INFECTION AND IMMUNITY, Jan. 1993, p. 64–70
0019-9567/93/010064-07$02.00/0

Vol. 61, No. 1

Purification of a Factor Which Provides a Costimulatory Signal for Gamma Interferon Production

KYOHSHI NAKAMURA,[1]* HARUKI OKAMURA,[1] KUMIKO NAGATA,[1] TOSHINORI KOMATSU,[2] AND TOSHIHIDE TAMURA[1]

Department of Bacteriology[1] and Department of Immunozoology,[2] Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo, Japan

Received 29 January 1992/Accepted 29 July 1992

A protein factor which induces high levels of gamma interferon (IFN-γ) in resting splenic nonadherent cells was isolated from the sera of mice with generalized inflammation caused by endotoxic shock. The factor was highly purified by ammonium sulfate precipitation followed by ion-exchange column chromatography on DEAE-Sepharose, molecular sieving on Ultrogel AcA 44, and hydrophobic column chromatography with phenyl-Sepharose. It was further purified to apparent homogeneity by polyacrylamide gel electrophoresis. It induced IFN-γ production in a dose-dependent manner in the presence of interleukin-2, monoclonal anti-CD3 antibody (anti-CD3 MAb), or concanavalin A (ConA) in spleen cells deprived of plastic plate- and nylon wool-adherent cells. Anti-CD3 MAb induced the highest level of production of the three. The factor, interleukin-2, anti-CD3 MAb, or ConA alone induced a trace of or no detectable IFN-γ in these cells. The factor also exhibited an accessory function during proliferation in these cells in the presence of a suboptimal dose of ConA. However, the factor failed to stimulate IFN-γ production when staphylococcal enterotoxin A, a superantigenic T-cell mitogen, was employed. Treatment with pronase or heat abolished these activities. These studies confirm the existence of a soluble protein factor which is able to exhibit a novel accessory function in IFN-γ production in resting T or natural killer cells. It will be of interest to compare this factor with the recently cloned human natural killer stimulatory factor (NKSF/IL-12).

It is believed that gamma interferon (IFN-γ) is involved in the immune system through regulation of the expression of surface antigens of mononuclear cells or by augmentation of antimicrobial and antitumor functions of lymphoid cells or macrophages (7). Recent investigations have also indicated a role for IFN-γ in the regulation of antibody formation for each class (29). The involvement of IFNs in inflammatory responses has been also demonstrated by using lipopolysaccharide (LPS) as an inflammatory stimulus (11). Thus, IFN-γ may be concerned with both inflammatory and immune responses in vivo.

As for the mechanism of IFN-γ production, interleukin-2 (IL-2) has been found to induce IFN-γ in both human and murine lymphoid cells (8, 10, 13, 25–28, 31), and a dependency on IL-2 receptors has also been suggested (15, 17, 21). Although the macrophage requirement for effective production has been indicated by investigations using antigens or lectins (2, 5, 6, 14), the roles of accessory cells in the induction of IFN-γ by IL-2 are still obscure. Some of the authors claiming a requirement for accessory cells have claimed that the functions of accessory cells are exhibited through cell-to-cell contact (3, 19), while others have stated that there are soluble factors which can substitute for accessory cells (1, 17, 34). Since IFN-γ is said to be produced by $CD4^+$ or $CD8^+$ T cells and natural killer (NK) cells, IFN-γ production by each cells may be induced in a different manner, thus leading to a different conclusion according to the different experimental systems.

Recently, the division of helper T (Th) cells into two subsets was proposed on the basis of distinct requirements for costimulatory signals or for different cytokine secretions (18, 20, 33). One subset, Th2, which secretes IL-5, IL-6, or IL-10, utilizes IL-4 as a growth factor and requires IL-1 as a cofactor. The other subset, Th1, which secretes IFN-γ and IL-2, is said to be concerned with cellular immunity, but there has been no definite conclusion about costimulatory signals for its differentiation. On the other hand, it has long been known that immune responses to some bacterial or protozoan antigens are biased to either cellular or humoral immunity, and the regulation of differentiation of such Th cell subsets may be concerned with the biased immune response (12). Thus, the effort to clarify the induction mechanism of IFN-γ, which plays a central role in cellular immunity, also becomes involved in the study of the differentiation of Th1 cells.

Apart from being used in studies on IFN-γ induction with specific antigens, LPS was also shown to induce IFN-γ both in vivo (23, 32) and in vitro (3, 17, 19, 24), being dependent on IL-2 in most cases, and IFN-γ was also demonstrated to be involved in inflammatory responses caused by LPS (11). We also found that sera obtained from mice variously pretreated with *Propionibacterium acnes* or *Mycobacterium bovis* BCG and challenged with LPS exhibited marked abilities to induce IFN-γ in resting mouse spleen cells deprived of plastic- and nylon-adherent cells (22). Some unidentified factor with a molecular size of approximately 70 kDa and able to stimulate IFN-γ secretion dependent on the presence of IL-2 was suggested as being involved.

In order to extend these observations and confirm whether a single factor exerted the costimulatory action, we aimed to purify this factor for characterization and clarification of its roles. In the present experiments, the purified factor exhibited a marked costimulatory effect on IFN-γ production in spleen nonadherent cells stimulated with monoclonal anti-CD3 antibody (anti-CD3 MAb) or concanavalin A (ConA). It will be of interest to investigate the relationship of the factor and the regulation of Th1-like responses in vivo. Recently,

* Corresponding author.

NK stimulatory factor (NKSF/IL-12) was characterized, and its cDNA was cloned from human B-cell lines (4, 9, 16, 30, 35). The molecular weight or other characteristics of biological actions are similar to those of the factor used in this study. It will also be interesting to examine whether NKSF/IL-12 exists experimental animals, since NKSF/IL-12 may play physiologically important roles in the mechanism of defense against infection.

MATERIALS AND METHODS

Mice. Inbred C3H/HeJ mice and outbred ICR mice (female, 7 to 8 weeks of age) were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan. Since *P. acnes*-treated ICR mice exhibited sensitivity to LPS equal to that of *P. acnes*-treated C57BL/6 mice and produced more IFN-γ-inducing factor per mouse, ICR mice were employed in the experiment described here (22).

Bacteria. *P. acnes* ATCC 11827 (formerly designated "*Corynebacterium parvum*") was grown in brain heart infusion medium supplemented with L-cysteine (0.03%) and Tween 80 (0.03%) by stand cultures. Cells were harvested, washed with phosphate-buffered saline (PBS) by centrifugation, suspended in PBS, and exposed to a temperature of 60°C for 1 h.

Reagents. LPS (*Escherichia coli* O127:B8; Difco Laboratories, Detroit, Mich.) and ConA (Pharmacia, Uppsala, Sweden) were obtained commercially. Recombinant human IL-2 (27,590 U/mg) was kindly donated by Takeda Chemical Industries, Osaka, Japan. Recombinant human IL-1α and recombinant tumor necrosis factor alpha were kindly donated by Dainippon Pharmaceutical Co., Osaka, Japan. Staphylococcal enterotoxin A (SEA) was obtained as described before (23).

Antibodies. Anti-murine IFN-γ antiserum and anti-IL-2 receptor MAb were obtained commercially from Lee Biomolecular Research Laboratories Inc., San Diego, Calif., and from Boehringer GmbH, Mannheim, Germany, respectively. Specific antiserum against murine IFN-α/β was kindly provided by Y. Kawade of the Institute for Virus Research, Kyoto University, Kyoto, Japan (23). Purified hamster anti-CD3 MAb was obtained from PharMingen, San Diego, Calif. Anti-Thy-1.2 MAb of the immunoglobulin M class (Serotec Ltd., Bicester, United Kingdom) was commercially obtained.

Treatment of mice and serum preparation. Mice were injected intraperitoneally with 0.2 ml of PBS containing 10 mg (wet weight) of heat-killed *P. acnes*. Mice were challenged intravenously with 1 μg of LPS in 0.2 ml of PBS 1 week after pretreatment by *P. acnes*, when massive hepatosplenomegaly was observed. Since IFN-γ and IFN-γ-inducing factor appeared in the serum as a function of time (22), blood was taken from the heart at 2 h after LPS challenge and pooled, and serum was isolated. This serum contained IFN-α/β but not IFN-γ, which appeared about 2 h later. Although live BCG was available for pretreatment (22), killed *P. acnes* was employed for the present study because the latter grew more rapidly and the incubation period for pretreatment was much shorter (23, 32).

Assay of the factor: induction assay for IFN-γ. The factor was assayed for its ability to induce IFN-γ in plastic plate- and nylon wool-nonadherent (NWNA) cells in the presence of 10 U of IL-2 or sometimes anti-CD3 MAb (1 μg/ml). The titer was tentatively expressed as the sample dilution required to induce 160 U of IFN-γ per ml in 10^7 lymphoid cells per ml. Spleens of normal untreated C3H/HeJ mice were excised, minced, and exposed to 0.144 M NH_4Cl in 0.017 M Tris hydrochloride (pH 7.2) to disrupt erythrocytes. The cells were washed with minimum essential medium (Flow Laboratories, Inc., Inglewood, Calif.) supplemented with 5% fetal bovine serum (Bocknek Laboratories Inc., Rexdale, Ontario, Canada) and then suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). For the preparation of plastic-nonadherent and NWNA cells, cell suspensions were incubated in plastic plates (Becton Dickinson Overseas Inc., Oxford, Calif.) for 60 min at 37°C, nonadherent cells were gently gathered and centrifuged, and 5-ml samples of the cell suspensions (5×10^7 cells per ml) were applied to a nylon wool column (15 ml) equilibrated with the same medium. Nonadherent cells were eluted with prewarmed medium, washed by centrifugation, and resuspended. Viable cells were counted by the trypan blue dye exclusion test, and the cell concentration was adjusted to 10^7 cells per ml for use. Most of the nonspecific esterase-positive cells were removed by these procedures. However, nonadherent non-T cells were still existent in a considerable ratio when analyzed by fluorescence-activated cell sorting (ratio of $CD4^+/CD8^+$/non-T nonadherent cells was 43:17:40), although the resulting T- or NK-cell-enriched suspension was able to produce no detectable or only trace amounts of IFN-γ upon stimulation with SEA or ConA. NWNA cells in 0.15 ml of the medium described above were placed in a 96-well flat-bottomed microtest culture plate (Becton Dickinson). IL-2, anti-CD3 MAb, ConA, or variously diluted samples of the factor dissolved in 0.05 ml of PBS were added to the cultures. Cultures were incubated for 24 or 40 h in an atmosphere containing 5% CO_2 at 37°C, and the culture fluid was then employed for IFN assay.

IFN-γ assay. Assay for IFN-γ was carried out as described previously (22) by measuring the capacity to inhibit the cytopathic effect of vesicular stomatitis virus on mouse L cells. Characterization of IFNs was performed by using specific anti-murine IFN antibodies. Titers were expressed in international reference units calibrated against National Institute of Health mouse reference IFN (G002-904-511).

Purification procedure. DEAE–Sepharose CL-6B (Pharmacia), Ultrogel AcA 44 (IBF Biotechnics, Villeneuve-la-Garenne, France), and phenyl–Sepharose CL-4B (Pharmacia) were all obtained commercially.

PAGE. Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE with 2-mercaptoethanol were performed on polyacrylamide gradient gel prepared by Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan. Gels were stained with silver by using a staining kit (Wako Pure Chemicals, Osaka, Japan).

Proliferation assays. C3H/HeJ spleen NWNA cells (4×10^5 cells per 130-μl total volume) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Samples of the factor together with a suboptimal dose of ConA (0.5 μg/ml) were added to cells and cultured for 56 h. Cultures were pulsed with 0.25 μCi of [^{3}H]thymidine per well during the final 8 h of culture and harvested. [^{3}H]thymidine uptake was measured in a Packard 460 scintillation counter.

RESULTS

Purification of the factor. The sera of endotoxin-shocked mice exhibited strong IFN-γ-inducing activity in T lymphocytes (22) and were therefore used for purification of the

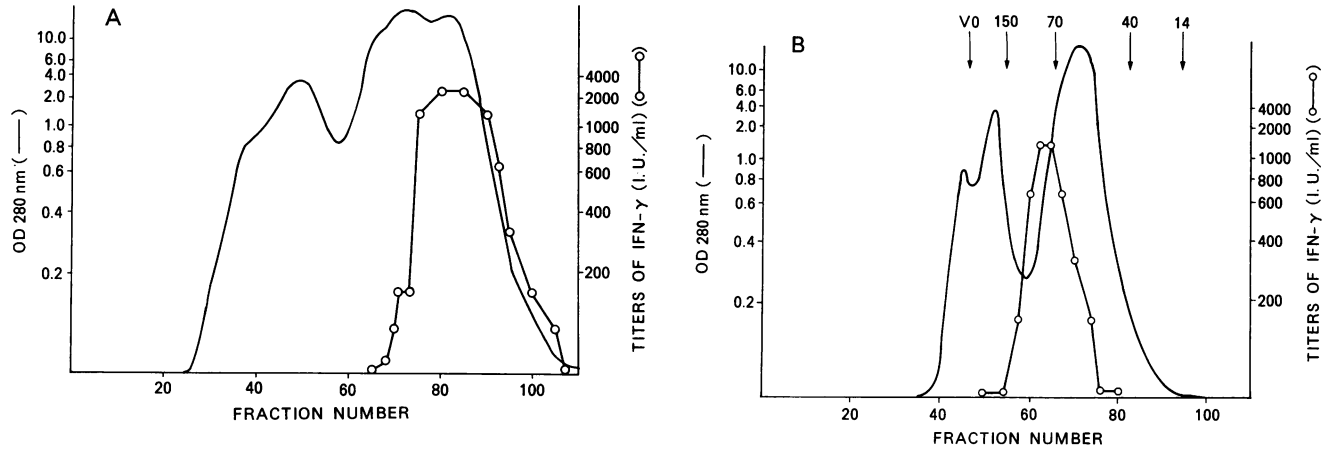

FIG. 1. (A) Fractionation of IFN-γ-inducing factor by DEAE-Sepharose column chromatography. Proteins were eluted from the column (3 by 35 cm) by using a gradient of 0 to 0.25 M NaCl in 0.02 M PB (300 ml each). Protein concentration was monitored by UV absorption. (B) Gel filtration of IFN-γ-inducing factor by Ultrogel AcA 44 column chromatography. Standard proteins used for estimation of molecular weights were RNase A, chymotrypsinogen A, ovalbumin, bovine serum albumin, and blue dextran 2000. Molecular weights (in thousands) are indicated at top of the figure. V0, void volume. (C) Elution pattern of IFN-γ-inducing factor in hydrophobic column chromatography with phenyl-Sepharose. Proteins were eluted with an ammonium sulfate gradient from 1.0 to 0.1 M (200 ml each). Each fraction was dialyzed against 0.02 M PB to remove ammonium sulfate and assayed for IFN-γ-inducing activity. A sample from each fraction was added to the culture of spleen NWNA cells (1.5×10^6 cells per well) at a dilution of 1/10 in the presence of IL-2 (1 U/ml), and the culture supernatant was assayed for IFN-γ activity after 24 h. OD 280 nm, optical density at 280 nm.

factor. Pooled mouse serum (300 ml) was first fractionated with ammonium sulfate ranging from 55 to 75% saturation. Almost 100% of the factor's activity was precipitated in this range. About one-third of the serum protein, probably containing immunoglobulin, was precipitated with a concentration of ammonium sulfate of less than 55%. The precipitate (4,212 mg) was dialyzed against 0.02 M sodium phosphate buffer (PB, pH 7.2) and applied to a DEAE-Sepharose column. The factor eluted at an ionic strength of about 0.15 M NaCl (Fig. 1A). Each fraction was assayed for the factor, and the active fractions were combined, concentrated with ammonium sulfate, dialyzed against 0.02 M PB, applied to an Ultrogel AcA 44 column (2.8 by 90 cm), and equilibrated with 0.02 M PB (pH 7.2) (Fig. 1B). The activity eluted a little faster than bovine serum albumin, exhibiting a single symmetric peak, and the molecular size was estimated at 70 to 75 kDa, as reported previously (22). The fractions containing activity were again precipitated with ammonium sulfate, dialyzed against 1.0 M ammonium sulfate in 0.02 M PB, applied to a phenyl-Sepharose column (3 by 10 cm), and equilibrated with 1.0 M ammonium sulfate in 0.02 M PB. The column was washed with the same buffer containing ammonium sulfate and eluted with ammonium sulfate at 1.0 to 0.1 M (Fig. 1C). Each fraction was assayed for the factor after removal of ammonium sulfate by dialysis. Most of the activity was recovered in the fractions which contained little protein according to UV detection. During all these purification procedures, the factor could be traced as a single component. The factor was concentrated by lyophilization when necessary. Data on the purification procedure are summarized in Table 1.

Gel electrophoresis analysis of the factor. To examine the purity of the factor prepared as described above, we electrophoresed the purified sample by gradient PAGE in Tris-glycine buffer (pH 8.6). One of the lanes was stained with silver, and the identical gel was sliced (1 mm) and extracted in PB (pH 7.2) for electrophoresis. As shown in Fig. 2A, lane a, the purified factor exhibited two bands on PAGE, and the biological activity corresponded to the slow-moving upper band. The active extract from the gel was concentrated and again electrophoresed (Fig. 2A, lane b). The stained band appeared homogeneous. The same extract was subjected to SDS-PAGE (Fig. 2B, lane c), and unexpectedly, an apparently single band with a molecular size of 50 to 55 kDa was observed.

Characterization of the factor. Physicochemical character-

TABLE 1. Summary of procedures for purification of INF-γ-inducing factor

Procedure	Protein (mg)	Total activity (U)[a]	Recovery (%)	Sp act (U/mg)
Serum	6,048	204,800	100	34
Ammonium sulfate	4,212	206,700	100	49
DEAE-Sepharose	1,428	146,200	71	102
Ultrogel	313	100,700	49	322
Phenyl-Sepharose	0.123	34,850	17	283,333

[a] The titer (1 U/ml) of IFN-γ-inducing factor was tentatively expressed as the maximum dilution necessary to induce 160 U of IFN-γ in the culture.

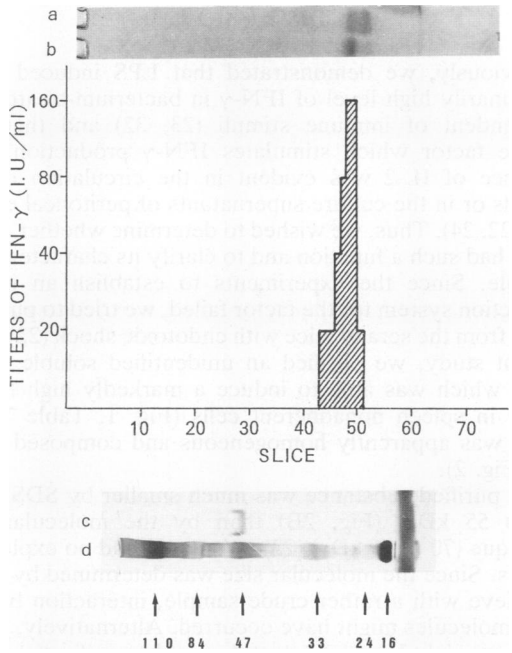

FIG. 2. Electrophoresis of IFN-γ-inducing factor. The eluate from phenyl-Sepharose column chromatography was subjected to PAGE (lane a) and stained with silver, the equivalent gel was sliced to 1-mm thickness, and the proteins were extracted by electrophoresis and assayed for IFN-γ-inducing activity. The active extract was again electrophoresed on the same gel by PAGE (lane b) and SDS-PAGE (lane c). Gels were stained with silver. Lane d represents molecular size standards (in kilodaltons).

ization of the purified factor regarding heat stability and sensitivity to protease, DNase, and 2-mercaptoethanol was carried out (Table 2). The factor was labile to heat treatment and protease treatment. Treatment with 2-mercaptoethanol resulted in augmented ability to induce IFN-γ. DNase treatment had no effect on the activity.

Dual requirement and effect of a dose. As shown in Table 3, the partially purified factor (phenyl-Sepharose eluate) alone, IL-2, a suboptimal dose of ConA, or anti-CD3 MAb alone failed to induce IFN-γ in NWNA cells. However, combined stimulation with partially purified factor and IL-2, ConA, or anti-CD3 MAb produced marked induction in these cells. This mutual dependency was strict, and almost no detectable amount was produced with a high dose of the factor alone. However, LPS, SEA, or anti-Thy-1 MAb together with the factor did not induce significant levels of IFN-γ. SEA alone induced a high level of IFN-γ when supplemented with adherent cells, while the factor failed to exhibit an enhancing effect. Interestingly, the amount of IFN-γ produced by these cells was dependent on the factor concentration when stimulator, IL-2, ConA (suboptimal dose), or anti-CD3 MAb was employed (Fig. 3). However, the requirement for a higher dose of the factor was observed when IL-2 or ConA was employed (Fig. 3), and anti-CD3 MAb induced a high level of IFN-γ in the smallest amount of the factor (Fig. 3). More

TABLE 2. Effects of various treatments on the factor

Treatment[a]	IFN-γ (mean IU/ml ± SD)
PBS	3,413 ± 1,478
Pronase K (50 μg/ml, 1 h)	133 ± 46
DNase (5 μg/ml, 1 h)	3,413 ± 1,478
RNase (5 μg/ml, 1 h)	2,560 ± 0
Heat (80°C, 60 min)	66 ± 23
pH 2 (overnight)	26 ± 11
2-Mercaptoethanol (0.1 mM)	4,266 ± 1,478
6 M urea	266 ± 92

[a] IFN-γ-inducing factor was variously treated and assayed for its costimulatory activity in NWNA cells in the presence of anti-CD3 MAb (1 μg/ml). Each enzyme treatment was carried out at 37°C. Phenyl-Sepharose eluate containing the factor was dialyzed to pH 2.0 against Tris-HCl, 2-mercaptoethanol, or 6 M urea in PB. After dialysis, the samples were dialyzed against 0.02 M PB (pH 7.2).

TABLE 3. Dual requirement for stimulant and IFN-γ-inducing factor[a]

Treatment	IFN-γ produced (mean IU/ml ± SD)
IL-2 (10 U/ml)	<10 ± 0
ConA	
0.5 μg/ml	<10 ± 0
5.0 μg/ml	52 ± 26
Anti-CD3 MAb (1 μg/ml)	20 ± 12
IFN-γ-inducing factor (1/20)	16 ± 5
+IL-2	1,280 ± 783
+ConA (0.5 μg/ml)	1,024 ± 350
+Anti-CD3 MAb	3,840 ± 1,810
+LPS (10 μg/ml)	<10 ± 0
+SEA (1 μg/ml)	<10 ± 0
+Anti-Thy-1 antibody (1/25)	<10 ± 0
SEA (whole spleen cells)	1,152 ± 286
ConA (whole spleen cells)	12 ± 4
Anti-CD3 MAb (whole spleen cells)	<10 ± 0

[a] Phenyl-Sepharose eluate was employed as the IFN-γ-inducing factor at the final dose (1/20). NWNA cells were prepared from the spleens of C3H/HeJ mice by carefully passing cells through nylon wool twice. Cells (10^7/ml) were stimulated with various stimulants, and culture supernatants were assayed for IFN-γ activity. Average values were calculated from five samples.

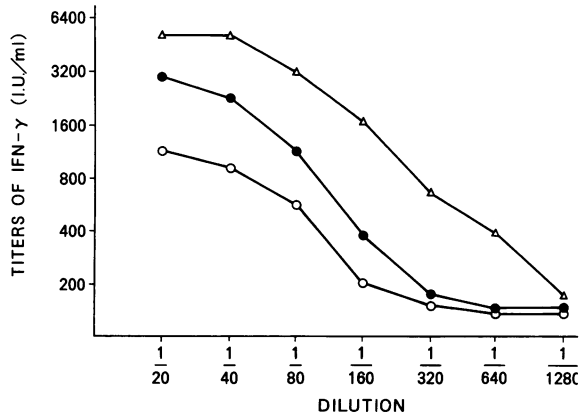

FIG. 3. Mutual dependency between various stimulants and IFN-γ-inducing factor. ○, Dose effect of the factor on IFN-γ production by spleen NWNA cells in the presence of IL-2 (10 U/ml). The partially purified factor (phenyl-Sepharose eluate) was diluted by serial twofold dilution and added to the culture to give the indicated final dose (by volume). IFN-γ in the cultures was assayed after 24 h as described in Materials and Methods. ●, Dose effect of the factor on IFN-γ production in the presence of a suboptimal dose of ConA (0.5 μg/ml); △, dose effect of the factor on IFN-γ production in the presence of anti-CD3 MAb (1 μg/ml).

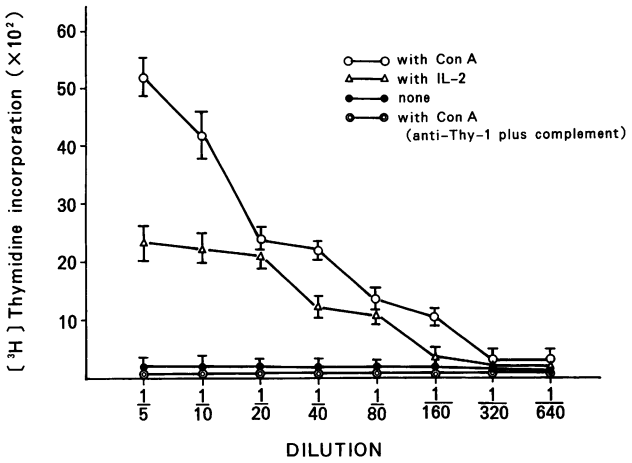

FIG. 4. Effect of IFN-γ-inducing factor on T-lymphocyte proliferation in the presence of IL-2 or ConA. The purified factor was serially diluted and added to the culture of NWNA cells (4×10^5 cells per ml) from normal spleens of C3H/HeJ mice as described in the legend to Fig. 3. Each sample was measured for thymidine incorporation in the presence of IL-2 (10 U/ml) or ConA (0.5 μg/ml). For the measurement of thymidine incorporation, NWNA cells (4×10^5 cells per ml) were placed in a microwell in 130 μl of medium, and 20 μl of [^{3}H]thymidine solution was added as described in Materials and Methods. Average value and standard deviation for each sample were calculated from the values for four wells.

than 1 μg of the dose of anti-CD3 MAb per ml was required in order to get the maximum level of induction.

Effect of the factor on cell proliferation. The effect of the factor on T-lymphocyte proliferation was then examined. When macrophage-depleted T lymphocytes from the normal spleen were stimulated with IL-2 alone, almost no cell proliferation was observed. However, the addition of partially purified factor stimulated significant proliferation (Fig. 4). Cells treated with anti-Thy-1 antibody and complement failed to take up thymidine when treated with the factor and ConA. In comparison with IFN-γ induction, T-lymphocyte proliferation was only slightly stimulated by the factor alone. Moreover, the partially purified factor exhibited accessory functions in IFN-γ production and proliferation in T lymphocytes when stimulated by a suboptimal dose of ConA (0.5 μg/ml) (Fig. 4). A suboptimal dose of ConA alone stimulated proliferation only slightly, while addition of the partially purified factor enhanced the uptake of radioactive thymidine by T lymphocytes about 50-fold at the maximum dose employed. When ConA was added to the culture at the optimal dose (5 μg/ml), proliferation was augmented eightfold.

Effect of antisera against various cytokines. Previously, we examined various cytokines to assess whether they could substitute for the present factor (22). We failed to find any cytokine which could induce IFN-γ in purified T lymphocytes in the presence of IL-2. In addition, since IL-1 is reported to be an essential cofactor for IL-2-dependent IFN-γ induction (34), we examined whether anti-IL-1 antisera inhibited IFN-γ production induced by the combination of IL-2 and the purified factor. No significant inhibition was observed, and likewise, antibodies against tumor necrosis factor, IL-4, IL-5, and IL-6 did not affect IFN-γ induction by the factor and IL-2 (data not shown).

DISCUSSION

Previously, we demonstrated that LPS induced an extraordinarily high level of IFN-γ in bacterium-treated mice independent of immune stimuli (23, 32) and that some soluble factor which stimulates IFN-γ production in the presence of IL-2 was evident in the circulation of such animals or in the culture supernatants of peritoneal exudate cells (22, 24). Thus, we wished to determine whether a single factor had such a function and to clarify its characteristics if possible. Since the experiments to establish an in vitro production system for the factor failed, we tried to purify the factor from the sera of mice with endotoxic shock (22). In the present study, we purified an unidentified soluble protein factor which was able to induce a markedly high level of IFN-γ in spleen nonadherent cells (Fig. 1, Table 1). The factor was apparently homogeneous and composed of one unit (Fig. 2).

The purified substance was much smaller by SDS-PAGE (50 to 55 kDa) (Fig. 2B) than by the molecular sieve technique (70 to 75 kDa) (22). We could find no explanation for this. Since the molecular size was determined by molecular sieve with a rather crude sample, interaction between other molecules might have occurred. Alternatively, a small fragment might have been lost during the purification procedures. The molecular shape may also have influenced the result. Since the factor lost its activity in SDS-PAGE, we also failed to definitely establish that the band revealed by SDS-PAGE was the factor. In any event, the molecular sizes of most cytokines range from 20 to 30 kDa, making this material comparatively large.

By using partially purified material, mutual dependence on both various stimulants and the factor was elucidated (Fig. 3, Table 3). IL-2, ConA, anti-CD3 MAb, or the factor alone failed to induce IFN-γ production in NWNA cells. Effective IFN-γ induction was observed only when the stimulants and the factor were used together. The apparent dose effect of the factor on IFN-γ induction was observed for every stimulant, and IL-2 needed the higher dose of the factor for its accessory function. This dose effect may reflect the fact that the factor is usually effective in the limited environment where it is synthesized and that it appears in the circulation only in case of systemic inflammatory reaction such as endotoxic shock. That the factor failed to exert its accessory function on NWNA cells stimulated with SEA may suggest that the factor reveals its function through CD3 antigen rather than T-cell receptor for antigens. Yet, detailed analysis on the dual requirement was not performed in the present study. Binding of the stimulants to lymphoid cells might occur prior to the binding of the factor, or the reverse might occur.

Since the deprivation of macrophages eliminated or decreased IFN-γ production induced by ConA in normal spleen cells (22), the participation of macrophages in the NWNA cells was considered negligible. In the present study, ConA (suboptimal dose), SEA, anti-CD3 MAb, IL-2, or LPS also failed to stimulate IFN-γ secretion in NWNA cells, which were carefully passed through nylon wool twice (Table 3). The addition of adherent cells to a culture of NWNA cells stimulated with SEA restored IFN-γ production, while addition to NWNA cells stimulated with a suboptimal dose of ConA or anti-CD3 MAb did not. The markedly high levels of IFN-γ induction may be due to the enrichment of IFN-γ-producing cells, since the usual levels produced in unseparated spleen cells by stimulation with an optimal dose of ConA were 320 U/ml or less. Another

possible explanation is that some adherent cells causing feedback regulation were removed from the cells during preparation of NWNA cells. The cytokines IL-1, IL-3, IL-4, IL-5, IL-6, and tumor necrosis factor, whose molecular weights are much lower than that of the factor, failed to substitute for the factor, and conversely, antibodies against these cytokines did not inhibit the induction of IFN-γ production (data not shown). However, details of the molecule such as its terminal amino acid sequence or amino acid composition to be compared with details of the known cytokines remain to be elucidated. We were unable to obtain sufficient amounts of the factor for these purposes.

The cells producing the factor have also not been identified. In order to get enough factor for a detailed analysis of its molecular characteristics, this problem of identification should be solved. Established macrophage cell lines such as P388D1 or RAW were examined, but no significant activity was observed, perhaps because of the differentiation stage of the macrophages or because cells other than macrophages are responsible for production of activity. Recently, NKSF/IL-12 was characterized and its cDNA was cloned from human B-cell lines (4, 9, 16, 30, 35). It is desirable also to examine B cells in murine systems, since the factor in the present study is induced by LPS and LPS is a strong stimulator for B cells. The apparent molecular weight of the active form of NKSF/IL-12; its IFN-γ inducibility; and the synergy with IL-2, anti-CD3 MAb, or a mitogenic lectin are all similar to those of the present factor. It will be of interest to examine whether B cells produce a factor regulating the function of T cells under physiological conditions.

Another difficulty concerns the cells responding to the factor by producing IFN-γ. Among IFN-γ-producing $CD4^+$ or $CD8^+$ T cells and NK cells, each type of cell may respond differently to different stimulants in the presence of the factor. It is interesting that there are no differences among mouse strains in their abilities to produce IFN-γ, whereas they show marked differences in their abilities to release the factor in the circulation (22). That is, the IFN-γ-inducing factor was released hardly at all in the circulation of mice genetically resistant to LPS, whereas there seemed to be no difference in the responsiveness to the factor among LPS-sensitive and -resistant strains.

In conclusion, we have successfully demonstrated the existence of a soluble factor which is able to stimulate IFN-γ production in T lymphocytes or NK cells in cooperation with various stimulants. It will be interesting to examine its relationship to NKSF/IL-12 and to speculate on its roles relavent to the regulation of the Th1 subset, which is said to be involved in cellular immunity in vivo.

REFERENCES

1. **Antonelli, G., F. Dianzani, J. V. Damme, P. Amicucci, F. DeMarco, and A. Cefaro.** 1988. A macrophage-derived factor different from interleukin 1 and able to induce interferon-γ and lymphoproliferation in resting T lymphocytes. Cell. Immunol. **113:**376–386.
2. **Arbeit, R. D., P. L. Leary, and M. J. Levin.** 1982. Gamma interferon production by combinations of human peripheral blood lymphocytes, monocytes, and cultured macrophages. Infect. Immun. **35:**383–390.
3. **Blanchard, D. K., J. Y. Djeu, T. W. Klein, H. Friedman, and W. E. Stewart II.** 1986. Interferon-γ induction by lipopolysaccharide: dependence on interleukin 2 and macrophages. J. Immunol. **136:**963–970.
4. **Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri.** 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J. Exp. Med. **173:**869–879.
5. **Croll, A. D., and A. G. Morris.** 1986. The regulation of γ-interferon production by interleukin 1 and 2. Cell. Immunol. **102:**33–42.
6. **Croll, A. D., M. F. Wilkinson, and A. G. Morris.** 1986. Gamma-interferon by human low-density lymphocytes induced by T-cell mitogens. Immunology **58:**641–646.
7. **Finter, N. B., J. Vilček, and E. DeMaeyer (ed.).** 1984. Interferon 2. Interferon and the immune system. Elsevier/North-Holland Publishing Co., Amsterdam.
8. **Grabstein, K., S. Dower, S. Gillis, D. Uradol, and A. Larsen.** 1986. Expression of interleukin 2, interferon-γ, and IL 2 receptor by human peripheral blood lymphocytes. J. Immunol. **136:** 4503–4508.
9. **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.
10. **Handa, K., R. Suzuki, H. Matsui, Y. Shimizu, and K. Kumagai.** 1983. Natural killer (NK) cells as a responder to interleukin 2 (IL 2). II. IL 2-induced interferon γ production. J. Immunol. **130:**988–992.
11. **Heremans, H., J. V. Damme, C. Dillen, R. Dijekins, and A. Billiau.** 1990. Interferon γ, a mediator of lethal lipopolysaccharide-induced Schwarzman-like shock reactions in mice. J. Exp. Med. **171:**1853–1869.
12. **Janeway, C. A., S. Carding, B. Jones, J. Murray, P. Portoles, R. Rasmussen, K. Saizawa, J. West, and K. Bottomly.** 1988. CD4+ T cells: specificity and function. Immunol. Rev. **101:**39–80.
13. **Kasahara, T., J. J. Hooks, S. Dougherty, and J. J. Oppenheim.** 1983. Interleukin 2-mediated immune interferon (IFN-γ) production by human T cells and T cell subsets. J. Immunol. **130:**1784–1789.
14. **Kawase, I., C. G. Brooks, K. Kuribayashi, S. Olabuenaga, W. Newman, S. Gillis, and C. S. Henney.** 1983. Interleukin 2 induces γ interferon production: participation of macrophages and NK-like cells. J. Immunol. **131:**288–292.
15. **Kelly, C. D., K. Welte, and H. W. Murray.** 1987. Antigen-induced human interferon-γ production: differential dependence on interleukin 2 and its receptor. J. Immunol. **139:**2325–2328.
16. **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.
17. **Le, J., J. Liu, D. Henricksen Destefano, and J. Vilček.** 1986. Bacterial lipopolysaccharide-induced interferon-γ production: Roles of interleukin 1 and interleukin 2. J. Immunol. **136:**4525–4530.
18. **Lichtman, A. H., J. Chin, J. A. Schmidt, and A. K. Abbas.** 1988. Role of interleukin 1 in the activation of T lymphocytes. Proc. Natl. Acad. Sci. USA **85:**9699–9703.
19. **Matsumura, N., and M. Nakano.** 1988. Endotoxin-induced interferon-γ production in culture cells derived from BCG-infected C3H/HeJ mice. J. Immunol. **140:**494–500.
20. **Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman.** 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. **136:**2348–2357.
21. **Mukaida, N., T. Kasahara, J. Hosoi, K. Shioiri-Nakano, and T. Kawai.** 1986. Effect of anti-Tac antibody on the response of large granular lymphocytes to interleukin-2. Immunology **57:** 137–143.
22. **Nakamura, K., H. Okamura, M. Wada, K. Nagata, and T. Tamura.** 1989. Endotoxin-induced serum factor that stimulates gamma interferon production. Infect. Immun. **57:**590–595.
23. **Okamura, H., K. Kawaguchi, K. Shoji, and Y. Kawade.** 1982. High-level induction of gamma interferon with various mitogens in mice pretreated with *Propionibacterium acnes*. Infect. Immun. **38:**440–443.

24. **Okamura, H., M. Wada, K. Nagata, T. Tamura, and K. Shoji.** 1987. Induction of murine gamma interferon production by lipopolysaccharide and interleukin-2 in *Propionibacterium acnes*-induced peritoneal exudate cells. Infect. Immun. **55:**335–341.
25. **Ortaldo, J. R., A. T. Mason, J. P. Gerard, L. E. Henderson, W. Farrar, R. F. Hopkins III, R. B. Heberman, and H. Rabin.** 1984. Effects of natural and recombinant IL 2 on regulation of IFN γ production and natural killer activity: lack of involvement of the Tac antigen for these immunoregulatory effects. J. Immunol. **133:**779–783.
26. **Pearlstein, K. T., M. A. Palladino, K. Welte, and J. Vilček.** 1983. Purified human interleukin-2 enhances induction of immune interferon. Cell. Immunol. **80:**1–9.
27. **Reem, G., and N. H. Yeh.** 1984. Interleukin 2 regulates expression of its receptor and synthesis of gamma interferon by human T lymphocytes. Science **225:**429–430.
28. **Shiiba, K., K. Itoh, Y. Shimizu, and K. Kumagai.** 1984. Interleukin 2 (IL-2)-dependent proliferation of human NK cells accompanied by interferon γ-production, p. 187–192. *In* T. Hoshina, H. S. Koren, and A. Uchida (ed.), natural killer activity and its regulation. Excerpta Medica, Amsterdam.
29. **Snapper, C. M., and W. E. Paul.** 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science **236:**944–947.
30. **Stern, A. S., F. J. Podlaski, J. D. Hulmes, Y. C. E. Pan, P. M. Quinn, A. G. Wolitzky, P. C. Familletti, D. L. Stremlo, and T. Truitt.** 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.
31. **Trinchieri, G., M. Matsumoto-Kobayashi, S. C. Clark, J. Seehra, L. London, and B. Perussia.** 1984. Response of resting human peripheral blood natural killer cells to interleukin 2. J. Exp. Med. **160:**1147–1169.
32. **Wada, M., H. Okamura, K. Nagata, T. Shimoyama, and Y. Kawade.** 1985. Cellular mechanisms in *in vivo* production of gamma interferon induced by lipopolysaccharide in mice infected with Mycobacterium bovis BCG. J. Interferon. Res. **5:**431–443.
33. **Weaver, C. T., C. M. Hawrylowitz, and E. R. Unanue.** 1988. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. Proc. Natl. Acad. Sci. USA **85:**8181–8185.
34. **Wilson, A. B., J. M. Harris, and R. R. A. Coombs.** 1988. Interleukin-2-induced production of interferon-γ by resting human T cells and large granular lymphocytes: requirement for accessory cell factors, including interleukin-1. Cell. Immunol. **113:**130–142.
35. **Wolf, S. F., P. A. Temple, M. Kobayashi, 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 heterodimetric cytokine with multiple biologic effects on T and natural killer cells. J. Immunol. **146:**3074–3081.