INFECTION AND IMMUNITY, May 1997, p. 1953–1955 Vol. 65, No. 5
0019-9567/97/$04.00+0

Arabinofuranosyl-Terminated and Mannosylated Lipoarabinomannans from *Mycobacterium tuberculosis* Induce Different Levels of Interleukin-12 Expression in Murine Macrophages

ATSUSHI YOSHIDA* AND YUKIO KOIDE

Department of Microbiology and Immunology, School of Medicine, Hamamatsu University, Hamamatsu 431-31, Japan

Received 26 August 1996/Returned for modification 17 September 1996/Accepted 6 February 1997

Lipoarabinomannan (LAM) is a major surface lipoglycan of *Mycobacterium tuberculosis*. In the present study, we demonstrated that arabinofuranosyl-terminated LAM (AraLAM) derived from a rapidly growing *Mycobacterium* sp., but not extensively mannosylated LAM derived from the Erdman strain, is capable of inducing interleukin-12 (IL-12) expression in murine macrophages. Since IL-12 is known to drive the differentiation of naive T cells toward T-helper type 1 (Th1) cell development, AraLAM may be an effective adjuvant in vaccines and immunotherapies that need Th1 responses.

Lipoarabinomannan (LAM) is the major antigen in the mycobacterial cell envelope (9). Two types of LAM have been described, arabinofuranosyl-terminated LAM (AraLAM) derived from a rapidly growing *Mycobacterium* sp. and heavily mannosylated LAM (ManLAM) derived from the Erdman strain (5). Although AraLAM potently induces expression of immediate-early genes such as those for c-fos, KC, JE, and tumor necrosis factor alpha in murine macrophages, ManLAM fails to do so (12, 14, 18). AraLAM is also known to elicit inducible nitric oxide synthase in macrophages (13), resulting in the production of nitric oxide, a potent inhibitor of various intracellular pathogens, including *Mycobacterium tuberculosis* (2, 7, 17). Thus, in the absence of mannosyl capping, AraLAM is capable of inducing several important functions of macrophages. In this context, we examined the effects of AraLAM and ManLAM on induction of interleukin-12 (IL-12) in macrophages since several investigators, including ourselves, have demonstrated that IL-12 has an obligatory role in the generation of T-helper type 1 cells that play a major role in potent T-cell-dependent protection against infection with intracellular pathogens, including *Mycobacterium* sp. (6, 8, 17). First, murine macrophage cell line J774, provided by the Japanese Cancer Research Bank, was treated with either AraLAM or ManLAM (AraLAM and ManLAM contained 12.7 and 3.7 ng of endotoxin [kindly provided by J. T. Belisle] per mg, respectively) (4, 5) at various concentrations and examined for IL-12 mRNA expression. Since the IL-12 heterodimer p35 subunit is constitutively expressed (3), we examined the expression of the p40 subunit mRNA of IL-12 by employing competitive quantitative reverse transcription (RT)-PCR. J774 cells were cultured in 12-well flat-bottom culture plates (Costar, Cambridge, Mass.) at 2×10^6/well in serum-free Cosmedium 004 (COSMO BIO Co., Ltd., Tokyo, Japan) at 37°C to exclude the effect of possible contamination with the lipopolysaccharide in fetal calf serum. After 8 h of incubation with either AraLAM or ManLAM, the cells were washed twice and total RNA was isolated. The RT-PCR method was performed as previously reported (17), except for the addition of competitor DNAs. Competitors were constructed with PCR MIMIC Construction Kit (CLONTECH Laboratories, Inc., Palo Alto, Calif.), and a 2.0-μl aliquot of each competitor was added to a 25-μl reaction mixture (0.05 amol of IL-12 or 0.2 amol of β-actin). The sizes of the competitive cDNAs amplified by PCR were approximately 606 and 602 bp for IL-12 and β-actin, respectively, although the sizes of wild-type cDNAs amplified by PCR were approximately 396 and 150 bp for IL-12 and β-actin, respectively. For each sample, the number of densitometric units (wild-type cDNA/constructed competitor DNA) in the IL-12 band was normalized to that found in the β-actin band, which was the internal standard. As shown in Fig. 1, AraLAM, but not ManLAM, appeared to induce the IL-12 transcript in a dose-dependent manner, producing a detectable increase at 2 μg/ml and maximal induction at 5 μg/ml. AraLAM-inducible IL-12 mRNA was not inhibited by polymyxin B, indicating that the induction was not due to contamination of LAM with endotoxin (data not shown). The possibility exists that a small amount of IL-12 cDNA in the sample, such as ManLAM-stimulated cells, is undetectable due to the presence of excess competitor. Therefore, titration experiments were performed in which constant amounts of IL-12 (p40) cDNA (0.005, 0.05, and 0.5 amol) were amplified in the presence of serially diluted IL-12 MIMIC competitors (0.2, 0.05, 0.01, and 0.002 amol). However, smaller amounts of competitors

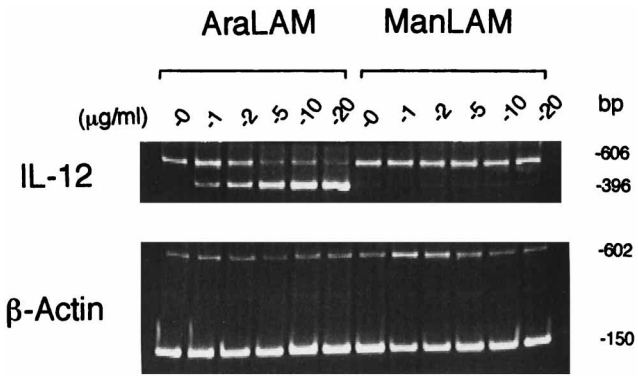

FIG. 1. Differential induction of IL-12 (p40) transcripts by AraLAM and ManLAM in J774 cells. J774 cells were incubated for 8 h with AraLAM or ManLAM at the concentrations shown. Total RNA was isolated for first-strand cDNA synthesis, and competitive RT-PCR was performed for IL-12 (p40) and β-actin. Ten microliters of the PCR product was electrophoresed in each lane. The wild-type cDNA band (lower band) and the construct band (upper band) were visualized by ethidium bromide staining.

* Corresponding author. Mailing address: Department of Microbiology and Immunology, School of Medicine, Hamamatsu University, 3600 Handa-cho, Hamamatsu 431-31, Japan. Phone: 81-53-435-2335. Fax: 81-53-435-2335. E-mail: yoshida@hama-med.ac.jp.

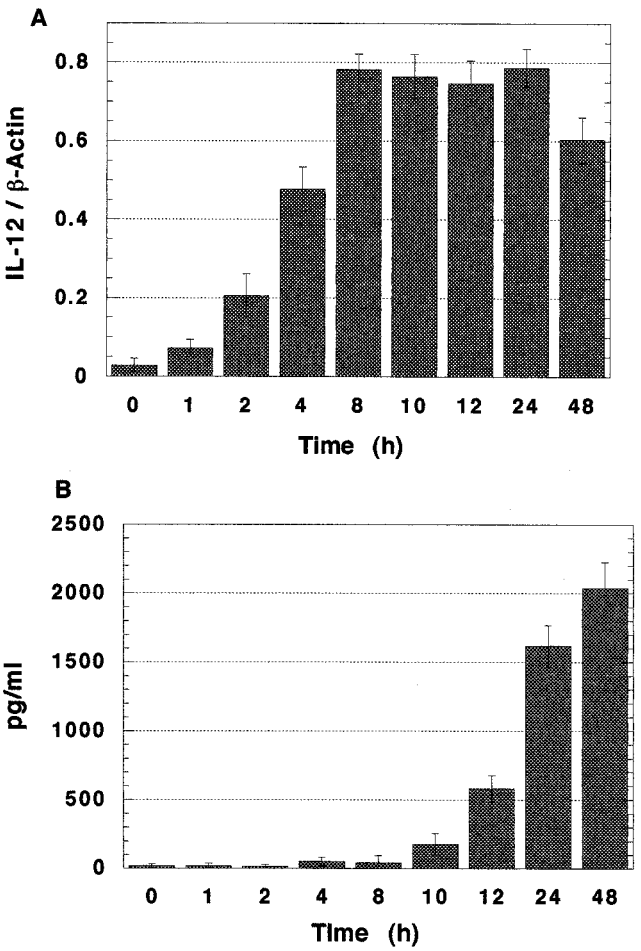

FIG. 2. Time course of IL-12 accumulation induced by AraLAM. (A) IL-12 (p40) mRNA accumulation. J774 cells were incubated with AraLAM (5 μg/ml) for the times indicated. Total RNA was isolated for first-strand cDNA synthesis, and competitive RT-PCR was performed. Ten microliters of the PCR product was electrophorsed in each lane. After ethidium bromide staining, quantitation of transcripts was accomplished by comparing amplification of the wild-type cDNA band with the construct band by using imaging densitometry. Each sample number (wild-type cDNA/constructed competitor DNA) of densitometric units in the IL-12 band was normalized to that found in the β-actin band, which was the internal standard. Results are expressed as the mean ± standard deviation for five independent experiments. (B) Time course of IL-12 production. J774 cells were incubated with AraLAM (5 μg/ml) for the times indicated. Culture supernatants were collected and then examined for IL-12 production by enzyme-linked immunosorbent assay. Results are expressed as the mean ± standard deviation for five independent experiments.

(0.01 and 0.002 amol) were incapable of increasing the density of amplified bands for 0.05 amol of IL-12 cDNA, the minimum amount needed to produce detectable bands after PCR amplification (data not shown).

Figure 2A shows the time course of IL-12 mRNA accumulation in J774 cells treated with 5 μg of AraLAM per ml. Induction of IL-12 mRNA was apparent by 2 h, although maximal induction required 8 h. We also evaluated IL-12 p40 production by J774 cells induced by AraLAM and ManLAM. IL-12 production was assessed by enzyme-linked immunosorbent assay with anti-murine IL-12 monoclonal antibodies (C15.1 and C15.6; kindly provided by Trinchieri) as previously described (16). Consistent with the finding on mRNA expression, AraLAM, but not ManLAM, was capable of inducing IL-12 production. Production of IL-12 induced by AraLAM was readily detectable by 10 h poststimulation, later than mRNA expression (Fig. 2B). To exclude the possibility that the IL-12 mRNA expression induced by AraLAM was unique to J774 cells, we examined the effects of AraLAM and ManLAM on induction of IL-12 mRNA and protein in glycogen-elicited peritoneal macrophages. Again, AraLAM, but not ManLAM, appeared to induce the IL-12 transcript and protein in a dose-dependent manner (data not shown).

Taken together, these data indicated that AraLAM, but not ManLAM, can induce IL-12 expression in both a macrophage cell line and glycogen-elicited peritoneal macrophages. Earlier studies suggested that the different abilities of LAMs to induce the expression of several cytokines and immediate-early genes is an important determinant of virulence (1, 12, 14, 18). However, LAM from avirulent *M. tuberculosis* H37Ra and that from vaccine strain *M. bovis* BCG have both been recently shown to be mannose capped; therefore, mannose capping may not be directly correlated with virulence (10, 15). Mannose capping is, rather, considered to be a common feature of LAMs from all strains of *M. tuberculosis* (10). Furthermore, it has been shown that BCG is a potent inducer of IL-12 in macrophages (17) and that tuberculosis patients have large numbers of IL-12 producers in their peripheral blood (11), suggesting that some component(s) other than LAM is also responsible for the induction of IL-12 in *Mycobacterium* sp. Nevertheless, AraLAM, a powerful inducer of IL-12, may be an effective adjuvant for initiation of protective T-helper type 1 responses against intracellular pathogens and tumors.

We thank John T. Belisle (Colorado State University, Fort Collins) for providing LAMs through National Institutes of Health contract No1-AI-25147, entitled "Tuberculosis Research Materials," and Giorgio Trinchieri (The Wistar Institute, Philadelphia, Pa.) for anti-murine IL-12 monoclonal antibodies (C15.1 and C15.6).

This work was supported in part by a grant-in-aid from the Ministry of Education, Science, and Culture, Japan.

REFERENCES

1. **Barnes, P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin.** 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. J. Immunol. **149:**541–547.
2. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.
3. **Chan, S. H., M. Kobayashi, D. Santoli, B. Perussia, and G. Trinchieri.** 1992. Mechanisms of IFN-γ induction by natural killer cell stimulatory factor (NKSF/IL-12). Role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. J. Immunol. **148:**92–98.
4. **Chatterjee, D., C. M. Bozic, M. McNeil, and P. J. Brennan.** 1991. Structural features of the arabinan component of the lipoarabinomannan of *Mycobacterium tuberculosis*. J. Biol. Chem. **266:**9652–9660.
5. **Chatterjee, D., K. Lowell, B. Rivoire, M. R. McNeil, and P. J. Brennan.** 1992. Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannosyl residues in some strains. J. Biol. Chem. **267:**6234–6239.
6. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
7. **Evans, T. G., L. Thai, D. L. Granger, and J. J. Hibbs.** 1993. Effect of in vivo inhibition of nitric oxide production in murine leishmaniasis. J. Immunol. **151:**907–915.
8. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom.** 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. **178:**2249–2254.
9. **Hunter, S. W., H. Gaylord, and P. J. Brennan.** 1986. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. J. Biol. Chem. **261:**12345–12351.
10. **Khoo, K. H., A. Dell, H. R. Morris, P. J. Brennan, and D. Chatterjee.** 1995. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. J. Biol. Chem. **270:**12380–12389.
11. **Munk, M. E., P. Mayer, P. Anding, K. Feldmann, and S. H. Kaufmann.** 1996. Increased numbers of interleukin-12-producing cells in human tuberculosis. Infect. Immun. **64:**1078–1080.

12. **Roach, T. I., C. H. Barton, D. Chatterjee, and J. M. Blackwell.** 1993. Macrophage activation: lipoarabinomannan from avirulent and virulent strains of *Mycobacterium tuberculosis* differentially induces the early genes c-fos, KC, JE, and tumor necrosis factor-α. J. Immunol. **150:**1886–1896.
13. **Roach, T. I., C. H. Barton, D. Chatterjee, F. Y. Liew, and J. M. Blackwell.** 1995. Opposing effects of interferon-γ on iNOS and interleukin-10 expression in lipopolysaccharide- and mycobacterial lipoarabinomannan-stimulated macrophages. Immunology **85:**106–113.
14. **Roach, T. I. A., D. Chatterjee, and J. M. Blackwell.** 1994. Induction of early-response genes KC and JE by mycobacterial lipoarabinomannans: regulation of KC expression in murine macrophages by *Lsh*/*Ity*/*Bcg* (candidate *Nramp*). Infect. Immun. **62:**1176–1184.
15. **Venisse, A., J. M. Berjeaud, P. Chaurand, M. Gilleron, and G. Puzo.** 1993. Structural features of lipoarabinomannan from *Mycobacterium bovis* BCG. Determination of molecular mass by laser desorption mass spectrometry. J. Biol. Chem. **268:**12401–12411.
16. **Wysocka, M., M. Kubin, L. Q. Vieira, L. Ozmen, G. Garotta, P. Scott, and G. Trinchieri.** 1995. Interleukin-12 is required for interferon-γ production and lethality in lipopolysaccharide-induced shock in mice. Eur. J. Immunol. **25:** 672–676.
17. **Yoshida, A., Y. Koide, M. Uchijima, and T. O. Yoshida.** 1995. Dissection of strain difference in acquired protective immunity against *Mycobacterium bovis* BCG: macrophages regulate the susceptibility through cytokine network and the induction of nitric oxide synthase. J. Immunol. **155:**2057–2066.
18. **Zhang, Y., M. Doerfler, T. C. Lee, B. Guillemin, and W. N. Rom.** 1993. Mechanisms of stimulation of interleukin-1β and tumor necrosis factor-α by *Mycobacterium tuberculosis* components. J. Clin. Invest. **91:**2076–2083.

Editor: S. H. E. Kaufmann