Endotoxin-Induced Serum Factor That Stimulates Gamma Interferon Production

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Gamma interferon (IFN-γ) is believed to be involved in the immunological system by regulating the expression of surface antigens of mononuclear cells (3, 6, 23) or by regulating the antitumoral or tumoricidal functions of macrophages or natural killer cells (17, 26). Therefore, elucidation of the regulatory mechanism of IFN-γ production would lead to a clearer understanding of the role of IFN-γ in immune reactions. The cellular sources and mechanism of IFN-γ secretion have hitherto been investigated by using either nonspecific stimulants, such as mitogens, or specific antigens (13). Most of the results obtained have shown that T lymphocytes or natural killer cells produce IFN-γ in the presence of accessory cells, and the participation of interleukin-2 (IL-2) has also been suggested (7, 11, 12, 16, 22). However, details of the mechanism, for example, the mode of participation of macrophages or IL-2, still remain to be clarified.

Previously, we found that high titers of IFN-γ were released into the circulatory system of bacteria-treated mice upon challenge with endotoxin (lipopolysaccharide [LPS]) (20, 28). LPS was also shown to induce IFN-γ in lymphoid cells with the help of IL-2 (5, 14, 21), and recently the involvement of IFN-γ in LPS-induced inflammatory reactions was suggested (4, 8). Because accessory cells were required for IFN-γ induction by LPS and it seemed unlikely that LPS directly stimulated the producer cells, we were prompted to search for a second soluble factor participating in the induction mechanism of IFN-γ production by LPS in vivo. Since high titers of IFN-γ induced by LPS in bacteria-treated mice seemed to appear in the later phase (20, 28), such a factor would probably exist in the circulation before the appearance of IFN-γ.

On the other hand, evidence has accumulated which suggests that differences exist among various strains of mice in their capacities to produce IFN-γ. The levels of IFN-γ production induced by antigens or by mitogens in vivo (18–20, 25) or those induced in vitro (9, 10, 27) are influenced by murine genotypes, as are the levels of IFN-α/β induction by LPS (2, 15). However, since various cell populations are involved in IFN-γ production, it is difficult to elucidate how the process is genetically controlled. It would thus be significant to investigate whether such control exists in the functions of accessory cells.

Here, we show that serum from Mycobacterium bovis BCG-infected mice challenged with LPS contains a soluble substance which is able to induce high levels of IFN-γ in spleen cell cultures depleted of plastic- and nylon wool-adherent cells, in cooperation with human recombinant IL-2. We also showed data suggesting that the synthesis of this factor is genetically controlled but that no strain variation is observable with regard to production of IFN-γ by purified T lymphocytes or natural killer cells.

MATERIALS AND METHODS

Mice. Inbred C57BL/6, BALB/c, and A/J mice and outbred ICR mice, female, 8 to 12 weeks of age, were obtained from Shizuoka Laboratory Animal Center, Hamamatsu, Japan. CBA/JN mice and C3H/HeJ mice were obtained from Charles River Japan Inc., Atsugi, Japan, and from Clea Japan Inc., Osaka, Japan, respectively.

Reagents. Live lyophilized BCG (Japan BCG Manufacturing Co., Tokyo, Japan), LPS (Escherichia coli O127:B8; Difco Laboratories, Detroit, Mich.), and concanavalin A (ConA; Pharmacia, Uppsala, Sweden) were commercially obtained. Recombinant human IL-2 was kindly donated by Takeda Chemical Industries, Osaka, Japan. Recombinant human IL-1α (IL-1α) and recombinant human tumor necrosis factor α (TNF-α) were kindly donated by Dainippon Pharmaceutical Co., Osaka, Japan.

Antibodies. Anti-murine IFN-γ antiserum and monoclonal anti-IL-2 receptor antibody were commercially obtained.
from Lee Biomolecular Research Laboratories Inc., San Diego, Calif., and from Boehringer GmbH, Mannheim, Federal Republic of Germany, respectively. Specific antiserum to murine IFN-α/β was kindly provided by Y. Kawade of the Institute for Virus Research, Kyoto University, Kyoto, Japan.

**Treatment of mice.** Mice were injected intraperitoneally with 1.5 mg of live BCG (1.5 × 10⁷ cells) in 0.2 ml of phosphate-buffered saline (PBS) and were then challenged intraperitoneally with 1 μg of LPS in 0.2 ml of PBS after 3 weeks, when massive hepatosplenomegaly was observed. Blood was taken from the hearts at different times after challenge, and the sera were assayed for IFN activity and the ability to induce IFN production (IFN-γ-inducing factor) in various spleen cell cultures. Control mice received the same amount of PBS.

**Cell preparation.** Spleens were excised, minced, and exposed to 0.144 M NH₄Cl in 0.017 M Tris hydrochloride (pH 7.2) to disrupt erythrocytes. Cells were washed with minimal essential medium (Flow Laboratories, Inc., Inglewood, Calif.) supplemented with 5% fetal bovine serum (Bocknek Laboratories Inc., Rexdale, Ontario, Canada) and with RPMI 1640 medium supplemented with 10% fetal bovine serum–2 mM 1-glutamine–penicillin (100 U/ml)–streptomycin (100 μg/ml). For the preparation of nylon wool-nonadherent (NWNA) cells, macrophages were removed from cell suspensions by incubation in plastic plates (Becton Dickinson Overseas Inc., Oxnard, Calif.) for 60 min at 37°C, and 5-ml samples of the cell suspensions (4 × 10⁹/ml) were applied to a nylon wool column (15 ml) equilibrated with the same medium. More than 90% of nonspecific esterase-positive cells were removed by nylon wool column, and the resulting T-cell-enriched fraction was not able to induce IFN-γ upon stimulation with ConA. After incubation at 37°C for 1 h, nonadherent cells were eluted with the prewarmed medium, washed by centrifugation (1,500 × g) twice, and suspended in RPMI 1640 medium. Viable cells were counted by the trypsin blue dye exclusion test. The cell concentration was adjusted to 10⁷ cells/ml for use.

**Induction of IFN-γ.** Spleen cells from normal C57BL/6 mice were used for the assay of the IFN-γ-inducing factor. Whole spleen or NWNA cells in 0.18 ml of RPMI 1640 medium as described above were placed in the wells of a 96-well flat-bottom microtest culture plate (Becton Dickinson). Inducers or variously diluted sera dissolved in 0.02 ml of PBS were added to the above cultures, which were then incubated for 24 h in an atmosphere containing 5% CO₂ at 37°C, and the culture fluid was harvested for IFN assay. The IFN-γ-inducing activity of the serum factor was tentatively expressed as the maximum dilution at which 160 U of IFN-γ per ml was induced in the culture.

**IFN assay and characterization of IFNs.** IFN assay was carried out by measuring the capacity to inhibit the cytopathic effect of vesicular stomatitis virus on mouse L cells, and characterization of IFNs was performed by using specific anti-mouse IFN antibodies as previously described (20). Titers were expressed in international reference units, as calibrated against National Institutes of Health mouse reference IFN (G002-904-511).

**Gel filtration of the IFN-γ-inducing factor.** Sera containing IFN-γ-inducing activity were combined and fractionated by ammonium sulfate (55 to 75% saturation). The precipitate was dissolved in 2 ml of phosphate buffer (0.02 M, pH 7.2) applied to a column of Ultrogel AcA 44 (2 by 75 cm) (LKB Instruments, Inc.), and eluted by the same buffer. A sample from each fraction (2 ml) was added to the culture of spleen cells, macrophages were removed from cell suspensions by incubation in plastic plates (Becton Dickinson Overseas Inc., Oxnard, Calif.) for 60 min at 37°C, and 5-ml samples of the cell suspensions (4 × 10⁹/ml) were applied to a nylon wool column (15 ml) equilibrated with the same medium. More than 90% of nonspecific esterase-positive cells were removed by nylon wool column, and the resulting T-cell-enriched fraction was not able to induce IFN-γ upon stimulation with ConA. After incubation at 37°C for 1 h, nonadherent cells were eluted with the prewarmed medium, washed by centrifugation (1,500 × g) twice, and suspended in RPMI 1640 medium. Viable cells were counted by the trypsin blue dye exclusion test. The cell concentration was adjusted to 10⁷ cells/ml for use.

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**RESULTS**

**Profile of the appearance of the IFN-γ-inducing factor in LPS-induced sera.** The induction kinetics of circulating IFN-γ and IFN-γ-inducing factor in sera of variously diluted sera from normal C57BL/6 mice after LPS challenge are shown in Fig. 1. Serum samples were taken at various times after LPS challenge, appropriately diluted, and added to cultures of spleen cells from untreated mice, along with IL-2. Titration and characterization of IFN in the culture supernatant were carried out after 24 h of incubation. The IFN-γ-inducing factor became detectable in the circulatory system about 90 min after LPS challenge, reached a peak after about 2 to 3 h, and disappeared at around 5 h. On the other hand, IFN-γ appeared in the circulatory system at around 3 h after LPS challenge and reached a peak after about 4 to 5 h. Most of the BCG-infected mice challenged with LPS died by 7 h. No IFN-γ-inducing factor or IFN-γ was observed in the sera of normal mice challenged with LPS or in those of BCG-infected mice challenged with PBS (data not shown).

**Effect of IL-2 on IFN-γ induction by diluted serum.** Appropriately diluted serum from a BCG-infected mouse challenged with LPS was added to the spleen cell culture and incubated for 24 h. Serum at a final dilution of between 1/40 and 1/160 effectively induced IFN-γ production in these cells (Fig. 2). At concentrations higher than a 1/20 dilution, the induction was suppressed, possibly because of an inhibitor(s). At concentrations lower than a 1/320 dilution, the induction by the serum alone was almost unobservable but was recovered effectively by the addition of IL-2. More than 2 U of IL-2 per ml was necessary for maximum induction. Thus, the decrease in IFN-γ induction seemed to be due to the decrease in endogenous IL-2 when the serum was diluted, suggesting the possible involvement of endogenous IL-2 activity in the serum. Because LPS and IL-2 induced
only a trace amount of IFN-γ (in spleen cells), the participation of LPS remaining in the serum was negligible.

**Effect of anti-IL-2 receptor antibody on IFN-γ induction.** Because IL-2 augmented IFN-γ induction by the LPS-induced serum factor, and because the participation of IL-2 was believed to occur through the function of the IL-2 receptor, the effect of anti-IL-2 receptor antibody was examined by using whole spleen cells. Appropriately diluted anti-IL-2 receptor antibody was added to the culture of spleen cells (10^7 cells per ml) on microtiter plates. After the plates were incubated for 1 h at 37°C, the LPS-induced serum factor from BCG-infected C57BL/6 mice 2 h after LPS inoculation with or without IL-2 (2 U/ml) was added at a final concentration of 1%. For cultures induced with the LPS-induced serum factor alone, the IFN-γ titers were 560 ± 160 and 30 ± 11 after treatment with PBS and anti-IL-2 receptor antibody, respectively; for cultures induced with the LPS-induced serum factor plus IL-2, the IFN-γ titers were 2,240 ± 640 and 140 ± 40 after treatment with PBS and anti-IL-2 receptor antibody, respectively (all values, mean ± standard deviation for four experiments). Thus, the antibody effectively suppressed the induction when added 1 h before the LPS-induced serum factor, both in the presence and absence of exogenous IL-2. Furthermore, induction by ConA was also suppressed by the antibody (data not shown). Thus, the participation of IL-2 receptor in IFN-γ induction in our system was strongly suggested.

**Effect of depletion of adherent cells on IFN-γ production.** Since participation of macrophages in IFN-γ production has been pointed out by numerous investigators (24), we examined whether the unknown serum factor could substitute for macrophages. IL-2 or ConA alone induced a low level of IFN-γ in cultures of whole spleen cells from untreated mice (Table 1). The known LPS-elicited macrophage product IL-1α or TNF-α, together with IL-2, was not able to induce IFN-γ. The depletion of adherent cells completely eliminated IFN-γ induction by ConA or by IL-2 alone. However, the induction by the LPS-induced serum factor in the presence of IL-2 in NWNA cells was somewhat augmented. Perhaps the augmented induction in these cells was a result of the enrichment of the IFN-γ-producing cells.

**Estimation of the molecular weight of the serum factor.** The IFN-γ-inducing factor was fractionated with ammonium sulfate (55 to 75% saturation) and was applied to a column of Ultrogel AcA 44 (LKB Instruments) for the determination of molecular weight (Fig. 3). Each fraction was examined for IFN-γ-inducing activity in NWNA cells in the presence or in the absence of IL-2. The activity exhibited a single and symmetric peak in the presence of IL-2, and the molecular weight of the fraction possessing the activity was estimated to be 70,000. Almost no activity was detected in the absence of added IL-2.

**Variations among different strains of mice in the production of IFN-γ and IFN-γ-inducing factor.** Various strains of mice infected with BCG were challenged with LPS, and both circulating serum factor and IFN-γ were assayed (Fig. 4). Blood was taken for assay of the IFN-γ-inducing factor 2 h after challenge with LPS, and the sera were used for induction of IFN-γ in the presence of IL-2. The intensity of

**TABLE 1. Effect of deprivation of adherent cells on IFN-γ induction with LPS-induced serum factor.**

<table>
<thead>
<tr>
<th>Inducer (conc)</th>
<th>IFN-γ titer produced by cells (10^5/ml)</th>
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<tbody>
<tr>
<td>LPS (10 µg/ml)</td>
<td>Whole spleen NWNA</td>
</tr>
<tr>
<td>IL-2 (2 U/ml)</td>
<td>80 &lt;20</td>
</tr>
<tr>
<td>ConA (5 µg/ml)</td>
<td>40 &lt;20</td>
</tr>
<tr>
<td>ConA (5 µg/ml) + IL-2 (2 U/ml)</td>
<td>160 &lt;20</td>
</tr>
<tr>
<td>IL-1α (1 to 100 U/ml) + IL-2 (2 U/ml)</td>
<td>40 &lt;20</td>
</tr>
<tr>
<td>TNF-α (1 to 100 U/ml) + IL-2 (2 U/ml)</td>
<td>40 &lt;20</td>
</tr>
<tr>
<td>LPS-induced serum factor (1%)</td>
<td>320 640</td>
</tr>
<tr>
<td>LPS-induced serum factor (1%) + IL-2 (2 U/ml)</td>
<td>640 2,560</td>
</tr>
</tbody>
</table>

* Adherent cells were removed by first adhering the spleen cells from normal C57BL/6 mice to a plastic plate for 1 h at 37°C and then applying the nonadherent cells to the nylon wool column as described in Materials and Methods.

* LPS-induced serum factor was obtained from BCG-infected C57BL/6 mice 2 h after LPS inoculation.
FIG. 4. Strain variations of in vivo IFN-γ production and IFN-γ-inducing factor. Symbols: Ⅰ, IFN-γ levels in the sera of BCG-infected mice obtained 4 to 5 h after LPS inoculation; Ⅱ, IFN-γ-inducing activity in sera obtained 2 h after LPS inoculation and maximally diluted to allow induction of 160 U of IFN-γ per ml in NWWA cells from normal C57BL/6 mice in the presence of IL-2 (2 U/ml). Each bar represents mean ± standard deviation for five mice.

the serum factor activity was compared in terms of the amount of IFN-γ produced in the culture of NWWA cells from C57BL/6 mice when the serum was added at a 100× dilution. Circulating IFN-γ was assayed with sera taken from mice 4 h after LPS challenge. There were pronounced differences among various strains of inbred or outbred mice in the amounts of circulating IFN-γ and IFN-γ-inducing activity, and an obvious parallelism was observed between the two parameters. Strains producing large amounts of IFN-γ, such as C57BL/6 or outbred ICR mice, were also high producers of the IFN-γ-inducing factor. CBA/JN was a moderate producer, whereas BALB/c, C3H/HeJ, and A/J mice were low producers.

Production of IFN-γ in NWWA cells from mice of various strains. To determine whether the strain differences in IFN-γ production in vivo were ascribable to variations in the capacities of cells to produce the cytokine, NWWA cells from mice of various strains were examined by using the serum factor from high-producer mice, i.e., C57BL/6. No significant differences were observed among the various strains (Table 2); cells from low-producer mice, C3H/HeJ, BALB/c, and A/J, produced the same level of IFN-γ as those from high-producer mice, C57BL/6 or ICR. Thus, genetic control of IFN-γ production in vivo seemed to be exerted not on the producing cells themselves, but rather on the production of a stimulating factor like that described above.

DISCUSSION

Recently, it was demonstrated that IFN-γ is involved in the LPS-induced inflammatory response (4, 8), and previ-ously, we found that LPS stimulated production of IFN-γ and IFN-α/β in BCG-infected mice or in mice treated with heat-killed Propionibacterium acnes (20, 28). Because these bacteria do not contain LPS, IFN-γ induction by LPS did not appear to be due to an antigen-specific stimulus but rather to the ability of LPS to cause inflammation. Therefore, the existence of some unidentified factor which was expected to stimulate IFN-γ production in vivo was anticipated. Recently, Wilson et al. indicated that IL-2-induced IFN-γ synthesis requires the synergistic activity of IL-1 and possibly of one or more other monokines (29). Antonelli et al. also claimed the existence of a macrophage-derived factor which differed from IL-1 and was able to induce IFN-γ in resting T lymphocytes (1).

In our present study, a factor stimulating IFN-γ production in spleen cells was shown to be present in the serum of BCG-infected mice challenged with LPS (Fig. 1 and 2). The appearance of the factor in the circulatory system preceded that of IFN-γ, indicating that the factor is probably concerned with IFN-γ production in vivo. As this serum also contained a strong inhibitor of IFN-γ induction, the serum had to be diluted (Fig. 2) or fractionated (Fig. 3).

Induction by the unknown factor was IL-2 dependent (Fig. 2), because it was augmented upon IL-2 addition and, conversely, was inhibited in the presence of anti-IL-2 receptor antibody. Although IL-2 alone induced a low level of IFN-γ in whole spleen cells, it failed to do so in NWWA cells (Table 1), and the partially purified serum factor alone could not induce IFN-γ in these cells (Fig. 3). The relationship between IL-2 and this factor is very interesting but puzzling. The serum factor may increase the expression of surface IL-2 receptor, or it may be involved with the release of synthesized IFN-γ from the cells stimulated by IL-2.

The cellular origin of the IFN-γ-inducing factor was not investigated in the present study, but activated macrophages may produce it, since the culture supernatant of LPS-stimulated adherent peritoneal exudate cells induced with P. acnes was able to induce IFN-γ in NWWA cells in the presence of IL-2 (21). From the beginning of investigations into IFN-γ, a macrophage requirement for effective production of IFN-γ has been indicated. However, details of the function of macrophages have not been elucidated. Contact between lymphocytes and macrophages may be necessary, or some soluble factor may substitute for macrophages. In the present study, such a macrophage requirement was overcome when IL-2 and the unknown factor were used for IFN-γ induction (Table 1). Induction by ConA was completely abolished by removal of splenic nylon wool-adherent cells, while induction by the serum factor plus IL-2 was somewhat augmented. Thus, production of IFN-γ by resting lymphocytes is possibly induced by soluble factors, some of which are released from macrophages. Although neither IL-1α nor TNF-α together with IL-2 was able to induce IFN-γ production in NWWA cells, participation of such

<table>
<thead>
<tr>
<th>Strain</th>
<th>IFN-γ titer (mean ± SD) produced by NWWA cells derived from:</th>
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<tbody>
<tr>
<td></td>
<td>C57BL/6</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>1,600 ± 640</td>
</tr>
<tr>
<td>BALB/c</td>
<td>80 ± 56</td>
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</tbody>
</table>

* Blood was taken from BCG-infected mice (C57BL/6 or BALB/c) 2 h after challenge with LPS. Sera were separated and added to the cultures of NWWA cells from mice of various strains at a concentration of 1% in the presence of IL-2 (2 U/ml).
* Each value represents mean ± SD for four experiments.
factors could not be eliminated since the active serum also contained IL-1 or TNF (data not shown). More than three factors may be required for effective induction as described by Wilson et al. (29). Such factors may not usually appear in the circulatory system but may be produced in local inflammatory sites. Preliminary experiments on the effect of the doses of both the partially purified IFN-γ-inducing factor and IL-2 indicated that in the presence of enough IL-2, the amount of IFN-γ induced by the factor increased linearly with the dose, whereas IFN-γ production by IL-2 in the presence of enough factor increased sigmoidally. This may suggest that the IFN-γ-inducing factor usually exhibits its functions in restricted environments.

Variations in the capacities of mouse strains to develop a cell-mediated immune response and also to produce IFN-γ have been reported (18, 19, 25). Investigations on such variations were carried out in vitro with mitogens and spleen cells (9, 10, 27). However, the basic mechanisms of these differences among mouse strains in vivo have not been documented. In the present study, sera obtained from various strains of BCG-infected mice after LPS challenge exhibited marked differences in their capacities to induce IFN-γ in NWNA cells prepared from mice of each strain (Fig. 4). This variation agreed well with the quantity of IFN-γ induced in the circulatory system, which appeared about 1 h after the serum factor. However, these NWNA cells elicited no significant difference among mouse strains (Table 2). Cells from the mouse strain showing the lowest response (C3H/HeJ) produced the same level of IFN-γ as that produced in the cells from the highest-responding strain upon stimulation with IL-2 and the serum factor. This indicates the possibility that the genetic control of IFN-γ production is not related to the control of the capacity of producing cells but to that of the function of accessory cells. Huygen and Palfiet (9) reported that CBA/Ca mice were low IFN-γ producers in vivo but high IFN-γ producers in vitro when stimulated with a specific antigen. Furthermore, they showed that in vitro IFN-γ induction by ConA was also influenced by mouse genotype (10). However, these studies were carried out with whole spleen cells, and the influence of accessory cells was not studied separately. Although we used different stimuli, our present results emphasize the influence of such accessory cells, which produce the IFN-γ-inducing factor.

Molecular characterization of the IFN-γ-inducing factor was not performed in the present study, but its roughly estimated molecular weight was 70,000, and the profile of its elution pattern on gel filtration was symmetric and not markedly spread (Fig. 3). This suggests that the IFN-γ-inducing factor is a single molecular species. Its physiological roles, characterization, cellular source, and relationship to other cytokines, such as IL-1 or IL-2, will need to be clarified.

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LITERATURE CITED


