species ([13]).

M. vaccae was used as a source of exochelins since it has been reported as being taxonomically the closest mycobacterium to *M. leprae* ([11]), although this has not been supported by more recent evidence ([8]). *M. neoaurum* was also used since it is closely related to *M. vaccae* ([2]). *M. bovis* BCG was used as a convenient source of chloroform-soluble exochelins.

MATERIALS AND METHODS

Organisms for exochelin production

M. neoaurum NCTC 10439, *M. bovis* BCG (Glaxo strain) and *M. vaccae* R877R, from J. L. Stanford, Middlesex Hospital Medical School, London, U.K., and *M. smegmatis* NCIB 8548 were grown iron-deficiently (0.02 μg Fe^{2+}/ml) in shaken culture at 37°C on a glycerol/asparagine/mineral salts medium as previously described ([10]). The exochelins were isolated from the culture filtrates as their ferri complexes ([12, 13]) and were partially purified but not fractionated into individual components except for *M. vaccae* where a single fraction was used (see text).

Preparation of ^{55}Fe-exochelin

^{55}Fe was substituted for the non-radioactive iron in the exochelins by a method based on that of Macham, *et al.* ([7]). Fer-

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[2] R. M. Hall, B.Sc., Ph.D., Department of Biochemistry, The University of Hull, Hull HU6 7RX, U.K. P. R. Wheeler, B.Sc., Ph.D., and C. Ratledge, B.Sc.Tech., AMSCT, Ph.D., C.Chem., FRSC, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

Reprint requests to Dr. Ratledge.

riexochelins (2 ml) were mixed with an equal volume of methanol and a few crystals of 8-hydroxyquinoline were added. The mixture was shaken and then allowed to stand for about 2 hr at 22°C to allow complete formation of ferri-8-hydroxyquinoline which was extracted with 4 × 10 ml chloroform. The remaining solution of desferriexochelin was then dried under vacuum in an "iron-free" flask ([10]) with the residue dissolved in approximately 50 μl of water with 200 μl of $^{55}FeCl_3$ (specific activity about 1 μCi per μg Fe in 0.1 N HCl). Unchelated $^{55}FeCl_3$ was removed after 16 hr from each sample by passage through Sephadex G10.

Isolation of *M. leprae* cells for uptake studies

Livers, stored at −80°C, from experimentally infected nine-banded armadillos (*Dasypus novemcinctus,* Linn) were used as a source of *M. leprae.* Bacteria, $>5 \times 10^9$ bacilli per g wet weight of tissue, were isolated by the method quoted by Wheeler and Gregory ([16]).

Uptake of ^{55}Fe into *M. leprae*

A scaled-down method based on that of Stephenson and Ratledge ([12]) was followed with several important differences. Each incubation was carried out in "iron-free," steam-sterilized glass vials (5 ml capacity) with caps fitted to prevent possible microbial contamination as well as evaporation. Iron uptake by *M. leprae* was assayed in 50 mM KH_2PO_4/NaOH, pH 7.1 buffer (0.49 ml) containing 100 units of penicillin, water (70 μl), cell suspension (70 μl ≅ 1 mg dry weight cells) and 10 μM ^{55}Fe-exochelin (70 μl). ^{55}Fe-exochelin and water were filter-sterilized before adding. Incubation was at 22°C in a negative flow cabinet. Both the initial *M. leprae* extract and the assay mixture at the end of the incubation were checked on nutrient agar for possible contamination by fast-growing organisms.

Iron uptake was stopped at various times (Table 1) by adding 1 ml 50 mM EDTA to each vial. The solutions were filtered through 2.5 cm Whatman GFC filters, using a Millipore multiport filtration unit, and the cells washed with 2 ml 50 mM EDTA. Filters were dried and placed in Soluene 350 (1 ml, Packard Instrument Co., Inc., Rockville, Maryland, U.S.A.) for 2 hr at 37°C which digested the bacteria. Glacial acetic acid (50 μl) and scintillation fluid (10 ml), comprising 10% (w/v) naphthalene and 1% (w/v) 2,5-diphenyloxazole in toluene, were added to each vial prior to counting. Rates of uptake were expressed as nmol ^{55}Fe accumulated per g dry weight, with duplicate samples taken in all cases and average values then calculated.

Uptake of ^{55}Fe into *M. neoaurum*

M. neoaurum was grown iron deficiently (0.02 μg Fe^{2+}/ml) on glycerol/asparagine medium for 7 days and then harvested by filtration through Whatman GFC filters and washed with 0.85% NaCl containing 1.0% Tween 80. Uptake of ^{55}Fe-exochelin was followed into the cells by the above method, except the solution was stirred with a 12 mm Teflon magnetic stirrer. Metabolic inhibitors (NaN_3 at 30 mM, $HgCl_2$ at 0.1 mM, and 2,4-dinitrophenol at 2 mM) were added 2 min prior to the ^{55}Fe-exochelin. All uptakes were followed for 10 min.

RESULTS AND DISCUSSION

Uptake of ^{55}Fe complexed to various exochelins into a suspension of *M. leprae* is given in Table 1. With this batch of *M. leprae,* two experiments were carried out which gave results in broad agreement: only the mixed fraction of exochelins from *M. neoaurum* donated iron to *M. leprae* to any significant extent. The purity of this preparation of *M. leprae* was such that there was not more than one contaminating bacterium per 2.5×10^7 cells of *M. leprae,* a level judged to be of no importance. Contamination by debris from host cells was judged to be negligible by the soluble blue counterstaining method of Wheeler and Draper ([15]).

A further experiment (not shown in Table 1) was carried out on a separate occasion with a new preparation of *M. leprae* isolated from another armadillo. The same activity with respect to the uptake of ^{55}Fe complexed to the exochelins from *M. neoaurum* was found: using 1.06 mg of *M. leprae* per incubation, 7.8 nmol Fe were taken up per g dry weight after 1 sec; after 15 hr, 292.2 nmol had been assimilated. The exochelins from the other sources listed in Table 1 were not re-examined in this second experiment, but the ability of the exochelins from *M. neoaurum* to donate iron to *M. leprae* was demonstrated to be clearly reproducible.

TABLE 1. *Uptake of ^{55}Fe complexed to various exochelins into washed suspension of* M. leprae. *Exochelins, at 10 μM, incubated with 1 mg dry weight of* M. leprae *(=approx. 10^{10} cells).*[a]

Source of exochelin	Exochelin fraction used	Expt. no.	^{55}Fe accumulated (nmol per g dry wt) in				
			Control[b]	1 sec[c]	15 hr	24 hr	40 hr
M. bovis BCG	total	1	28.4	32.6	42.5	—	—
		2	—	—	22.4	17.5	21.2
M. smegmatis	total	1	44.3	42.8	59.3	—	—
		2	—	—	47.7	49.2	51.0
M. vaccae	fraction 3[d]	1	54.3	32.8	44.3	—	—
		2	—	—	15.0	24.0	37.1
M. neoaurum	total	1	46.9	38.0	246.5	—	—
		2	—	—	122.4	102.4	178.6

[a] Each incubation (one per determination) carried out in duplicate. Two separate experiments were carried out with the same preparation of *M. leprae*. (A further experiment with a new batch of *M. leprae* was carried out—see text.)

[b] No cells added, sample taken immediately (1 sec); this determines residual ^{55}Fe collected by sampling process.

[c] Zero time sample; this determines amount of ^{55}Fe non-specifically adsorbed to cells if above control value.

[d] Single monocomponent (main fraction).

Although the times for following uptake were long, they are necessary in view of the very slow growth and metabolism of *M. leprae*. Uptake of other nutrients, such as glucose, have been found to be equally slow ([4, 5]).

The possible mode of uptake of iron into *M. leprae* was examined by carrying out an additional incubation in the presence of 30 mM NaN_3, which has been shown to inhibit the active transport of ^{55}Fe-exochelin MS (i.e., the exochelin from *M. smegmatis*) into *M. smegmatis* ([12]). In the presence of this energy poison, there was little if any inhibition of uptake: after 1 sec, 10.6 nmol ^{55}Fe per g dry weight had been assimilated; after 15 hr, 279.4 nmol had been taken up. The mode of uptake would therefore appear to be by a facilitated diffusion mechanism rather than by active transport, i.e., coupled to the provision of metabolic energy. This appears similar to the process of iron uptake from exochelin of the MB type into *M. bovis* BCG and *M. intracellulare* ([13]).

Since the mode of uptake of ^{55}Fe-exochelin MN (i.e., from *M. neoaurum*) has not been previously studied, we followed its uptake into a washed suspension of iron-deficiently grown *M. neoaurum* in the presence and absence of several inhibitors: NaN_3; 2,4-dinitrophenol; and $HgCl_2$ (Table 2). Over 10 min incubation with 10 μM ^{55}Fe-exochelin, there was no evidence for any inhibition of uptake. If anything, each inhibitor caused a slight increase in the

TABLE 2. *Uptake of ^{55}Fe-exochelin into* M. neoaurum.[a]

Addition to incubation	^{55}Fe accumulated (nmol per g dry wt) (in seconds)						
	1[c]	10	30	60	180	300	600
None	50	51	86	67	81	103	120
NaN_3 (30 mM)	41	62	58	85	66	146	147
$HgCl_2$ (0.1 mM)	60	132	143	169	131	174	206
DNP[b] (2 mM)	50	89	96	134	163	168	186

[a] *M. neoaurum*, grown iron-deficiently, was suspended in 40 mM KH_2PO_4/NaOH buffer, pH 7.1, and purified, but unfractionated; exochelins from the culture filtrates of the same organism, complexed with ^{55}Fe, were added at 10 μM. Each incubation (one per determination) was stirred continuously and carried out in duplicate.

[b] 2,4-dinitrophenol.

[c] Taken as zero time sample and value taken as an indication of the amount of ^{55}Fe non-specifically adsorbed to the cells.

amount of iron taken up, although the reason for this is not obvious. We therefore conclude that the iron from the water-soluble exochelins of *M. neoaurum* is also taken up by a facilitated diffusion process. Whether this corresponds exactly to the mechanism demonstrated in *M. leprae* is yet to be investigated.

This is the first report of how *M. leprae* may possibly acquire the iron which is essential for its growth. The process appears to be a specific one in that only the exochelins from *M. neoaurum* proved to be effective agents for this process to occur. Of course, it remains to be established whether *M. leprae* is capable of elaborating any exochelin for itself. Should it prove not to do so, our results will then be taken as fortuitous but the problem of elucidating the mechanism of iron acquisition by *M. leprae* will still remain. It is not, however, an easy matter to establish if *M. leprae* does, or does not, produce exochelin, and hence we have had to resort to this indirect method of inferring what is the most likely route for iron assimilation. Exochelins from *M. neoaurum* do not, however, act as a growth factor for *M. leprae* in Hart-Valentine elongation medium at 32°C and 37°C for 5 and 10 weeks (R. M. Hall and P. R. Wheeler, unpublished results), but this cannot be taken as implying that *M. leprae* must therefore produce its own exochelins since the failure of *M. leprae* to grow *in vitro* is probably a multifactorial problem.

The exochelins of *M. neoaurum* are undoubtedly of interest in their promotion of iron uptake into *M. leprae.* The exochelins used in the present study, although purified, were not fractionated into single compounds. It therefore cannot be said, as yet, whether iron uptake into *M. leprae* would be mediated by one or more of the three exochelins known to be present in this preparation (R. M. Hall, unpublished work). Although the exochelins from *M. neoaurum* are superficially similar to those from *M. vaccae* and *M. smegmatis,* based on ion-exchange, chromatography (R. M. Hall, unpublished work), there must be a significant difference between them to account for their difference in ability to donate iron to *M. leprae. M. bovis* BCG, unlike *M. neoaurum,* is capable of successful *in vivo* growth. Therefore, one might expect the former to produce exochelins more akin to those of *M. leprae.* However, as shown, this appeared not to be the case. Evidently the ability of a mycobacterium to be a pathogen—if *M. leprae* is a pathogen in the accepted sense—must not be related to the type of exochelin it uses to acquire iron.

Further work to clarify the nature of the active exochelin from *M. neoaurum* and the process of iron acquisition by *M. leprae* is currently under way.

SUMMARY

Iron chelated to the exochelins from *Mycobacterium neoaurum* was taken up by a suspension of *M. leprae,* prepared from the liver of an infected armadillo, over 15 hr. No uptake occurred when the iron was chelated with exochelins from *M. bovis* BCG or *M. smegmatis* or to a single exochelin from *M. vaccae.* Uptake appeared to be by facilitated diffusion since it was not inhibited by either $HgCl_2$, NaN_3, or 2,4-dinitrophenol. This was similar to the mode of uptake of ferriexochelin into *M. neoaurum* itself.

RESUMEN

El hierro quelado a las exoquelinas del *Mycobacterium neoaurum* fue incorporado, en un periodo de 15 horas, por una suspensión de *M. leprae* preparada a partir del hígado de un armadillo infectado con el bacilo. No ocurrió la incorporación cuando el hierro fue quelado a exoquelinas del *M. bovis* BCG, o del *M. smegmatis,* o a una sóla exoquelina del *M. vaccae.* La incorporación pareció ocurrir por difusión facilitada puesto que no fue inhibida por el $HgCl_2$, la NaN_3, ni por el 2,4-dinitrofenol. Esto fue similar al modo de incorporación de ferriexoquelina por el *M. neoaurum* mismo.

RÉSUMÉ

Le fer lié (par chelation) aux exochélines de *Mycobacterium neoaurum* a été incorporé par une suspension de *M. leprae* préparée à partir du foie d'un tatou infecté, sur une période s'étalant sur 15 heures. Aucune incorporation n'est survenue lorsque le fer était lié aux exochélines de *M. bovis* BCG ou de *M. smegmatis,* ou bien à une seule exochéline de *M. vaccae.* L'incorporation paraît dépendre d'une facilitation de la diffusion, car elle n'était pas inhibée par le $HgCl_2$, le NaN_3, ou le 2,4-dinitrophenol. Ceci est similaire au mode d'incorporation de la ferriexochéline dans *M. neoaurum* lui-même.

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