Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 8619–8622, December 1987
Medical Sciences

Modulation of endogenous hormone action by recombinant human tumor necrosis factor

(amino acid transport/glucagon/acute phase response)

ROBERT S. WARREN*, DAVID B. DONNER†, H. FLETCHER STARNES, JR.*, AND MURRAY F. BRENNAN*‡

Departments of *Surgery and †Cell Biology, Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021

Communicated by Paul A. Marks, July 16, 1987

ABSTRACT **Tumor necrosis factor (TNF) has been implicated in the toxic manifestations of overwhelming bacterial infection and in the tissue wasting that often accompanies prolonged infections and malignancy. We have examined a possible role of TNF in the early metabolic alterations following acute tissue injury or sepsis. Recombinant human TNF stimulated rat liver amino acid uptake up to 5-fold *in vivo* and there was a concomitant increase in plasma glucagon. *In vitro* TNF had no direct effect on hepatocyte amino acid uptake, but it markedly enhanced the stimulation of amino acid transport by glucagon, without an alteration in binding of glucagon to hepatocytes. This permissive effect of TNF on glucagon action represents an interrelationship between the immune and endocrine systems, and it may help to explain the mechanism of hormonal regulation of both the anabolic and catabolic responses to acute injury.**

The systemic reaction to tissue injury or infection often consists of a stereotypic pattern of metabolic and hormonal alterations that maintain physiologic homeostasis and have been collectively termed the "acute phase response" (1). Characteristically, there is a shift from energy storage to energy utilization. Accelerated net breakdown of skeletal muscle (2, 3) occurs with a concomitant shift to anabolic metabolism in the liver (4). Hepatic uptake of amino acids released by peripheral tissues increases dramatically for use as gluconeogenic substrates (5–7) and to support synthesis of the acute phase reactants, a set of hepatocyte secretory proteins whose plasma levels rise several- to 1,000-fold in sepsis and injury (8). This exchange of amino acids from muscle to liver, as well as to bone marrow and wounds, to support the anabolic activity of the hepatocyte, for cellular proliferation within the immune system, and for healing of injured tissue, represents a reprioritization of carbon and energy utilization in the acute phase response as an adaptation of the injured or septic animal for survival (9). Regulation of the acute phase response has been attributed, in part, to elevation in glucagon, insulin, cortisol, and the catecholamines, but the complex panoply of metabolic events cannot be explained fully by the actions of these "stress hormones" (10).

Recently, experimental support has accumulated for the involvement of the immune system in the endocrine and metabolic changes of the acute phase response through the action of monokines, the secreted protein products of activated macrophages (11). Particular interest has developed in the activity of one such monokine, tumor necrosis factor (TNF), a macrophage-derived polypeptide initially described by Carswell *et al.* (12) as the agent responsible for hemorrhagic necrosis in transplanted murine sarcomas after administration of endotoxin, the lipopolysaccharide (LPS) portion of the Gram-negative bacterial cell wall. Subsequently, Beutler and co-workers reported the amino acid sequence homology of TNF with cachectin, the LPS-induced murine macrophage factor that inhibits transcription of the adipocyte lipoprotein lipase gene (13–15). It has been proposed that TNF/cachectin may be responsible for the syndrome of body wasting that frequently accompanies chronic infection and malignancy (16), and recent emphasis has been placed on the ability of TNF to produce lethal shock in animals (17). In the present investigation, we have explored the possibility that TNF may have a physiologic role in mediating the acute metabolic homeostatic adaptations to tissue injury and sepsis. As a measure of the anabolic alterations characteristic of acute injury *in vivo*, we have examined amino acid uptake by the liver in response to recombinant human TNFα (rHu-TNFα).

MATERIALS AND METHODS

Animals. Male Fischer 344 rats (Charles River Breeding Laboratories) weighing 200–250 g were housed individually in metal cages in a temperature-controlled room with alternating 12-hr light/dark cycles and allowed free access to water and food (Purina Rat Chow). *In vivo* studies were carried out between 10:00 a.m. and 2:00 p.m., during which period both food and water were withheld.

Monokines. rHuTNFα produced in *Escherichia coli* and purified to homogeneity was obtained from Genentech (San Francisco). Specific activity was 5×10^7 units/mg of protein, and the preparation contained less than 0.4 ng of endotoxin per mg of protein. TNF was diluted with pyrogen-free NaCl (0.9% wt/vol) just prior to use *in vivo* and with culture medium for use *in vitro*. Recombinant human interleukin 1β (rHuIL-1β; specific activity, 10^7 units/mg of protein) was obtained from Cistron Technology (Pine Brook, NJ).

Hepatic Amino Acid Uptake *in Vivo*. Liver uptake of α-amino[1-^{14}C]isobutyric acid (New England Nuclear), a nonmetabolized analogue of alanine, was determined by the method of Walker and Whitfield (18). Briefly, α-amino[^{14}C]-isobutyrate was given by intracardial injection (8.5 μCi, 0.2 μmol/kg of body weight; 1 Ci = 37 GBq) to rats under light pentobarbital anesthesia 3 hr after intraperitoneal administration of rHuTNFα. Sixty minutes later, animals were decapitated and trunk blood was collected. Livers were excised and homogenized in phosphate-buffered saline. α-Amino[^{14}C]isobutyrate was extracted from liver homogenates or serum with 0.2 M perchloric acid, and radioactivity in aliquots was measured in Biofluor (New England Nuclear). Liver amino acid uptake is expressed by the distribution ratio, calculated as the dpm per g of liver/dpm per ml of serum. Plasma glucagon levels were measured by radio-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TNF, tumor necrosis factor; r-, recombinant; Hu-, human; IL-1β, interleukin 1β.

‡To whom reprint requests should be addressed.

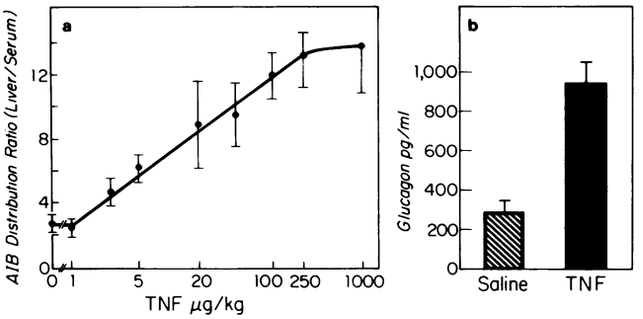

FIG. 1. (*a*) Hepatic amino acid uptake *in vivo* 4 hr after intraperitoneal injection of rHuTNFα. Each value represents the mean ± SEM for duplicate determinations in 4–8 animals at each rHuTNFα dose and in 18 saline-injected controls. AIB, α-aminoisobutyrate. (*b*) Plasma glucagon 4 hr after injection of saline ($n = 6$) or rHuTNFα (500 μg/kg) ($n = 8$). Values are mean ± SEM.

immunoassay (kit from Cambridge Medical Diagnostics, Billerica, MA).

Hepatocyte Amino Acid Transport *in Vitro*. Hepatocytes were isolated by *in situ* liver perfusion with collagenase as previously described (19) and resuspended in Eagle's minimal essential medium (MEM) containing nonessential amino acids, penicillin, streptomycin, 10% (vol/vol) fetal calf serum, and 170 mM insulin (Humulin, Genentech). Six-well plastic culture plates (Falcon) were inoculated to a density of 10^5 cells per cm^2 (fewer than 1% Kupffer cells by latex bead uptake) and cultured at 37°C in an atmosphere of 95% air/5% CO_2. After 4 hr cultures were washed and media were replaced with fresh MEM minus insulin but containing rHuTNFα or rHuIL-1β at various concentrations.

Twenty hours after the medium change, α-aminoisobutyrate transport in a 10-min interval was measured by the method of Pariza *et al.* (20). In other experiments, glucagon (10^{-9}–10^{-6} M in 1.6% glycerol and 0.2% phenol) or dibutyryl-cAMP (0.1 μM in phosphate-buffered saline) was added directly to the cultures (10 μl/ml of culture medium) 14 hr after the medium change and the 10-min transport rates were measured after an additional 6-hr incubation. Rates for three to six replicate wells were obtained in each experiment and expressed as the percent of basal α-aminoisobutyrate uptake (cells incubated for 20 hr after medium change in the absence of TNF, IL-1, glucagon, or cAMP). Cellular protein was assayed in control and TNF-treated hepatocytes by the method of Bradford, using bovine serum albumin as a standard.

RESULTS

Liver Amino Acid Uptake *in Vivo*. rHuTNFα was administered by intraperitoneal injection to fed Fischer 344 rats and hepatic amino acid uptake was determined as the accumulation of α-amino[1-^{14}C]isobutyrate, a nonmetabolized analogue of alanine, the principal amino acid released by muscle in stress (4) and the major gluconeogenic amino acid in the liver (21). rHuTNFα-produced a dose-dependent increase in hepatic α-aminoisobutyrate uptake (Fig. 1*a*). A measurable effect occurred with as little as 2.5 μg/kg of body weight, and maximal stimulation was nearly 5-fold over the saline-injected controls. Because glucagon is a potent stimulator of hepatic amino acid uptake (22) and its plasma concentrations are elevated in sepsis and injury (7, 24), we measured the systemic level of this hormone after TNF injection. Plasma glucagon rose more than 3-fold compared to controls (Fig. 1*b*). Concomitantly, there was a modest increase in serum insulin (55 ± 2 vs. 33 ± 5 microunits/ml, TNF vs control, respectively; $P < 0.001$ by unpaired Student's *t* test) with no net change in serum glucose concentration (data not shown). Thus the *in vivo* stimulation of hepatic amino acid uptake by rHuTNFα can be explained in part by the known action of glucagon.

Hepatocyte Amino Acid Uptake *in Vitro*. To investigate a possible direct action of TNF on the liver, we characterized the influence of rHuTNFα on the rate of α-aminoisobutyrate transport by cultured rat hepatocytes. A 16-hr incubation of hepatocytes with rHuTNFα at various concentrations produced no significant stimulation of amino acid uptake over basal values (cells cultured in media supplemented with 10% heat-inactivated fetal calf serum but no other additions) (Fig. 2*a*). As expected, glucagon (5 nM) stimulated α-aminoisobutyrate uptake 2-fold. An unexpected finding was an enhanced response of hepatocyte amino acid transport to glucagon by cells cultured in the presence of as little as 30 fM

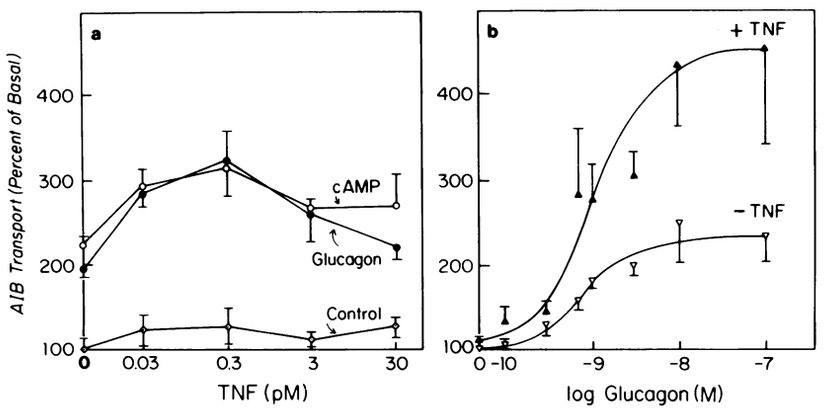

FIG. 2. (*a*) Effects of rHuTNF alone or in combination with glucagon or dibutyryl-cAMP on amino acid transport by rat hepatocytes. Rates of α-amino[^{14}C]isobutyrate (AIB) transport were determined in hepatocytes cultured for 20 hr in the presence of various concentrations of rHuTNF alone (◇) or with the addition of 5 nM glucagon (●) or 0.1 mM dibutyryl-cAMP (○) during the final 6 hr of the TNF incubation. Rates for three to six replicate wells were obtained in each experiment and expressed as the percent of basal α-aminoisobutyrate uptake (cells incubated for 20 hr in the absence of TNF, glucagon, or cAMP). Values represent the mean ± SEM for 3–10 separate experiments. Parallel experiments demonstrated no differences in protein content per well after the 20-hr incubation at any TNF concentration. Average rate of basal α-aminoisobutyrate transport in four experiments was 4.35 ± 0.72 nmol/10 min per mg of protein. (*b*) Response of α-aminoisobutyrate transport to glucagon. Hepatocytes were cultured in the absence (−) or presence (+) of 0.3 pM rHuTNFα for 20 hr. During the final 6 hr, glucagon was added as described for *a* to the indicated final concentration, and 10-min α-aminoisobutyrate transport rates were determined and expressed as percent of basal (hepatocytes cultured in the absence of TNF or glucagon).

TNF. Likewise, prior incubation of hepatocytes with TNF increased α-aminoisobutyrate uptake in response to the addition of dibutyryl-cAMP (0.1 mM). To further examine this "permissive" effect of TNF on α-aminoisobutyrate transport, a glucagon dose–response study was performed. Hepatocytes were incubated in the absence or presence of 0.3 pM TNF and α-aminoisobutyrate uptake was measured after an additional 6-hr incubation of cells with rHuTNFα and various concentrations of glucagon (Fig. 2*b*). Exposure to the rHuTNFα doubled the maximal hepatocyte response to glucagon, while the concentration of glucagon giving half-maximal response was unchanged.

Because TNF has recently been shown to induce the production of IL-1 (25, 26), another monokine thought to participate in the acute phase response, we tested the capacity of rHuIL-1β to directly stimulate α-aminoisobutyrate transport by cultured rat hepatocytes. In three separate experiments, rHuIL-1β over a range of IL-1 concentrations (0.3–3000 pM) failed to stimulate hepatocyte AIB transport (data not shown).

DISCUSSION

Modulation of hepatocyte function in a manner similar to that induced by TNF has previously been observed in studies with dexamethasone. This synthetic glucocorticoid exerts a permissive effect both on the induction of α-aminoisobutyrate transport by glucagon (27) and in the synthesis of α_2-macroglobulin, an acute phase protein, by rat hepatocytes in response to stimulatory factors secreted by human squamous carcinoma cells in culture (28). Glucocorticoids may also contribute to TNF-induced hepatic amino acid transport, since serum corticosterone levels rise significantly in TNF-injected rats concomitant with the elevation in glucagon (unpublished data).

In the present investigation we demonstrate a permissive effect of rHuTNFα on glucagon action, as well as an interrelationship between the immune system, the endocrine system, and a common target cell. TNF appears to stimulate liver amino acid uptake in the rat in two ways: by increasing circulating glucagon concentrations and subsequently by enhancing the action of glucagon upon the hepatocyte. The mechanism by which TNF exerts this permissive effect on the hepatocyte is not evident from these data. Specific binding of ^{125}I-labeled glucagon to hepatocytes cultured for 20 hr with 0.3 pM TNF is unchanged (data not shown). While glucagon action is known to be mediated in part through an increase of intracellular cAMP, the physiological relevance of the chosen cAMP concentration used *in vitro* has not been established. The comparable effects of glucagon and dibutyryl-cAMP-induced α-aminoisobutyrate uptake suggest that the target of TNF action may be after the glucagon receptor. In support of this interpretation, we have observed that both glucagon and TNF induce changes in the phosphorylation state of common intracellular proteins in the hepatocyte (unpublished data). Our observations also raise the possibility that TNF may exert a similar permissive effect on the known catabolic actions of the combination of glucagon, cortisol, and epinephrine on skeletal muscle (10), thus linking the acute anabolic and catabolic components of the injury response.

TNF has recently been shown to induce the synthesis of IL-1 by macrophages (25, 26) and by vascular endothelial cells (29). Both TNF and IL-1 are synthesized as 17,500-Da monomers, principally by cells of the reticuloendothelial system in response to endotoxin, and are members of distinct families of polypeptides (TNF and lymphotoxin/Il-1α and IL-1β) that are biologically active in both secreted and membrane-bound forms (30, 31). TNF and IL-1 share a wide variety of biologic actions important in the acute host response to injury and infection [reviewed in detail by Le and Vilcek (32)], and the functional relationship *in vivo* between these hormones is of considerable interest. In the present study we were unable to demonstrate a direct stimulation of hepatocyte amino acid transport by rHuIL-1β. In light of the recent observation that rHuIL-1β is toxic to cultured pancreatic islet cells and leads to a decreased islet content of glucagon and insulin (33), the *in vivo* effect we have observed with TNF is unlikely to be mediated by IL-1.

Few of the multiple and diverse biological effects of TNF demonstrated *in vitro* have been assessed *in vivo* (34). The ability to cause necrosis of tumors *in vivo* as well as to produce lysis of certain transformed cells in culture has led to intense interest in the clinical utility of TNF as an antineoplastic agent. The studies of Beutler and co-workers have implicated TNF in the lethal effects of endotoxin (35) and the chronic catabolic state (cachexia) characteristic of patients with certain chronic infections and malignancy (15). Further, Tracey *et al.* have recently demonstrated that rHuTNF infusion into dogs reproduces certain of the hemodynamic and counterregulatory hormone perturbations seen in septic shock (36). In contrast, the present data indicate that TNF may have a role in the integrated, acute metabolic adaptations to tissue injury and sepsis, consisting of catabolic changes in certain tissues (muscle and fat) and a concomitant shift to anabolic metabolism in higher priority tissues (liver, bone marrow, healing wounds). Lending support to this view of TNF as an anabolic hormone in specific target tissues in the acute phase of tissue injury is the recent demonstration that TNF stimulates growth of human diploid fibroblasts, either alone or in a synergistic manner with insulin (23), suggesting that TNF might contribute to wound healing and tissue repair. Two recent studies have shown that TNF stimulates the synthesis of certain acute phase proteins by hepatoma cells *in vitro* (38, 39). Further, we have recently found that a single injection of recombinant human TNF in patients with cancer induces fever, leukocytosis, decreased serum trace metals, and increased concentrations of the hepatic acute phase reactant C-reactive protein (unpublished data), all characteristics of the acute phase response.

The mechanism by which TNF exerts its pleiotropic actions is unclear. TNF binds to both high- and low-affinity membrane receptors in adipocytes (40) and hepatocytes (unpublished data), while a single class of receptors is present on fibroblasts (40) and a variety of transformed cell lines (23). Elucidation of the specific biological responses in different cell types as a consequence of TNF binding to such receptors, as well as a characterization of the proximal postreceptor events following ligand binding to a variety of both normal and neoplastic tissues, will undoubtedly help to clarify this issue.

We thank L. J. Old for many helpful discussions and critical evaluation of the manuscript, P. Leo for technical assistance, and M. Palladino (Genentech) for the generous supply of rHuTNFα and Cistron Technology for the rHuIL-1β. This work was supported by National Institutes of Health Grants CA 38858 and T32 CA 09501 (to R.S.W.). D.B.D. is the recipient of Research Career Development Award AM 01045 from the National Institutes of Health.

1. Kushner, I. (1986) *Ann. N.Y. Acad. Sci.* **389**, 39–48.
2. Goldberg, A. F., Baracos, V., Rodeman, P., Waxman, L. & Dinarello, C. A. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 1301–1306.
3. Millward, D. J. (1986) *Biochem. Soc. Trans.* **13**, 1023–1026.
4. Beisel, W. R. (1975) *Annu. Rev. Med.* **26**, 9–20.
5. Beisel, W. R. & Wannemacher, R. W. (1980) *JPEN J. Parenter. Enteral Nutr.* **4**, 277–285.
6. Long, C. L., Kinney, J. M. & Geiger, J. W. (1976) *Metabolism* **25**, 193–201.

7. Lang, C. H., Bagby, G. L. & Spitzer, J. J. (1984) *Metabolism* **33,** 959–963.
8. Pepys, M. B & Baltz, M. L. (1983) *Adv. Immunol.* **34,** 141–212.
9. Rosenblatt, S., Clowes, G. H. A., George, B. C., Hirsch, E. & Lindberg, B. (1983) *Arch. Surg. (Chicago)* **118,** 175–176.
10. Bessey, P. Q., Watters, J. M., Aoki, T. T & Wilmore, D. W. (1984) *Ann. Surg.* **200,** 264–280.
11. Filkins, J. P. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44,** 300–304.
12. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. & Williamson, B. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3666–3670.
13. Beutler, B., Greenwald, D., Hulmes, J. D., Chang, M., Pan, C. E., Mathison, R., Ulevitch, R. & Cerami, A. (1985) *Nature (London)* **316,** 552–554.
14. Beutler, B., Mahoney, J., LeTrang, N., Pekala, P. & Cerami, A. (1985) *J. Exp. Med.* **161,** 984–995.
15. Torti, F. M., Dieckman, B., Beutler, B., Cerami, A. & Ringold, G. M. (1985) *Science* **229,** 867–869.
16. Beutler, B. & Cerami, A. (1986) *Nature (London)* **320,** 584–588.
17. Tracey, K. J., Beutler, B., Lowry, S. F., Merryweather, J., Wolpe, S., Milsark, I., Hariri, R. J., Fahey, T. J., Zentella, A., Albert, J. D., Shires, G. T. & Cerami, A. (1986) *Science* **234,** 470–474.
18. Walker, P. R. & Whitfield, J. F. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1394–1398.
19. Warren, R. S., Jeevanandam, M. & Brennan, M. F. (1985) *Surgery* **98,** 275–281.
20. Pariza, M. W., Butcher, F. R., Kletzein, R. F., Becker, J. E. & Potter, V. R. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4511–4515.
21. Felig, P., Pozefsky, T., Marliss, C. & Cahill, G. F. (1970) *Science* **167,** 1003–1004.
22. Mallette, L. E., Exton, J. H. & Park, C. R. (1969) *J. Biol. Chem.* **244,** 5713–5723.
23. Sugarman, B. J., Aggarwal, B. B., Hass, P. E., Figari, I. S., Palladino, M. A. & Shepard, H. M. (1985) *Science* **230,** 943–945.
24. Jahoor, S., Herndon, D. N. & Wolfe, R. R. (1986) *J. Clin. Invest.* **78,** 807–814.
25. Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B. & Cerami, A. (1986) *J. Exp. Med.* **163,** 1433–1450.
26. Bachwich, P. R., Chensue, S. W., Larrick, J. W. & Kunkel, S. L. (1986) *Biochem. Biophys. Res. Commun.* **136,** 94–101.
27. Kletzein, R. F., Pariza, M. W., Becker, J. E. & Potter, V. R. (1975) *Nature (London)* **256,** 46–47.
28. Baumann, H., Hill, R. E., Sauder, D. N. & Jahreis, J. (1986) *J. Cell Biol.* **102,** 370–383.
29. Nawroth, P. P., Bank, I., Handley, D., Cassimeris, J., Chess, L. & Stern, D. (1986) *J. Exp. Med.* **163,** 1363–1375.
30. Decker, T., Lohmann-Mathes, M.-L. & Gifford, G. E. (1987) *J. Immunol.* **138,** 957–962.
31. Kurt-Jones, E. A., Virgin, I. V. & Unanue, E. R. (1985) *J. Immunol.* **135,** 3652–3659.
32. Le, J. & Vilcek, J. (19[illegible]) *Lab. Invest.* **56,** 234–248.
33. Bendtzen, K., Mandrup-Paulsen, T., Nerup, J., Nielsen, J. H., Dinarello, C. A. & Svenson, M. (1986) *Science* **232,** 1545–1547.
34. Nathan, C. F. (1987) *J. Clin. Invest.* **79,** 319–326.
35. Beutler, B., Milsark, I. W. & Cerami, A. C. (1985) *Science* **229,** 869–871.
36. Tracey, K. T., Lowry, S. F., Fahey, T. J., Albert, J. D., Fong, Y., Hesse, D., Beutler, B., Manoge, K. R., Calvano, S., Wei, H., Cerami, A. & Shires, T. (1987) *Surg. Gynecol. Obstet.* **164,** 415–422.
37. Vilcek, J., Palombella, V. J., Heriksen, I., DeStefano, D., Swenson, C., Feinman, R., Hirai, M. & Tsujimoto, M. (1986) *J. Exp. Med.* **163,** 632–643.
38. Darlington, G. J., Wilson, D. R. & Lachman, L. B. (1986) *J. Cell Biol.* **103,** 787–793.
39. Perlmutter, D. H., Dinarello, C. A., Punsal, P. J. & Colten, H. R. (1986) *J. Clin. Invest.* **78,** 1349–1354.
40. Patton, J. S., Shepard, H. M., Wilking, H., Lewis, G., Aggarwal, B. B., Eessalu, T. E., Gavin, L. A. & Grunfeld, C. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8313–8317.