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Inflammation Triggers Hypoferremia and De Novo Synthesis of Serum Transferrin and Ceruloplasmin in Mice

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Oil of turpentine was used to induce an artificial inflammation so that we could study its effect on iron metabolism and on synthesis of serum transferrin and ceruloplasmin in mice. It was found that turpentine-induced inflammation triggered the establishment of a hypoferremic state characterized by low levels of serum iron, followed by recovery and a gradual return to normal plasma iron levels. This turpentine-induced hypoferremia and its subsequent recovery paralleled the hypoferremia obtained during meningococcal infection. Moreover, serum transferrin and ceruloplasmin activity levels increased drastically during the recovery from hypoferremia. [^{14}C]leucine incorporation studies revealed a de novo synthesis of both transferrin and ceruloplasmin. Turpentine-induced hypoferremia was also found to provide a protective effect against meningococcal infection which could be partially reversed by exogenous iron. The results of this study suggest that transferrin and ceruloplasmin may be synthesized partly in response to the altered iron metabolism observed during hypoferremia.

The hypoferremic response involves a marked reduction in the levels of extracellular iron associated with circulating serum transferrin. This response has been shown to occur during a wide variety of pathological states such as neoplastic disease and inflammation (6, 10, 26, 30, 40), as well as in viral, parasitic, and bacterial infections (5, 11, 20, 29, 39).

The hypoferremia during acute bacterial infection has been regarded as an important defense mechanism whereby the host attempts to limit the availability of essential iron to the invading pathogens (39). The hypoferremia with experimental *Neisseria meningitidis* infection in mice occurs early after the onset of the infection and appears to control the proliferation of the meningococcus through iron deprivation (11), especially since transferrin (Tf) iron is important for the growth of this organism (3, 12).

During the hypoferremic stage of meningococcal infection in mice, it appears that heme iron processed by the reticuloendothelial system (RES) is not returned to the plasma Tf pool but rather is incorporated into intracellular hepatic ferritin. This diversion of iron accounts for the reduction in plasma transferrin iron levels and the establishment of the hypoferremic state (21, 22).

The exact mechanism leading to the hypoferremia is not yet clearly understood. Evidence suggests that a leukocytic endogenous mediator, also believed to be interleukin-1 (IL-1), released by macrophages at the site of inflammation, provides the triggering signal for hypoferremia (16, 28).

The two plasma glycoproteins Tf and ceruloplasmin (Cp) play central roles in iron metabolism. Tf acts in transporting iron among extracellular sites of absorption, utilization, and storage (1). Cp acts by its ferroxidase activity which is implicated in the mobilization of iron from intracellular stores to the extracellular circulating Tf pool (23, 27, 31, 34). Profound changes in iron metabolism as a result of hypoferremia could influence the metabolism of both Tf and Cp.

In this study, we have investigated inflammation induced artificially by turpentine.

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MATERIALS AND METHODS

Mice. C57BL/6 HPB male mice were obtained from the Animal Resources Division, Health and Welfare Canada (Ottawa, Ontario, Canada) or from Charles River Breeding Laboratories, Inc. (St -Constant, Quebec, Canada). They were maintained on standard rat chow, watered ad libitum, and used for experiments when they were 6 to 8 weeks of age and 20 to 21 g.

Bacterium. *N. meningitidis* M1011, a serogroup B, serotype 2 disease strain, was used. This strain was cultivated on blood agar and suspended in Neisseria Chemically Defined Medium (GIBCO Canada, Montreal, Quebec, Canada) for infection studies as described before (11, 13). The 50% lethal dose for this strain in mice given iron dextran has been shown to be <2 CFU (13). Bacteria were enumerated as CFU on Mueller-Hinton agar (GIBCO) after 18 h of growth at 37°C in an atmosphere containing 5% CO_2–95% air and 100% relative humidity.

Inflammation studies. Inflammation was produced by the subcutaneous injection of 0.05 ml of oil of turpentine (Fisher Scientific Co., Montreal, Quebec, Canada) in each of two separate bilateral sites on the back during light ether anesthesia. Control mice were anesthetized and treated similarly with sterile saline. Six mice from each group were bled by cardiac puncture into 3.0-ml Vacutainer tubes (Becton Dickinson, Montreal, Quebec, Canada) at intervals over 72 h. The collected blood was allowed to clot at room temperature for 30 min and then at 4°C for 8 h. The serum samples obtained after centrifugation were assayed for total iron-binding capacity, unsaturated iron-binding capacity, and transferrin iron with a ^{59}Fe radioassay kit (Becton Dickinson). Ceruloplasmin ferroxidase [iron(II):O_2 oxidoreductase, EC 1.16.3.1] activity was also measured by a colorimetric assay described by Ravin (32).

Synthesis of Tf and Cp in normal and turpentine-treated mice. Mice were divided into two groups; one group was turpentine treated and the other was saline treated as de-

scribed above. Three mice from each group were bled at 0, 12, 24, 36, 48, and 72 h posttreatment from the retroorbital sinus with a Pasteur pipette (Fisher Scientific Co.) dipped first into a heparin solution (150 U/ml) (Sigma Chemical Co., St. Louis, Mo.) or by cardiac puncture into a heparinized 3.0-ml Vacutainer (Becton Dickinson). At 36 h posttreatment, 15 mice of each group were put under a white incandescent bulb to dilate their tail veins and then were injected intravenously with 0.1 ml of L-[U-^{14}C]leucine (342 mCi/mmol) (New England Nuclear [NEN], Lachine, Quebec, Canada). Thereafter, three mice from each group were bled at 0, 15, 30, 45, and 60 min. These blood samples were centrifuged for 3 min at 12,800 × g at room temperature, and the plasma samples obtained were kept frozen until used for electrophoresis.

Electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the samples was carried out in slabs of 1.5 mm thickness by the method of Laemmli (19). Running gels were 12.0 cm in length and consisted of 10% (wt/vol) acrylamide and 0.26% (wt/vol) bisacrylamide, whereas the stacking gels consisted of 5% (wt/vol) acrylamide and 0.13% (wt/vol) bisacrylamide. Gels were prerun at 17 mA per slab in a buffer system consisting of 0.025 M Tris-hydrochloride (pH 8.3) containing 0.19 M glycine and 0.1% (wt/vol) SDS. The protein content of the samples was determined by the method of Lowry et al. (25), and the samples were diluted to 1 mg of protein per ml with phosphate-buffered saline (pH 7.3). Samples were prepared by being boiled for 5 min in 0.08 M Tris-hydrochloride (pH 6.8) containing 2% (wt/vol) SDS, 10% (wt/vol) glycerol, 0.04% (wt/vol) bromophenol blue, and 5% (vol/vol) β-mercaptoethanol. Standards consisted of a range of low- and high-molecular-weight proteins (Pharmacia, Dorval, Quebec, Canada), purified mouse Tf (21), and human Cp (Sigma).

Gels were run at 25 mA per slab for a period of 2.5 h after the elution of the tracking dye. Gels were stained in a solution of methanol, acetic acid, and water (40:5:55) containing 0.0625% (wt/vol) Coomassie brilliant blue R-250 (ICN Pharmaceuticals Inc., Plainview, N.Y.) overnight at 22°C, followed by destaining in methanol–acetic acid–water (40:5:55) for 1 day. The gels were then dried on filter paper at 70°C for 1.5 h, using a Bio-Rad gel slab dryer (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

Fluorography. Slab gels used for fluorography were first fixed in a solution of 10% (wt/vol) trichlorocetic acid (TCA), 10% (vol/vol) glacial acetic acid, and 30% (vol/vol) methanol for 1 h with gentle agitation. They were then stained and destained as above and impregnated with En^{3}Hance (NEN) for 1 h with agitation. The En^{3}Hance was then discarded, and cold water was added to the gels to precipitate the impregnated fluors during a further 1 h with agitation. Exposure of the dried gel was done at −76°C for 15 days with Kodak X-Omat AR film.

Incorporation of [^{14}C]leucine into Tf and Cp. After electrophoresis, staining, and destaining, the Tf, Cp, and albumin bands were identified and cut out of the gels and counted in 10 ml of a toluene-based cocktail (Fisher) containing 1% (wt/vol) NCS tissue solubilizer (0.6 N in toluene) (Amersham/Searle, Arlington Heights, Ill.), 1% (vol/vol) 1 M hyamine chloride in ethanol (Packard Instrument Co., Inc., Downers Grove, Ill.), and 0.6% (wt/vol) 2,5-diphenyloxazole (PPO) (NEN) by a modification of the method of Aloyo (2). Vials were counted with a Beckman liquid scintillation counter LS 8000 (Beckman Instruments, Inc., Fullerton, Calif.) with automatic quench compensation.

Infection studies. Groups of saline- and turpentine-treated hypoferremic mice (12 h after saline and turpentine treatment) were infected intraperitoneally with ca. 10^4 CFU of *N. meningitidis* M1011 as described before (11). Other groups of saline- and turpentine-treated mice were injected intraperitoneally with iron dextran at a dosage of 250 mg/kg of body weight just before the infection. Two mice of each group were sacrificed at regular intervals by cardiac puncture into heparin-containing Vacutainer tubes (Becton-Dickinson). 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).

Statistical analysis. Data were analyzed by the Student t test, with probability levels of $P = 0.01$ or less considered significant.

RESULTS

Turpentine-induced hypoferremia. Oil of turpentine, when injected subcutaneously in the back of C57 black mice, induced a sterile inflammation resulting in the formation of a subcutaneous abscess. In addition, a marked hypoferremic response was observed 12 h after the injection of turpentine, and the transferrin iron reached its lowest level by 24 h (Fig. 1). This rapid and intense turpentine-induced hypoferremia was followed by a gradual return to normal plasma iron levels, which were attained between 48 and 60 h after turpentine treatment. Control mice injected subcutaneously with sterile saline maintained fairly constant levels of transferrin iron over the 72-h period of observation (data not shown). The reduction in transferrin iron levels observed between 12 and 36 h after turpentine treatment was found to be highly significant ($P < 0.001$). Interestingly, this turpentine-induced hypoferremia and its subsequent recovery par-

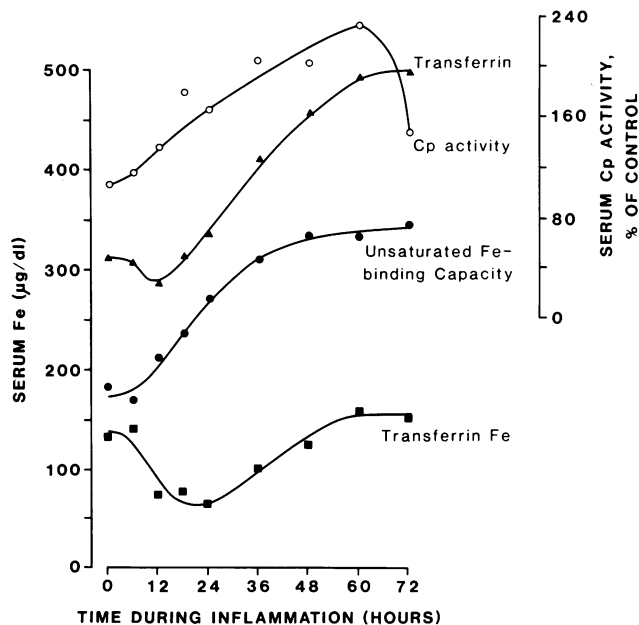

FIG. 1. Hypoferremia during turpentine-induced inflammation in mice. Mice were anesthetized and injected subcutaneously in the back with 0.05 ml of turpentine at two separate sites. At the indicated times, six mice were bled by cardiac puncture for determinations of serum transferrin or total iron-binding capacity, unsaturated iron-binding capacity, transferrin iron, and ceruloplasmin ferroxidase activity. Values shown represent the average data of two identical experiments.

alleled the hypoferremia obtained when mice were infected with 10^4 CFU of *N. meningitidis* M1011 (11, 21; D. L. Beaumier and B. E. Holbein, unpublished data).

A dramatic increase in transferrin levels was also observed 24 h after the turpentine treatment (Fig. 1). The transferrin level had increased to ca. 160% of its initial and control values by 72 h ($P < 0.001$). Transferrin increases paralleled the return to normal serum iron values. A similar pattern of transferrin increase was observed during meningococcal infection, although the maximum levels were lower and this occurred after the infection was cleared (Beaumier and Holbein, unpublished data). The increase in serum unsaturated iron-binding capacity paralleled the initial hypoferremia and then the later increase in total serum transferrin. It is important to note in this regard that serum transferrin iron levels returned to pretreatment levels even though there was much more available serum transferrin iron binding capacity after recovery.

Ceruloplasmin ferroxidase activity also increased markedly during the inflammation, with a significant increase observed as early as 12 h and a maximum level (235% of control) at 60 h posttreatment. The decrease in ferroxidase activity between 60 and 72 h was consistent but remains unexplained.

Plasma protein synthesis during inflammation. It was important to establish whether there was de novo synthesis of transferrin and ceruloplasmin or a redistribution of preformed Tf or Cp from other pools to the vascular system. Preliminary studies with normal mice had shown a fairly linear incorporation of L-[*U*-^{14}C]leucine into total TCA-precipitable plasma protein between 10 and 60 min after label

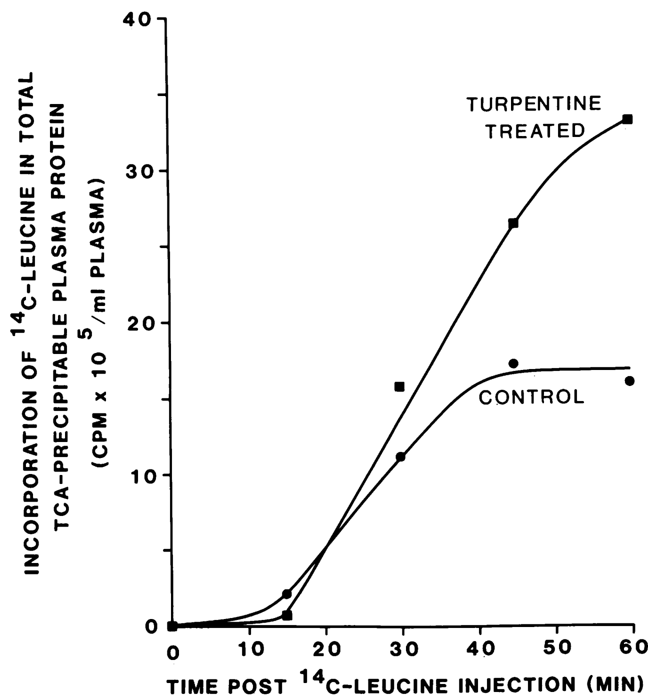

FIG. 2. Incorporation of [^{14}C]leucine in total TCA-precipitable plasma protein. Mice were injected intravenously with 50 μCi of L-[*U*-^{14}C]leucine 36 h after the subcutaneous injection of turpentine or saline. Mice were bled from the retroorbital sinus, and the radioactivity in plasma proteins was determined after precipitation by 5% TCA (final concentration). Data shown are the means of triplicate samples for one experiment but are typical of the results obtained in three separate experiments.

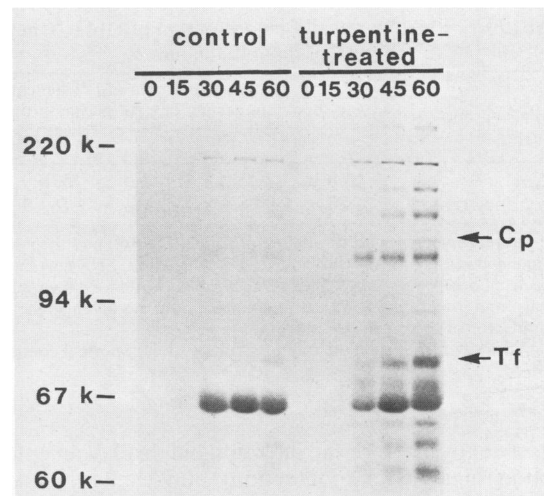

FIG. 3. Fluorograph of the incorporation of [^{14}C]leucine into Tf and Cp. Mice were treated and sampled as described in the legend to Fig. 2. SDS-polyacrylamide gel electrophoresis was performed on the plasma samples obtained at 0, 15, 30, 45, and 60 min after [^{14}C]leucine injection; 35 μg of total plasma protein was applied to each lane. Numbers at the left indicate the positions of molecular weight markers from adjacent lanes.

addition (data not shown). There was a higher rate of incorporation of [^{14}C]leucine in total TCA-precipitable plasma protein for turpentine-treated mice than for control mice (Fig. 2) at 36 h posttreatment. Incorporation of label was examined at 36 h posttreatment because Tf and Cp levels in serum were increasing at this time (Fig. 1). Thus, there was a higher rate of synthesis of serum proteins in turpentine-treated mice.

Electrophoresis coupled with fluorography revealed that the incorporation of [^{14}C]leucine was greater for transferrin and ceruloplasmin in the inflamed mice than in control mice (Fig. 3). In addition, several other plasma proteins were synthesized at higher rates (Fig. 3), although no attempt was made to identify these proteins.

Measurements of the relative rates of incorporation of [^{14}C]leucine into transferrin, ceruloplasmin, and albumin (control nonacute phase protein) revealed a rate of transferrin synthesis 4.4-fold higher in turpentine-treated hypoferremic mice than in control mice (Table 1). Furthermore, the relative rate of ceruloplasmin synthesis was found to be 5.5-fold higher in turpentine-treated mice. These results were highly significant ($P < 0.001$). In contrast, the rate of incorporation of [^{14}C]leucine into albumin was similar in both control and turpentine-treated mice.

Turpentine-induced resistance to meningococcal infection. Mice were rendered hypoferremic by the injection of turpentine and were then examined for their susceptibility to infection with *N. meningitidis* M1011 (Fig. 4). The peak of infection was significantly lower for the hypoferremic mice ($P < 0.01$), and the time for clearance of the infection (average of 5.2 h for the turpentine-treated mice and 10.4 h for the control mice) was also significantly lower ($P < 0.001$).

Another group of mice was rendered hypoferremic and received iron dextran just before the injection of bacteria. It has been shown previously that supplemental iron in the form of iron dextran enhances meningococcal infection, causing a uniformly lethal infection in normal mice (11, 13). Interestingly, hypoferremic turpentine-treated mice receiv-

TABLE 1. Relative rates of incorporation of L-[U-^{14}C]leucine into Tf, Cp, and albumin[a]

Mouse group	Rate of incorporation of [^{14}C]leucine (cpm/min per mg of total plasma protein)		
	Tf	Cp	Albumin
Control	10.8 ± 2.7	1.5 ± 0.2	178.9 ± 17.6
Turpentine treated	48.0 ± 5.8[b]	8.1 ± 1.4[b]	163.9 ± 22.0

[a] Mice were injected with turpentine or saline (control) and then 36 h later were injected intravenously with 50 μCi of [^{14}C]leucine. SDS-polyacrylamide gel electrophoresis was performed on the plasma samples taken at various intervals, and the protein bands were counted. Data are given as the mean ± standard error of the mean (n = 8).

[b] Significantly higher than the rate of incorporation of normal controls (P < 0.001).

ing iron dextran before the infection did display an enhanced infection (higher peak of infection), but despite the additional iron these mice were eventually able to control and clear the infection (Fig. 4). Control mice given the same dose of iron dextran experienced an uncontrolled infection resulting in the death of all the animals by 48 h (data not shown).

DISCUSSION

Hypoferremia and infection. Hypoferremia has been shown previously to occur during meningococcal infection of mice and to be an important component of host defense in the successful control of this infection (11, 12, 21, 22). The mechanism of this hypoferremia has been shown to involve an altered processing of heme-derived iron within the RES which limits the supply of iron to the extracellular plasma transferrin pool (21, 22). The exact mechanism triggering the onset of hypoferremia during meningococcal infection of

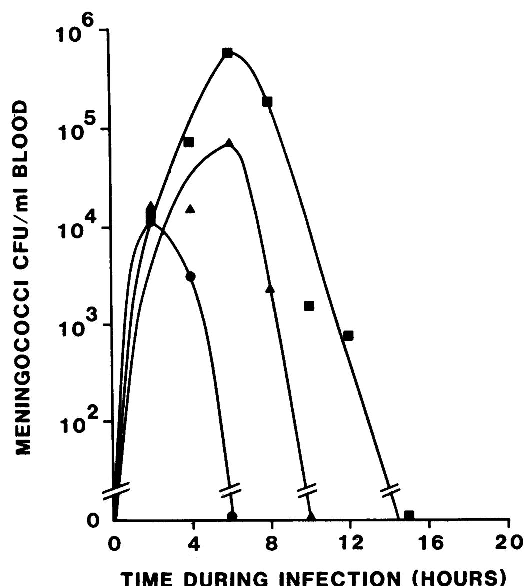

FIG. 4. Effects of turpentine-induced hypoferremia on *N. meningitidis* infection in mice. Groups of turpentine- (●) and saline-treated (▲) mice were infected by an intraperitoneal injection of ca. 10^4 CFU of *N. meningitidis* M1011 12 h after the first treatment. Another group of turpentine-treated mice was injected with 5 mg of iron dextran (■) just before the infection. Two mice were removed at intervals from each group, and the numbers of bacteria in their blood were determined by plate counting. The data represent the average values of three separate experiments.

mice has been obscure, although we had suggested that the inflammatory process and the involvement of phagocytic cells and their products seemed likely to be involved (22).

Here, we have shown that an artificially induced sterile inflammation from turpentine also induces a rapid and pronounced hypoferremia in mice of this strain. The intensity of the response was somewhat greater with turpentine-induced inflammation than had been observed in infection (11). It has been shown that infection often produces a transient inflammatory state (33), and this alone may account for the lower intensity of the response during meningococcal infection. Indeed, meningococcal infection in these mice, when not augmented by exogenous iron, is transient itself, with the infection disappearing completely after ca. 18 h. The subcutaneous injection of turpentine creates a rapid, intense, and prolonged inflammation at the site of the injection and presumably provides a better stimulus, resulting in a stronger response.

The present findings strengthen the contention that the inflammatory process, initiated during infection, is responsible for the induction of the hypoferremia. The resistance of turpentine-treated, hypoferremic mice to meningococcal infection adds further evidence to the importance of transferrin iron (3, 12) in meningococcal growth in vivo and to the importance of the hypoferremia (11–13, 21, 22) in the control of meningococcal infection. In addition, mice rendered hypoferremic by a dietary Cu deficiency, which limits the availability of Cp, are also resistant to meningococcal infection (23). The finding that exogenous iron supplied to turpentine-treated, hypoferremic mice stimulated infection but did not overcome the ability of the mouse to eventually control and clear the infection is particularly noteworthy. Iron dextran is largely unavailable to the meningococcus, as such, but rather must be processed through the RES along a pathway similar to that of heme iron (11, 12, 23). Thus, hypoferremic mice, impaired in RES turnover of iron, would be expected to accumulate the iron from iron dextran within the RES and not route it to the plasma transferrin iron pool. Therefore, little of this exogenous iron may have become available for meningococci proliferating in the extracellular compartments and dependent upon transferrin iron. The opposite situation has been found in the case of *Trypanosoma cruzi*, which is an intracellular parasite and proliferates within the RES of these mice (20).

Previous studies have demonstrated that hypoferremia induced by the injection of sham infectious materials is effective in lowering the intensity or the incidence or both of infection in experimental animals and that added iron neutralizes the protective action of the sham materials (9, 17, 36; G. C. Chandlee and G. M. Fukui, Bacteriol. Proc., p. 45, 1965). Leukocytic endogenous mediator (LEM), also called IL-1, is a low-molecular-weight protein produced by stimulated phagocytes and has been implicated in the hypoferremic response (4, 15, 38). Hypoferremia induced by the injection of LEM also increases host defense against infection (14). However, crude preparations of LEM also produce a variety of other physiological effects, including hypotension, tachycardia, and transient hepatic dysfunction (24). LEM preparations of better purity should better delineate the specificity of LEM effects.

Partially purified LEM (or IL-1) has been shown to trigger a reduction in plasma iron levels in mice (15). Lactoferrin may be involved in the local sequestration of iron at the site of inflammation (38) or in the overall mechanism producing an impaired release of iron from the RES (21). Furthermore, according to Bornstein (4), the acute-phase reaction taking

place during inflammation is initiated and regulated by IL-1, which produces a variety of physiological changes including alterations in hepatic protein synthesis and hypoferremia. However, the exact mechanism of action of IL-1 is still obscure. It may act as a direct or an indirect mediator of the onset of the hypoferremic state. Further studies involving turpentine-induced hypoferremia or meningococcus-induced hypoferremia in C57 mice might facilitate studies directed at discerning the roles of LEM and other mediators in the regulation of the hypoferremic response.

Tf and Cp in iron metabolism. Turpentine-induced inflammation was also found to trigger a de novo synthesis of both Tf and Cp but not of albumin. In addition, other unidentified proteins were synthesized at enhanced rates after turpentine treatment. A number of proteins, collectively termed acute-phase proteins, are synthesized in response to infection and other stimuli including experimental inflammation (4, 7, 18, 35). It appears likely that the majority of the proteins which were synthesized at elevated rates during turpentine-induced inflammation are of the acute-phase class. However, we have reasons to question whether Tf and Cp should be regarded merely as typical acute-phase proteins. Transferrin has been observed to increase markedly in the plasma of mice infected with *Listeria monocytogenes* (37) or *Mycoplasma arthritidis* (8) but has not been regarded thus far as an acute-phase protein. Cp has been shown to be much more important to iron metabolism in mice than as an acute-phase reactant (23). Mice deficient in Cp were resistant to infection, whereas the absence of Cp as an acute-phase protein in host defense might be expected to increase susceptibility (23).

Both Tf and Cp are involved in the regulation of the extracellular transferrin iron pool (Fig. 5). Mice develop a hypoferremia in response to an inducer that might be LEM or IL-1. This hypoferremia involves the withholding of heme iron after its processing within the RES (22; Fig. 5). The result of this alteration in iron metabolism is to deny incoming iron to the dynamic transferrin iron pool. The recovery from meningococcal infection or turpentine-induced hypoferremia involves the appearances of elevated amounts of Tf and Cp (23; Beaumier and Holbein, unpublished data) (Fig. 1). We suggest on the basis of these findings that the synthesis of Tf and Cp, under these conditions, might in part be related to iron regulation (Fig. 5). This working hypothesis places importance on the level of iron in the transferrin pool in the regulation of two major proteins, Tf and Cp, involved in this pool.

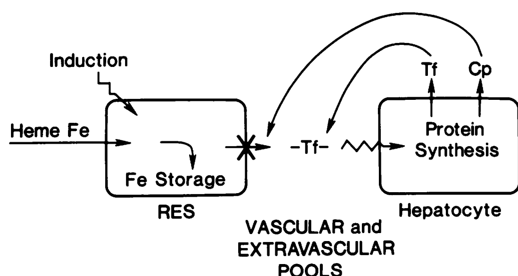

FIG. 5. Working hypothesis for the hypoferremic response in mice. *N. meningitidis* infection or turpentine-mediated inflammation induces the RES to reroute Fe from incoming senescent erythrocytes to storage instead of recycling it to the Tf pool. This would decrease the level of iron associated with Tf in the vascular and extravascular pools, thereby producing hypoferremia. Low transferrin iron would in turn induce hepatocyte synthesis of Tf and Cp. Elevated Tf and Cp would facilitate a return to normal transferrin iron levels.

We are currently examining the factors involved in the regulation of Tf and Cp synthesis, as well as other hepatic proteins, to investigate this hypothesis. Any direct involvement of iron in the regulation of the synthesis of Tf, Cp, or other proteins of iron metabolism would have important implications in a number of situations involving altered iron metabolism.

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