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Acquisition of Iron from Host Proteins by the Group A Streptococcus

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To identify mammalian iron-binding proteins that can serve as iron sources for *Streptococcus pyogenes*, the group A streptococcus (GAS), we used a plate assay. Ferritin, hemin, hemoglobin, myoglobin, and catalase can support growth of GAS on iron-depleted medium. However, growth was not detected when iron was provided as iron-saturated transferrin or lactoferrin or bound to cytochrome *c*. Therefore, it appears that GAS can use the intracellular iron sources available in the human body, which is consistent with its ability to cause tissue destruction during infection.

The process of infectious disease as it relates to pathogenic microorganisms depends largely upon the efficiency with which these organisms gain access to the host, colonize, cause pathology, and disseminate to a new host (9). Colonization involves adherence and multiplication. The latter requires acquisition from the host of nutrients essential for growth, including iron. Little free iron (ca. 10^{-18} M) is present in the human host because most is complexed to proteins, which represent the primary reservoir of iron for pathogenic bacteria (4, 12, 13). Approximately 95% of the iron in the body is located intracellularly in developing or mature erythrocytes or in cells of the liver, spleen, or muscle. The small amount of extracellular iron that is found in body fluids (ca. 4%) is bound to the glycoproteins transferrin (in serum and lymph) and lactoferrin (in mucosal secretions) (5, 24). The ability of a pathogenic microorganism to obtain iron from its host's environment is a fundamental requirement for the production of disease.

Group A streptococci (GAS; *Streptococcus pyogenes*) are obligate human pathogens. In addition to causing common infections of the pharynx and skin, GAS also cause invasive deep-tissue infections, including myositis, fasciitis, and streptococcal toxic shock syndrome (2, 6, 19, 20). In the last 15 years, an increase in the incidence and severity of these invasive GAS infections has been observed (2, 3, 21). Such life-threatening infections cause considerable tissue damage, resulting in the release of intracellular iron-containing compounds.

Very little is known about the iron requirements and uptake systems of the different species of streptococci. Recently, we reported that the growth of GAS was inhibited by the addition of the chelating agent nitrilotriacetate (NTA) to Todd-Hewitt broth medium supplemented with additional cations (ZTHYB) (7). This inhibition was the result of iron limitation, as growth inhibition was reversed by the addition of either ferric citrate or ferrous sulfate to the NTA-containing (ZTHYB) medium. In the present study, we used a plate assay employing NTA-containing medium to ascertain which host iron sources could be used by GAS for in vitro growth.

The M6 GAS strain JRS4 was used in this study (17). The iron-restricted medium (TH-NTA agar) contained Todd-Hewitt broth (30 g/liter), agar (14 g/liter), 10 mM NTA (trisodium salt), and a mixture of $MgCl_2$, $CaCl_2$, $MnCl_2$, and $ZnCl_2$ (0.55 mM each). This strain did not grow on the TH-NTA medium unless it was supplemented with 5 mM ferric citrate. Thus, the growth restriction resulted from iron limitation.

The ability of GAS strain JRS4 to use various host iron sources was determined by inoculating approximately 10^6 CFU on the surface of TH-NTA plates with a glass spreader. Wells were then punched in the agar, and each was filled with 50 μl of a solution containing an iron source (Table 1). The plates were incubated at 37°C overnight and then examined for growth. No growth was observed around wells containing transferrin, lactoferrin, or cytochrome *c*. However, growth was observed around the wells containing a positive control (ferric citrate or ferrous sulfate) (24-mm growth zone diameter), as well as around wells containing ferritin (16-mm growth zone diameter), hemin (10-mm growth zone diameter), or the heme-containing protein hemoglobin (10-mm growth zone diameter), myoglobin (10-mm growth zone diameter), or catalase (14-mm growth zone diameter). This is consistent with a previous report which demonstrated that GAS removes iron from hemoglobin and from a complex of hemoglobin with its serum carrier protein, haptoglobin (10). However, this previous report did not examine the ability of GAS to use these compounds as sources of iron for growth. Our results show that the sources of iron used by GAS for growth are those found within host cells.

On solid medium, diffusion of the iron source is expected to limit iron availability. However, the largest growth zones resulted around wells containing the high-molecular-weight proteins ferritin and catalase. It seems possible that the iron is released from these proteins, perhaps by an extracellular protease produced by GAS. In support of this idea of diffusion limitation, in liquid medium, hemoglobin supported a growth rate equal to that of the controls (ZTHYB alone or ZTHYB with 18 mM NTA and 13 mM ferric citrate) (data not shown).

Heme is associated to hemoglobin, myoglobin, and catalase through hydrogen bonding but is covalently bound in cytochrome *c* (15, 23). Thus, the ability of heme-containing proteins to serve as sources of heme for GAS appears to be correlated with the noncovalent attachment of heme, as has been observed for the sexually transmitted pathogen *Haemophilus ducreyi* (11). Although the mechanism by which GAS acquires iron from hemoglobin, myoglobin, and catalase is unknown, it is possible that these compounds interact directly with the cell surface of GAS and that either the intact hemin molecule is internalized or the heme is removed from the heme protein.

Under our experimental conditions, neither iron-saturated human transferrin nor lactoferrin was used as a source of iron for the growth of GAS. The same results were obtained when

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TABLE 1. Utilization of host iron sources by group A streptococcus[a]

Iron source[b]	GAS growth
Ferric citrate	+
Ferrous sulfate	+
Ferritin	+
Hemin	+
Hemoglobin	+
Myoglobin	+
Catalase	+
Transferrin	−
Lactoferrin	−
Cytochrome *c*	−

[a] About 10^6 GAS CFU were plated on TH-NTA plates. Wells were punched in the agar, and each was filled with 50 μl of the iron compound. Each experiment was repeated at least three times. +, growth stimulation occurred in wells containing the iron source; −, no growth occurred.

[b] All iron compounds were obtained from Sigma. Solutions with the following concentrations were used: 0.1 M ferric citrate, 0.1 M ferrous sulfate, 100 mg of horse spleen ferritin per ml, 2 mg of bovine hemin (in 20-mg/ml bovine serum albumin) per ml, 20 mg of human hemoglobin per ml, 20 mg of horse skeletal myoglobin per ml, 20 mg of bovine liver catalase per ml, 50 mg of iron-saturated human transferrin per ml, 50 mg of iron-saturated human lactoferrin per ml, and 20 mg of horse heart cytochrome *c* per ml. *c* Apotransferrin and apolactoferrin were saturated with FeCl (18). Fresh solutions of hemin and hemoglobin were prepared for each experiment.

the assay was performed in liquid medium. Addition of iron-saturated transferrin or lactoferrin (18 μM) to an iron-starved culture grown for 5 h in ZTHYB supplemented with 18 mM NTA (7) did not reverse the growth inhibition, while addition of 13 mM ferric citrate (or 10 μM hemoglobin) did. Since NTA is unable to remove iron from either transferrin or lactoferrin and is unable to effectively compete with these substances for free iron (14), it is unlikely that the presence of NTA in the medium affected the ability of GAS to acquire iron from these proteins. A recent report (22) indicated findings similar to what we observed in GAS: *Streptococcus pneumoniae* was able to use hemoglobin as an iron source but was unable to acquire transferrin-bound iron.

The ability of GAS to bind human transferrin and lactoferrin conjugated to peroxidase was determined by dot assay (16). Consistent with the inability of transferrin to support GAS growth under iron-depleted conditions, we found that GAS did not bind this protein. Human lactoferrin, however, did bind GAS cells; nevertheless, since it did not support GAS growth under iron-restricted conditions, its binding by GAS was likely to be nonspecific and not related to iron acquisition. Lactoferrin is a basic protein with a high isoelectric point, which probably enables it to bind nonspecifically to many target cells or proteins (1).

The apparent inability of GAS to acquire iron from human transferrin and lactoferrin suggests that GAS do not produce a siderophore capable of removing iron from these glycoproteins. Neither of the related organisms *S. pneumoniae* or *Streptococcus mutans* produces siderophores (8, 22). In fact, no species of streptococcus has been reported to produce such iron-chelating compounds.

The ability of GAS to lyse cells during infection provides this pathogen with a strategy other than siderophore production for acquiring iron from its human host. GAS produce several extracellular products (e.g., streptokinase, streptolysins O and S, proteases, and superantigens) that cause cell lysis and tissue damage. These activities result in the release of intracellular iron-containing compounds, including heme, hemoglobin, myoglobin, and ferritin. It is not surprising that we find that these compounds can be used by GAS as sources of iron. The availability of these compounds at the site of infection will allow GAS to multiply, which in turn can result in further tissue damage and release of intracellular iron sources. Thus, once tissue damage begins, iron may rapidly increase at the site of infection. In the absence of other factors inhibiting the growth of GAS, this ability to utilize iron sources released by cell lysis may therefore facilitate rapid progression of the severe invasive disease caused by the GAS.

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