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Mechanism of Impaired Iron Release by the Reticuloendothelial System During the Hypoferremic Phase of Experimental *Neisseria meningitidis* Infection in Mice

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Hypoferremia, the reduction of plasma transferrin iron levels during infection, has been shown to control *Neisseria meningitidis* infection in mice. The exact nature of the mechanism that regulates this response has been obscure. We have previously shown that hypoferremia does not result from an accelerated removal of iron from the plasma transferrin pool. In this study, we have examined the processing of iron by the reticuloendothelial system during infection. Normal and hypoferremic meningococcus-infected mice were injected with ^{59}Fe-labeled erythrocytes. Kinetics of uptake and redistribution of the label indicated that during the hypoferremic phase of the infection, reticuloendothelial system-processed iron was not returned to the plasma transferrin pool. Fractionation of hepatic cellular compartments showed that this impaired release of iron resulted from a preferential incorporation of heme-derived iron into the intracellular ferritin pool during the hypoferremic phase of the infection. These findings indicate that this withholding of iron within the intracellular pool leads to hypoferremia and therefore denies the extracellular pathogen its essential iron.

Hypoferremia, the reduction of plasma transferrin iron levels, has been reported for a variety of pathological states ranging from neoplasms and inflammation (2, 8, 16, 20) to viral, parasitic, and bacterial infections (9, 19, 22; R. G. Lalonde and B. E. Holbein, J. Clin. Invest., in press). The role of this response has been described more specifically for bacterial infection in which the hypoferremic phase of infection has been regarded as a host attempt to withhold essential iron from the invading pathogens (22). With experimental *Neisseria meningitidis* infection in mice, hypoferremia commences 6 h after the initiation of infection and is maintained for approximately 15 h (9). This hypoferremia controls meningococcal infection in mice through iron deprivation (9) as this organism appears dependent on transferrin (Tf) iron for its growth in the host (10).

The role of hypoferremia in other disease states is still obscure as is the exact nature of the mechanism that regulates it. The triggering signal appears to be provided by a leukocytic endogenous mediator released by phagocytes at sites of inflammation (12, 18). However, the mechanism that leads to the reduction of plasma iron levels has yet to be defined. We have previously reported that there is not an accelerated removal of iron from the plasma Tf pool during the hypoferremic phase of infection (14). We have now investigated the processing of iron within the reticuloendothelial system (RES) during the hypoferremic phase of *N. meningitidis* infection in mice. This study presents evidence for an altered processing of iron by the RES as the mechanism that leads to the establishment of hypoferremia during infection.

MATERIALS AND METHODS

Mice. C57BL/6, HPB strain, male weanling mice were obtained at 4 weeks of age from the University of Calgary Medical Vivarium (Calgary, Canada) or from the Animal Resources Division, Health and Welfare Canada (Ottawa, Canada).

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Bacterium. *N. meningitidis* strain M1011, a serogroup B, serotype 2 disease strain, was used. Maintenance of stock cultures and routine examinations were as described before (11).

Infection studies. Mice 6 to 8 weeks of age were injected intraperitoneally with 10^4 CFU of a 17-h culture of *N. meningitidis* strain M1011. Mice were sacrificed at regular time intervals by cardiac puncture into heparin-containing Vacutainers (Becton-Dickinson Co., Montreal, Canada). Bacteria in plasma samples were enumerated as CFU 24 h after growth on Mueller-Hinton plates containing an antibiotic mixture (7.5 μg of colistimethate, 12.5 U of nystatin, and 3 μg of vancomycin per ml).

Preparation of ^{59}FeDRBC. Heat-denatured ^{59}Fe-labeled erythrocytes (^{59}FeDRBC) for use in labeling the iron compartments of the RES were prepared as follows. One mouse was injected intravenously with ^{59}Fe citrate to effect high-activity labeling of RBC. The labeled mouse was sacrificed 24 h later by cardiac puncture into a citrate-containing vacutainer. The ^{59}Fe-labeled RBC were harvested and washed three times with physiological saline. ^{59}Fe-labeled RBC were then resuspended in 10 ml of an ACD solution (4.4 g of citric acid, 13.2 g of trisodium citrate, and 14.7 g of glucose per liter of distilled water) and heated at 41°C for 12 min. ^{59}FeDRBC were washed in an ACS solution (prepared as for ACD solution except glucose was replaced by 14.7 g of sorbitol). The washed ^{59}FeDRBC were resuspended in ACS at an appropriate concentration (approximately 10^6 cells per ml and 3×10^5 cpm per ml) for injection into mice.

Kinetics of processing of RES iron in normal and infected mice. Groups of noninfected and infected hypoferremic mice (12 h postinfection) were warmed under a white incandescent lamp to dilate their tail veins. The mice were then injected intravenously with 100 μl of the ^{59}FeDRBC suspension. Animals from both groups were then sacrificed every 30 min over 7 consecutive h by cardiac puncture into heparin-containing Vacutainers. The animals were dissected immediately after exsanguination. The liver and the spleen of each mouse were removed and placed in individual

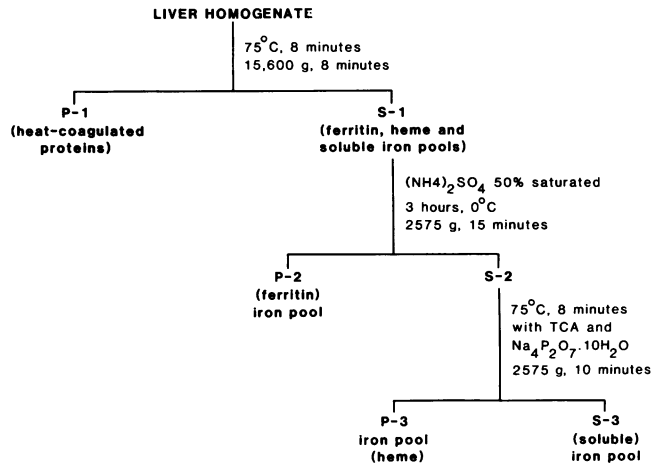

FIG. 1. Subcellular fractionation scheme for liver RES iron. The livers of normal and infected hypoferremic mice, previously injected with ^{59}Fe-labeled RBC, were homogenized and fractionated as shown. TCA, Trichloroacetic acid.

scintillation vials for counting. ^{59}Fe activity in 100 μl of blood and 100 μl of plasma was also determined. All samples were counted in a Beckman 8000 gamma spectrometer. Computational and correction factors were applied as described before (14).

Fractionation of hepatic iron pools. Groups of noninfected and 12-h-infected hypoferremic mice were injected intravenously with 100 μl of ^{59}FeDRBC. Two mice from each group were sacrificed by cardiac puncture every 20 min for 3 consecutive h. ^{59}Fe activity in 100 μl of blood and 100 μl of plasma was determined. The liver of each sacrificed mouse was removed immediately after exsanguination, rinsed in cold physiological saline, and blotted dry. Samples of each liver were excised, weighed, and counted. The remainder of the liver was weighed and homogenized in 4 volumes of ice-cold glass-distilled water over 8 min, using a Potter-Elvejhem homogenizer with a Teflon pestle (1,000 rpm and with continuous up-and-down strokes). Fractionation of liver homogenates into their iron pools was performed as outlined in Fig. 1. Fractions containing the major iron pools (S-1) were obtained from liver homogenates by heating at 75°C for 8 min, followed by removal of the heat-coagulated proteins by centrifugation at 15,600 × *g* for 8 min. Ferritin was precipitated from these fractions by a 50% (wt/vol) saturation with ammonium sulfate, with standing for 3 h at 0°C. The ferritin fractions (P-2) were collected by centrifugation at 2,575 × *g* for 15 min and counted. The supernatant fractions (S-2) were treated with 4 ml of a 20% trichloroacetic acid solution and 4 ml of a saturated sodium pyrophosphate solution for 8 min at 75°C as described by Gale et al. (6). After centrifugation at 2,575 × *g* for 10 min, the supernatant fractions (S-3) and the pellets (P-3) were counted individually. ^{59}Fe in these fractions represented the nonprotein, nonheme, soluble iron pool (S-3) and the heme iron pool (P-3).

SDS-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the S-1 fractions obtained from liver homogenates was carried out in slabs of 1.5-mm thickness, using the method of Lugtenberg et al. (15). Running gels were 10 cm in length and consisted of 10% (wt/vol) acrylamide and 5% (wt/vol) bisacrylamide, whereas the stacking gel consisted of 3% (wt/vol) acrylamide and 0.2% (wt/vol) bisacrylamide. Gels were run at 25 mA per slab in a buffer system consisting of 0.25 M Tris-hydrochloride (pH 8.3) containing 0.19 M glycine and 0.1% (wt/vol) SDS.

Samples were prepared by boiling for 5 min in 0.0625 M Tris-hydrochloride (pH 6.8) containing 0.5% (wt/vol) SDS, 10% (vol/vol) glycerol, and 0.001% (wt/vol) bromophenol blue. Samples, 80 μl per well, were electrophoresed until the tracking dye was within 1 cm of the end of the running gel for investigation of low-molecular-weight proteins. Identical samples were electrophoresed under the same conditions for 4 h after the disappearance of the tracking dye (approximately 7 h total) for the investigation of high-molecular-weight proteins. Standards consisted of a range of low-molecular-weight and high-molecular-weight proteins (Pharmacia Fine Chemicals, Dorval, Canada) and of purified horse spleen ferritin (Sigma Chemical Co., St. Louis, Mo.).

Gels were stained in a solution of methanol, acetic acid, and water (50:10:40) containing 0.125% (wt/vol) Coomassie brilliant blue R-250 (ICN Pharmaceuticals Inc., Plainview, N.Y.) for 12 h at room temperature followed by destaining in methanol-acetic acid-water (50:10:40) and then methanol-acetic acid-water (5:7:88). The gels were then dried on filter paper for 1.5 h, using a Bio-Rad gel slab dryer (Bio-Rad Laboratories, Mississauga, Ontario).

Autoradiography. Identification of ^{59}Fe-containing proteins was obtained by autoradiography of dried gels placed in Titan cassettes with Kodak X-Omat film and stored at −76°C for 2 weeks.

RESULTS

Processing of iron by the RES in normal and infected mice. The hypoferremic response to meningococcal infection in mice begins at approximately 6 h of infection and is maximal by approximately 12 h (9, 14). We elected to study the processing of iron by the RES at 12 h postinfection, i.e., at a time when the events that lead to hypoferremia should be fully expressed. Age-matched control mice were injected

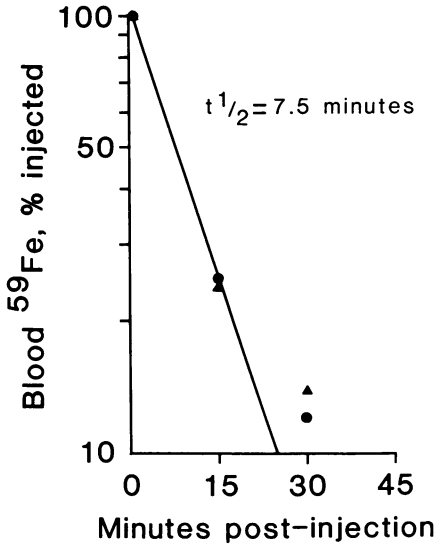

FIG. 2. Clearance of ^{59}FeDRBC from the vascular compartments in normal and hypoferremic, meningococcus-infected mice. Control (●) and 12-h-infected (▲) mice were injected intravenously with 100 μl of a suspension of ^{59}FeDRBC. Mice were sacrificed at 15-min intervals after label injection for determination of blood ^{59}Fe. ^{59}Fe activity is expressed as a percentage of time zero-injected activity. All points represent averages of the data obtained with four mice at each sampling time of five separate identical experiments.

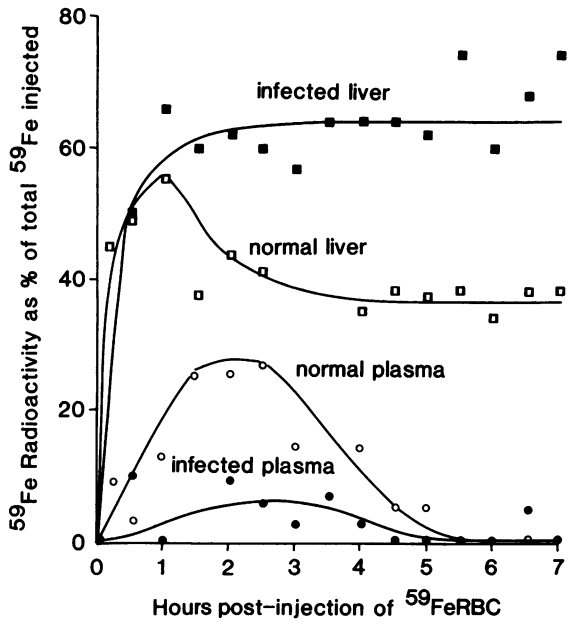

FIG. 3. Kinetics of ^{59}Fe turnover by the liver RES in normal and infected mice. Control (open symbols) and 12-h-infected hypoferremic mice (closed symbols) were injected intravenously with 100 μl of a suspension of $^{59}FeDRBC$. Mice from both groups were sacrificed at 30-min intervals after label injection for determination of ^{59}Fe activity in the plasma Tf pool (●) and the liver compartment (■). Radioactivity in the compartments was expressed as a percentage of the total ^{59}Fe injected. All points represent averages of the data obtained with four mice at each sampling time of five separate identical experiments.

with physiological saline 12 h before the investigation. Clearance rates of $^{59}FeDRBC$ from the blood were similar in control and infected mice with a $t_{1/2}$ of 7.5 min (Fig. 2).

The rapid removal of $^{59}FeDRBC$ from the blood was virtually complete by 30 min after injection, indicating that the surface-denatured RBC were recognized as being senescent and were taken up efficiently by the RES. Livers of control and infected mice took up similar amounts of the label, with maximal uptake being achieved by approximately 30 min postinjection. This liver RES ^{59}Fe represented approximately 55% of the total label initially injected. The rest of the label was taken up by the RES components of other organs and its fate was not followed (data not shown).

There was a rapid turnover of approximately 35% of the liver ^{59}Fe in the case of control mice (Fig. 3). This portion of the label left the RES and entered the plasma Tf pool of normal mice, whereas approximately 65% of the label which had been taken up initially by the liver remained RES associated over the 7-h observation period. Iron in the plasma Tf pool presumably left at a high rate ($t_{1/2}$ of 0.7 h) to enter the bone marrow as shown previously (14). This sequence represents the expected normal movement of iron from the RES, to the plasma Tf pool, and then to erythropoietic sites of the bone marrow. Thus, a substantial portion of the ^{59}Fe taken up by the RES as heme-^{59}Fe was promptly processed and routed to the plasma Tf iron pool.

The fate of RES-associated ^{59}Fe was very different in infected mice during the hypoferremic phase of infection. In infected mice, virtually all of the iron remained associated with the RES during the 7-h observation period. Only small amounts of ^{59}Fe were found in the plasma Tf pool (Fig. 3). These results indicated that during the hypoferremic phase of infection the normal recycling of iron from the RES to the plasma Tf pool did not occur.

Subcellular distribution of RES-associated iron in normal and infected mice. The processing of RES-associated iron, as revealed by ^{59}Fe distribution within subcellular iron pools, was investigated in normal and hypoferremic infected mice as outlined in Fig. 1. Autoradiography of polyacrylamide gels after electrophoresis revealed that the S-1 fractions contained only one ^{59}Fe-containing protein which was identified as ferritin (data not shown). Hence, radioactivity within the P-2 fraction was used as a direct measure of ferritin-associated ^{59}Fe. Removal of heme from the S-2 fractions yielded supernatant fractions which were presumed to consist of nonprotein, nonheme iron intermediates and were termed soluble iron fractions. ^{59}Fe present in each fraction was expressed as a percentage of total counts per minute in the corresponding S-1 fraction which contained the three major iron pools of interest.

Release of iron by the RES has been shown to occur in two distinct phases in other experimental systems (4, 7). The early phase of iron release occurs as a consequence of heme catabolism, with a portion of this iron being readily available for release to the extracellular circulating Tf. This pool is comparatively very dynamic ($t_{1/2}$ of 30 min in dogs [4]) and is generally believed to consist of low-molecular-weight soluble intermediates (1). The S-3 pool was presumed to represent this early iron release pool. The second, late phase of iron release (not examined) represents iron release from the intracellular ferritin pool (1) and is comparatively slow moving ($t_{1/2}$ of 7 days in dogs [4]). This pool was

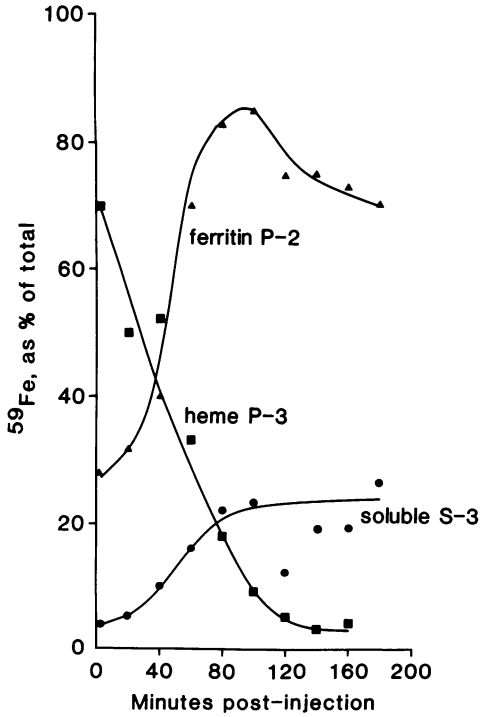

FIG. 4. Subcellular distribution of RES-associated iron in normal mice. Normal mice were injected intravenously with 100 μl of a suspension of $^{59}FeDRBC$. Two animals were sacrificed every 20 min by cardiac puncture. Liver of each animal was immediately removed, rinsed in cold physiological saline, and blotted dry. The major iron pools were obtained after liver homogenization as outlined in Fig. 1. ^{58}Fe activity in each fraction was expressed as a percentage of total ^{59}Fe activity in the corresponding S-1 fraction.

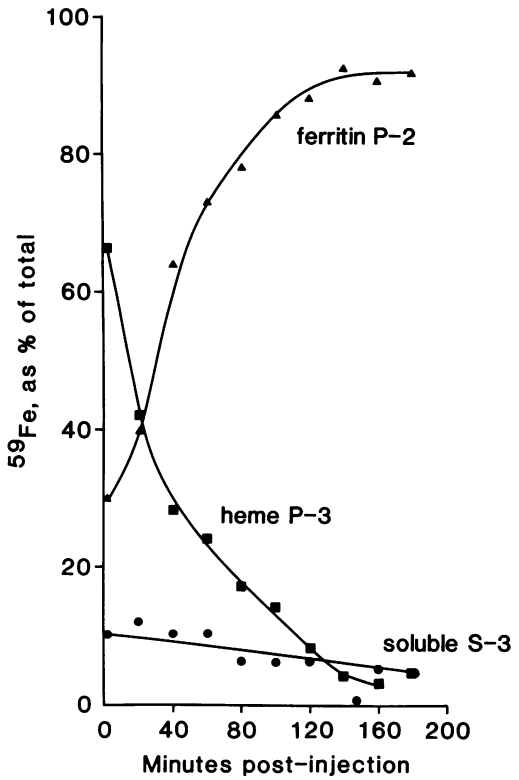

FIG. 5. Subcellular distribution of RES-associated iron in 12 h-infected hypoferremic mice. Twelve-hour-infected hypoferremic mice were injected with 100 μl of a suspension of ^{59}FeDRBC. Two animals were sacrificed every 20 min by cardiac puncture. Liver of each animal was immediately removed, rinsed in cold physiological saline, and blotted dry. The major iron pools were obtained after liver homogenization as outlined in Fig. 1. ^{59}Fe activity in each fraction was expressed as a percentage of total ^{59}Fe activity in the corresponding S-1 fraction.

contained in the P-2 fraction as confirmed by electrophoresis and autoradiography (data not shown).

In normal mice, heme iron catabolism by the RES was accompanied by a simultaneous increase of ^{59}Fe within the soluble and the ferritin iron fractions (Fig. 4). Hence, heme-derived iron was directed to both the early and the late iron release pools of the RES. A portion of the ^{59}Fe associated with the soluble iron pool was regarded as iron en route to the plasma Tf compartment (see Fig. 3). A portion of ferritin-associated ^{59}Fe of control animals was turned over shortly after label incorporation. The significance of this movement of iron remains to be determined.

In hypoferremic infected mice, heme was catabolized at control rates, indicating that this process was not altered at this stage of hypoferremia (Fig. 5). However, iron derived from heme catabolism was preferentially directed to the ferritin pool (Fig. 5). The retention of ^{59}Fe within the ferritin pool was confirmed by autoradiography (Fig. 6). Little iron remained in the soluble iron pool, consistent with the absence of iron movement to the plasma Tf pool.

These results suggested that the hypoferremic phase of infection resulted from an impaired release of iron to the circulating Tf as a consequence of the preferential incorporation of heme-derived iron into the ferritin iron pool of the RES.

DISCUSSION

This study reports on the processing of iron by the RES during the hypoferremic phase of *N. meningitidis* infection in mice. Under normal conditions, iron metabolism operates as a closed cycle characterized by a very efficient reutilization of endogenous iron pools. Tf operates as an iron transport protein within the plasma pool and delivers the metal to various utilization sites, primarily to the erythropoietic tissues of the bone marrow (3). Iron is then incorporated into hemoglobin and is returned to the vascular compartment in RBC. Senescent RBC are subsequently removed by the RES. Iron derived from heme catabolism within the RES is then returned to the plasma Tf pool to repeat the cycle (17).

Bacterial invasion triggers a marked reduction in plasma Tf iron levels. This hypoferremia controls *N. meningitidis* infection in mice (9). Since iron metabolism operates as a closed cycle, the hypoferremic response of infection could result from an accelerated removal of iron from the plasma Tf pool or an impaired return of RES-associated iron to the plasma compartment or both. Van Snick et al. (21) have previously reported evidence for an accelerated removal of iron from the plasma Tf pool during inflammation. In their model, lactoferrin released by neutrophils at sites of inflammation removed iron from circulating Tf or competed with Tf for iron released into the plasma pool. Subsequently, lactoferrin was rapidly taken up by macrophages of the RES where iron became incorporated into stores. Little evidence has been obtained to support this model. However, whereas it seems reasonable that lactoferrin could obtain iron from Tf under possible conditions of low pH at localized sites of inflammation, it is far less likely to operate in this manner in the plasma compartment at physiological pH.

We have previously investigated the existence of an accelerated removal of iron from the plasma Tf pool during the hypoferremic response to experimental *N. meningitidis* infection in mice (14). Iron supplied specifically as Tf-Fe was found to leave the plasma compartment at normal rates in infected animals. We concluded (14) that there was not an accelerated removal of iron from the plasma Tf pool during infection and that hypoferremia must result from an impaired

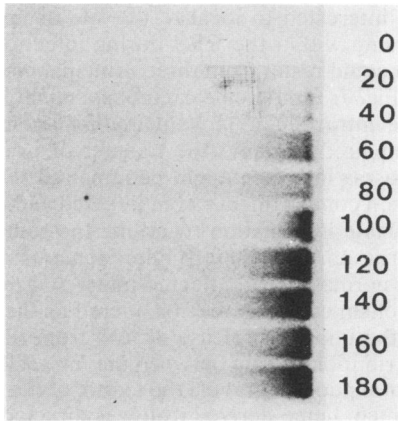

FIG. 6. Accumulation of heme-derived ^{59}Fe in the ferritin pool of hepatic RES cells during the hypoferremic phase of meningococcal infection. Hypoferremic infected mice were sacrificed at 20-min intervals after label injection. S-1 fractions obtained from liver homogenates were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiographed as described in the text.

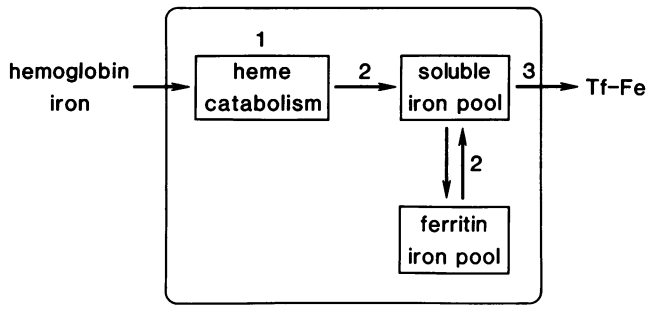

FIG. 7. Possible mechanisms for impaired processing of iron by the RES during infection. Impaired release of RES-associated iron during infection could result from impaired heme catabolism (1), impaired intracellular movement of heme-derived iron (2), or impaired transfer of iron across the RES cell-plasma interface (3). The kinetics of iron processing by the RES and the intracellular distribution of heme-derived iron identified intracellular iron movement as the underlying mechanism for impaired RES iron release during infection.

release of RES-processed iron. In this study, we have investigated directly the processing of iron by the RES during *N. meningitidis*-induced hypoferremia in mice.

The kinetics of iron release by RES cells can be studied by specifically labeling the compartment with ^{59}Fe-labeled heat-damaged surface-denatured RBC. Heme iron acquired by the RES apparently enters a labile iron pool, part of which is immediately returned to circulating Tf (early release) while the rest becomes incorporated into a more slowly moving ferritin pool. In dogs, the early and late release pools of iron are approximately equal in size, but this ratio varies from one species to another (4).

In normal mice, a portion of the heme-derived iron was processed and promptly released to the extracellular plasma Tf pool. The remainder of the heme-derived iron remained liver associated within the ferritin pool for later release. During the hypoferremic phase of infection, all of the heme-derived label remained liver associated. Thus, hypoferremia was readily explained by an impaired release of heme-derived iron from the RES to the plasma Tf iron pool. Such an impaired release of RES-associated iron has been reported previously during inflammation (5).

We were interested to localize the site of impairment of iron processing within the RES during infection. Impaired iron release could result from three principal mechanisms as outlined in Fig. 7. First, heme catabolism might be impaired. Second, the intracellular movement of heme-derived iron might be impaired. Finally, the transfer of iron across the RES cell-plasma interface might be impaired. Fractionation of hepatic iron compartments from infected and normal mice yielded valuable information regarding the routing of RES-processed iron during hypoferremia. Heme catabolism proceeded at a normal rate in infected mice, and thus impaired heme catabolism could not be considered as the mechanism underlying the impaired release of iron from the RES. The relative distribution of iron between the intracellular soluble and the ferritin pools did reveal the nature of the impairment. In normal mice, heme-derived iron was directed to both the soluble and the ferritin pools simultaneously. However, during the hypoferremic phase of infection, iron was incorporated only into ferritin with very low levels entering the soluble pool. Consequently, no iron was available for release to the circulating Tf pool. This routing of heme-derived iron to the ferritin pool was regarded as the underlying mechanism for impaired RES iron release during hypoferremia.

Assuming that heme-derived iron first enters the soluble pool for release or incorporation into ferritin, the results suggest an accelerated rate of incorporation of iron into ferritin during hypoferremia. The cause(s) for this shift in iron routing during hypoferremia remains obscure. It is interesting to note in this regard that a stimulation of ferritin synthesis during inflammation has been reported previously and has been suggested to be part of the mechanism that diverts iron into ferritin stores, thus reducing the pool of soluble iron available for release to circulating Tf (13). An impaired transfer of iron across the RES cell-plasma interface does not appear to be involved in this hypoferremic response. One would expect a buildup of intracellular soluble iron intermediates if this were the case. Interestingly, the hypoferremia which occurs during ceruloplasmin deficiency (Letendre and Holbein, unpublished data) does appear to involve impaired iron movement across the RES cell-plasma interface, as copper-deficient, ceruloplasmin-deficient mice accumulate soluble iron within the liver RES, which is readily releasable by exogenous ceruloplasmin addition.

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