Brief Definitive Report

Passive Immunization of Mice against D Factor Blocks Lethality and Cytokine Release during Endotoxemia

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Summary

D factor, also known as leukemia inhibitory factor, is a pleiotropic cytokine whose role during acute injury and inflammation is not known. Intraperitoneal administration of *Escherichia coli* endotoxin induced D factor gene expression in mice, and passive immunization against D factor protected them from the lethal effects of endotoxin and blocked endotoxin-induced increases in serum levels of interleukin 1 and 6. Peak levels of tumor necrosis factor and interferon γ were not affected. These results indicate that D factor is an essential early mediator of the inflammatory cytokine response and therefore may be important in the pathogenesis of the many inflammatory conditions, such as sepsis, arthritis, allograft rejection, and cancer immunotherapy.

Differentiation-inducing factor/leukemia inhibitory factor (D factor/LIF) is a recently identified cytokine with an impressive array of seemingly unrelated activities (1, 2). First described because of its ability to induce differentiation of murine M1 leukemia cells (3, 4), D factor has been independently identified under several synonyms: leukemia inhibitory factor (LIF), differentiation-inhibiting factor (D factor), osteoclast-activating factor (OAF), human interleukin for DA-1 cells (HILDA), cholinergic neuronal differentiation factor (CNDF), hepatocyte-stimulating factor (HSF-II and III), and melanocyte-derived lipoprotein lipase inhibitor (MLPLI), and is now recognized to have pleiotropic actions in regulating metabolism, growth, and differentiation. Although diverse effects of D factor have been identified in vitro (5–11) and in vivo (12–16), little is known about its role during acute and chronic inflammation. It can be produced by a variety of cells, including fibroblasts (3, 17), monocytes, macrophages (18), and T lymphocytes (19), and its synthesis can be induced by TNF, IL-1, and endotoxin (LPS) (17, 20, 21). In addition, elevated levels of D factor have been identified in the body fluids of patients with a variety of acute and chronic inflammatory conditions, such as septic shock, rheumatoid arthritis, renal allograft rejection, and cancer (20, 22, 23).

These findings suggest that D factor participates in the regulation of immune system function and is a mediator of inflammation. However, pretreatment of rodents with D factor appears to have antiinflammatory consequences, protecting them against a lethal dose of LPS (24) and enhancing host resistance against radiation injury (25) and oxygen toxicity (26). Exogenous TNF (27, 28) and IL-1 (29) similarly protect rodents against subsequent lethal endotoxemia, yet when these cytokines are produced in response to LPS they mediate toxicity. Therefore, although exogenous D factor is protective, endogenous D factor produced during an inflammatory response, analogous to endogenous TNF and IL-1, may contribute to toxicity.

We hypothesized D factor is produced as part of the host response to an immune system stimulus and participates in the network of cytokine interactions activated during acute injury and inflammation. To test this hypothesis we conducted experiments using endotoxemia in adult mice as a model of sepsis and investigated: (*a*) tissue D factor gene expression before and after intraperitoneal administration of LPS; (*b*) the effect of passive immunization against D factor on survival after a lethal dose of LPS; and (*c*) the effect of passive immunization against D factor on levels of circulating TNF, IL-1, IL-6, and IFN-γ after LPS administration.

Materials and Methods

Animals. Female C57Bl/6 mice weighing 19–21 g were group housed five to six per cage in the animal care facility of the National Institutes of Health (NIH; Bethesda, MD), with access to food and water ad libitum. All experiments were conducted in compliance with the guidelines of the Animal Care and Use Committee of the NIH.

Reagents. Recombinant murine D factor was a gift of Genentech, Inc. (South San Francisco, CA). Rabbit nonimmune IgG (IgG) was purchased from ICN Immunobiologicals (Costa Mesa, CA). Endotoxin (*Escherichia coli* LPS, serotype 0127:B8) was purchased from Sigma Chemical Co. (St. Louis, MO).

Total RNA Preparation. Mice were injected intraperitoneally with LPS (35 mg/kg body weight). Livers, lungs, and spleens were harvested from two mice at each of the time points indicated and snap frozen in liquid nitrogen. Tissues were then pulverized, homogenized in guanidinium thiocyanate buffer, and total RNA was isolated by phenol/chloroform extraction. RNA was also isolated from cultured RAW 264.7 cells (American Type Culture Collection, Rockville, MD) before and 6 h after stimulation with LPS (1 μg/ml).

Reverse Transcriptase (RT) PCR. Primers specifying a 601-bp fragment of murine D factor cDNA for amplification were synthesized (392 DNA/RNA Synthesizer; Applied Biosystems, Inc., Foster City, CA) based on the published sequence of murine D factor (GenBank no. M63419) and corresponded to sequences from exon 1 (sense primer: 5'-GGAGTCCAGCCCATAATGAAGGTCTTGGC-3') and exon 3 (antisense primer: 5'-TGACTTGCTTGTATGTCCCCAGAAGCTGG-3').

Total RNA (0.5 μg) from each sample was reverse transcribed using the antisense primer (1.0 μM) and the entire product (10 μl) amplified in a final volume of 50 μl PCR buffer for 38 cycles (95°C for 40 s, 70°C for 30 s). A 30% portion of the reaction products (15 μl) was analyzed by electrophoresis on an ethidium bromide–stained 1.5% agarose gel. Visualized bands were of expected size (601 bp) and identity was confirmed by restriction endonuclease digestion with MboI and SmaI (Bethesda Research Laboratories, Gaithersburg, MD). Reagents for RT-PCR were purchased from Perkin Elmer Cetus (GeneAmp® RNA PCR kit; Norwalk, CT).

D Factor Antibody. Using a protocol that has been previously described (30), we generated polyclonal antibodies against murine D factor (DF Ab) by inoculating New Zealand White rabbits with recombinant murine D factor. Immune serum was collected at intervals after boosting and the IgG fraction was purified over a protein G column (Clinetics Corp., La Jolla, CA). Endotoxin content of this preparation was <0.1 ng/mg protein (limulus amebocyte lysate assay; Whittaker Bioproducts, Walkersville, MD). DF Ab and IgG were brought to a final concentration in sterile saline and administered intraperitoneally.

Western Blot. Murine cytokines (25 ng each: D factor and TNF-α [Genentech, Inc., South San Francisco, CA]; IL-1α, IL-1β, and IL-6 [R & D Systems, Minneapolis, MN]; IFN-γ [Amgen Biologicals, Thousand Oaks, CA]) and LPS were separated on a 14% polyacrylamide gel (Novex, San Diego, CA) under reducing conditions. Proteins were transferred to a nitrocellulose membrane and incubated with DF Ab (31.5 μg/ml final concentration), then with horseradish peroxidase–conjugated affinity-purified goat anti-rabbit IgG (Bio-Rad Laboratories, Richmond, CA), diluted 1:2,000. Blots were developed using an enhanced chemiluminescence procedure (Amersham Corp., Arlington Heights, IL) and exposed to photographic film for ~3 min.

M1 Assay. M1-T22 murine leukemia cells (gift of Genentech, Inc.) were added to wells of a 96-well plate (10^4 cells/well) in 100 μl of culture medium (Eagle's MEM with 10% heat-inactivated FCS, 1× L-glutamine, 1× sodium pyruvate, 2× nonessential amino acids, vitamins, penicillin, streptomycin, and gentamicin sulfate [Biofluids, Inc., Rockville, MD]). DF Ab was diluted in culture medium and 90 μl was added to each well to give final concentrations of 0.01, 0.1, 1.0, 10.0, and 50.0 ng/ml. After 15 min, D factor was added in 10 μl of medium to give final concentrations of 1.0 or 10.0 ng/ml. Control wells received equivalent volumes of medium alone. Cells were incubated at 37°C, 5% CO_2 for 68 h, followed by the addition of 1 μCi [^{3}H]thymidine per well (NEN Research Products, DuPont Co., Boston, MA). After 4 h, cells were harvested with a cell harvester (1295–001; LKB Instruments, Gaithersburg, MD) and [^{3}H]thymidine incorporation was measured on a beta plate liquid scintillation counter (1205; LKB Instruments).

Effect of D Factor Antibody on Survival after LPS. Mice were treated with 1 mg of either DF Ab or IgG (reconstituted to 2 mg/ml in sterile saline and 0.5 ml administered intraperitoneally) 6 h before, 30 min after, or 1 h after administration of LPS (reconstituted to 3.5 mg/ml in sterile saline and 35 mg/kg body weight administered intraperitoneally). Survival was recorded at regular intervals, and statistical significance was assessed by the Breslow modification of Kruskal-Wallis test. After 4 d, surviving animals were normally active and appeared fully recovered. All mice were ear-tagged and randomized, and reagents were administered in a blinded fashion.

Effect of D Factor Antibody on Circulating Cytokine Levels after LPS. LPS was reconstituted to 0.2 mg/ml in sterile saline and given by intravenous tail vein injection (2 mg/kg body weight) 6 h after a 1-mg intraperitoneal dose of either DF Ab or nonimmune IgG. Blood was collected from naive (time = 0) and endotoxemic mice (1, 3, and 6 h after intravenous LPS) by retroorbital puncture and allowed to clot at 0–4°C. Samples were centrifuged for 15 min at 4°C and the supernatant (serum) was collected. Determinations of cytokine levels were made by ELISA: murine (mu)TNF-α, muIL-1α, and muIFN-γ (Genzyme, Boston, MA), and muIL-6 (Endogen Inc., Boston, MA).

Results and Discussion

Comparison of product yields from RT-PCR analyses of RNA from resting and LPS-stimulated RAW 264.7 cells shows the ability of RT-PCR to detect D factor gene induction (Fig. 1). Similar analysis of RNA isolated from tissues of naive and endotoxemic mice shows that D factor gene expression was induced in both liver and spleen tissue within 1 h of intraperitoneal administration of LPS. D factor mRNA was detected in the lung tissue of naive mice, indicating some constitutive expression. Consistent with the findings of others (1, 2), Northern blot analyses were insufficiently sensitive to detect D factor mRNA in total RNA and mRNA preparations derived from tissues (data not shown). This suggests that D factor transcripts were present either in low abundance in all parenchymal cells, in only a small population of parenchymal cells, or in circulating cells trapped in the organ at the time of death.

Despite the ability to detect induction of D factor gene expression during endotoxemia, synthesis of the protein has been difficult to confirm. We have used both Western blot and ELISAs to detect LPS-induced D factor production by RAW 264.7 cells in vitro (M. I. Block, K. G. Billingsley, and H. R. Alexander, unpublished observations). However, using these techniques, we have been unable to detect D factor in the serum of endotoxemic mice. The more sensitive but

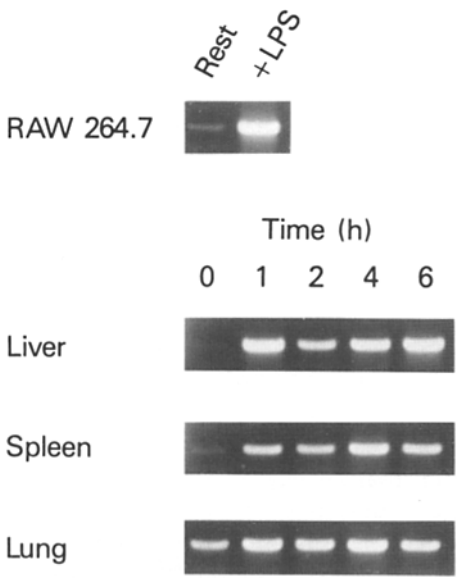

Figure 1. Induction of D factor gene expression during endotoxemia in mice. Comparison of product yields shows the ability of RT-PCR to detect D factor gene induction in RAW 264.7 cells exposed to LPS (*top*). Similar analysis of RNA isolated from naive (time = 0) and endotoxemic mice (time after LPS indicated) shows induction of gene expression in both the liver and spleen during endotoxemia; constitutive expression was detected in the lung.

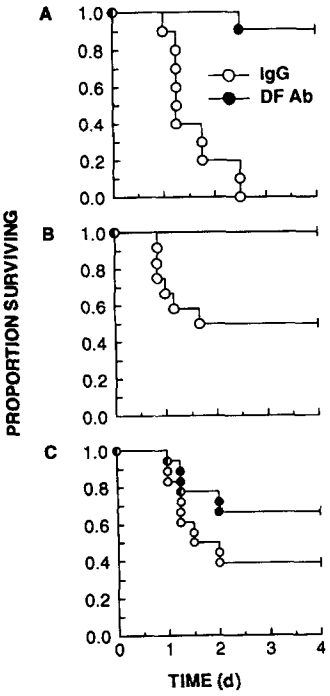

Figure 3. Passive immunization against D factor protects mice from the lethal effects of endotoxemia. Kaplan-Meier survival curves for endotoxemic mice treated with either DF Ab or control nonimmune rabbit IgG. (*A*) Treatment with either DF Ab (n = 11) or IgG (n = 10) 6 h before LPS; $p < 0.001$. (*B*) DF Ab (n = 11) or IgG (n = 12) was given 30 min after LPS; p = 0.008. Control mice treated after LPS (*B*) had improved survival compared with control mice treated before LPS (*A*), consistent with a resuscitative benefit from the fluid bolus alone. (*C*) DF Ab (n = 18) or IgG (n = 18) was given 1 h after LPS; p = 0.09.

potentially less specific bioassay was used by Metcalf (31), who measured circulating D factor in the same model and detected peak levels occurring 2 h after intravenous LPS. These findings suggest that circulating levels are either episodic or remain low during endotoxemia, and that D factor may act primarily at the tissue level.

The observation that D factor gene transcription can be induced by administration of LPS supports the hypothesis that D factor plays an important role in the host response to an inflammatory stimulus. To better understand this role, antibodies against murine D factor (DF Ab) were generated in rabbits and characterized for use in vivo. Sensitivity and specificity were tested by Western blot analyses (Fig. 2, *A* and *B*), which demonstrated that DF Ab was capable of detecting as little as 10 ng of recombinant murine D factor (Fig. 2 *A*), and did not bind to other inflammatory murine cytokines or LPS (Fig. 2 *B*). The neutralizing ability of DF Ab was demonstrated by dose-dependent inhibition of the antiproliferative effects of D factor on M1 cells (Fig. 2 *C*). In the absence of DF Ab, increasing doses of D factor promoted the differentiation of M1 cells and decreased [^{3}H]thymidine incorporation. Increased concentrations of DF Ab progressively inhibited D factor–induced M1 cell differentiation and restored proliferation to control levels. DF Ab alone had no detectable effect on M1 cell differentiation.

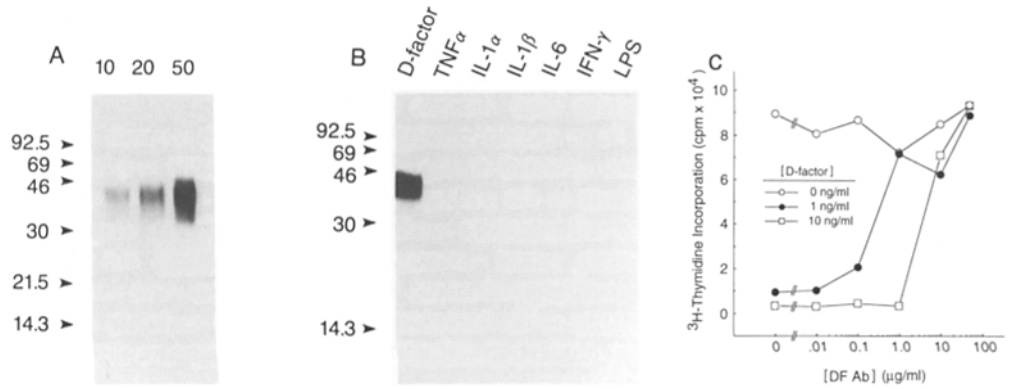

Figure 2. Sensitivity, specificity, and neutralizing ability of DF Ab. (*A*) Western blot of 10, 20, and 50 ng of recombinant murine D factor shows sensitivity of DF Ab. (*B*) Western blot showing specific binding of DF Ab to D factor, but not to TNF-α, IL-1α, IL-1β, IL-6, IFN-γ, or LPS. Positions of molecular weight markers are shown. (*C*) Proliferating M1 cells were treated with varying amounts of D factor and DF Ab in the presence of [^{3}H]thymidine. Increasing factor activity promoted differentiation and reduced [^{3}H]thymidine incorporation. Neutralization of D factor activity by DF Ab restored M1 cell proliferation to control levels.

Passive immunization of mice with DF Ab protected them from the subsequent effects of lethal endotoxemia and identified an optimum antibody dose of 1 mg per mouse (data not shown). In subsequent studies, mortality among mice given DF Ab 6 h before (Fig. 3 *A*) and 2 h before (data not shown) intraperitoneal LPS was significantly lower than mortality among control mice. Use of a different, affinity-purified rabbit anti-murine D factor antibody (gift of Genentech) achieved similar results (data not shown). DF Ab also improved survival when given 30 min after LPS (Fig. 3 *B*); this therapeutic effect is similar to that seen during endotoxemia with administration of the IL-1 receptor antagonist (IL-1ra) (32), an agent currently being evaluated in clinical trials for the treatment of septicemia. Although the survival difference was not statistically significant when DF Ab was administered 1 h after LPS (Fig. 3 *C*), multiple repeated experiments demonstrated a small but consistent improvement. These results indicate that D factor produced by the host in response to LPS is necessary for the lethal consequences of endotoxemia.

Critical early mediators of systemic toxicity during endotoxemia, such as TNF and IL-1, are released into the serum quickly and can reproduce the manifestations of endotoxic shock when administered alone or in combination. Administration of D factor has not been shown to reproduce the acute toxicity associated with these cytokines. Although D factor alone may not induce a fulminant inflammatory response, the improvement in survival with passive immunization against D factor suggests that D factor may be a necessary component in the network of cytokine interactions activated during endotoxemia. To assess the role of D factor in modulating the cytokine response to LPS, we measured circulating levels of TNF, IL-1, IL-6, and IFN-γ during endotoxemia in passively immunized (DF Ab) and control (nonimmune IgG) mice after intravenous LPS (Fig. 4). In repeated experiments, no deaths were seen among mice pretreated with DF Ab, whereas the mortality rate of control mice was consistently near 30% (data not shown). In DF Ab–pretreated mice the initial peak in serum levels of TNF was unaffected, although the return to baseline was more rapid compared with controls (Fig. 4 *A*). In contrast, pretreatment with DF Ab significantly attenuated the increase in circulating levels of IL-1 and IL-6 seen after LPS administration (Figs. 4, *B* and *C*, respectively). Serum levels of IFN-γ increased later and were unaffected by blocking D factor activity (Fig. 4 *D*). These results indicate that D factor is an early mediator of cytokine release in response to LPS, necessary for IL-1 and IL-6, but not IFN-γ, secretion. D factor does not appear to be necessary for the initial production of TNF in response to LPS, but may be required either directly, or indirectly through modulation of IL-1 levels, for sustained TNF production.

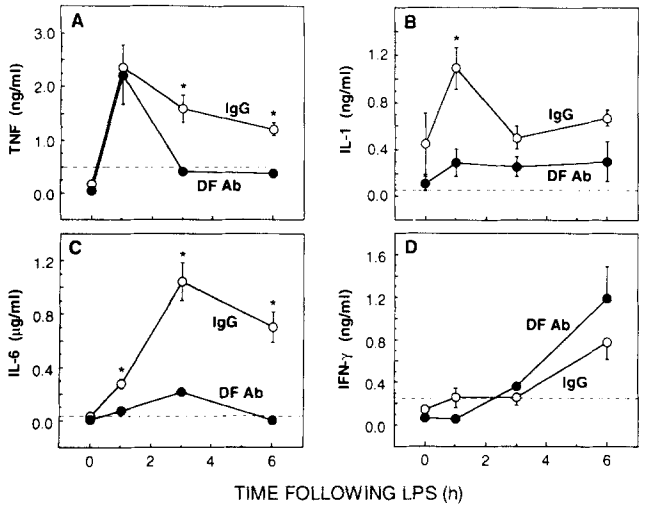

Figure 4. D factor mediates cytokine appearance in the serum of endotoxemic mice. Serum cytokine levels in mice pretreated with either DF Ab or IgG (mean ± SE) are plotted vs. time after intravenous LPS; $n \geq 5$ for all groups. Horizontal dashed lines represent the minimum level detectable; $*p < 0.05$ by unpaired, two tailed Student's t test.

The hose response during sepsis involves complex interactions between endogenous protein and lipid mediators. TNF is recognized to be an essential component of this network because inhibition of its activity improves survival and decreases production of other mediators (IL-1 and IL-6) (33–35). The current studies show that D factor, like TNF, plays a critical early role in the pathophysiology of endotoxemia. Endotoxin administration induced D factor gene expression in vivo and inhibition of endogenous D factor activity improved survival and decreased IL-1 and IL-6 production. The systemic manifestations of septic shock therefore may require both TNF and D factor activity. This effect is consistent with the hypothesis of Layton et al. (36), who recently identified a neutralizing soluble receptor (LIF binding protein [LBP]) present in the serum of normal mice and suggested that it acts to limit the systemic effects of locally produced D factor. LBP, analogous to DF Ab, may represent a natural inhibitory mechanism to protect the host against systemic effects of D factor produced during inflammation. A human LBP has not yet been identified.

The protection afforded rodents against radiation injury, oxygen toxicity, and endotoxemia by pretreatment with D factor seems inconsistent with the results of the present study. As noted, similar results have been observed with both TNF and IL-1. Concerning D factor, changes in the level of circulating LBP may be important. Pregnant mice, which produce greater amounts of D factor than normal mice, have much higher levels of circulating LBP than do normal mice (36). This suggests that pretreatment of a mouse with D factor might induce production of LBP. The resulting increased amount of circulating LBP could then neutralize subsequently produced D factor (e.g., in response to LPS) and thereby limit systemic toxicity.

As an essential participant in the complex network of mediators activated during endotoxemia, D factor may also contribute to the pathophysiology of other cytokine-mediated and inflammatory conditions, such as arthritis, allograft rejection, cancer cachexia, autoimmune disease, and the immunotherapy of cancer. Although its role in these conditions remains to be determined, the ability to modulate endogenous production and activity of D factor may have therapeutic application.

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