

Desferal Attenuates TNF Release Following Hepatic Ischemia/Reperfusion^{1,2}

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Iron chelators have been shown to protect against oxygen free radical injury occurring in association with ischemia/reperfusion (I/R). Tumor necrosis factor alpha (TNF) represents a major mediator of the pulmonary and hepatic injury occurring after hepatic I/R since pretreatment with anti-TNF antibody results in significant protection against both the lung and liver injury following this insult. We were therefore interested in the possible association of the protective actions of deferoxamine (Desferal) following hepatic I/R and subsequent TNF release. A rat model of hepatic I/R was used to evaluate this; four experimental groups were studied. Animals in I/R underwent 90 min of hepatic ischemia with subsequent reperfusion. DES-I/R animals were pretreated with 200 mg of deferoxamine and VEH-I/R rats were given an equivalent amount of vehicle prior to hepatic I/R. SHAM animals underwent sham laparotomy alone. Plasma specimens were obtained and analyzed for TNF using a cytolytic bioassay based on the WEHI 164 subclone 13 cell line. Mean peak TNF levels following deferoxamine pretreatment was 110.38 ± 24.68 pg/ml, as compared to mean peak TNF levels of 213.64 ± 38.09 pg/ml in the VEH-I/R group ($P < 0.01$). Lung injury following hepatic I/R was evaluated by assessment of pulmonary microvascular permeability and by evaluation of pulmonary neutrophil infiltration as measured by pulmonary myeloperoxidase (MPO) content. Pretreatment with deferoxamine resulted in a significant decrease in lung leak as compared to animals pretreated with vehicle prior to I/R (DES-I/R = 0.192 ± 0.013 , VEH-I/R = 0.690 ± 0.050 ; $P < 0.005$). Deferoxamine had a similar significant protective effect against neutrophil influx: DES-I/R MPO = 0.318 ± 0.078 and VEH-I/R MPO = 0.600 ± 0.060 ($P < 0.005$). Liver injury following I/R was measured with SGPT levels 24 hr postreperfusion and a

significant protective effect was seen in animals receiving deferoxamine prior to hepatic I/R (DES-I/R = 1816 ± 528 , VEH-I/R = 7367 ± 922 ; $P < 0.001$). Although deferoxamine's protective actions can be attributed to inhibition of oxygen free radical generation, these experiments also document a decrease in TNF release. The decrease in organ injury may be due to a combination of fewer toxic oxygen metabolites and less TNF. © 1994 Academic Press, Inc.

INTRODUCTION

Ischemia/reperfusion injury to the liver remains an important clinical problem and includes situations such as hepatic failure following shock, liver surgery, and liver preservation and transplantation. Experimental work in this area has focused mainly on the role of toxic oxygen species in the pathophysiology of hepatic ischemia/reperfusion injury, and the Kupffer cell appears to be the major source of the reactive oxygen species generated following hepatic ischemia/reperfusion [1]. Tumor necrosis factor- α (TNF)³ is involved in many of the immunologic responses to inflammation, injury, and infection. The cytotoxic potential of TNF is thought to require oxygen [1, 2], and it appears to involve the production of oxygen-derived free radicals such as hydrogen peroxide, the superoxide anion, singlet oxygen, and hydroxyl radicals [1, 2]. TNF may also prime the host system against infection by priming macrophages and neutrophils to generate increased amounts of toxic oxygen species when subsequently challenged with invading organisms [3-5]. However, oxygen free-radical release may also be responsible for the organ damage and failure seen with severe systemic infections and endotoxemia. We have previously demonstrated that TNF is intimately involved in both the pulmonary and hepatic injury

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² All experiments were approved by the University of Michigan Committee on the Use and Care of Laboratory Animals and their guidelines for animal use and care were followed.

³ Abbreviations used: I/R, ischemia/reperfusion; TNF, tumor necrosis factor- α ; MPI, mean permeability index; MPO, myeloperoxidase; ¹²⁵I-BSA, ¹²⁵I-labeled bovine serum albumin; PMNs, polymorphonuclear leukocytes.

which occurs following hepatic ischemia/reperfusion [6, 7]. The current experiments were undertaken in order to attempt to ascertain whether the protective effects of deferoxamine (Desferal) were in part related to inhibition of TNF or if these protective actions were based purely on inhibition of oxygen free radical generation.

MATERIALS AND METHODS

Animal Model

A rat model of lobar, rather than total, hepatic ischemia, was used in order to produce a severe ischemic insult without inducing mesenteric venous hypertension [8]. All experiments utilized 200- to 300-g adult male pathogen-free Sprague-Dawley rats (Charles River, Portage, MI). Anesthesia for laparotomy was induced with intramuscular (im) ketamine hydrochloride (100 mg/kg) and an indwelling intravenous line was placed via the right external jugular vein for serial blood sampling and administration of intravenous fluids and medications. By positioning the catheter tip in the suprahepatic vena cava just above the dome of the liver, immediately posthepatic blood samples were obtained; correct catheter position was confirmed at laparotomy. Following intravenous heparinization with 100 units/100 g body wt, midline laparotomy was performed. Hepatic ischemia was initiated by application of an atraumatic microaneurysm (Heifitz) clip across the portal venous and hepatic arterial blood supply to the cephalad three lobes of the liver; the three caudal lobes retained an intact portal inflow and venous outflow, preventing intestinal venous congestion. Lobar ischemia was maintained for 90 min and the Heifitz clip was then removed at a second laparotomy. Intravenous lactated Ringer's solution in a dose of 0.75 ml was administered to replace operative fluid and blood losses. Sham-operated control animals were treated in an identical fashion with the omission of vascular occlusion.

Experimental Design

Four experimental groups were constructed. Animals in I/R underwent 90 min of lobar hepatic ischemia with subsequent reperfusion. DES-I/R rats received 100 mg of deferoxamine im 12 and 2 hr prior to the induction of 90 min of hepatic ischemia and reperfusion. VEH-I/R received an equivalent volume of vehicle, 0.5 cc of normal saline, im 12 and 2 hr prior to hepatic ischemia/reperfusion, and SHAM animals underwent sham laparotomy alone. Blood specimens were obtained and analyzed for TNF prelaparotomy and at 30, 60, 90, 120, 150, 180, and 210 min following reperfusion; animals undergoing sham laparotomy had blood sampled at equivalent time points following the conclusion of sham laparotomy. Three hundred-microliter aliquots of blood were removed via the indwelling suprahepatic catheter at each

time point. Intravenous lactated Ringer's solution was administered as blood volume replacement in a ratio of 3 cc of Ringer's solution for each 1 cc of blood lost. Plasma was separated from the samples and stored at 4°C. Of note, separate groups of animals were used to obtain TNF levels and assess lung and liver injury in order to avoid problems with excessive blood loss due to repeated sampling.

TNF Assay [9]

TNF activity in the plasma specimens was measured using a sensitive bioassay with the WEHI 164, subclone 13 cell line as previously described [9]. Briefly, plasma samples were serially diluted directly into 96-well flat-bottom cell culture trays and 100 μ l of WEHI cells (5×10^4 cells) with 0.5 μ g/ml actinomycin D (Calbiochem, LaJolla, CA) was added to each well. The plates were incubated for 20 hr at 37°C. Following the addition of 20 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO) at a concentration of 5 mg/ml in phosphate-buffered saline to each well, the plates were again incubated at 37°C for 4 hr. Next, 150 μ l of medium was removed and 100 μ l of isopropanol with 0.04 N hydrochloric acid was added to each well to dissolve the purple formazan crystals. The plates were read on a Bio-Tek ELISA Reader (Winooski, VT) at an absorbance of 550 nm. Standard curves utilizing human recombinant TNF were generated with each assay; a medium only well served as a negative control. The standard curve was used to calculate TNF values in the experimental samples. TNF values are expressed in pg/ml. The assay was repeated after the positive samples were neutralized with a polyclonal rabbit anti-murine TNF antiserum which cross reacts with rat TNF [10]. A negative assay following this treatment confirmed that the cytotoxic activity was due to TNF.

Assessment of Lung Injury

Lung injury was assessed in this model in two ways. Changes in pulmonary microvascular permeability were evaluated using an 125 I-labeled bovine serum albumin (125 I-BSA) washout technique [11]. Previous studies in our laboratory analyzed the kinetics of lung injury following 90 min of hepatic ischemia with reperfusion and the maximal increase in pulmonary microvascular permeability occurs following 12 hr of hepatic reperfusion [6]; therefore, changes in lung permeability in these experiments were assessed following a 12-hr period of hepatic revascularization. Myeloperoxidase (MPO) activity, as a measure of pulmonary neutrophil influx, was also used to evaluate lung injury in this model. This assay is based on a spectrophotometric reaction with *O*-dianisidine hydrochloride [12]. Once again, previous studies in our laboratory have determined that the time of peak neutrophil influx in this model occurs following

1 hr of hepatic reperfusion [7]; therefore, lung neutrophil influx in these experiments was evaluated after 1 hr of hepatic reperfusion.

The ^{125}I -BSA washout technique was performed as follows [11]: 3 hr prior to sacrifice, 1 ml of ^{125}I -BSA (800,000–1,000,000 cpm) was administered intravenously. At sacrifice, 1 ml of blood was aspirated from the inferior vena cava and the vena cava was transected. The heart and lungs were removed *en bloc* and 10 ml of saline was injected into the spontaneously beating right ventricle in order to remove any remaining blood from the pulmonary vascular bed. The lungs were then excised from the heart and mediastinal structures. Both the lungs and blood sample were weighed and counted in a gamma scintillation counter (Beckman, Fullerton, CA); cpm for each sample were normalized by their respective weights. The mean permeability index (MPI) was calculated by dividing the cpm/g of lung tissue by the cpm/g of blood and represents the degree of extravasation of intravascular ^{125}I -BSA into the extravascular pulmonary compartment.

The lung MPO assay was performed as follows: at the time of sacrifice, 10 ml of saline was injected into the spontaneously beating right ventricle in order to flush the lungs of intravascular pulmonary blood. The left lung was excised and placed in a 50 mM potassium phosphate buffer (pH 6.0) with 5% hexadecyltrimethyl ammonium bromide (Sigma). The pulmonary tissue was homogenized, sonicated, and centrifuged at 12,000g for 15 min at 4°C. The resultant supernatant was assayed for myeloperoxidase activity using a spectrophotometric reaction with *O*-dianisidine hydrochloride (Sigma) at 460 nm [12].

Assessment of Liver Injury

Liver injury was evaluated by measurement of serum SGPT and blood samples for measurement of serum SGPT were obtained following 24 hr of hepatic revascularization. SGPT was quantitated using standard clinical automated analysis. Again, previous studies from our laboratory have demonstrated several peaks in SGPT release following hepatic ischemia/reperfusion; statistically significant increases, compared to sham-operated control animals, occur following 3, 12, and 24 hr of hepatic reperfusion [7]. SGPT levels in these experiments were obtained following 24 hr of hepatic reperfusion.

Statistical Analysis

TNF levels are expressed in pg/ml as the mean \pm the standard error of the mean. Mean permeability index as an indicator of lung injury is expressed as the mean \pm the standard error of the mean; the MPI represents a ratio and therefore has no units associated with it. Lung myeloperoxidase levels are expressed in mean units \pm the standard error of the mean. SGPT levels are ex-

pressed in international units (IU) and are expressed as the mean \pm the standard error of the mean. Statistical analysis was performed using an unpaired Student *t* test unless otherwise stated.

RESULTS

TNF Release Following Hepatic Ischemia/Reperfusion

Peak TNF release occurred between 30 and 150 min of hepatic reperfusion. Sham-operated control animals ($n = 5$) had undetectable TNF levels. Rats subjected to hepatic ischemia/reperfusion alone ($n = 6$) had mean peak TNF levels of 242.24 ± 39.37 pg/ml. Animals receiving vehicle ($n = 6$) prior to hepatic ischemia/reperfusion had a similar mean peak TNF level of 213.64 ± 38.09 pg/ml ($P = \text{NS}$). In contrast, the mean peak TNF level following hepatic ischemia/reperfusion in animals pretreated with deferoxamine ($n = 10$) was 110.38 ± 24.68 pg/ml ($P < 0.01$, as compared to VEH-I/R, Table 1).

Lung Injury

Lung injury following hepatic I/R was evaluated in two ways: changes in pulmonary MPI were measured using an ^{125}I -BSA washout technique and pulmonary neutrophil infiltration was assessed with a spectrophotometric MPO assay. As previously discussed, MPI was measured following 12 hr of hepatic reperfusion and MPO was measured following 1 hr of reperfusion. The MPI in rats undergoing hepatic ischemia/reperfusion alone ($n = 6$) was 0.601 ± 0.102 and was similar to levels seen in previous experiments [6] and was also similar to the MPI in rats pretreated with vehicle prior to ischemia/reperfusion ($n = 6$, MPI = 0.690 ± 0.050 , $P = \text{NS}$ compared to I/R). In contrast, MPI in rats pretreated with deferoxamine ($n = 6$) was significantly decreased and was 0.192 ± 0.013 ($P < 0.005$ compared to VEH-I/R). The MPI in the sham-operated control animals ($n = 6$) was 0.114 ± 0.017 (Fig. 1).

Deferoxamine had a similar significant protective effect on neutrophil influx. Pulmonary MPO in rats undergoing hepatic ischemia/reperfusion alone ($n = 6$) was similar to MPO levels in animals pretreated with vehicle ($n = 5$), being 0.654 ± 0.041 and 0.600 ± 0.060 , respectively ($P = \text{NS}$). MPO levels were significantly decreased following deferoxamine pretreatment ($n = 5$, MPO = 0.318 ± 0.078 ; $P < 0.005$ compared to VEH-I/R). Sham-operated control animal ($n = 5$) MPO levels were 0.213 ± 0.015 (Fig. 2).

Liver Injury

Liver injury following hepatic ischemia/reperfusion in these experiments was evaluated by measuring SGPT levels 24 hr postreperfusion. SGPT in I/R ($n = 6$) was 7205 ± 552 IU and in VEH-I/R ($n = 6$) was 7367 ± 922

TABLE 1

TNF Levels Following Hepatic Ischemia/Reperfusion

Animal	Peak TNF level (pg/ml)	Mean peak TNF level (pg/ml) \pm SEM
I/R-1	352.81	
I/R-2	246.89	
I/R-3	77.39	242.24 \pm 39.37
I/R-4	266.32	
I/R-5	310.60	
I/R-6	199.47	
DES-I/R-1	94.10	
DES-I/R-2	76.37	
DES-I/R-3	75.92	
DES-I/R-4	101.38	
DES-I/R-5	153.20	110.38 \pm 24.68*
DES-I/R-6	295.76	
DES-I/R-7	138.34	
DES-I/R-8	7.87	
DES-I/R-9	119.61	
DES-I/R-10	38.23	
VEH-I/R-1	114.56	
VEH-I/R-2	264.58	
VEH-I/R-3	219.12	213.64 \pm 38.09
VEH-I/R-4	237.30	
VEH-I/R-5	346.41	
VEH-I/R-6	100.01	
SHAM-1	0.00	
SHAM-2	0.00	
SHAM-3	0.00	0.0 \pm 0.0
SHAM-4	0.00	
SHAM-5	0.00	

Note. This table outlines peak TNF levels in rats undergoing hepatic I/R alone, pretreatment with deferoxamine or vehicle prior to hepatic I/R, or sham laparotomy. TNF levels peak between 30 and 150 min of hepatic reperfusion. Mean peak TNF levels \pm standard error of the mean are also listed for each group. There is a statistically significant decrease in mean peak TNF release in animals pretreated with deferoxamine prior to hepatic I/R compared to animals pretreated with vehicle (* $P < 0.01$).

IU ($P = NS$). Again, a significant protective effect was seen in animals receiving deferoxamine prior to hepatic I/R ($n = 9$, SGPT = 1816 ± 528 IU; $P < 0.001$ as compared to VEH-I/R). SGPT in the sham-operated control animals ($n = 6$) was 61 ± 6 IU (Fig. 3).

DISCUSSION

Many investigators have shown that the generation of toxic oxygen species is intimately involved with the organ injury which occurs following ischemia/reperfusion and this has also been demonstrated following ischemia/reperfusion injury to the liver [13-15]. Some controversy exists with respect to the origin of these reactive oxygen species. In many organs, xanthine oxidase is the source for the toxic oxygen radicals [16]; however, this has been

excluded in the liver and other studies have suggested that the mitochondrial respiratory chain [17, 18] or activated leukocytes and Kupffer cells [19, 20] are the source of the oxygen free radicals in the liver. The hepatic parenchymal cells and Kupffer cells appear to differ in their responses to hypoxia and reoxygenation in primary culture. While hepatocytes are sensitive to hypoxia [21], the Kupffer cells appear to be stable under hypoxic conditions, but are particularly vulnerable to reoxygenation injury; there appears to be a self-destruction mechanism related to activation of these cells [22]. Following hypoxia and reoxygenation, Kupffer cells generate a burst of superoxide anion radicals which may be directly related to their destruction *in vitro* [23]. TNF has been demonstrated to prime the Kupffer cells to produce more superoxide anion following challenge with either phorbol 12-myristate 13-acetate or opsonized zymosan [24]. It is important to note, however, that TNF treatment alone does not induce Kupffer cells to produce reactive oxygen species [25], and the effect of TNF on these cells appears to be a sensitizing or priming effect and not a direct stimulus. Conversely, although it is not clear whether oxidative stress alone can induce TNF release, it has been demonstrated that reactive oxygen species can

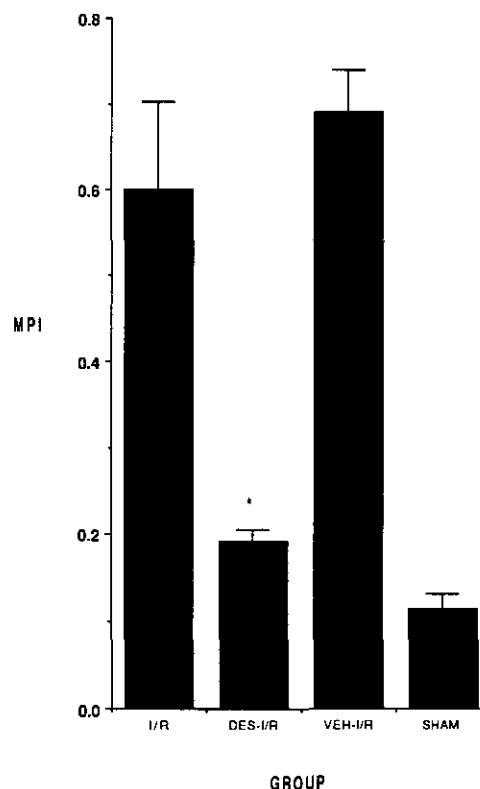


FIG. 1. Pulmonary MPI in rats following 90 min of hepatic ischemia and 12 hr of reperfusion. There is a significant protective effect against the pulmonary microvascular leak in animals pretreated with deferoxamine compared to those animals receiving vehicle prior to hepatic I/R (* $P < 0.005$).

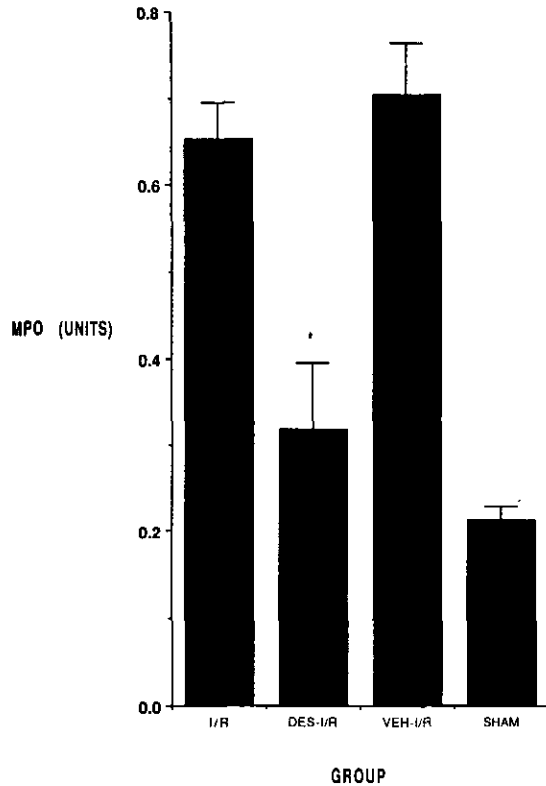


FIG. 2. Pulmonary MPO levels following 90 min of hepatic ischemia and 1 hr of reperfusion. Deferoxamine pretreatment results in a significant decrease in pulmonary neutrophil influx, as measured by pulmonary MPO levels, compared to animals pretreated with vehicle ($*P < 0.005$).

upregulate macrophages to elaborate larger quantities of TNF in response to a second stimulus [26].

Previous studies have demonstrated that TNF is involved in both the pulmonary and hepatic injuries which occur following hepatic ischemia/reperfusion and that pretreatment with anti-TNF antibody affords at least partial protection from both the pulmonary and hepatic injuries which follow reflow [6, 7]. It is important to note that the lung injury in this model is precipitated by the liver injury and prevention of the initiating liver insult would prevent the development of lung injury. The current experiments demonstrate a decrease in hepatic-derived TNF release and a subsequent decrease in both the pulmonary and hepatic injuries following hepatic ischemia/reperfusion in animals pretreated with the iron-chelating agent, deferoxamine. The protective actions of deferoxamine are at least in part attributable to an inhibition of the generation of oxygen free radicals per se; however, the protective actions may also be related to a decrease in TNF release and there may be a synergistic action of both a decrease in TNF release and a decrease in the generation of toxic oxygen species. First, oxygen free radicals appear to have a priming effect on Kupffer cells, i.e., Kupffer cells exposed to oxy-

gen free radicals appear to release more TNF in response to a subsequent challenge, although free radicals in and of themselves do not appear to induce Kupffer cells to produce TNF [26]. In addition, Kupffer cells have been shown to be the major source of reactive oxygen species following hypoxia and reflow [1, 20] and deferoxamine may exert protective actions against the generation of toxic oxygen radicals. In this setting, TNF again primes the Kupffer cell to generate more superoxide anion following ischemia/reperfusion [24]. In addition, although the Kupffer cells appear to be the major source of the extracellular reactive oxygen species formed during early reperfusion [1, 20], there is evidence that polymorphonuclear leukocytes (PMNs) contribute to the generation of free radicals in the later phases of reflow [27]. TNF may be involved in two parts of the subsequent neutrophil-dependent portion of the hepatic injury: first, TNF may be important in the generation of chemotactic agents to recruit neutrophils into the area of injury [28–30]; it may also be responsible for subsequent PMN activation [30–33]. Other data have also demonstrated an accumulation of neutrophils in the lung [10] and in the nonischemic lobes of the liver following hepatic ischemia/reperfusion [20], suggesting an upregulation of the adhesion molecules on circulating PMNs

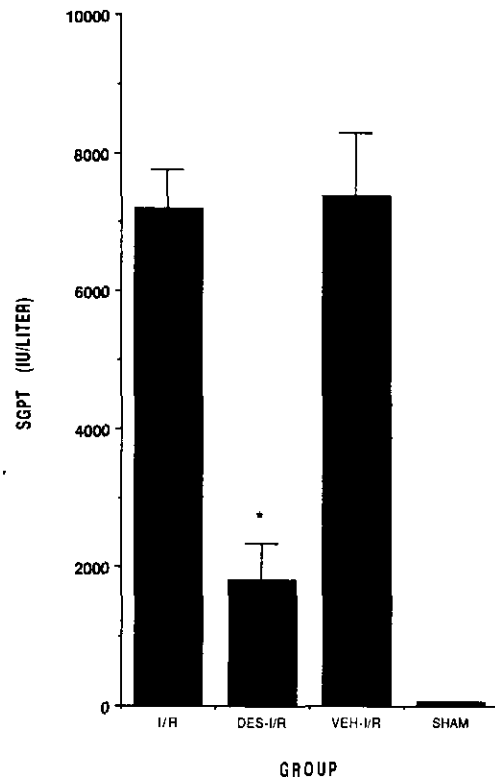


FIG. 3. SGPT levels following 90 min of hepatic ischemia and 24 hr of reperfusion. Again, animals pretreated with deferoxamine are afforded significant protection from liver injury as assessed by SGPT levels as compared to animals pretreated with vehicle ($*P < 0.001$).

leading to an increase in their adherence [34, 35]. Deferoxamine could provide protection in several areas of the neutrophil-mediated portion of this injury: first, it would protect against the oxygen free radicals generated by the activated PMNs, and second, whether by direct or indirect actions, a decrease in TNF release would decrease neutrophil activation and recruitment. Furthermore, the cytotoxic potential of TNF is thought to require oxygen and it appears to involve the production of oxygen-derived free radicals [2, 36]. This is also supported by the fact that susceptibility of various cell types to *in vitro* TNF killing appears to be related to the free radical scavenging ability of the cell [36, 37]. Again, because of the dependence on oxygen-derived free radicals, deferoxamine would again provide protection against the TNF-mediated cellular injury. Thus, deferoxamine may provide protection against the hepatic and pulmonary injuries which follow hepatic ischemia/reperfusion through several mechanisms; it decreases the generation of toxic oxygen species via the chelation of iron. In addition, it appears to decrease the production and release of TNF which also has beneficial effects.

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