INFECTION AND IMMUNITY, Apr. 1993, p. 1496–1499
0019-9567/93/041496-04$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 61, No. 4

Protective Role of Interleukin 6 in the Lipopolysaccharide-Galactosamine Septic Shock Model

BEVERLY E. BARTON* AND JAMES V. JACKSON
Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, New Jersey 07033

Received 21 September 1992/Accepted 11 January 1993

C57BL/6J mice given low doses of lipopolysaccharide (LPS) (100 ng per mouse) plus D-galactosamine (8 mg per mouse) die within 24 h following LPS administration. We used this septic shock model to confirm the role of tumor necrosis factor in mortality using a monoclonal antibody to tumor necrosis factor to prevent lethality. Furthermore, we demonstrated that interleukin 6, rather than playing a lethal role, protected mice against death in this septic shock model. Antibody to interleukin 6 did not affect the fatal outcome in this low-LPS-dose model. However, pretreatment with antibody to tumor necrosis factor did protect the mice against death, in a dose-dependent manner. Moreover, mortality was enhanced by pretreatment with antibody to interleukin 6 when tumor necrosis factor was partly limited by anti-tumor necrosis factor treatment. Mortality was significantly reduced by pretreatment with both recombinant interleukin 6 and low doses of antibody to tumor necrosis factor; in the absence of the low dose of antibody to tumor necrosis factor, interleukin 6 alone did not confer any protection. These data demonstrate in vivo antagonistic activities of tumor necrosis factor and interleukin 6 and show that interleukin 6 can play a protective role against death from septic shock.

Septic shock is an often-fatal condition usually resulting from gram-negative bacteremia. In the absence of overt infection, it can be caused by lipopolysaccharide (LPS). Investigators have shown that the in vivo activity of LPS is due to the mediators produced by the host's cells. Among these mediators are tumor necrosis factor (TNF), interleukin 1 (IL-1), IL-8, and IL-6.

Passive administration of recombinant TNF was shown to induce shock in rats (20), while antibodies (Abs) to TNF protected mice against the lethal effects of TNF (5, 7, 10). Moreover, it had been noted that high levels of IL-6 in meningococcal septic shock were associated with fatal outcome (21). Since recombinant TNF alone did not induce shock in patients undergoing clinical trials with the cytokine in one study (21), interaction with another cytokine, such as IL-6, may play an important role in human septic shock. In another study, TNF did induce shock symptoms in patients (15), but the possibility of interaction with another cytokine, such as IL-6, had not been explored. LPS is known to potentiate recombinant TNF-induced shock (16), and LPS induces IL-6 synthesis (6).

On the basis of these observations, we decided to investigate the role each cytokine plays in a murine septic shock model. We confirmed the role of TNF in this septic shock model and found that IL-6 antagonized TNF in vivo, protecting mice from death.

MATERIALS AND METHODS

Mice. Male C57BL/6J mice, 5 weeks of age, were obtained from Jackson Laboratory. They were rested at least 5 days prior to use.

Reagents. Endotoxin-free phosphate-buffered saline (PBS) was purchased from GIBCO.

LPS (*Escherichia coli* O111:B4) was purchased from List Biological Laboratories, Inc. It was dissolved in PBS at 1 mg/ml and stored in 500-μl aliquots at −80°C until use. Prior to being frozen, the mixture was sonicated for 5 min in a sonifying bath. After being thawed, the mixture was resonicated for 5 min. Appropriate dilutions were made in PBS, in polypropylene tubes.

D-Galactosamine (Sigma Chemicals) was dissolved at 32 mg/ml in PBS and mixed with an equal volume of diluted, sonicated LPS. The LPS-galactosamine mixture (LPS-GalN) was used immediately, and fresh batches were made for each experiment.

Procedure. Each mouse received 0.5 ml of LPS-GalN (consisting of 100 ng of LPS plus 8 mg of D-galactosamine) (see Table 1) intraperitoneally (i.p.) between 1 and 3 p.m. Twenty mice per experimental (treatment) group were used; each experiment was repeated at least twice. The animals were scored for mortality at 24 h. In early experiments it was observed that if the animals survived for 24 h, they survived the LPS challenge. Thus, all experiments were terminated at 24 h. Ab to IL-6 was given at doses used by Starnes et al. (19), at either 1 or 18 h before the LPS-GalN challenge. Ab to TNF was given 18 h prior to the LPS-GalN challenge at the doses described in Results.

Analysis of serum cytokines. Blood samples were collected from anesthetized mice into Capiject (Terumo) serum separator tubes and allowed to clot. Sera were removed after microcentrifugation for 5 min. The IL-6 and TNF contents in serum samples obtained 90 min after LPS-GalN administration were measured; this time point was found to be associated with the peak concentrations of TNF in serum after LPS administration in earlier experiments (data not shown). Cytokine-specific enzyme-linked immunosorbent assays (ELISAs) were performed as described in the original papers (18, 19). Purified recombinant TNF (Genzyme) and IL-6 (Biosource) were used as standards.

Monoclonal Abs to cytokines. Monoclonal Ab 20F-3 (anti-IL-6) was obtained from John Abrams, DNAX Research Institute of Cellular and Molecular Biology (19). It was made by Verax, New Lebanon, N.H., according to Good Manufacturing Procedures and was ascertained by Verax to be endotoxin free by the *Limulus* lysate assay. The lyophilized Ab was reconstituted in endotoxin-free PBS (Sigma) prior to use. Purified TN3-19.12 Ab (anti-TNF) was also diluted in

* Corresponding author.

TABLE 1. Effects of LPS doses on mortality[a]

LPS (ng)/mouse	Ab (mg)/mouse	% of mice dead[b]
0	0	0
6.25	0	57 ± 8
12.5	0	57 ± 10
25	0	64 ± 20
50	0	78 ± 14
100	0	90 ± 13
150	0	70 ± 4
200	0	70 ± 8
100	1[c]	85 ± 8
100	2[c]	95 ± 5
100	1[d]	100 ± 0

[a] The table shows that Ab to IL-6 does not protect against mortality. C57BL/6J mice were given various doses of LPS-GalN i.p. as indicated. Twenty-four hours later, percent mortality was assessed. At least 20 mice were included in each group.

[b] The data are means and standard deviations from three independent experiments.

[c] Ab was anti-IL-6, as described in Materials and Methods.

[d] Control Ab, anti-IL-5, used as described in Materials and Methods.

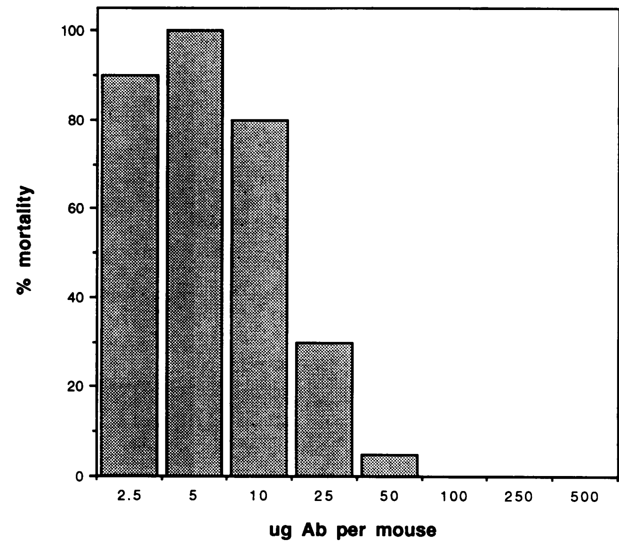

FIG. 1. Protection by anti-TNF was dose dependent. Various doses of anti-TNF were given. Eighteen hours after Ab or control treatment, mice were given LPS-GalN, and mortality was assessed 24 h later. Administration of 0.5 mg of control protein (purified hamster gamma globulin) per mouse resulted in 100% mortality.

endotoxin-free PBS for all experiments (18). As protein controls, purified hamster gamma globulin (Cappel) and purified anti-IL-5 (obtained from Robert Coffman, DNAX Research Institute of Cellular and Molecular Biology; same isotype as anti-IL-6 [17]) were used.

Statistical analysis. Data were analyzed by Student's t test.

RESULTS

Determination of the 90% lethal dose for LPS-GalN. Doses of LPS ranging from 6.25 to 200 ng per mouse were injected i.p. with D-galactosamine as described above. Mortality was assessed 24 h later. As shown in Table 1, a dose relationship was observed with doses up to 100 ng per mouse (90% mortality). In parallel experiments in which D-galactosamine was not coadministered with the LPS, animals survived doses of 1.5 mg for more than 72 h (data not shown).

Treatment with anti-IL-6 did not affect mortality. To identify the role of IL-6 in this lethal septic shock model, we injected mice i.p. with 1 or 2 mg of anti-IL-6 1 to 2 h prior to LPS-GalN treatment. These doses were shown previously to protect mice from death in a lethal bacteremia model (19). Isotype control Ab anti-IL-5 was used as a control protein in equal amounts. Table 1 shows that there was no protective effect against mortality with anti-IL-6 treatment. Because of the long circulating half-life of the isotype of anti-IL-6 (rat γ1κ; half-life, 10 days to 2 weeks), we gave the Ab the night before LPS-GalN administration in case 1 h was not long enough for all the anti-IL-6 to enter the general circulation from the peritoneal cavity. Even with overnight treatment, anti-IL-6 failed to confer any protection against mortality (data not shown). We concluded from these experiments that IL-6 was not playing a pathological role in this lethal septic shock model.

Treatment with anti-TNF protected mice against mortality. We measured the protective effect of the TN3-19.12 Ab in this septic shock model, which had not been determined previously for this particular antibody to TNF. Figure 1 shows the relationship between mortality and the average dose of anti-TNF administered. We found that 25 μg of anti-TNF given overnight routinely conferred 50 to 70% protection from death in our model. Lower doses (10 μg and below) conferred very little protection.

Treatment with anti-IL-6 potentiated mortality when TNF was partially neutralized. We wanted to see whether treatment with anti-IL-6 could confer any protection under conditions in which the circulating TNF concentration was extremely limited. Therefore, we treated mice with 1 or 2 mg of anti-IL-6 simultaneously with 25 μg of anti-TNF 18 h prior to LPS-GalN administration. Figure 2 summarizes the results of two such experiments. To our surprise, we found that treatment with anti-IL-6 enhanced mortality significantly. In one experiment, anti-TNF treatment alone resulted in 25% mortality whereas the combination of anti-IL-6 and anti-TNF Abs conferred 65% mortality. In another

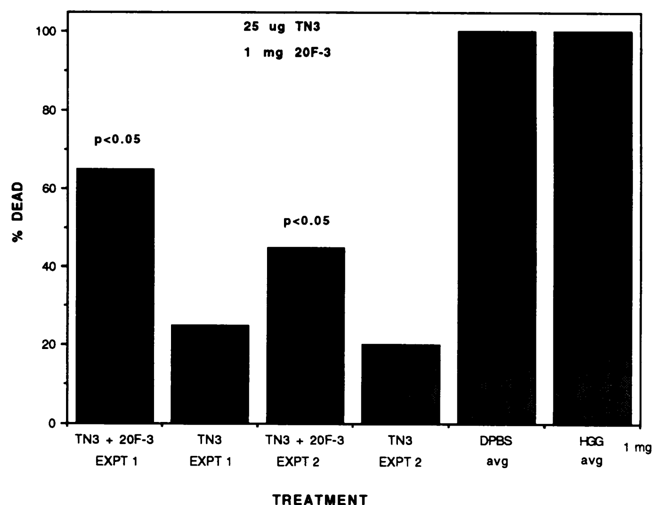

FIG. 2. Treatment with anti-IL-6 (20F-3) enhanced mortality when TNF was partially neutralized with anti-TNF (TN3). Mice (20 per group) were treated overnight with 25 μg of anti-TNF alone or supplemented with 1 mg of anti-IL-6 (20F-3) and then challenged with LPS-GalN. Twenty-four hours later, mortality was assessed. Data were analyzed by Student's t test. DPBS, Dulbecco's phosphate-buffered saline; HGG, hamster gamma globulin.

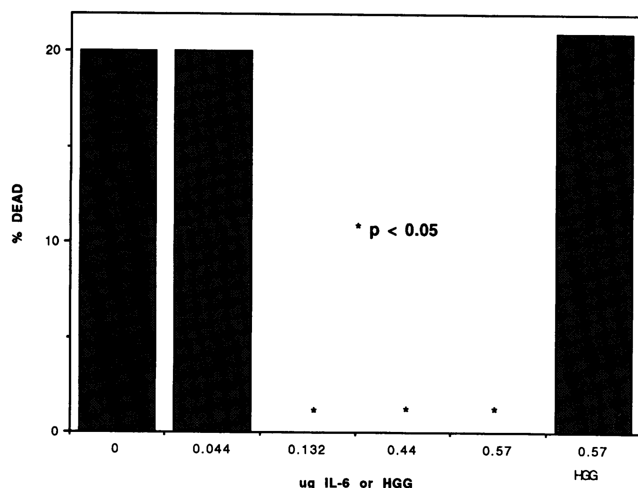

FIG. 3. Treatment with IL-6 enhanced protection from mortality when TNF was partially neutralized by anti-TNF. Mice (20 per group) were given IL-6 at the indicated doses 1 h before receiving LPS-GalN. Twenty-four hours later, mortality was assessed. Data were analyzed by Student's t test. HGG, hamster gamma globulin.

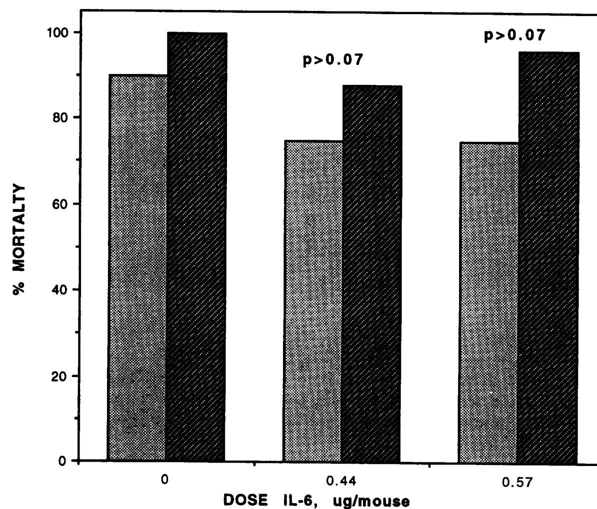

FIG. 4. IL-6 treatment without anti-TNF treatment did not enhance protection from mortality. Mice (20 per group) were given IL-6 at the indicated doses 1 h prior to receiving LPS-GalN. Twenty-four hours later, mortality was assessed. Data were analyzed by Student's t test. Shaded and hatched bars, experiments 1 and 2, respectively.

experiment, anti-TNF treatment alone resulted in 20% mortality but the combination of Abs gave 45% mortality. When only 10 μg of anti-TNF was given, there was no effect on mortality (data not shown). These data indicated to us that IL-6 may be playing a protective, rather than lethal, role in this septic shock model.

Treatment with rIL-6 protected against mortality when TNF was limited. On the basis of the data shown in Fig. 2, we administered recombinant IL-6 (rIL-6) i.p. to mice 1 h prior to LPS-GalN administration. Each of these mice had been treated the night before with 25 μg of anti-TNF. The doses of rIL-6 were decided upon as follows. Mice injected with 100 ng of LPS-GalN were bled 90 min later. The sera obtained were analyzed for TNF and IL-6 content at 90 min, because that is the time of peak TNF (the lethal cytokine) concentration. At 90 min after LPS-GalN administration, the concentration of TNF was 2 ± 1 ng/ml and the IL-6 concentration was 44 ± 13 ng/ml. Assuming a maximum total body fluid volume of 10 ml per mouse, we calculated that treatment with 100 ng of LPS-GalN resulted in 440 ng of IL-6 per mouse. Therefore, we gave this dose and higher and lower doses in half-log increments to see the effect of rIL-6 when TNF was limited by anti-TNF pretreatment.

Figure 3 shows that at doses of 132 to 570 ng per mouse, rIL-6 conferred significant protection against mortality. In these experiments, mortality was lowered from an average of 20% to 0 ($P < 0.05$) at 132, 440, and 570 ng per mouse. With lower doses tested (44 ng and less), no effect was observed. Thus, when TNF was partially neutralized, rIL-6 protected mice against death.

In experiments in which TNF was not partly neutralized with 25 μg of Ab to TNF 18 h prior to LPS-GalN treatment, rIL-6 failed to confer any effect (Fig. 4). Thus, the effect of IL-6 was protective only in situations in which TNF was limited by pretreatment with low doses of anti-TNF.

DISCUSSION

We have confirmed that TNF plays the major pathological role in the LPS-GalN septic shock model. Moreover, when TNF is partially limited, IL-6 protects against TNF-mediated mortality. The data are significant for several reasons. First, they emphasize the potential therapeutic value of limiting TNF levels in the treatment of septic shock. Second, they indicate that adjunct therapy with rIL-6 may be beneficial in the treatment of patients with septic shock. Third, the data demonstrate that TNF and IL-6 are not parallel-acting proinflammatory cytokines but, rather, can antagonize each other in vivo.

Because we wanted to use a murine model of septic shock requiring low LPS doses, we decided to use the LPS-GalN model developed by Galanos et al. (12, 15). These authors had shown that treatment with D-galactosamine affected UDP ribosylation in the liver but the other organs were not affected by the treatment nor was D-galactosamine treatment in the absence of LPS lethal. Other models using low doses of LPS, such as adrenalectomy (4) and actinomycin D treatment (3), affect many organs. While investigators had previously shown that macrophage products are involved in septic shock following LPS-GalN treatment (9, 11), they did not definitively show the role of TNF nor did they examine the role of IL-6 in this model.

Our data support the findings of Aderka et al. (1). These authors found that IL-6 inhibited TNF production by LPS-stimulated human monocytes and by mice in vivo. However, they did not show actual antagonism of a biological effect of TNF by IL-6, as we have shown. It is possible that treatment with rIL-6 1 h before LPS-GalN administration likewise attenuated TNF production but that the inhibition was not sufficient to affect fatality in the absence of anti-TNF pretreatment (Fig. 3 and 4). In any event, TNF and IL-6 form a complete feedback loop whereby TNF induces IL-6 synthesis (13) and IL-6 in turn inhibits TNF production (1). More recently, Franks et al. (8) showed that in mice pretreated with carrageenan there was an increase in survival and an increase in the levels of IL-6 in serum. In their model, IL-6 may be playing a protective role as well. Alcorn et al. (2) showed that inducing the acute-phase response with turpentine 24 h prior to challenge with LPS-GalN had a protective effect. This could be explained, in light of the data presented

here, by the generation of circulating IL-6 by the turpentine. The IL-6 would then generate the acute-phase response, and the mechanism of action for the protection could be due to the acute-phase proteins themselves. We are currently investigating this question. Since LPS-GalN did not inhibit IL-6 synthesis in vivo but D-galactosamine treatment is known to inhibit acute-phase protein transcription in the liver (14), it is likely that protection from lethal sepsis by IL-6 is mediated by the acute-phase response. These data indicate that IL-6 is not so much a proinflammatory cytokine like IL-1 or TNF but, rather, is part of a natural anti-inflammatory response, which includes a feedback loop on TNF synthesis. Furthermore, IL-6 induces adrenocorticotropin release (22) and thereby induces cortisol synthesis. Thus, it possible that IL-6 plays a major beneficial role in homeostasis via induction of glucocorticoids, as well as via the induction of the acute-phase response.

ACKNOWLEDGMENTS

We are indebted to Robert D. Schreiber, Washington University School of Medicine, for providing us with the anti-TNF Ab and for helpful discussions. We also thank J. P. Jakway, Department of Immunology, Schering-Plough Research, for performing the TNF ELISA on the serum samples.

REFERENCES

1. **Aderka, D., J. Le, and J. Vilcek.** 1989. IL-6 inhibits lipopolysaccharide-induced tumor necrosis factor production in cultured human monocytes, U937 cells, and in mice. J. Immunol. **143:** 3517–3523.
2. **Alcorn, J. M., J. Fierer, and M. Chojkier.** 1992. The acute-phase response protects mice from D-galactosamine sensitization to endotoxin and tumor necrosis factor-α. Hepatology **15:**122–129.
3. **Berry, L. J., and D. S. Smythe.** 1964. Effects of bacterial endotoxins on metabolism. VII. Enzyme induction and cortisone protection. J. Exp. Med. **120:**721–732.
4. **Bertini, R., M. Bianchi, and P. Ghezzi.** 1988. Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. J. Exp. Med. **167:**1708–1712.
5. **Beutler, B., I. W. Milsark, and A. C. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229:**869–871.
6. **Fong, Y., L. L. Moldawer, M. Marano, et al.** 1989. Endotoxemia elicits increased circulating β_2-IFN/IL-6 in man. J. Immunol. **142:**2321–2324.
7. **Fong, Y., K. J. Tracey, L. L. Moldawer, et al.** 1989. Antibodies to cachectin/tumor necrosis factor reduce interleukin 1β and interleukin 6 appearance during lethal bacteremia. J. Exp. Med. **170:**1627–1633.
8. **Franks, A. K., K. I. Kujawa, and L. J. Yaffe.** 1991. Experimental elimination of tumor necrosis factor in low-dose endotoxin models has variable effects on survival. Infect. Immun. **59:**2609–2614.
9. **Freudenberg, M. A., and C. Galanos.** 1988. Induction of tolerance to lipopolysaccharide (LPS)–D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. Infect. Immun. **56:**1352–1357.
10. **Freudenberg, M. A., and C. Galanos.** 1991. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. Infect. Immun. **59:**2110–2115.
11. **Freudenberg, M. A., D. Keppler, and C. Galanos.** 1986. Requirement for lipopolysaccharide-responsive macrophages in galactosamine-induced sensitization to endotoxin. Infect. Immun. **51:**891–895.
12. **Galanos, C., M. A. Freudenberg, and W. Reutter.** 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76:**5939–5943.
13. **Kohase, M., D. Hendriksen-DeStefano, L. T. May, J. Vilcek, and P. B. Sehgal.** 1986. Induction of β_2-interferon by tumor necrosis factor: a homeostatic mechanism in the control of cell proliferation. Cell **45:**659–666.
14. **Koj, A., and A. Dubin.** 1976. Inhibition of the liver and plasma protein acute-phase response in mice by D-galactosamine. Br. J. Exp. Pathol. **57:**733–741.
15. **Lehmann, V., M. A. Freudenberg, and C. Galanos.** 1987. Lethal toxicity of lipopolysaccharide and tumor necrosis factor in normal and D-galactosamine-treated mice. J. Exp. Med. **165:** 657–663.
16. **Rothstein, J. L., and H. Schreiber.** 1988. Synergy between tumor necrosis factor and bacterial products causes hemorrhagic necrosis and lethal shock in normal mice. Proc. Natl. Acad. Sci. USA **85:**607–611.
17. **Schumacher, J. H., A. O'Garra, B. Shrader, A. van Kimmenade, M. W. Bond, T. R. Mosmann, and R. L. Coffman.** 1988. The characterization of four monoclonal antibodies specific for mouse IL-5 and development of mouse and human IL-5 enzyme-linked immunosorbent assay. J. Immunol. **141:**1576–1581.
18. **Sheehan, K. C. F., N. H. Ruddle, and R. D. Schreiber.** 1989. Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. J. Immunol. **142:**3884–3893.
19. **Starnes, H. F., Jr., M. K. Pearce, A. Tewari, J. H. Yim, J.-C. Zou, and J. S. Abrams.** 1990. Anti-IL-6 monoclonal antibodies protect against lethal *Escherichia coli* infection and lethal tumor necrosis factor-α challenge in mice. J. Immunol. **145:**4185–4191.
20. **Tracey, K. J., Y. Fong, D. G. Hesse, et al.** 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature (London) **330:**662–664.
21. **Waage, A., P. Brandtzaeg, A. Halstensen, P. Kierulf, and T. Espevik.** 1989. The complex pattern of cytokines in serum from patients with meningococcal septic shock. J. Exp. Med. **169:** 333–338.
22. **Woloski, B. M. R. N. J., E. M. Smith, W. J. Meyer, G. M. Fuller, and J. E. Blalock.** 1985. Corticotropin-releasing factor activity of monokines. Science **230:**1035.