Brief Definitive Report

Differentiation Factor/Leukemia Inhibitory Factor Protection Against Lethal Endotoxemia in Mice: Synergistic Effect with Interleukin 1 and Tumor Necrosis Factor

By H. Richard Alexander,* Grace G. H. Wong,§ Gerard M. Doherty,* David J. Venzon,‡ Douglas L. Fraker,* and Jeffrey A. Norton*

*From the *Surgical Metabolism Section, Surgery Branch, ‡the Biostatistics and Data Management Section of the National Cancer Institute, Bethesda, Maryland 20892; and §the Department of Molecular Biology, Genentech, Inc., South San Francisco, California 94080*

Summary

Differentiation factor (D factor), also called leukemia inhibitory factor (LIF), is a glycoprotein that has been increasingly recognized to possess a wide range of physiological activities. We examined the possibility that the administration of D factor may confer beneficial effects and enhance host resistance against lethal endotoxemia. A single intravenous dose of recombinant human D factor completely protected C57/Bl6 mice from the lethal effect of *Escherichia coli* endotoxin (lipopolysaccharide [LPS]). The protective effects were dose dependent and observed when administered 2–24 h before LPS. Previous work has shown that interleukin 1 (IL-1) and tumor necrosis factor (TNF) also protect against a subsequent LPS challenge in a dose-dependent manner. When human D factor was combined with sub-protective doses of IL-1β or TNF-α, there was dramatic synergistic protection against a subsequent lethal LPS challenge.

Differentiation factor (D factor), also known as leukemia inhibitory factor (LIF), is a pluripotent glycoprotein with important regulatory physiological activities which include the ability to induce maturation and decrease clonagenicity of murine and human leukemia cells (1, 2); direct phenotype expression in neuronal tissue (3); induce changes in calcium metabolism (1, 4); and promote acute phase protein synthesis in hepatocytes (5). In vivo, D factor has a remarkable variety of other potent activities (1, 6). A role for D factor in the host response against bacterial infection is suggested by the finding that serum levels of D factor are detectable in mice after the administration of endotoxin (7).

IL-1 and TNF are similarly pluripotent, highly conserved peptide cytokines which, when administered before an insult, will confer in vivo protection against the lethal effects of radiation (8), endotoxin or Gram-negative sepsis (9, 10), ischemia/reperfusion injury (11), and oxygen toxicity (12, 13). Because TNF and IL-1 require a pretreatment interval, this suggests that the cytokines may confer protective effects indirectly. It is interesting that TNF and IL-1 have been shown to induce the synthesis of D factor in vitro (14). The current studies were performed to investigate the possibility that D factor may also have an important protective role against the lethality of endotoxin, and to investigate the effect of D factor in modulating the protective properties of IL-1 and TNF.

Materials and Methods

Animals. Female C57/Bl6 mice weighing 19–21 g were housed five to six per cage and kept in a controlled environment. All experiments were conducted in compliance with the Animal Care and Use Committee of the National Institutes of Health.

Reagents. Human rTNF-α and IL-1β were produced from *Escherichia coli*, and had an endotoxin content of <2 Eu/mg (15). Human and murine rD factor were produced as previously described, and had an endotoxin content of <2 Eu/mg (16). All reagents were brought to final concentration in PBS with 0.5% fatty acid-poor endotoxin-free BSA (Calbiochem-Behring Corp., La Jolla, CA), and kept at 2–8°C until administered. Control mice received inert protein carrier solution (saline). *E. coli* endotoxin (LPS, serotype 0127:B8) was purchased from Sigma Chemical Co. (St. Louis, MO), and reconstituted in saline to a final concentration of 4 mg/ml.

Survival Experiments. All survival experiments followed the same general format. In initial experiments animals (n = 10–12/group) were injected with increasing concentrations of D factor by tail vein (0.4 ml/mouse), followed 24 h later by either 35 or 40 mg/kg *E. coli* LPS intraperitoneally. In the kinetics studies, D factor injections were staggered at various intervals before LPS. Control animals received an equal volume of saline. In the synergy experiments, all animals received two tail vein injections of the pertinent reagents or an equal volume of saline. Survival was assessed at frequent intervals for 72 h, and then daily for 6 d. In this model, LPS lethality occurs from 24–72 h, at which time surviving animals are recovering and appear stable (9).

Statistics. In the initial survival experiments differences in survival at 72 h were determined using Fisher's exact test. Other significant differences in survival between groups were determined by the Breslow modification of the Kruskal-Wallis test. In synergy experiments, the logarithms of the survival times were assumed to be the sums of the effects of D factor, IL-1, or TNF, and an interaction term with a logistic error distribution. The likelihood ratio test was used to assess the significance of the interaction after adjusting for the two main effects.

Results and Discussion

To test the protective effects of human rD factor against LPS, mice received an intravenous injection of increasing concentrations of the protein 24 h before LPS (Table 1, *A*). The protective effects of D factor were dose dependent and 500 μg/kg completely protected mice from a 95% lethal dose (LD_{95}) or greater of LPS. In initial experiments, comparable improvements in survival were also observed with murine D factor (data not shown), and subsequent experiments were performed with the human homologue. The similar protection was not unexpected in light of the fact that the human and murine proteins share 78% amino acid homology and have equally high affinity for specific cellular receptors on murine cells (2, 17). The protective effects of D factor were observed at doses up to 1,500 μg/kg, and did not produce any observable toxicity. The protein used in these experiments was expressed in CHO cells and had an endotoxin content of <2 Eu/mg, minimizing the possibility that any toxic or protective effects were produced by endotoxin contamination. Protection was produced rapidly and observed with a pretreatment interval of 2 h or greater (Table 1). After the injection of LPS, mice in both groups initially appeared ill with hudded posture and piloerection, which suggested that pretreatment with D factor did not simply inactivate the LPS.

Our laboratory has previously reported that a single intravenous dose of IL-1 will protect mice against a subsequent lethal challenge with endotoxin (9). The profile of protective effects is similar to that of D factor, requiring only a 2 h pretreatment interval, and having a broad range of nontoxic therapeutic doses. Single intravenous sub-protective doses of D factor and IL-1, when given in combination, showed synergistic protection against LPS lethality (Fig. 1).

Pretreatment with TNF has also been shown to protect against lethal endotoxemia or bacterial sepsis (10). When a single intravenous dose of TNF-α, 25 μg/kg, was administered with D factor, 10 μg/kg, there was also synergistic protection against a subsequent lethal LPS challenge (Fig. 2). However, complete protection against LPS with a combination of D factor and TNF was not observed because dose escalation of TNF alone produced a significant survival advantage against LPS lethality. The combination of D factor with either IL-1 or TNF did not appear toxic under these experimental conditions, but clearly synergized the therapeutic effects. In contrast, low doses of TNF and IL-1 have been shown to result in synergistic lethality (18). It appears that IL-1 and D factor are ideally suited for use in combination because of their minimal toxicity in association with the synergistic effects.

The mechanism by which IL-1 or TNF exert their beneficial effects is not understood, but may be related to their ability to selectively induce gene expression of manganous superoxide dismutase (MnSOD) (19), an inducible mitochondrial metalloenzyme that protects cells against injury from reactive oxygen species (20). D factor alone does not induce this antiox-

Table 1. *D Factor Protection against E. Coli LPS Lethality in Female C57/Bl6 Mice*

Exp.	Group	Dose	Time of Rx	Intraperitoneal LPS	72 h survival	P_2
		μg/kg	*h*	*mg/kg*		
A	D factor	1	−24	40	0/10	–
	D factor	10	−24	40	3/12	–
	D factor	100	−24	40	4/10	–
	D factor	500	−24	40	10/10	<0.001*
	Saline	–	−24	40	2/13	–
B	D factor	500	−24	35	9/9	0.02*
	D factor	500	−6	35	11/11	0.01*
	Saline	–	−6	35	8/15	–
C	D factor	500	−2	40	11/12	<0.001*
	Saline	–	−2	40	0/12	–
D	D factor	500	0	35	6/15	–
	Saline	–	0	35	7/15	–

* Versus saline

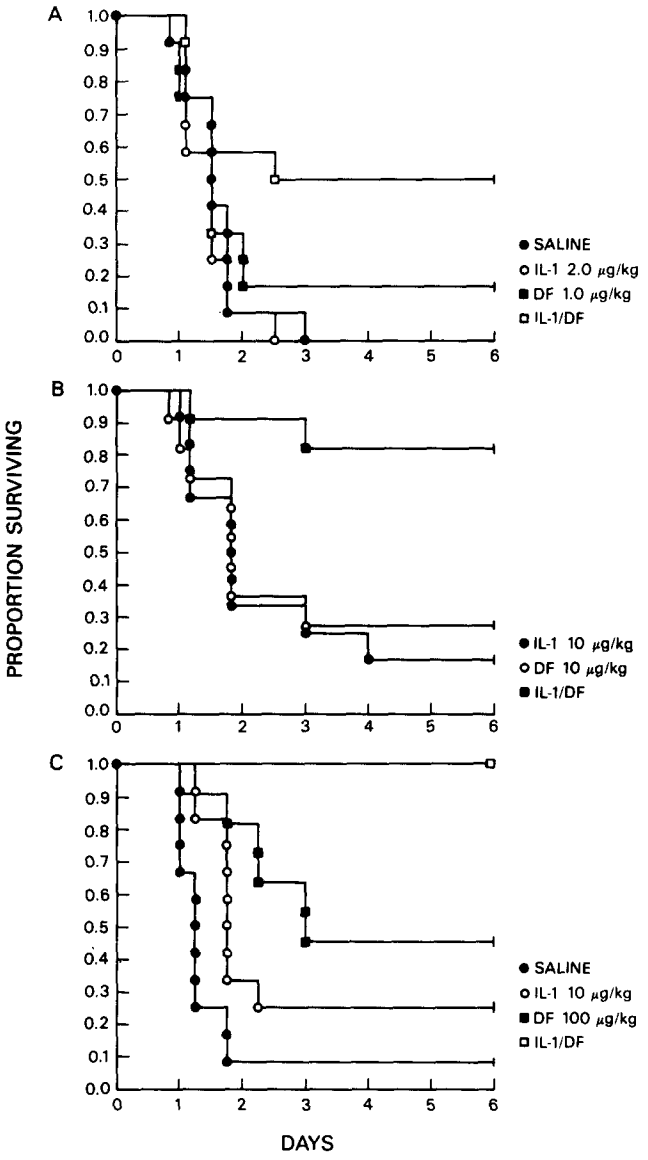

Figure 1. Synergistic protection of D factor and IL-1 against LPS lethality. Mice were treated with increasing doses of IL-1 or D factor alone, or in combination 24 h before LPS, 40 mg/kg, intraperitoneally as described. Control mice received an equal volume of saline. (*A*) IL-1 or D factor did not confer protection against LPS compared with controls. The combination of cytokines resulted in a significantly improved survival compared with any other treatment ($p = 0.025$). (*B*) Dose escalation of both cytokines resulted in synergistic protection against LPS ($p = 0.0014$). In this experiment, control lethality was 7:12 and not significantly different than IL-1 or D factor alone. (*C*) D factor, 100 μg/kg, conferred significant protection against controls ($p = 0.002$). In combination with a nonprotective dose of IL-1, there was significantly improved survival against LPS compared with D factor or IL-1 alone ($p = 0.01$).

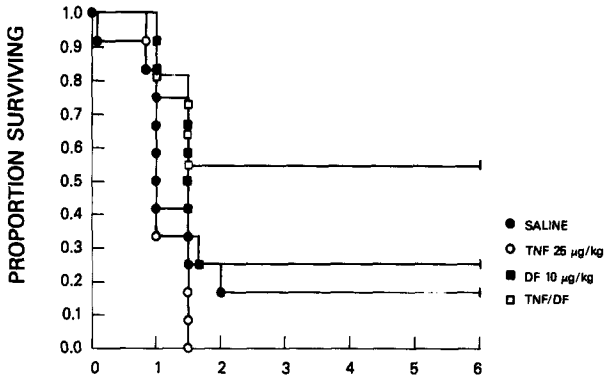

Figure 2. Synergistic protection of D factor and TNF against LPS lethality. TNF or D factor were given 24 h before LPS as described. Survival with either TNF or D factor alone was not different than controls. Combination treatment resulted in significantly improved survival ($p = 0.04$). Horizontal axis represents time in days.

idant enzyme, but does act synergistically with TNF and IL-1 on the induction of MnSOD in M1 cells (G. Wong, unpublished data). D factor is synonymous with hepatocyte stimulating factor III (2, 5, 7) and the modulation of acute phase protein synthesis before or during endotoxemia may also be a contributing beneficial effect. Recently, the binding, internalization, and partial degradation of endotoxin by scavenger receptors on macrophages has been implicated as a mechanism by which circulating endotoxin is cleared and detoxified in animals (21). Because macrophages and monocytes appear to be the primary target cell responsible for binding of D factor (22, 23), the function of these cell types may be altered after exposure to D factor and result in an accelerated rate of endotoxin clearance or augmented production of TNF or IL-1 in response to LPS.

In conclusion, these experiments demonstrate that D factor has important physiological effects that may enhance the host defense against a variety of acute disease processes as demonstrated in this model of endotoxin shock. The studies expand the known in vivo properties of exogenously administered D factor and demonstrate therapeutic synergism with IL-1 or TNF. Recent work has also shown that D factor has synergistic activity with TNF against radiation lethality in mice (24). The synergism of D factor with IL-1 or TNF suggests that D factor is unlikely to be the sole mediator of the protective activity of IL-1 and TNF. The application of these cytokines in combination with D factor may be extremely useful against a variety of disease processes ameliorated by IL-1 or TNF treatment.

Address correspondence to Dr. H. Richard Alexander, Surgical Metabolism Section Surgery Branch/NCI, Building 10, Room 2B01, National Institutes of Health, Bethesda, MD 20892.

Received for publication 2 December 1991 and in revised form 23 January 1992.

References

1. Metcalf, D. 1991. The leukemia inhibitory factor (LIF). *Int. J. Cell Cloning.* 9:95.
2. Gough, N.M., and R.L. Williams. 1989. The pleiotropic actions of leukemia inhibitory factor. *Cancer Cells (Cold Spring Harbor).* 1:77.
3. Yamamori, T., K. Fukada, R. Aebersold, S. Korsching, M.-J. Famm, and P.H. Patterson. 1989. The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. *Science (Wash. DC)* 246:1412.
4. Reid, I.R., C. Lowe, J. Cornish, J.M. Skinner, D.J. Hilton, T.A. Willson, D.P. Gearing, and T.J. Martin. 1990. Leukemia inhibitory factor: a novel bone-active cytokine. *Endocrinology.* 126:1416.
5. Baumann, H., and G.G. Wong. 1989. Hepatocyte-stimulating factor III shares structural and functional identity with leukemia-inhibitory factor. *J. Immunol.* 143:1163.
6. Metcalf, D., and D.P. Gearing. 1989. Fatal syndrome in mice engrafted with cells producing high levels of the leukemia inhibitory factor. *Proc. Natl. Acad. Sci. USA.* 86:5948.
7. Hilton, D.J., and N.M. Gough. 1991. Leukemia inhibitory factor: A biological perspective. *J. Cell. Biochem.* 46:21.
8. Neta, R., J.J. Oppenheim, and S.D. Douches. 1988. Interdependence of the radioprotective effects of human recombinant interleukin 1α, tumor necrosis factor α, granulocyte colony-stimulating factor, and murine recombinant granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 140:109.
9. Alexander, H.R., G.M. Doherty, D.L. Fraker, M.I. Block, J.A. Swedenborg, and J.A. Norton. 1991. Human recombinant interleukin-1a protection against the lethality of endotoxin and experimental sepsis in mice. *J. Surg. Res.* 50:421.
10. Sheppard, B.C., D.L. Fraker, and J.A. Norton. 1989. Prevention and treatment of endotoxin and sepsis lethality with recombinant human tumor necrosis factor. *Surgery (St. Louis).* 106:156.
11. Brown, J.M., C.W. White, L.S. Terada, M.A. Grosso, P.F. Shanley, D.W. Mulvin, A. Banerjee, G.J.R. Whitman, A.H. Harken, and J.E. Repine. 1990. Interleukin-1 pretreatment decreases ischemia/reperfusion injury. *Proc. Natl. Acad. Sci. USA.* 87:5026.
12. White, C.W., P. Ghezzi, C.A. Dinarello, S.A. Caldwell, I.F. McMurty, and J.E. Repine. 1987. Recombinant tumor necrosis factor/cachectin and interleukin-1 pretreatment decreases lung oxidized glutathione accumulation, lung injury, and mortality in rats exposed to hyperoxia. *J. Clin. Invest.* 79:1868.
13. Tsan, M.-F., J.E. White, T.A. Santana, and C.Y. Lee. 1990. Tracheal insufflation of tumor necrosis factor protects rats against oxygen toxicity. *J. Appl. Physiol.* 68:1211.
14. Wetzler, M., M. Talpaz, D.G. Lowe, G. Baiocchi, J.U. Gutterman, and R. Kurzrock. 1991. Constitutive expression of leukemia inhibitory factor RNA by human bone marrow stromal cells and modulation by IL-1, TNF-α, and TGF-β. *Exp. Hematol. (NY)* 19:347.
15. Pennica, D.G., G.E. Nedwin, J.S. Hayflick, P.H. Seeburg, R. Derynck, M.A. Palladino, W.J. Kowr, B.B. Aggarwal, and D.V. Goeddel. 1984. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature (Lond.).* 312:724.
16. Lowe, D.G., W. Nunes, M. Bombara, S. McCabe, G.E. Ranges, W. Henzel, M. Tomida, Y. Yamamoto-Yamaguchi, M. Hozumi, and D.V. Goeddel. 1989. Genomic cloning and heterologous expression of human differentiation-stimulating factor. *DNA (New York).* 8:351.
17. Gough, N.M., D.P. Gearing, J.A. King, T.A. Willson, D.J. Hilton, N.A. Nicola, and D. Metcalf. 1988. Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia inhibitory factor. *Proc. Natl. Acad. Sci. USA.* 85:2623.
18. Waage, A., and T. Espevik. 1988. Interleukin-1 potentiates the lethal effect of tumor necrosis factor alpha/cachectin in mice. *J. Exp. Med.* 167:1987.
19. Wong, G.H.W., and D.V. Goeddel. 1988. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science (Wash. DC).* 242:941.
20. Bannister, J.V., W.H. Bannister, and G. Rotilio. 1987. Aspects of the structure, function, and applications of superoxide dismutase. *CRC Crit. Rev. Biochem.* 22:111.
21. Hampton, R.Y., D.T. Golenbock, M. Penman, M. Krieger, and C.R.H. Raetz. 1991. Recognition and plasma clearance of endotoxin by scavenger receptors. *Nature (Lond.).* 352:342.
22. Abe, T., M. Muradami, T. Sato, M. Kajiki, M. Ohno, and R. Kodaira. 1989. Macrophage differentiation inducing factor from human monocytic cells is equivalent to murine leukemia inhibitory factor. *J. Biol. Chem.* 264:8941.
23. Hilton, D.J., N.A. Nicola, and D. Metcalf. 1988. Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells. *Proc. Natl. Acad. Sci. USA.* 85:5971.
24. Wong, G.H.W., R. Neta, and D.V. Goeddel. 1992. Protective roles of MnSOD, TNF-α, TNF-β and D-factor (LIF) in radiation injury. *In* Eicosanoids and other Bioactive Lipids in Cancer, Inflammation and Radiation Injury. 2nd International Conference. Klinikum Steglitz, editor. Berlin. In press.