INFECTION AND IMMUNITY, Apr. 1996, p. 1437–1440 Vol. 64, No. 4
0019-9567/96/$04.00+0

Endogenous and Exogenous Interleukin-12 Augment the Protective Immune Response in Mice Orally Challenged with *Salmonella dublin*

TAMMY KINCY-CAIN, JOHN D. CLEMENTS, AND KENNETH L. BOST*

Department of Microbiology and Immunology, Tulane University Medical Center, New Orleans, Louisiana 70112-2699

Received 8 November 1995/Returned for modification 15 December 1995/Accepted 3 January 1996

Following oral challenge with *Salmonella dublin*, we observed significant increases in interleukin-12 (IL-12) protein expression in the mesenteric lymph nodes. The importance of this endogenous cytokine production in the immune response against *S. dublin* was demonstrated by in vivo depletion of IL-12 with an anti-IL-12 monoclonal antibody prior to oral *S. dublin* challenge. Mice pretreated with anti-IL-12 antibody had increased salmonellosis and reduced survival times compared with mice receiving control antibody. Furthermore, administration of exogenous murine recombinant IL-12 dramatically increased survival times of mice challenged orally with *S. dublin*. Together, these results demonstrate that endogenous and exogenous IL-12 significantly augment the mucosal immune response against the intracellular pathogen *S. dublin*.

Interleukin-12 (IL-12) is a heterodimer composed of 40- and 35-kDa subunits (8, 28) and has been suggested to play a critical role in the development of cell-mediated immune responses to intracellular pathogens (2, 10, 22). IL-12 not only augments gamma interferon (IFN-γ) production by Th1 lymphocytes (14–16, 23, 24) and natural killer (NK) cells (28) but also has been shown to diminish Th2 lymphocyte development (16, 23). These characteristics suggest that this cytokine plays an important role in directing the initial immune response toward cell-mediated immunity. Consistent with this hypothesis, exogenously administered IL-12 greatly augments immune responses against intracellular pathogens, including *Leishmania* (1, 9, 18, 25), *Toxoplasma* (7, 12, 13), and *Mycobacterium* (5, 6) spp. and several viruses (19, 21) whereas treatment with anti-IL-12 antibodies reduces immunity against *Leishmania* (25), *Listeria* (26), and *Brucella* (30) spp.

Despite these numerous investigations, the contribution of IL-12 to the initiation of mucosal immune responses is not clear. With the goal of beginning to understand the molecular mechanisms which contribute to protective immunity at mucosal surfaces, we recently reported for the first time (3) the ability of a live attenuated *Salmonella* strain expressing a foreign protein antigen to stimulate expression of IL-12 mRNA in the Peyer's patches and mesenteric lymph nodes of mice within hours of oral inoculation. These results suggested that IL-12 may play an important role in the cellular immune response against *Salmonella*-based vaccine delivery systems. The studies described in this report extend these findings by demonstrating that endogenously produced IL-12 is involved in the survival of mice challenged orally with virulent wild-type *Salmonella* strains. Furthermore, we show that administration of exogenous recombinant IL-12 enhances survival of mice challenged with *Salmonella* strains. Together, these findings support the importance of IL-12 in induction of protective cell-mediated immune responses at mucosal sites.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Tulane University Medical Center, 1430 Tulane Ave., New Orleans, LA 70112. Phone: (504) 582-7956. Fax: (504) 588-5144.

Increased levels of IL-12 in the mesenteric lymph nodes of mice challenged orally with *S. dublin*. The quantification of cytokine production in vivo is often difficult, since many cytokines have significant effects at very low concentrations and since quantification by certain techniques (e.g., immunocytochemical staining) can be difficult. We took advantage of the sensitive and quantitative nature of a bioassay for IL-12 (29) to demonstrate increases in the production of this cytokine in the mesenteric lymph nodes of mice challenged orally with *S. dublin*. This bioassay is based on the ability of IL-12 to induce the secretion of IFN-γ by mononuclear splenocytes in a quantitative fashion (29). Furthermore, the sensitivity and specificity of this bioassay are ensured by capturing IL-12 present in supernatants or biologic fluids onto microtiter wells coated with a monoclonal anti-IL-12 antibody (designated C15.1) prior to determining IFN-γ induction. This antibody has the unique property of binding IL-12, but does not effectively inhibit IL-12 biologic activity.

To determine the quantitative nature of this bioassay and to generate a standard curve, 96-well plates were coated with 20 μg of protein G-purified monoclonal anti-IL-12 antibody, C15.1 (a generous gift of G. Trinchieri, Wistar Institute, Philadelphia, Pa.), per ml. After unbound antibody was washed off, different dilutions of murine recombinant IL-12 (mrIL-12) (batch MRB 02294-2; kindly provided by J. Sypek, Genetics Institute, Andover, Mass.) were added and allowed to bind. BALB/c splenic mononuclear cells (2×10^6) were added to each well, and the plates were incubated at 37°C for 48 h to allow IL-12-induced IFN-γ production. Culture supernatants were then assayed for IFN-γ production by capture enzyme-linked immunosorbent assay (ELISA) (Intertest-γ; Genzyme Corp., Cambridge, Mass.), and the amount of IFN-γ produced was determined by extrapolation from standard curves generated with recombinant IFN-γ (rIFN-γ). Figure 1 shows the results of one such bioassay, in which the amount of IFN-γ produced is plotted against the amount of mrIL-12 added. This bioassay was quite sensitive since as little as 5 pg of mrIL-12 per ml could induce significant IFN-γ production. Furthermore, there was a linear relationship for IL-12-induced IFN-γ production between 10 and 1,000 pg of added mrIL-12 per ml.

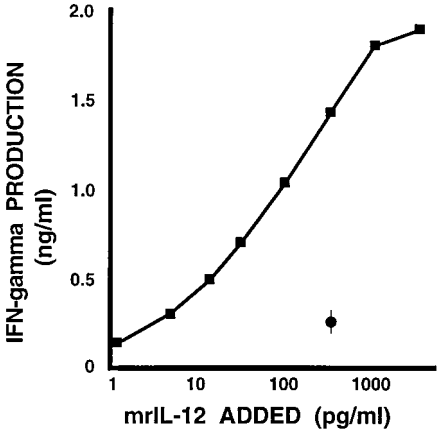

FIG. 1. Quantitative bioassay for IL-12 based on IFN-γ production. The ability of IL-12 to induce the production of IFN-γ by splenic mononuclear cells was used to quantify IL-12 secretion. The results of one representative bioassay are shown here. The amount of IFN-γ produced is plotted against the amount of mrIL-12 added (■). The specificity of the assay was ensured since the addition of the neutralizing monoclonal anti-IL-12 antibody, C17.8, to wells containing 300 pg of mrIL-12 blocked >90% of the IFN-γ production (●).

The specificity of the assay was ensured, since the addition of a neutralizing monoclonal anti-IL-12 antibody (designated C17.8; kindly provided by G. Trinchieri) to wells containing 300 pg of mrIL-12 per ml blocked >90% of the IFN-γ production (Fig. 1).

By using this bioassay, it was possible to demonstrate upregulated production of IL-12 in the mesenteric lymph nodes of mice orally challenged with *S. dublin*. For this study, groups of female BALB/c mice (Charles River, Wilmington, Mass.) were inoculated orally with 0.5 ml of sterile saline or with 10^7 log-phase wild-type *S. dublin* cells (strain SL1363 [11]) suspended in 0.5 ml of sterile saline. At 36 h after oral inoculation, mesenteric lymph nodes were removed and gently homogenized in cold, sterile saline. Cellular debris was removed by centrifugation (13,000 × *g* for 10 min), and supernatants were stored at −70°C for quantification of IL-12 by the bioassay. By using the bioassay, IL-12 production in the mesenteric lymph nodes of mice challenged orally with *S. dublin* (40.1 ± 16.7 pg/mg of tissue) was significantly increased over that in control mice given only saline (9.8 ± 3.8 pg/mg of tissue). Similar results were obtained in two separate studies containing four mice per group. These results not only substantiate our previous findings of increased IL-12 mRNA expression in vivo following oral challenge with *S. dublin* (3) but also demonstrate secretion of biologically active IL-12 in situ. This is an important consideration, since it has been recently suggested that the presence of free IL-12p40 subunits may function as an antagonist of IL-12 activity (17).

Pretreatment of mice with anti-IL-12 antibody increases systemic salmonellosis. Because of the observed increase in IL-12 levels in mucosal tissues following oral *Salmonella* challenge, we questioned whether endogenously produced IL-12 was an important component of the mucosal immune response against this pathogen. Groups of mice were given intraperitoneal injections of 1 mg of rat anti-mouse IL-12 (C17.15; generously provided by G. Trinchieri), which has previously been shown to be an effective antagonist of IL-12 activity in vivo (29), or (as a control) 1 mg of rat immunoglobulin G (IgG) 4 days and 1 day prior to oral challenge with 10^6 *S. dublin* SL1363 cells. This dose of bacteria, which is approximately the 50% lethal dose (LD_{50}) for BALB/c mice, was selected for these studies, so that any increase in the development of salmonellosis in mice treated with anti-IL-12 antibodies could be observed.

Table 1 demonstrates the effect of such antibody treatment on the development of salmonellosis in mice. Animals treated with anti-IL-12 antibodies had advanced disease by 4 days postchallenge, as indicated by significant decreases in body weight as well as significant increases in spleen weights when compared with those of control mice receiving rat IgG. Furthermore, dissemination of *S. dublin* into the peripheral organs was greatly enhanced in mice treated with anti-IL-12, as shown by bacterial colony counts in the spleen.

Pretreatment of mice with anti-IL-12 antibody reduces survival after oral *Salmonella* challenge. To further investigate the importance of endogenously produced IL-12 in the mucosal immune response against *Salmonella* spp., groups of mice (six per group) were injected with 1 mg of anti-IL-12 (C17.15) or 1 mg of rat IgG 4 days and 1 day prior to oral challenge with 10^7 *S. dublin* cells.

Figure 2 shows that mice treated with anti-IL-12 antibody had dramatically reduced rates of survival compared with controls. In fact, all six mice in the anti-IL-12 treatment group died before any of the six control animals did. Taken together, the results in Table 1 and Fig. 2 strongly support a role for endogenous IL-12 production in the protective mucosal immune response against oral challenge with *Salmonella* spp.

Administration of mrIL-12 enhances survival of mice challenged orally with *S. dublin*. Since endogenously produced IL-12 was important in the response against *Salmonella* spp., we examined whether exogenously administered mrIL-12 could also augment survival. Groups of six mice were implanted subcutaneously with osmotic pumps (ALZET model 2002; Alza Corporation, Palo Alto, Calif.) containing 10 μg of mrIL-12 (Genetics Institute) or sterile saline 2 days prior to oral challenge with 10^7 *S. dublin* cells. These osmotic pumps provided a continual release of mrIL-12 (approximately 710 ng of mrIL-12 per day) over a 14-day period.

TABLE 1. Increased salmonellosis in mice treated with anti-IL-12 antibody

Antibody treatment[a]	*Salmonella* challenge (no. of cells)[b]	Body wt (g)[c]	Spleen wt[d] (mg)	Colony counts[e] (no. of colonies/spleen)
Anti-IL-12 (C17.15)	10^6	15.93 ± 1.30*[f]	185 ± 25*	(8.3 ± 7) × 10^7*
Rat IgG	10^6	19.24 ± 0.39	90 ± 10	0
None[g]	0	21.40 ± 0.50	96 ± 6	0

[a] BALB/c mice were given intraperitoneal injections with either 1 mg of anti-IL-12 antibody (C17.15) or 1 mg of rat IgG 4 days and 1 day prior to challenge with *S. dublin* SL1363. Results are representative of three separate experiments with two mice per group.

[b] Mice were inoculated orally with 10^6 log-phase *S. dublin* SL1363 cells in 0.5 ml of sterile saline.

[c] Mice were weighed immediately before sacrifice at 4 days after inoculation with *S. dublin*. Results are presented as mean body weight ± standard deviation.

[d] Spleens were excised and weighed. Results are presented as mean spleen weight ± standard deviation.

[e] Spleen fragments were dispersed in sterile saline over a mesh screen. Various dilutions of the spleen suspensions were spread onto Luria agar plates, and the numbers of *Salmonella* colonies were determined by counting the bacterial colonies after incubation for 24 h at 37°C. Data represent the mean number of bacterial colonies per spleen ± standard deviation.

[f] Asterisks indicate that values obtained from anti-IL-12-treated mice were significantly different from values obtained from mice treated with control antibody ($P < 0.05$).

[g] Values for age- and sex-matched mice which were not treated with antibody and not challenged with *S. dublin* are given for comparison purposes.

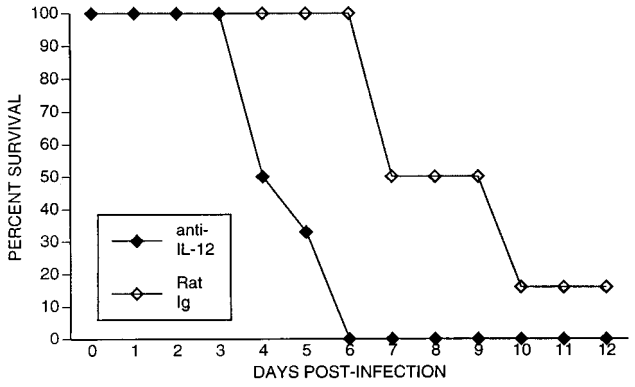

FIG. 2. Depletion of endogenous IL-12 exacerbates *Salmonella* infection in mice. BALB/c mice were given intraperitoneal injections of 1 mg of rat anti-murine IL-12 or rat IgG 4 days and 1 day prior to oral challenge with 10^7 log-phase wild-type *S. dublin* SL1363 cells. Results are presented as percent survival of mice in each group over time. Each group consisted of six mice, and this entire experiment was performed three times with similar results.

As shown in Fig. 3, treatment with exogenous mrIL-12 markedly increased the survival times of mice challenged with a lethal dose of *S. dublin*. In fact, all of the control mice died by day 6 postchallenge, while none of the IL-12-treated mice died until day 11. These findings clearly demonstrate a therapeutic role for mrIL-12 in resistance to orally administered *Salmonella* cells and suggest that such treatments augment cell-mediated immunity at mucosal sites.

It is clear, however, that while our method of administering mrIL-12 had protective effects, it did not cure mice orally inoculated with this dose of *S. dublin* (i.e., a dose representing approximately 10 times the LD_{50}). We reasoned that if treatment with mrIL-12 could affect survival after administration of such an overwhelming inoculum, this would demonstrate the importance of IL-12 in resistance to *Salmonella* spp. It is not clear at present if treatment with mrIL-12 would cure mice inoculated with a lower dose of *Salmonella* spp. or whether administration of mrIL-12 at "optimal" doses or kinetics would facilitate the survival of inoculated mice. Such studies are under way in our laboratories.

Initially, studies in murine models of other diseases caused by intracellular pathogens such as *Leishmania* (9, 18, 25), *Listeria* (26), *Mycobacterium* (5, 6), *Brucella* (30), and *Toxoplasma* (7, 12, 13) spp. established a critical role for IL-12 in the generation of a protective immune response against these pathogens. This report demonstrates that IL-12 is also important in the immune response to *Salmonella* spp., which are gut mucosal pathogens. The mechanism by which IL-12 provides a protective effect in mice infected with *Salmonella* spp. is not known. However, because of the intracellular nature of *Salmonella* spp., it is probable that early protective immunity involves IL-12-elicited production of IFN-γ by NK cells, as reported previously (20). Therefore, it appears that IL-12 most probably influences initiation of immune responses against this intracellular pathogen by non-antigen-specific mechanisms.

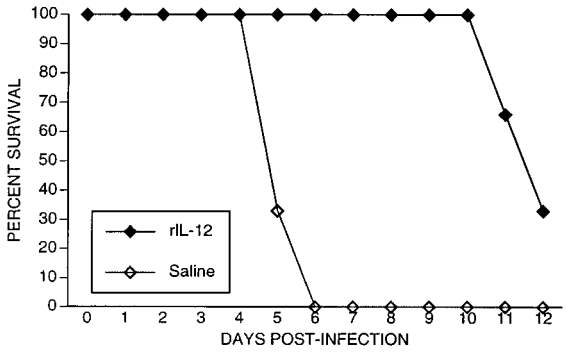

FIG. 3. Treatment with mrIL-12 increases the survival time of mice infected with *S. dublin*. Osmotic pumps containing 10 μg of mrIL-12 or sterile saline were implanted in BALB/c mice 2 days prior to oral challenge with 10^7 log-phase wild-type *S. dublin* SL1363 cells. Results are presented as percent survival of mice in each group over time. Each group consisted of six mice, and this entire experiment was performed three times with similar results.

It does not appear likely that *Salmonella* lipopolysaccharide contributes significantly to increased IL-12 expression in vivo. Support for this notion includes the induction of increased IL-12 production both in vivo and in vitro by intracellular pathogens which do not contain lipopolysaccharide (5, 7, 9, 27), as well as the lack of excessive expression of constitutive IL-12 despite the presence of a substantial number of gram-negative organisms expressing lipopolysaccharide in the normal gut flora. Moreover, we have recently demonstrated, by using in vitro macrophage cultures, that LPS does not contribute significantly to *Salmonella*-induced IL-12 expression (4).

This report establishes a role for endogenously produced IL-12 in the induction of a protective mucosal immune response against *Salmonella* infections. Furthermore, we demonstrated that administration of exogenous mrIL-12 augmented the mucosal immune response against *Salmonella* spp., suggesting that such treatments have some therapeutic value. While our previous work suggested a role for IL-12 in the initiation of mucosal immunity against *Salmonella* constructs used as delivery systems for subunit vaccines (3), the present study extends these findings to show that endogenously produced IL-12 contributes to protection against wild-type *Salmonella* infections. Whether these findings can be used to enhance the protective effects of candidate mucosal vaccines is the focus of ongoing investigations in our laboratory.

This work was supported by Public Health Service grants AI28835, AI36519, and AI32976 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

1. **Afonso, L. C. C., T. M. Scharton, L. Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major*. Science **263:**235–237.
2. **Biron, C. A., and R. T. Gazzinelli.** 1995. Effects of IL-12 on immune responses to microbial infections: a key mediator in regulating disease outcome. Curr. Opin. Immunol. **7:**485–496.
3. **Bost, K. L., and J. D. Clements.** 1995. In vivo induction of interleukin-12 mRNA expression after oral immunization with *Salmonella dublin* or the B subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **63:**1076–1083.
4. **Chong, C., K. L. Bost, and J. D. Clements.** 1996. Differential production of interleukin-12 mRNA by murine macrophages in response to viable or killed *Salmonella* spp. Infect. Immun. **64:**1154–1160.
5. **Cooper, A. M., A. D. Roberts, E. R. Rhoades, J. E. Callahan, D. M. Getzy, and I. M. Orme.** 1995. The role of IL-12 in acquired immunity to *Mycobacterium tuberculosis* infection. Immunology **84:**423–432.
6. **Flynn, J. L., M. M. Goldstein, K. J. Triebold, J. Sypek, S. Wolf, and B. R. Bloom.** 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. J. Immunol. **155:**2515–2524.
7. **Gazzinelli, R. T., S. Hieny, T. A. Wynn, S. Wolf, and A. Sher.** 1993. IL-12 is required for the T-lymphocyte-independent induction of interferon-gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. Proc. Natl. Acad. Sci. USA **90:**6115–6119.
8. **Gubler, U., A. O. Chua, D. S. Schoenhaut, C. M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A. G. Wolitzky, P. M. Quinn, P. C. Familletti, and M. K. Gately.** 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. Proc. Natl. Acad. Sci. USA **88:**4143–4147.

9. **Heinzel, F. P., D. S. Schoenhaut, R. M. Rerko, L. E. Rosser, and M. K. Gately.** 1993. Recombinant IL-12 cures mice infected with *Leishmania major*. J. Exp. Med. **177:**1505–1509.
10. **Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra, and K. M. Murphy.** 1993. Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. Science **260:**547–549.
11. **Hsu, H. S.** 1989. Pathogenesis and immunity in murine salmonellosis. Microbiol. Rev. **53:**390–409.
12. **Hunter, C. A., C. S. Subauste, V. H. Van Cleave, and J. S. Remington.** 1994. Production of gamma interferon by natural killer cells from *Toxoplasma gondii*-infected SCID mice. Infect. Immun. **62:**2818–2824.
13. **Khan, I. A., T. Matsuura, and L. H. Kasper.** 1994. IL-12 enhances murine survival against acute toxoplasmosis. Infect. Immun. **62:**1639–1642.
14. **Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri.** 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J. Exp. Med. **170:**827–845.
15. **Manetti, R., F. Gerosa, M. G. Giudizi, R. Biagiotti, P. Parronchi, M.-P. Piccinni, S. Sampognaro, E. Maggi, S. Romagnani, and G. Trinchieri.** 1994. IL-12 induces stable priming for interferon gamma production during differentiation of human T helper (Th) cells and transient interferon-gamma production in established Th2 cell clones. J. Exp. Med. **179:**1273–1283.
16. **Manetti, R., P. Parronchi, M. G. Giudizi, M. P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani.** 1993. Natural killer cell stimulatory factor (interleukin-12) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4 producing cells. J. Exp. Med. **177:** 1199–1204.
17. **Mattner, R., S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rude, and T. Germann.** 1993. The p40 subunit of IL-12 inhibits bioactivity of the IL-12 heterodimer. Eur. J. Immunol. **23:**2202–2208.
18. **Murray, H. W., and J. Hariprashad.** 1995. Interleukin-12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. J. Exp. Med. **181:**387–391.
19. **Orange, J. S., S. F. Wolf, and C. A. Biron.** 1994. Effects of IL-12 on the response and susceptibility to experimental viral infections. J. Immunol. **152:** 1253–1264.
20. **Ramarathinam, L., D. W. Neisel, and G. R. Klimpel.** 1993. *Salmonella typhimurium* induces interferon-gamma production in murine splenocytes. J. Immunol. **150:**3973–3981.
21. **Schijns, V. E. C. J., B. L. Haagmans, and M. C. Horzinek.** 1995. IL-12 stimulates an antiviral type 1 cytokine response but lacks adjuvant activity in interferon gamma receptor deficient mice. J. Immunol. **155:**2525–2532.
22. **Scott, P.** 1993. IL-12: initiation cytokine for cell-mediated immunity. Science **260:**496–497.
23. **Seder, R. A., R. Gazzinelli, A. Sher, and W. E. Paul.** 1993. IL-12 acts directly on CD4+ T cells to enhance priming for gamma-interferon production and diminishes IL-4 inhibition of such priming. Proc. Natl. Acad. Sci. USA **90:** 10188–10192.
24. **Stern, A. S., F. J. Podlaski, J. D. Hulmes, Y. E. Pan, P. M. Quinn, A. G. Wolitsky, C. Familletti, D. L. Stremlo, T. Truitt, R. Chizzonite, and M. K. Gately.** 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc. Natl. Acad. Sci. USA **87:**6808–6812.
25. **Sypek, J. P., C. L. Chung, S. E. H. Mayor, J. M. Subramanyam, S. J. Goldman, D. S. Sieburth, S. F. Wolf, and R. G. Schaub.** 1993. Resolution of cutaneous leishmaniasis: interleukin-12 initiates a protective T helper type 1 immune response. J. Exp. Med. **177:**1797–1802.
26. **Tripp, C. S., M. K. Gately, J. Hakimi, P. Ling, and E. R. Unanue.** 1994. Neutralization of IL-12 decreases resistance to *Listeria* in SCID and C.B-17 mice. Reversal by gamma-interferon. J. Immunol. **152:**1883–1887.
27. **Vieira, L. Q., B. D. Hondowicz, L. C. C. Afonso, M. Wysocka, G. Trinchieri, and P. Scott.** 1994. Infection with *Leishmania major* induces interleukin-12 production in vivo. Immunol. Lett. **40:**157–161.
28. **Wolf, S. F., P. A. Temple, M. Kobayashe, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, K. Kelleher, S. H. Herrmann, S. C. Clark, L. Azzoni, S. H. Chan, G. Trinchieri, and B. Perussia.** 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J. Immunol. **146:** 3074–3081.
29. **Wysocka, M., M. Kubin, L. Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri.** 1995. Interleukin-12 is required for interferon-gamma production and lethality in lipopolysaccharide-induced shock in mice. Eur. J. Immunol. **25:**672–676.
30. **Zhan, Y., and C. Cheers.** 1995. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. Infect. Immun. **63:**1387–1390.

Editor: R. E. McCallum