Mechanisms of the protective action of diethylthiocarbamate-iron complex on acute cardiac allograft rejection

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ACUTE ALLOGRAFT REJECTION is a complex phenomenon that involves various potential inflammatory mediators arising from the interaction of antigen-presenting cells and lymphocytes. The potential mediators include lymphokines, cytokines, and nitric oxide (NO) derived from inducible NO synthase (iNOS). Increased iNOS activity, protein, or mRNA has been documented before rejection in cardiac allografts but not isografts (3, 32, 52, 55). The role of iNOS in acute cardiac allograft rejection is supported by experimental studies showing that treatment with classical immunosuppressive drugs decreased iNOS mRNA or protein (3, 53, 54). Also, iNOS is upregulated in rejecting allografts in humans despite immunosuppressant therapy (18, 46). Supporting a role of iNOS in acute cardiac allograft rejection, several investigators have used treatments in vivo with nonselective and selective iNOS enzyme inhibitors but with variable findings (2, 5, 30, 43, 51). The importance of iNOS was supported as well by studies showing that gene deletion of iNOS decreased histological rejection scores (11).

An alternative approach is to evaluate agents that limit the action of excess NO rather than inhibit enzyme production of NO. This may be accomplished by the design of therapeutic agents that complex NO with a suitable ligand and remove it from the body. Iron complexes of dithiocarbamate derivatives have been used for therapeutic (6, 14) and diagnostic purposes for counteracting or detecting NO (16, 17, 36), respectively, in experimental models of endotoxic shock. Less information is known about the efficacy of dithiocarbamate-iron complexes in acute organ rejection. Previous studies in our laboratory revealed that a hydrophilic dithiocarbamate derivative enhanced acute cardiac allograft survival (33, 37).

Despite this therapeutic efficacy, other studies using electron paramagnetic resonance (EPR) spectroscopy reveal that hydrophilic derivatives such as iron complexes of N-methyl-d-glucamine dithiocarbamate (MGD-Fe) display chemistry that is different from hydrophobic derivatives such as iron complexes of diethyldithiocarbamate (DETC-Fe) (13, 25). Essentially, these studies suggest that DETC-Fe is more resistant to redox changes that might alter the efficiency of NO scavenging in vivo. On the basis of differences in the partitioning characteristics, DETC-Fe would be expected to act intracellularly and in membrane regions, whereas hydrophilic agents like MGD-Fe would likely act primarily in the extracellular space.

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To date, no studies have examined the actions of lipophilic derivatives in rejection in any model of acute organ rejection. The purpose of the present study was to evaluate the actions of a lipophilic derivative, DETC-Fe, on scavenging of NO in vivo, nitrosylation of heme protein, intragraft infiltration of inflammatory cells, activation of key transcription factors, and gene expression for iNOS and interferon (IFN)-γ in acute cardiac transplant rejection.

MATERIALS AND METHODS

Transplantation model and protocol. Rats weighing ~210–230 g were obtained from Harlan (Indianapolis, IN). The Lewis (Lew: RT1<sup>1</sup>) and Wistar-Furth (WF: RT1<sup>1</sup>) rat strains chosen for donor-to-recipient combinations were Lew → Lew (for isografts) or WF → Lew (for allografts). In rats anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium, heterotopic cardiac transplantation was performed according to techniques established in our laboratory (33, 37). Graft function was monitored twice daily by standard external palpation with acute rejection defined as a loss of palpable contractile activity. Loss of graft function was confirmed on direct inspection after laparotomy.

Experimental groups and biopsy procedures. Studies were terminated at either postoperative day 4, postoperative day 6, or upon the day of rejection. Beginning the day of surgery until the day of tissue harvest or rejection, a subset of allograft recipients received twice daily intraperitoneal injections of 400 mg/kg DETC plus 7.5 mg FeSO<sub>4</sub> including the first dose on the day of tissue harvest or blood samples. This dosage is equivalent to or lower than that routinely used to detect NO in a variety of experimental models of endotoxemia: 0.5 μg of labeled oligonucleotide was desalted and resuspended in Tris-EDTA buffer. DNA binding reactions were performed at room temperature using 12 μg of nuclear extract, 0.5 ng of labeled oligonucleotide, and 3 μg of poly(dI-dC) (Pharmacia-Uppsala; Kalamazoo, MI) and electrophoresed using published procedures (37). Specificity for NF-κB or AP-1 binding activity was verified by competition with 100-fold excess of unlabeled mutant or wild-type oligonucleotides. Antibodies for NF-κB supershift assays including p50, p65, c-Rel, and Rel B were obtained from commercial sources (Santa Cruz Biotechnology; San Diego, CA). Gels were dried on Whatman 3-mm filter paper and exposed to Kodak XAR film (Eastman Kodak; Rochester, NY). Band intensity was determined by phosphorimaging (Molecular Dynamics; Sunnyvale, CA) or by an AlphalImager 2000 image-analysis system (Alpha Innotech; San Leandro, CA).

Western blotting. Frozen tissue was homogenized in ice-cold PBS with 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 35 ng/ml pepstatin A, and 10 ng/ml leupeptin. Homogenates were centrifuged at 10,000 g for 10 min at 4°C. Protein concentration of the supernatant was determined using the Bio-Rad DC protein assay. Fifty micrograms of each sample were precipitated with 12.5% trichloroacetic acid containing 0.5 mg/ml deoxycholate. After the pellet was washed in ice-cold acetone, it was resuspended in SDS-PAGE loading buffer, neutralized with 1 mol/l Tris base, and electrophoresed on 7.5% SDS-polyacrylamide gels (for iNOS) or 12% SDS-PAGE gels (for IkB) using a Bio-Rad Mini-Transphorer system (Gels were dried on Whatman 3-mm filter paper and stored in liquid N<sub>2</sub>. Tissue was stored at −80°C (for Western blotting and gel shift assays). In a few cases, the native heart of the recipient was used as an internal control. For EPR analysis, samples were frozen in 4.0-mm quartz EPR tubes and stored in liquid N<sub>2</sub>. Plasma was obtained for determination of NO by-products, nitrate + nitrite, using a commercial kit (Cayman Chemical; Ann Arbor, MI).

Histological rejection scoring. Tissue from a portion of grafts was fixed at postoperative day 6 in 4% phosphate-buffered formalin, and paraffin-embedded sections were stained with hematoxylin and eosin. Rejection scoring was performed in a blinded manner based on six-point graded criteria established by the International Society for Heart and Lung Transplantation (ISHLT) as described in our previous studies (34).

EPR spectroscopy. Nitrosylation of myocardial heme protein was detected at liquid N<sub>2</sub> temperature using X-band EPR spectroscopy using a liquid N<sub>2</sub> finger dewar in a Varian E-109 spectrometer (Palo Alto, CA). Samples from each group were analyzed on the same day under similar instrument settings consisting of a 1,000-G scan range, 4-min scan time, 0.25-s time constant, 2-G modulation amplitude, 100-kHz modulation frequency, and 5-mW microwave power. The magnetic field was calibrated with Fremy’s salt, giving a g value of 2.0055 ± 0.0001.

Electrophoretic mobility gel shift assay for the nuclear proteins NF-κB and AP-1. Nuclear protein from homogenates of cardiac allografts was extracted as described (37). Double-stranded nuclear factor (NF)-κB or activator protein (AP)-1 oligonucleotides (Promega; Madison, WI) were end labeled with [γ-<sup>32</sup>P]ATP and T4 polynucleotide kinase (Promega) for 10 min at 37°C. After incubation, the labeled oligonucleotide was desalted and resuspended in Tris-EDTA buffer. DNA binding reactions were performed at room temperature using 12 μg of nuclear extract, 0.5 ng of labeled oligonucleotide, and 3 μg of poly(dI-dC) (Pharmacia-Uppsala; Kalamazoo, MI) and electrophoresed using published procedures (37). Specificity for NF-κB or AP-1 binding activity was verified by competition with 100-fold excess of unlabeled mutant or wild-type oligonucleotides. Antibodies for NF-κB supershift assays including p50, p65, c-Rel, and Rel B were obtained from commercial sources (Santa Cruz Biotechnology; San Diego, CA). Gels were dried on Whatman 3-mm filter paper and exposed to Kodak XAR film (Eastman Kodak; Rochester, NY). Band intensity was determined by phosphorimaging (Molecular Dynamics; Sunnyvale, CA) or by an AlphalImager 2000 image-analysis system (Alpha Innotech; San Leandro, CA).

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Gene expression. Total RNA was purified from ~60 μg of frozen tissue per sample using the Promega SV Total RNA Isolation System according to the manufacturer’s directions. RNA concentration was determined spectrophotometrically. cDNA was synthesized from 500 ng of total RNA using oligo-(dT) primer using the Invitrogen Superscript First-Strand Synthesis System for RT-PCR (Carlsbad, CA) according to the manufacturer’s directions. Amplification of iNOS was performed as previously described (15) with modifications. The 1 μl of cDNA was mixed with 25 pмол of each primer and Invitrogen PCR Supermix to a volume of 25 μl, and the reaction was incubated in a Bio-Rad iCycler under the following conditions: for iNOS, 94°C (60 s), 60°C (60 s), and 72°C (60 s) for 30 cycling times; and for IFN-γ, 95°C (30 s), 60°C (30 s), and 72°C (60 s) for 32 cycling times. One micro-liter of the PCR product was resolved on a 1.5% agarose gel in 0.5 × Tris borate-EDTA buffer (pH 8.4, final concentration 44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA) in the presence of SyBr green I (Sigma; St. Louis, MO). The PCR product was visualized by 300-nm ultraviolet transillumination using 5-actin as the control.

Data analysis. EPR spectra were processed using SUMSPEC and Grapher programs (Golden Software; Golden, CO). Data are presented as means ± SE. Statistics were performed by ANOVA for multiple group means or by Student’s t-test for comparisons between two group means. Statistical significance was set at the level of P < 0.05.
RESULTS

Isografts remained functional indefinitely, whereas most allografts were rejected by postoperative day 7. Chronic treatment of allograft recipients with DETC-Fe significantly (P < 0.01) prolonged graft survival (Fig. 1). Histological examination of grafts harvested at postoperative day 6 revealed pronounced cellular infiltration in untreated allografts compared with isograft controls (Fig. 1). ISHLT histological score was decreased by treatment with DETC-Fe at both postoperative day 5 (untreated: 4.25 ± 0.2, n = 8, and DETC-Fe: 3.2 ± 0.5, n = 4; P < 0.03) and postoperative day 6 (Fig. 1).

On the basis of previous studies in our laboratory, we noted a pronounced increase in plasma or urine concentrations of nitrate + nitrite and/or iNOS protein in allografts on postoperative day 4 but not on postoperative day 3. The increase in plasma nitrate + nitrite concentration at postoperative day 4 was decreased in allograft recipients treated chronically with DETC-Fe (Fig. 2).

X-band EPR analysis of isografts (Fig. 3) or native hearts of allograft recipients (data not shown) at postoperative day 6 revealed background signals for reduced iron-sulfur (Fe-S) cluster complexes at g = 2.02 and 1.94 and a signal characteristic of semiquinone at g = 2.004. In contrast, EPR analysis of untreated allografts revealed a strong, broad EPR signal at g = 2.08 and a triplet signal at g = 2.014 with a hyperfine splitting of 17.5 G. This signal is seen infrequently in any allografts before postoperative day 4 and is attributed to the nitrosylation of myocardial heme protein (primarily myoglobin) (2, 33). Treatment with DETC-Fe decreased the amplitude of the nitrosylheme protein signal in allograft tissue (Fig. 3).

To verify in vivo scavenging of NO by DETC-Fe, untreated allograft recipients received a single-pulse intraperitoneal injection of DETC-Fe on postoperative day 6. Plasma nitrate + nitrite concentration was decreased to near-baseline levels 30 min later (isografts: 10.24 ± 1.22 μM, n = 9; allografts: 31.49 ± 2.99 μM, n = 9; DETC-Fe

Fig. 1. Daily treatment with diethyldithiocarbamate-iron complex (DETC-Fe) prolongs cardiac allograft (Allo) survival (top: untreated, n = 9; DETC-Fe, n = 6) and decreases International Society for Heart and Lung Transplantation (ISHLT) rejection scores measured at postoperative day (POD) 6 (bottom: untreated, n = 9; DETC-Fe, n = 4). ‡P < 0.01 vs. untreated.

Fig. 2. Chronic treatment with DETC-Fe (n = 4) decreases plasma NO by-products (nitrate + nitrite) obtained from Allo recipients at postoperative day 4 compared with isografts (Iso, n = 6) or untreated Allo (n = 9). ‡P < 0.01 vs. Iso or treated Allo.

Fig. 3. Examples of spectra obtained from an Iso (top spectra) vs. individual Allo measured by X-band electron paramagnetic resonance (EPR) spectroscopy. Baseline signals in the Iso are for Fe-S cluster protein at g = 2.02 and g = 1.94 (●) and semiquinone radical at g = 2.004 (○). Examples show decreased nitrosylation of myocardial heme protein (g = 2.08 and 2.014) in Allo recipients treated with DETC-Fe. Each spectra was obtained on the same day with identical instrument settings.

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pulse: 13.98 ± 0.54 μM, n = 2). EPR analysis of DETC-Fe-pulsed isograft controls revealed a four-line EPR signal with g = 2.025 (Fig. 4). In these spectra, the background signal at g = 1.94, representing reduced Fe-S cluster protein, is also apparent. The four-line EPR signal has been described in normal brain tissue after administration of DETC-Fe to represent the g signal for the copper (II) complex of DETC (44). In contrast to this four-line EPR signal in isografts, a new signal was clearly distinguished in DETC-Fe-pulsed allografts that was superimposed between the composite nitrosylheme signal (i.e., g = 2.08 and the triplet at g = 2.014). The superimposed signal in allograft recipients was assigned to an anisotropic triplet signal with resonance positions of g factors of $g_\parallel = 2.038$ and $g_\perp = 2.02$ representing the mononitrosyl iron complex of DETC (denoted as DETC-Fe-NO). This signal is similar to that detected by EPR analysis after the addition of NO solutions containing DETC-Fe or in cells (27). To document the source of production of this signal, we observed that the DETC-Fe-NO adduct was inhibited by a single intravenous injection of 5 mg/kg L-(1-iminoethyl)lysine, an iNOS inhibitor (4), 30 min before the pulse injection with DETC-Fe (Fig. 5).

NF-κB binding activity was detected by electrophoretic mobility shift assay (EMSA). Supershift analysis of untreated allograft samples revealed that dimers were shifted by incubation with antibodies to p50 and p65 but not to Rel B or cRel (Fig. 6). Specificity for NF-κB binding in this assay was verified by the abolition of the band with excess cold wild-type (Fig. 6, lane 2) but not mutant (Fig. 6, lane 1) oligonucleotide.

![Fig. 4](image-url)  
**Fig. 4.** Direct evidence showing in vivo trapping of nitric oxide (NO) by DETC-Fe within cardiac Allo at postoperative day 6 based on formation of a DETC-Fe-NO adduct EPR signal at g = 2.038 superimposed on the broad nitrosylated heme protein signal.

![Fig. 5](image-url)  
**Fig. 5.** Lack of DETC-Fe-NO adduct formation after a single pulse injection of DETC-Fe on postoperative day 6 in Allo pretreated with the inducible NO synthase (iNOS) inhibitor L-(1-iminoethyl)lysine (L-NIL) vs. DETC-Fe without L-NIL.

![Fig. 6](image-url)  
**Fig. 6.** Supershift electrophoretic mobility shift assay (EMSA) showing nuclear factor (NF)-κB binding activity of nuclear extract of untreated Allo (lane 3) at postoperative day 4. Specificity is shown by elimination of band by incubation in the presence of 100-fold excess wild-type (WT; lane 2) but not mutant (Mut; lane 1) oligonucleotide. The NF-κB specific activity was shifted by incubation with antibody to p50 and p65 subunit proteins (lanes 4 and 5) but not by Rel B or cRel (lanes 6 and 7).
NF-κB binding activity was increased in nuclear extracts derived from untreated allografts versus isograft controls (Fig. 7). NF-κB binding activity was normalized in nuclear extracts isolated from allograft recipients treated with DETC-Fe (Fig. 7). Western blot analysis indicates that treatment with DETC-Fe increased IκBα protein levels in allografts versus untreated allografts (Fig. 8). In addition to activation of NF-κB, allotransplantation was associated with activation of the transcription factor AP-1 (Fig. 9). Specificity for AP-1 binding was verified by elimination of the band in the presence of excess cold wild-type (Fig. 9, lane 2) but not NF-κB consensus oligonucleotide (Fig. 9, lane 1). Chronic treatment of recipients with DETC-Fe inhibited AP-1 activation due to allogeneic transplantation.

Western blot analysis of allografts at postoperative day 4 revealed significant upregulation in iNOS protein in untreated allografts compared with isograft controls (Fig. 10). Previous studies have shown that iNOS protein increases at postoperative day 4 and remains at this plateau up to postoperative day 6 (unpublished observations). No increase in iNOS protein was found in the native heart of allograft recipients (data not shown). This upregulation was nearly blocked or decreased in each recipient treated with DETC-Fe. RT-PCR revealed increased gene expression of iNOS and the cytokine IFN-γ in allografts compared with isografts (Fig. 11). Gene expression for both iNOS and IFN-γ was either decreased or absent in allograft recipients treated with DETC-Fe.

**DISCUSSION**

The findings of the present study support the hypothesis that excessive production of NO via the upregulation of iNOS plays a significant role in acute cardiac allograft rejection. Treatment with DETC-Fe complex was shown to inhibit heme protein nitrosylation, decrease peripheral NO by-products, and enhance graft survival. The significance of these findings is that the degree of enhancement in graft survival was similar in magnitude to that observed in our laboratory using low-dose cyclosporine (33, 37). Enhancement of graft survival is roughly equivalent in magnitude to that seen using specific or nonspecific NOS inhibitors (43, 51, 53), although some have reported either no benefit or detrimental effects of these agents on graft survival (2, 30). The enhanced survival by DETC-Fe is consistent with our findings of improved graft survival using either a ruthenium-based NO scavenger (34) or a water-soluble, dithiocarbamate-based iron complex (33, 37), although the potential mechanisms of action of
these other water-soluble derivatives have not been examined in detail previously.

DETC-Fe was concluded to cause this benefit, in part, via NO scavenging. In this context, the rate constant of reaction of NO with iron-dithiocarbamate compounds in solution has been estimated to be $1.1 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ (29). Consistent with this property, we showed that chronic treatment with DETC-Fe decreased plasma nitrate + nitrite concentration. Our subsequent finding that chronic treatment with DETC-Fe also decreased iNOS protein levels can partially explain the decreased plasma NO metabolites. To negate the action on iNOS protein levels, we performed additional studies to document the in vivo scavenging properties of DETC-Fe. This was accomplished by giving a single pulse injection of DETC-Fe in untreated allograft recipients followed by tissue and plasma harvesting after 30 min. In this strategy, DETC-Fe normalized plasma NO metabolites, providing indirect evidence of intrinsic activity to scavenge NO in vivo. In addition, EPR analysis provided direct evidence of the formation of DETC-Fe-NO adduct after pulse injection with DETC-Fe. The detection of DETC-Fe-NO adduct in allograft tissue is facilitated by the superior cell-penetrating property of DETC-Fe compared with more hydrophilic derivatives, such as MGD-Fe. In the present study, we provided unequivocal evidence that DETC-Fe scavenges NO under in vivo conditions. Furthermore, we showed that the NO formation is specific to allograft tissue. This technique cannot distinguish whether the NO trapped is derived from cardiac muscle or from trapped infiltrating cells. The amount contributed from the latter is expected to be limited because we noted that the amount of iNOS-containing ED1$^+$-positive macrophage cells peaks at postoperative day 2 and diminishes to baseline by postoperative day 4 (unpublished findings). On the basis of our knowledge of the amount of iNOS protein and peripheral NO metabolite levels at the early time period, detection of DETC-Fe-NO adducts by EPR would be impractical due to sensitivity considerations. This does not preclude the possibility that DETC-Fe may act at earlier time periods.
Previously, the detection of iron-nitrosyl complexes ex vivo in isolated perfused rat hearts after administration of iron-dithiocarbamates such as MGD-Fe or DETC-Fe to ischemic myocardium has been attributed to trapping of NO derived from NOS as the mononitrosyl iron adduct was inhibited by the nonselective NOS inhibitor Nω-nitro-L-arginine methyl ester (13, 57). In contrast, under certain conditions, detection of NO by such trapping agents may be independent of NO derived from NOS due to nonenzymatic conversion of nitrite to NO (56). We found that the formation of DETC-Fe-NO adduct within allograft tissue was blocked by prior treatment of recipients with L-(1-iminoethyl)lysine, a selective iNOS inhibitor. Thus we conclude that detection of DETC-Fe-NO within allograft tissue arises from NO produced enzymatically from iNOS rather than from nonenzymatic sources.

We also evaluated the effects of treatment with DETC-Fe on intragraft infiltration of inflammatory cells. Previous studies using a variety of NOS inhibitors in acute cardiac allograft rejection have yielded varying findings. For example, Nω-monomethyl-L-arginine, a nonselective NOS inhibitor that enhanced survival, decreased intragraft infiltration of inflammatory cells (51). Also, treatment with the iNOS inhibitor aminoguanidine decreased cell infiltration (52, 53). In the present study, we observed that treatment with DETC-Fe inhibited inflammatory cell infiltration. Our findings are consistent with the findings of decreased cell infiltration with another water-soluble dithiocarbamate derivative used in experimental hemorrhagic shock (23). Thus the benefits of the lipophilic derivative DETC-Fe on acute cardiac allograft rejection may be related, in part, to inhibiting intragraft infiltration of inflammatory cells. This anti-inflammatory property may not be unique to DETC-Fe as our laboratory recently observed a reduction in histological rejection scores indicative of decreased cell infiltration by chronic treatment with a ruthenium-based class of NO scavenger (34). The nature of this effect is incompletely understood but may relate to actions on alloimmune activation, including cytokine gene expression, that may be unrelated to NO scavenging (see below). In this context, diethyldithiocarbamate derivatives have been shown to possess potent immunosuppressive activity on macrophage or T-lymphocyte function/activation under in vitro conditions (9, 35) but have yet to be documented in vivo. Thus it is possible that DETC-Fe had action in vivo to directly inhibit inflammatory cell activation as a potential mechanism to limit recruitment and infiltration.

T-cell activation and production of inflammatory mediators is believed to be mediated, in part, by activation of the transcription factor NF-κB (1). The inactive NF-κB transcription factor exists as a cytosolic protein consisting of a trimeric structure of NF-κB dimers bound to the inhibitory protein IκBα. Phosphorylation of IκBα results in dissociation of the NF-κB dimer subunits usually consisting of homodimers or heterodimers of p50 and p65 subunits. The dissociated dimers translocate to the nuclear compartment. These dimers bind to sites in the promoter regions of genes that are important in the development of inflammatory diseases. NF-κB dimers may bind to a plethora of promoters for a variety of gene products that are believed to be implicated in allograft rejection, including cell adhesion molecules, major histocompatible antigen-binding complexes, iNOS, and various inflammatory cytokines.

In the present study, we found that treatment with DETC-Fe normalized activation of myocardial NF-κB. The inhibition of NF-κB activation by DETC-Fe is more pronounced than the modest or partial inhibition previously shown by our laboratory for water-soluble iron chelators such as pyrrolidine dithiocarbamate (PDTC) (5) and a water-soluble dithiocarbamate-based derivative (33). This enhanced activity of DETC-Fe may be related, in part, to more efficient scavenging of NO by DETC-Fe based on the data of the relative decrease in NO by-products and inhibition of heme nitrosylation compared with these other derivatives. It is possible that this is related, in part, to the higher lipophilicity of DETC-Fe. Indeed, metal complexes of DETC are six orders of magnitude more lipophilic than similar complexes of MGD (12). Furthermore, chelation of NO by DETC-Fe in lipid fractions may be facilitated by the estimated ninefold increased sequestration of NO in lipid compartments (20). It is also possible that DETC-Fe might act via other mechanisms independent of NO scavenging such as an antioxidant mechanism. Indeed, free and iron-nitrosylated dithiocarbamate derivatives can exhibit antioxidant potential (19, 21, 48). This action may account for the inhibition of activation of redox-sensitive transcription factors as discussed below.

The decrease in NF-κB binding activity by treatment with DETC-Fe is potentially explained, in part, by upregulation of total content of IκBα protein levels. Indeed, overexpression of IκBα protein is associated with decreased activation of NF-κB and decreased NF-κB-dependent gene expression (7). Furthermore, increased myocardial IκBα protein is associated with an increased tolerance to endotoxin (40). Reassociation of NF-κB dimers with the increased levels of IκBα inhibitory protein in allograft recipients treated with DETC-Fe may serve as a negative control to limit NF-κB activation and downstream effects.

The inhibition of activation of NF-κB is likely to counteract rejection as NF-κB is known to participate in T-lymphocyte activation (26). Furthermore, the importance of NF-κB was evidenced in studies showing that NF-κB decay oligodeoxynucleotides limited cell infiltration in renal allografts (49) and enhanced cardiac allograft survival (8, 45). Our findings that DETC-Fe prevented NF-κB activation, decreased cell infiltration, and prolonged graft survival are consistent with a link in NF-κB activation and acute cardiac allograft rejection.

We also evaluated the activation of another redox-sensitive transcription factor, AP-1 (39). This transcription factor consists of dimers of Jun and Fos family proteins. DNA binding of these dimers target
expression of AP-1-dependent genes. The role of AP-1 activation and its regulation by treatment regimens have not been previously examined in acute cardiac allograft rejection, although AP-1 activation is believed to regulate cell proliferation (39) and AP-1 sites positively regulate transcription of iNOS (22). We found that AP-1 activation occurred in untreated allografts but not in isografts or native hearts of allograft recipients. This indicates that AP-1 activation is specific to alloimmune activation and not related to surgery per se.

The actions of dithiocarbamates on activation of AP-1 in vitro are complex and incompletely understood as both activation (24, 38) and inhibition (31) have been reported in other experimental models using the iron chelator derivative PDTC. In our in vivo study, AP-1 activation was prevented by chronic treatment with DETC-Fe. As there are binding sites for both NF-κB and AP-1 in the iNOS promoter (22), the profound action of DETC-Fe to inhibit activation of both AP-1 and NF-κB may provide a concerted dual mechanism to limit inflammatory cell infiltration and inflammatory gene expression.

Accordingly, to examine the potential action of limiting activation of redox-sensitive transcription factors on the downstream effects on gene expression, we examined both iNOS protein and mRNA in allograft recipients treated with DETC-Fe. Previously, the iron chelator PDTC has been shown to decrease iNOS protein and expression in vitro in lipopolysaccharide- or cytokine-stimulated macrophage cells (10, 28). Actions of dithiocarbamate-related analogs in vivo on iNOS have not been examined to date in allograft rejection. Consistent with a decrease in activation of NF-κB and AP-1 by DETC-Fe, we found that iNOS protein and gene expression were decreased. To our knowledge, this is the first in vivo study to show that DETC-Fe is able to decrease iNOS protein in any model of autoimmune or inflammatory disease.

IFN-γ is released from activated T cells. This cytokine enhances alloantigen recognition and trafficking of CD4+ T lymphocytes, and it is a potent stimulant of macrophage-derived NO production. Thus its expression may indicate an important marker of early rejection. We also found that treatment with DETC-Fe produced a marked decrease in IFN-γ expression. To our knowledge, this is the first report to show that this class of agents limits expression of this potent proinflammatory cytokine in acute organ rejection in vivo. The promoter region for the IFN-γ gene contains NF-κB binding domains (41). Thus a reduction in iNOS protein by DETC-Fe may arise secondarily to the reduction in NF-κB-dependent IFN-γ gene expression. A decrease in IFN-γ gene expression may indicate that DETC-Fe also acts on early stages of rejection.

It is important to note that the ability of DETC-Fe to decrease iNOS protein and mRNA distinguishes it from the known action of NOS inhibitors that do not alter iNOS protein levels (42). For example, we noted that an NOS inhibitor does not alter NF-κB binding activity (unpublished observations). These observations suggest that NO may not be the molecule that causes activation of NF-κB. Nevertheless, the actions on activation of transcription factors important for iNOS and IFN-γ suggest that DETC-Fe has action at early stages of rejection. These findings raise the hypotheses that agents such as DETC-Fe might act differently from or are superior to NOS inhibitors in limiting the upstream and downstream actions of iNOS and may alter the expression of other unknown mediators of rejection that might be mediated via NF-κB-dependent pathways.

In summary, we conclude that treatment with DETC-Fe prolongs acute cardiac allograft survival by multiple mechanisms. DETC-Fe appears to act at upstream and downstream actions of mediators of rejection. These mechanisms include NO scavenging, decreased intragraft cell infiltration, inhibited activation of transcription factors (notably NF-κB and AP-1), and limited gene expression for iNOS and IFN-γ. Thus DETC-Fe possesses significant immunosuppressive and anti-inflammatory activities.

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