INFECTION AND IMMUNITY, Sept. 1991, p. 3185–3190
0019-9567/91/093185-06$02.00/0

Vol. 59, No. 9

Relative Availability of Transferrin-Bound Iron and Cell-Derived Iron to Aerobactin-Producing and Enterochelin-Producing Strains of *Escherichia coli* and to Other Microorganisms

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Received 22 May 1990/Accepted 1 July 1991

A method is described for determination of the relative availability of transferrin-bound iron and cell-derived iron to microbial iron-scavenging mechanisms. This involved incubation of parallel cultures of microorganisms in dialysis tubes placed in RPMI 1640 tissue culture medium containing 30%-iron-saturated transferrin and K562 erythroleukemia cells. In one culture the transferrin was labelled with ^{59}Fe and in the other the cells were labelled, and the relative uptake of radioiron by the microorganisms determined. The results showed that *Staphylococcus epidermidis* and *Staphylococcus aureus* acquired iron predominantly from cells, while *Candida albicans* and the enteropathogenic *Escherichia coli* NCTC 8623 tended to acquire iron from transferrin. *E. coli* K-12 strains W3110 and LG1705, which (like NCTC 8623) produce the siderophore enterochelin but not aerobactin, acquired predominantly transferrin-bound iron, whereas the related *E. coli* strains LG1315 and LG1628, which produce aerobactin but not enterochelin, showed a preference for cell-derived iron. When the cells were incubated in the presence of ^{59}Fe-labelled transferrin and ^{55}Fe-labelled ferritin, no difference in relative availability of iron to *E. coli* was observed, suggesting that differences in the ability of aerobactin and enterochelin to remove iron from intracellular ferritin were not responsible for this preference. These results may help to explain why production of aerobactin, despite its relatively low affinity for iron, is more closely associated with invasiveness in *E. coli* than is enterochelin production. Reduced availability of cell-bound iron during inflammation may contribute to antimicrobial defenses.

The ability to obtain iron during in vivo growth is an important virulence determinant for many microbial species (35). Since iron is not normally freely available in body tissues and fluids, many microorganisms produce low-molecular-weight, high-affinity iron chelators, known as siderophores (15), when growing under iron-limiting conditions, and the presence of siderophores during in vivo growth has been detected (13). Many studies have investigated the ability of siderophores to remove iron from transferrin, and it is generally assumed that transferrin-bound iron is the normal target of scavenging by siderophores in vivo (12).

However, some observations are not consistent with such a mechanism. Many coliforms secrete two siderophores, enterochelin (28) (also known as enterobactin), a catechol, and aerobactin, a hydroxamate (4). Enterochelin has by far the higher affinity constant for iron (14), yet it is production of aerobactin that is closely associated with invasiveness (34). In addition, the mechanisms involved in iron scavenging by desferrioxamine, a hydroxamate-type siderophore produced by *Streptomyces pilosus*, have been extensively studied because of its clinical use in the relief of iron overload and appear to involve removal of iron from intracellular sources rather than from transferrin (29).

During the invasive process bacteria tend to become intimately associated with host tissues rather than remaining free in the bloodstream. If other siderophores can, like desferrioxamine, scavenge iron from cells, then cell-associated iron might form a more readily available source than transferrin. In addition, many mammalian cells, particularly macrophages and hepatocytes, are continually releasing iron as part of the normal pattern of iron recirculation (6). Although the mechanisms of iron release by mammalian cells are incompletely understood, there is little evidence that iron is released attached to a carrier molecule, and we have previously shown in vitro that iron is released by macrophages in a form that can be readily bound by even a low-affinity chelator such as nitrilotriacetate (7). Any siderophores present in iron-releasing tissues might therefore readily intercept such iron before it is able to bind to transferrin.

We have previously shown that an enteropathogenic strain of *Escherichia coli* (NCTC 8623) can acquire iron from transferrin (9). In this paper we report the development of a method designed to compare the relative availability of transferrin-bound iron and cellular iron to microbial siderophores; we have used this method to investigate the relative availability of these two sources of iron to *E. coli* NCTC 8623, to mutant derivatives of the laboratory strain *E. coli* K-12 which differ in their ability to make aerobactin and enterochelin, and to some other species of microorganisms.

MATERIALS AND METHODS

Bacteria. *Staphylococcus epidermidis* was a fresh isolate from a peritoneal infection of a renal failure patient in the Western Infirmary, Glasgow, United Kingdom, and has been used in previous work (24). *Staphylococcus aureus* NCTC 8532 and *E. coli* NCTC 8623 were obtained from the National Collection of Type Cultures. *Candida albicans* was a fresh isolate from a patient in the Western Infirmary. The staphylococcal strains and *C. albicans* were all found to produce siderophores when tested by the method of Schwyn and Neilands (30), and *E. coli* NCTC 8623 was found to

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produce enterochelin but not aerobactin (37). *E. coli* K-12 strains LG1315 and LG1628 are aerobactin-producing (Iu^+) derivatives of the enterochelin-deficient (Ent^-) mutant AN1937 (*ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl* [34]). LG1315, which has been described previously (36), is a transconjugant harboring the prototypical aerobactin plasmid ColV-K30. However, to rule out the possibility that the other phenotypes associated with this large natural plasmid are involved in the phenomena reported here, we also used the transformant strain LG1628, which carries recombinant plasmid pABN1. This plasmid comprises a 16-kbp *Hin*dIII fragment encoding the entire aerobactin system of ColV-K30 cloned into the vector plasmid p*Plac* (3). *E. coli* W3110 is a streptomycin-resistant (*rpsL*) prototrophic strain of *E. coli* K-12 which can make enterochelin but not aerobactin (1, 36). LG1705 is an enterochelin-producing strain derived from AN1937 into which the $entA^+$ allele of W3110 was introduced by P1 transduction but which carries only the vector plasmid p*Plac* and so is unable to make aerobactin (i.e., Ent^+ Iu^-). All of the organisms were routinely subcultured in nutrient broth and maintained on nutrient agar slopes.

Cell line. The K562 erythroleukemic cell line was used throughout this work and was routinely cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 100 μg of streptomycin per ml, and 100 U of penicillin per ml (all from Flow Laboratories, Rickmansworth, England).

Radiolabelling. (i) Transferrin. A solution of human apotransferrin (Behringwerke, Hounslow, England) at 10 mg/ml in phosphate-buffered saline (PBS) containing 0.1% $NaHCO_3$ was labelled by adding sufficient ferric nitrilotriacetate trace labelled with ^{59}Fe-citrate (Amersham; specific activity, 10 μCi/μg) to give 30% iron saturation and 2 μCi of ^{59}Fe per mg of transferrin. Labelling was carried out at least 24 h before use to ensure complete binding of iron. An unlabelled sample of transferrin was prepared in the same way but omitting the ^{59}Fe.

(ii) Cells. K562 cells were labelled by addition of 10 μCi of ^{59}Fe-citrate 2 days prior to use. The cells were then spun down, counted, and washed twice in Hanks balanced salt solution. An identical unlabelled culture was set up simultaneously, with sodium citrate added to compensate for the citrate in the ^{59}Fe-citrate.

(iii) Ferritin. Horse spleen ferritin (Sigma, Poole, England) was rendered iron free by dialysis against two changes of 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7, containing 1% thioglycolic acid followed by two changes of PBS. The resulting apoferritin was then labelled with ^{55}Fe by adding 100 μl of ferrous ammonium sulfate (5 mg/ml) trace labelled with $^{55}FeCl_3$ (Amersham) and containing 14 μg of ascorbic acid to 10 mg of apoferritin in 0.5 ml of PBS. After overnight incubation at 4°C, unbound iron was removed by passing through a column of Chelex 100 resin (Bio-Rad, Gloucester, England). Fractions of the labelled ferritin and the resin were assayed for ^{55}Fe activity by scintillation counting, which showed that >99.7% of the radioiron had been incorporated into ferritin, which thus contained about 9 μg of iron per mg of protein.

Preparation of bacterial cultures. In order to obtain bacteria which had previously been adapted to an iron-deficient environment, 10 μl of an overnight culture in nutrient broth was inoculated into 3 ml of antibiotic-free, serum-free RPMI 1640 medium (RPMI 1640-ASF) containing 1 mg of human apotransferrin per ml. The culture was then incubated overnight (48 h in the case of *E. coli* W3110, LG1628, and LG1705, which grew more slowly than the other organisms in this medium).

Measurement of iron uptake by bacteria from labelled cells and transferrin. Two parallel cell cultures were set up in sterile plastic 20-ml containers. One contained labelled K562 cells, suspended in 10 ml of RPMI 1640-ASF containing 1 mg of unlabelled 30%-iron-saturated transferrin per ml. In the other, unlabelled cells were resuspended in medium containing labelled transferrin. The number of cells in both cultures was counted and was normally about 10^7 per culture. Meanwhile 0.2 ml of the bacterial culture was diluted to 4 ml in RPMI 1640-ASF containing 1 mg of apotransferrin per ml and split into two 2-ml aliquots. Each of these was placed in a dialysis sac, and one sac was added to each of the transferrin and cell cultures in the containers. These were then incubated for 4.5 h at 37°C in an atmosphere of 5% CO_2.

At the end of this time, the dialysis sacs were opened and the bacterial suspensions were removed and centrifuged at 5,500 × *g* for 20 min to sediment the organisms. These were then washed once in Hanks balanced salt solution, and ^{59}Fe activity in the supernatant, wash, and bacteria was determined on an LKB Compugamma counter (LKB, Croydon, England). The cells in the containers were recovered by centrifugation and washed twice in Hanks balanced salt solution, the number and viability were assessed by eosin exclusion, and ^{59}Fe activity in the cell pellets, supernatants, and washes was determined.

Determination of iron uptake from ferritin and transferrin. The determination of iron uptake from ferritin and transferrin was performed by a modification of the technique described above. Cultures of *E. coli* were prepared and introduced into dialysis sacs, and each was placed in a plastic container containing 10 ml of RPMI 1640-ASF with 1 mg of 30% saturated ^{59}Fe-transferrin per ml and 50 μg of ^{55}Fe-ferritin per ml. The cultures were then incubated for 4.5 h as described above, and the amounts of ^{55}Fe and ^{59}Fe incorporated into the bacteria were determined simultaneously by dual-channel scintillation counting of both isotopes.

Determination of the iron content of K562 cells. Approximately 10^7 K562 cells, grown as described above, were spun down and washed twice in Hanks balanced salt solution. Iron in the pellet was then estimated by the nonheme liver iron assay of Torrance and Bothwell (33), as modified by Mainou-Fowler and Brock (22).

Statistical methods. Groups were compared by Student's *t* test.

RESULTS

Appraisal of the method. In any procedure designed to measure iron removal from cells or iron-binding proteins, it is important to ensure that the results are not influenced by artifacts such as leakage of iron from moribund cells or release from transferrin due to a reduction in pH. The former aspect was controlled firstly by checking cell viability, which was always >98%, by eosin exclusion and secondly by ensuring that any iron lost passively from the cells was bound by the transferrin in the culture medium, which was only 30% saturated. The amount of iron so acquired by the transferrin would be negligible compared with the amount already bound to the protein prior to the incubation, as the total amount of transferrin-bound iron exceeded total cellular iron by a factor of about 12 (see below). As a further precaution, apotransferrin was included in the medium in which the bacteria were suspended. These measures ensured that no extracellular freely available source of iron was

TABLE 1. Distribution of ^{59}Fe activity before and after a trial incubation carried out in the absence of bacteria

Labelled contents of sample	Amt (ng) of ^{59}Fe at:			
	Start of expt (total)	End of expt in:		
		Interior of dialysis tube	K562 cells	Cell supernatant
K562 cells	957	2	869	114
Transferrin	5,000	47	25	4,282

present at any stage and that only iron scavenged by microbial siderophores was able to cross the dialysis membrane. In addition, the separation of cells and bacteria by a dialysis membrane ensured that any cytotoxic macromolecules produced by the microorganisms did not come into contact with the cells.

To ensure that transferrin-bound iron was not labilized by a reduction in pH resulting from bacterial metabolism, the incubation period was limited to 4.5 h, during which time no detectable change in pH occurred, as assessed by the lack of color change of the phenol red indicator in the tissue culture medium. Longer periods of incubation produced a significant drop in pH, which was invariably accompanied by an increase in the amount of iron acquired from transferrin (data not shown).

The results of a control experiment carried out in the absence of bacteria (Table 1) show that only negligible amounts of ^{59}Fe entered the dialysis tubing—about 1% of transferrin-bound radioiron and <0.02% of iron from labelled cells. Approximately 0.5% of transferrin-bound ^{59}Fe was transferred to the cells during the incubation, presumably because of receptor-mediated endocytosis of the labelled transferrin by the cells. Rather more iron—about 12%—was transferred from the cells to transferrin. However, this was not associated with a loss of viability and, as discussed below, may be a physiological phenomenon.

Iron content of K562 cells. The iron content of K562 cells grown under the standard conditions described above was found to be relatively constant, four separate assays giving a mean ± standard deviation of 87.1 ± 6.5 ng/10^6 cells. This value, which is comparable to values reported by others (32) for cells in tissue culture, was used for calculating absolute figures for iron uptake by bacteria.

Uptake of iron by various bacteria from transferrin and K562 cells. Table 2 shows the relative amounts of iron acquired from K562 cells and from transferrin by *S. aureus*, *S. epidermidis*, *E. coli* NCTC 8623, and *C. albicans*. It is evident that iron acquired by the staphylococci came predominantly from the cells, whereas *C. albicans* and *E. coli* NCTC 8623 tended to obtain more iron from transferrin. However, assuming an iron content of 87 ng/10^6 K562 cells (see above), the total amount of iron available from transferrin was always greater than (up to 12 times) the amount available in K562 cells (the ratio varied somewhat from one experiment to another because of differences in the total number of cells present). If this is taken into account, it is evident that the preference of the staphylococci for cell-derived iron becomes even more pronounced, while *E. coli* NCTC 8623 and *C. albicans* scavenge a significant proportion of iron from both sources. Thus, different species of microorganism appear to scavenge iron preferentially from different sources.

Iron uptake from transferrin and K562 cells by *E. coli* K-12 strains. Because many enterobacteria produce two siderophores (aerobactin and enterochelin) and because of the differences in iron scavenging demonstrated above, it was of interest to determine whether these two siderophores acquire iron from different sources. Accordingly, similar experiments were carried out using the previously characterized (1, 36) strains *E. coli* W3110, which produces only enterochelin, and LG1315, which produces only aerobactin. The results (Table 3) show that while both strains could acquire iron both from transferrin and from K562 cells, the enterochelin-producing strain, W3110, showed a significant ($P < 0.02$) preference for iron from transferrin in terms of the total amount of iron acquired. In contrast, the aerobactin-producing strain, LG1315, acquired rather similar amounts of iron from both sources, but when related to the amount of iron available from each source the relative uptake was significantly ($P < 0.05$) greater from K562 cells than from transferrin.

To rule out the possibility that other mechanisms associated with the ColV-K30 plasmid in strain LG1315 influence the results, similar experiments were carried out with strain LG1628, which carries the aerobactin operon as part of a multicopy recombinant plasmid. The isogenic enterochelin-producing strain LG1705 (harboring the vector plasmid alone) was included for comparison. As before, aerobactin-producing strain LG1628 acquired predominantly iron originating from the K562 cells, while enterochelin-producing strain LG1705 acquired iron mainly from transferrin (Table 3).

TABLE 2. Uptake of iron from transferrin and K562 cells by various microorganisms

Organism	Expt no.	Iron uptake[a] from:		Ratio of uptake values (transferrin/K562 cells)
		Transferrin	K562 cells	
S. epidermidis	1	1.6 (0.03)	11.0 (0.70)	0.15 (0.04)
	2	0.9 (0.02)	3.1 (0.21)	0.29 (0.09)
S. aureus	1	1.0 (0.02)	4.7 (0.45)	0.21 (0.04)
	2	2.1 (0.04)	3.6 (1.04)	0.58 (0.04)
C. albicans	1	16.6 (0.33)	0.9 (0.13)	18.4 (2.54)
	2	7.2 (0.14)	1.5 (0.21)	4.8 (0.67)
E. coli NCTC 8623[b]		62.5 ± 11.8 (1.25 ± 0.24)	6.4 ± 2.3 (1.82 ± 0.87)	9.8 (0.68)

[a] Uptake is expressed as nanograms of Fe per 10^9 microorganisms or (figures in parenthesis) as the percentage of total iron available from each source.
[b] *E. coli* values are means ± standard errors of the mean ($n = 4$).

TABLE 3. Uptake of iron from transferrin and K562 cells by *E. coli* strains

E. coli strain	Iron uptake[a] from:		Ratio of uptake values (transferrin/K562 cells)
	Transferrin	K562 cells	
W3110	22.9 ± 5.0[b] (0.46 ± 0.10)	0.62 ± 0.20[b] (0.07 ± 0.03)	36.9 (6.4)
LG1315	18.5 ± 7.1 (0.75 ± 0.06[c])	28.3 ± 4.0 (4.04 ± 1.46[c])	0.65 (0.18)
LG1705	30.3 ± 4.7[b] (0.61 ± 0.09[c])	0.57 ± 0.19[b] (0.07 ± 0.03[c])	53.2 (8.7)
LG1628	6.8 ± 4.4[c] (0.20 ± 0.10[b])	39.0 ± 11.3[c] (2.64 ± 0.71[b])	0.17 (0.08)

[a] Results are in nanograms of Fe per 10^9 cells (or, for values in parentheses, the percentage of total Fe available), expressed as means ± standard errors of the mean; $n = 4$ for W3110 and LG1315; $n = 3$ for LG1715 and LG1628.
[b] $P < 0.02$ for the difference between uptake from transferrin and uptake from K562 cells.
[c] $P < 0.05$ for the difference between uptake from transferrin and uptake from K562 cells.

Iron uptake from transferrin and ferritin by *E. coli* W3110 and LG1315. The results of two individual experiments with *E. coli* W3110 and LG1315 are shown in Table 4. Although the absolute amount of iron taken up by both strains varied in the two experiments, the ratio of uptake from transferrin to uptake from ferritin was similar in both. Both strains showed about a 10-fold preference for transferrin-bound iron in all cases, but there was also approximately 10 times more transferrin-bound iron present than ferritin-bound iron. *E. coli* W3110 showed a marginally greater uptake from transferrin than did *E. coli* LG1315, but the difference was far less than that seen when comparing uptake from transferrin with uptake from K562 cells.

DISCUSSION

It is generally thought that in microbial infection the key role in iron withholding is played by the serum iron-binding protein transferrin. As a result, the ability of siderophores to scavenge host iron has generally been related to their capacity to remove iron from transferrin, and little attention has been paid to the possibility that iron from cells or tissues is more readily available. The work reported here describes a method for determining in vitro whether indeed siderophore-producing bacteria do acquire iron from transferrin or whether iron derived from cells or tissues is also available to the chelators.

The results strongly suggest that in some cases cell-derived iron may be more readily available than iron bound to transferrin. In particular, *S. aureus* and *S. epidermidis* showed a marked preference for cell-derived iron. In contrast, *C. albicans* tended to acquire transferrin-bound iron. These results probably reflect the differing ability of various siderophores to acquire iron from the two sources. (It should be noted that the experimental system excludes the possibility of a direct contact between bacteria and iron-binding proteins of the type described for a *Neisseria* sp. [31].) The strains of *S. aureus*, *S. epidermidis*, and *C. albicans* used in this study all produced siderophores, as shown by a positive reaction in the detection method described by Schwyn and Neilands (30), but little is known about the siderophores produced by staphylococci or *C. albicans*. There is evidence that siderophore-mediated iron uptake by staphylococci can occur (8, 23), and a novel carboxylate siderophore has recently been described (25). Likewise, a survey of 11 strains of *C. albicans* demonstrated that all produced a hydroxamate-type siderophore and some also produced a phenolate-type siderophore (17), but their structures have not been investigated. Further work will be required to relate differing iron acquisition characteristics of these organisms to the type of siderophore produced.

TABLE 4. Uptake of iron from transferrin and ferritin by *E. coli* W3110 and LG1315

E. coli strain	Expt no.	Fe uptake (ng/10^9 organisms) from:		Ratio of uptake values (transferrin/ferritin)
		Transferrin	Ferritin	
W3110	1	18.1	1.45	12.4
	2	5.1	0.43	11.8
LG1315	1	50.9	5.15	9.8
	2	13.7	1.54	8.9

The ability of many strains of *E. coli* to produce both enterochelin and aerobactin has given rise to speculation that each may fulfill somewhat different functions (12). In particular, it is not obvious why invasiveness of *E. coli* correlates well with production of aerobactin (34), whose affinity for iron is marginally less than that of transferrin (12), rather than with enterochelin, which has a much higher iron affinity (14). Various explanations have been advanced, such as inactivation of enterochelin by albumin (20) or antibodies (26), the ability of aerobactin but not enterochelin to be reutilized (5), or differential availability of iron on the two iron-binding sites of transferrin (11). The work reported here indicates that the two siderophores may acquire iron from different sources, enterochelin scavenging predominantly transferrin-bound iron and aerobactin obtaining iron preferentially from cells or tissues. *E. coli* NCTC 8623 tended to obtain most iron from transferrin, which agrees with our earlier demonstration of iron removal from transferrin by this strain (9) and the fact that it produces enterochelin but not aerobactin (unpublished observations).

The relatively low affinity of aerobactin for iron compared with that of transferrin may therefore be irrelevant if this siderophore scavenges most iron from elsewhere. It is of particular relevance that aerobactin production by *E. coli* correlates with invasiveness, as strains producing systemic infections are likely to localize in tissues and thus come into intimate contact with a potential source of iron.

The way in which aerobactin and other siderophores acquire iron from cells will require further investigation. In particular, it needs to be established whether the siderophore enters the cell to remove intracellular iron or whether it is particularly adept at intercepting iron released by cells before it can be bound by transferrin. The latter mechanism suggests that the siderophore might bind to cell membranes. In the present work either of the two postulated mechanisms could be operating, as a significant proportion of iron initially associated with K562 cells had moved to transferrin at the end of the experiment. Such a transfer is probably not an in

vitro artifact caused by poor cell viability, as no loss of viability was detected, and a significant proportion of iron taken up by erythroleukemic cells is released again even in very short-term incubations (16). Moreover, iron release is a normal function of cells such as macrophages and hepatocytes which are involved in erythrocyte catabolism and iron storage and can thus be considered a normal physiological process.

It seems unlikely that the differences in availability of iron from cells and from transferrin to the two *E. coli* strains can be explained on the basis of a difference in their ability to scavenge intracellular ferritin-bound iron, as there was little difference in their ability to remove iron from either protein. Indeed, it seems likely that cells contain a form of iron that is much more readily available than that bound to ferritin, as siderophore-defective mutants of *Shigella flexneri* were able to grow in HeLa cells (27) or cell lysates (21). This probably corresponds to the as yet unidentified chelatable transit pool, which is thought to be the source of iron scavenged by desferrioxamine (18).

The ability of certain siderophores to acquire iron from cells may help to explain the physiological role of the hypoferremia of inflammation. It is generally considered that reduced transferrin saturation impedes microbial iron acquisition (35), though direct evidence is scarce and some have disputed this view (2). However, inflammation also reduces the intracellular chelatable transit pool (19), and we have previously shown that inflammatory macrophages release much less iron to the extracellular environment than do normal macrophages (10). Thus, reduced availability of intracellular iron or a reduced efflux of iron from cells during inflammation may be of greater importance in limiting microbial growth than the reduction in transferrin saturation.

In conclusion, the method reported in this work allows the relative availability to microorganisms of cellular and transferrin-bound iron to be assessed and suggests that for some microorganisms the ability to acquire iron from transferrin may be of less importance than was previously thought to be the case.

ACKNOWLEDGMENTS

K.G.W. acknowledges receipt of a Medical Council Research Studentship.

We are grateful to J. B. Neilands and A. Bindereif for supplying plasmid pABN1.

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