INTERRELATIONSHIPS OF HUMAN INTERFERON-GAMMA WITH LYMPHOTOXIN AND MONOCYTE CYTOTOXIN

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Interferons (IFN) affect many cell functions in addition to their inhibitory action on virus replication. Among these effects on cell functions are the inhibition of cell growth in vitro, augmentation or inhibition of natural killer (NK) cell-mediated cytotoxicity, induction of major histocompatibility antigens, and the activation of macrophage-mediated cytotoxicity (reviewed in 1–3). Different types of IFN, however, may vary in their capacities to mediate these biological responses. For example, the mitogen-induced “immune” (gamma) IFN appears to show stronger immunoregulatory (4–8) and cell growth inhibitory (9–11) activities than alpha or beta IFN. While recent studies used recombinant IFN-gamma (12) to demonstrate immunoregulatory activities of this IFN, most studies of antiproliferative activity used either crude or only partially purified IFN-gamma preparations, thus leaving open the possibility that this biological activity may be at least partially due to the presence of other cytotoxic lymphokines in these preparations.

Lymphotoxins (LT) are a group of lymphocyte-derived glycoproteins possessing direct cytostatic and cytolytic activity for a variety of tumor cells (reviewed in 13–15). LT inhibit the growth of transformed cells more than that of normal cells from the same species (16, 17). In addition to their effects on cell growth, LT may play a role in cell-mediated immune reactions. LT has been implicated in cytolytic T lymphocyte-mediated cytotoxicity (18, 19), antibody-dependent, cell-mediated cytotoxicity (ADCC) (20), and NK cell cytotoxicity reactions (21–23). Diverse forms of LT with molecular weights ranging from 12,000 to 200,000

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1 Abbreviations used in this paper: Con A, concanavalin A; FBS, fetal bovine serum; FPLC, fast protein liquid chromatography; IFN, interferon; LT, lymphotoxin; MAb, monoclonal antibody; MEM, Eagle’s minimum essential medium; NaDodSO4, sodium dodecyl sulfate; NK, natural killer; PBL, peripheral blood leukocytes; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; recIFN-gamma, recombinant interferon-gamma; SA, silicic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.
and somewhat different lytic capabilities have been separated by gel filtration (24, 25). In addition to LT, a number of other cytotoxins, apparently derived from monocytes or macrophages, have been described (25–29). Their relationship to classic LT (which itself has not been fully defined in molecular terms) needs to be clarified.

We recently demonstrated that LT and IFN-gamma are induced in parallel after various forms of mitogenic stimulation (30). In another report (31), we demonstrated that crude IFN-gamma preparations produced by human peripheral blood leukocytes (PBL) upon stimulation with 12-O-tetradecanoylphorbol-13-acetate (TPA) and phytohemagglutinin (PHA) exert a marked growth-inhibiting effect on HeLa cells and human foreskin fibroblasts (FS-4) that was not observed with more highly purified IFN-gamma preparations (31). The present study was undertaken to determine whether LT and/or other cytotoxins produced along with IFN-gamma contribute to the cytotoxic activity of crude IFN-gamma preparations.

**Materials and Methods**

**Materials.** Plateletpheresis residues were obtained through the courtesy of M. Wiebe from the New York Blood Center. TPA (LC Services Corp., Woburn, MA) was dissolved in 100% ethanol at a concentration of 1 mg/ml and stored at -20°C. PHA was prepared in the laboratory of J. D. Oppenheim at New York University Medical Center; stock solutions at 1 or 10 mg/ml in phosphate-buffered saline (PBS), pH 7.4, were kept frozen at -20°C. RPMI 1640 and Eagle’s minimal essential medium (MEM) were purchased from Gibco Laboratories, Grand Island, NY. Silicic acid (SA) (100 mesh) was obtained from Mallinckrodt Inc., St. Louis, MO. Concanavalin A (Con A)-Sepharose 4B, DEAE-Sepharose, and AH-Sepharose were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Biogel P200 was obtained from Bio-Rad Laboratories, Rockville Center, NY. Panisorbin, a preparation of the Cowan I strain of *Staphylococcus aureus*, was obtained from Calbiochem-Behring Corp., San Diego, CA. Rabbit antiserum against mouse Ig was purchased from Cappel Laboratories, Cochranville, PA. Highly purified recombinant IFN-gamma from *E. coli* (recIFN-gamma) was prepared by Genentech, Inc., South San Francisco, CA; the specific activity of this material ranged from ~10^6 to 10^7 laboratory units/mg of protein, depending on the assay system used. IFN-alpha purified on a monoclonal antibody affinity column to a specific activity of 2 × 10^8 IU/mg of protein was a gift from Bernard Horowitz of the New York Blood Center. IFN-beta was prepared by Rentschler Arzneimittel, Laupheim, Federal Republic of Germany and further purified in this laboratory on Blue-Sepharose to a specific activity of ~10^7 IU/mg of protein. All other chemicals were of the highest purity grade from standard sources.

**Preparation of Conditioned Medium Containing IFN-gamma and Lymphotoxin.** The procedure for obtaining high yields of IFN-gamma by combined stimulation of human PBL with the tumor-promoting agent, TPA, and the T cell mitogen, PHA, has been described (32). Briefly, plateletpheresis residues were washed once to remove plasma and seeded at a density of 6 × 10^6 white blood cells/ml in serum-free RPMI 1640 medium supplemented with gentamycin (50 μg/ml), Hepes (6 mM), Tricine (3 mM), and 20 ng/ml TPA. After a 2–3 h incubation period at 37°C in a humidified CO2 incubator, PHA was added to a final concentration of 5 μg/ml and the cultures were incubated for an additional 48–72 h, at which time the conditioned medium was harvested, centrifuged to remove cells and debris, and stored at 4°C until further processing.

**Isolation of Nonadherent and Adherent Cells.** Lymphocyte-rich plateletpheresis residues or buffy coats obtained from the New York Blood Center and from New York University Medical Center Blood Bank, respectively, were used for these studies within 24 h of collection. The heparinized blood concentrates were diluted twofold with RPMI 1640 medium and the initial separation of leukocytes from erythrocytes was achieved by
centrifugation on Ficoll-Hypaque gradients at 400 g for 30 min. Cells collected at the interface were washed twice with serum-free RPMI 1640 and were subsequently resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS). Removal of adherent cells was accomplished according to the method of Kumagai et al. (33). Tissue culture flasks (175 cm²) previously coated for 2 h with heat-inactivated FBS and washed once to remove serum were seeded with 50 ml of a white blood cell suspension adjusted to 4 × 10⁹ cells/ml and incubated for 1.5 h at 37°C in a CO₂ incubator. The remainder of the Ficoll-Hypaque-purified cells was set aside to be used as the "total" cell population. After the incubation period, nonadherent cells were removed and incubated in a second set of FBS-treated flasks for an additional 1.5 h. The nonadherent cells from the second set of flasks were collected and used in experiments as "nonadherent cells". Firmly adherent cells from both sets of FBS-treated flasks were detached after incubation with Ca²⁺, Mg²⁺-free PBS, pH 7.4, containing 0.2% EDTA and 5% FBS for 30 min at 37°C. Adherent cells recovered after this procedure, which represented 10–20% of the total number of mononuclear cells present in the original unseparated cell population, were washed twice with serum-free RPMI 1640 to remove EDTA and serum. Populations of nonadherent, adherent, and total cells seeded in 24-well Linbro plates at a final cell concentration of 6 × 10⁶ cells/ml were treated with TPA (20 ng/ml) or PHA (5 μg/ml), alone or in combination, as described in the preceding section. Cell-free supernatants were harvested at 72 h and assayed for both IFN-gamma and LT activities.

Assay for IFN Activity. The antiviral activity of IFN-gamma was assayed by inhibition of the cytopathic effect of encephalomyocarditis virus in human FS-4 fibroblasts in 96-well microtiter tissue culture plates as described elsewhere (32). An internal laboratory standard of human IFN-gamma was included in each assay and titers of IFN-gamma were expressed in laboratory units.

Assay for Lymphotoxin Activity. This assay is a modification of that developed by Spofford et al. (34). Briefly, L 929 cells (alpha subline, obtained from Gale Granger, University of California at Irvine, CA) were seeded at a density of 10⁴ cells/well in 96-well plastic tissue culture plates in RPMI 1640 medium containing 5% FBS in the presence of 0.5 μg/ml mitomycin C. After a 16–24 h incubation period at 37°C in a humidified CO₂ incubator, twofold serial dilutions of the test samples were prepared in separate 96-well plates. Mitomycin C-containing media from L 929 cell cultures were then discarded and replaced with the dilutions of the test samples. After a second incubation period of 24–48 h, the plates were examined microscopically for LT-induced cytolysis (rounding-up and detachment of cells). The highest dilution producing 50% cytolysis was taken as the endpoint of LT activity. LT activity is expressed in U/ml based on a laboratory standard provided by Gale Granger, as well as on an internal laboratory standard. Human IFN-gamma (natural or recombinant) neither mimicked LT activity nor did it affect the action of LT in this assay using murine cells. TPA and PHA, at concentrations used for the induction of PBL cultures, were also without effect in the LT assay.

HeLa Cell Clonal Survival Assay. A cloned HeLa cell line was used. Cells were seeded in triplicate at a density of 100 cells per well in 24-well Linbro plates or 300 cells per 60-mm petri dish, in MEM supplemented with 5% FBS, gentamycin, fungizone, and Hepes. After allowing a minimum incubation period of 4 h for cell attachment, test samples of different IFN-gamma and LT preparations were added and the plates were returned to the incubator for a period of 1 wk or until colonies were visible macroscopically. The colonies were washed with PBS, fixed with methanol, stained with Giemsa staining solution, and the number of surviving colonies was scored.

Partial Purification of IFN-gamma and Lymphotoxin. The three-step purification procedure developed in our laboratory for human IFN-gamma has been described in detail elsewhere (35, 36). This procedure includes sequential adsorption and elution of conditioned medium on SA, Con A-Sepharose, and DEAE-Sephacel. The processed material was then concentrated by a Millipore CX-10 ultrafiltration unit (Millipore Corp., Bedford, MA).

Gel Filtration Chromatography. Further purification of IFN-gamma and LT was performed using Biogel P200 filtration chromatography as previously described (35). A 1-ml
sample of concentrated material purified by the three-step process was applied to a Biogel P200 column (1.5 X 86 cm) that was previously equilibrated with 20 mM sodium phosphate buffer, pH 8.0. The flow rate was adjusted to 20 ml/h and 0.5-ml fractions were collected and assayed for biological activity.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).** Electrophoresis was performed on a linear 10–20% acrylamide gradient slab gel using the Laemmli procedure (37). Samples used for electrophoresis were purified as described above and concentrated. For treatment with sodium dodecyl sulfate (NaDodSO4), sample solutions were adjusted to contain 0.1% of the detergent and were incubated for 10–30 min at 25°C before application to the gel. Upon completion of electrophoresis, the slab was cut into 1-mm slices with the aid of a razor blade gel slicer. About ¾ portions of each slice were used for assays of IFN-gamma and LT, and ¼ of each slice still attached to the molecular weight markers (low molecular weight markers; Bio-Rad Laboratories) was used for staining with Coomassie blue stain.

**Isoelectric Focusing.** Conditioned medium from TPA-PHA-stimulated PBL cultures containing both IFN and LT activities was processed through the DEAE-Sephacel step (see above), concentrated, and loaded into the middle portion of a glass cylindrical tube gel (2 X 110 mm) containing 7.5% acrylamide plus 0.2% ampholine (pH 3.5–10.0). Glycerol concentrations in the bottom, middle, and top portions of the gel were 15, 11.5, and 6.5%, respectively. Electrophoresis was performed at 500 V for 3–4 h at 4°C. After electrophoresis, extruded gels were frozen at −70°C and then separated into 1-mm slices with a razor blade gel slicer. Each gel slice was eluted overnight at 4°C in 1 ml of MEM containing 5% FBS, and assayed the following day for LT and IFN-gamma activities. For pH determinations, two 1-mm slices were placed in 1 ml of degassed H2O and allowed to equilibrate for 1 h at 4°C before taking pH readings.

**Fast Protein Liquid Chromatography (FPLC).** Conditioned medium containing both lymphokine activities was processed through the Biogel P200 step, concentrated, and further purified by FPLC separation, performed by Larry Schwartz at the Pharmacia Applications Laboratory, Piscataway, NJ. The sample, equilibrated with 20 mM Tris-HCl, pH 7.0, was first chromatographed on a Mono Q (anionic) exchanger (Pharmacia Fine Chemicals). The unbound flow-through fraction containing close to 100% of the LT and IFN-gamma activities present in the starting material was subsequently chromatographed on a Mono S (cationic) exchanger. Elution in both cases was with a linear salt gradient ranging from 0 to 0.5 M NaCl in 20 mM Tris-HCl. Final elution was with 1 M NaCl. Fractions of 0.5 ml were collected, diluted 1:10 in MEM with 5% FBS, and assayed for LT and IFN-gamma activities.

**Neutralization of IFN-gamma and Lymphotoxin by Antisera.** Appropriate dilutions of supernatants from TPA-PHA-induced PBL cultures, partially purified through the DEAE-Sephacel or Biogel P200 steps, were incubated with polyclonal antisera against human IFN-gamma or LT for 1 h at 37°C. Residual IFN-gamma or LT activity was then determined by biological assays. Control samples included the lymphokine preparation or antiserum alone treated in a similar manner. Antibody titers are expressed in neutralizing units/ml, i.e., reciprocals of the highest dilution that completely neutralized 10 U/ml of IFN-gamma or LT. The antisera used in the experiments shown in Table II were as follows: (a) Rabbit anti-IFN-gamma partially pure was prepared at NYU against partially purified preparations of IFN-gamma that also contained LT activity (processed through the DEAE-Sephacel step). It had a neutralizing titer of 2,000 U/ml for IFN-gamma. (b) Rabbit anti-IFN-gamma pure was prepared at NYU against IFN-gamma extracted from SDS-PAGE gel slices corresponding to the 20,000 mol wt species of IFN-gamma as previously described (31). The neutralizing titer was 200 U/ml for IFN-gamma. (c) Rabbit anti LT was prepared against unfractionated whole supernatants (WS) of PHA-stimulated human PBL by the laboratory of Gale Granger, University of California at Irvine, CA (25). According to the producing laboratory, 2 µl neutralized 1 U/ml of LT activity. (d) Goat anti-LT alpha-2 was prepared against the “alpha-2” subclass of LT purified from PHA-stimulated human PBL after Ultragel, DEAE-Cellulose, and native PAGE (18) by Gale Granger’s laboratory. 1 µl neutralized 1 U/ml of homologous LT activity. (e) Rabbit
anti-LT<sub>1788</sub> was prepared against human LT purified from human lymphoblastoid 1788 cells at Genentech, Inc. The titer of this antiserum measured with homologous LT was ~7,500 neutralizing U/ml.

Preparation of Highly Purified Lymphotoxin by Use of Affinity Chromatography with Monoclonal Antibody to IFN-gamma. An affinity column was prepared using a recently described monoclonal antibody (MAb) to IFN-gamma (38). An AH-Sepharose mini-column (bed volume, 5 ml) equilibrated with PBS was activated by very slowly pumping 10 ml of 12.5% glutaraldehyde (vol/vol in PBS) through the column for a period of 1 h. Excess glutaraldehyde was removed by several washes with PBS. 7 ml of hybridoma culture fluid containing enough MAb to neutralize 35,000-70,000 U of human IFN-gamma activity was carefully pumped onto the column and recycled a total of three times over a period of 2 h. The column was washed with PBS until no more protein was detectable in the eluate. The column was then washed with 10 vol of 0.1 M ethanolamine in PBS and equilibrated with 10 vol of PBS. A partially purified preparation of IFN-gamma and LT processed through the Con A-Sepharose step was slowly added onto this column and the flow-through material was assayed for both lymphokine activities.

Immunoprecipitation of IFN-gamma Using MAb to Human IFN-gamma. Two hybridomas secreting MAb to human IFN-gamma were recently prepared by Le et al. (39). B3 is a cloned mouse hybridoma that secretes a neutralizing MAb with titers of ~1,000 U/ml in tissue culture supernatants and 1 x 10<sup>8</sup> U/ml in ascites fluid. Another hybridoma, B1, secretes a nonneutralizing MAb that binds human IFN-gamma with high avidity. The procedure for immunoprecipitation was briefly as follows: a 100 µl aliquot of either B3 or B1 hybridoma supernatant was incubated for 90 min at 37°C with 100 µl of partially purified conditioned medium sequentially processed through the DEAE-Sephacel step. A 100 µl aliquot of rabbit antiserum to mouse Ig (24 µg/ml) was added and the mixture was further incubated for 30 min. Finally 100 µl of washed Pansorbin was added and the mixture was incubated for another 50 min. The immunoprecipitate was sedimented by centrifugation in a Beckman microcentrifuge (Beckman Instruments, Inc., Fullerton, CA) for 2 min. The supernatants were then assayed for LT and IFN-gamma activities.

Results

Inhibitory Effects of Different IFN Preparations in the HeLa Cell Clonal Survival Assay. To evaluate the relative potencies of various IFN, we compared preparations of IFN-gamma of increasing purity, including highly purified recIFN-gamma, with purified preparations of IFN-alpha and IFN-beta in their effects on HeLa cell clonal survival (Fig. 1). Crude IFN-gamma preparations from TPA-PHA-induced PBL supernatants showed the strongest dose-related inhibition of clonal survival, while highly purified recombinant IFN-gamma, as well as natural IFN-alpha and beta, showed none. IFN-gamma partially purified by sequential adsorption and elution on SA, Con A-Sepharose, and DEAE-Sephacel showed intermediate activity. The inhibitory action of the crude and partially purified IFN-gamma was not due to extraneous agents used for induction, such as TPA and PHA, or to chemicals introduced during the purification procedures (data not shown). These results imply that a factor different from IFN-gamma present in these preparations is responsible for the potent cytotoxic effect in the HeLa cell clonal survival assay used in our experiments.

We recently reported (30) that high levels of other lymphokines including LT are induced in plateletpheresis-derived PBL cultures after stimulation with TPA and PHA. It was, therefore, conceivable that LT or some unidentified cytotoxic(s) might play a role in the cytotoxic effect of crude and partially purified IFN-gamma preparations demonstrated in the HeLa cell clonal survival assay.

Cytotoxin Production by Various Cell Populations Present in PBL. To determine
FIGURE 1. Comparative effects of human IFN on HeLa cell clonal survival. The HeLa cell clonal survival assay is described in Materials and Methods. Cells were seeded in 60-mm dishes in triplicate per condition. After cell attachment, the appropriate IFN preparation was added to the cultures at different concentrations. Crude IFN-gamma (○) was a 72-h supernatant collected from cultures of plateletpheresis residue cells stimulated with TPA and PHA. Partially purified IFN-gamma (●) was a preparation processed sequentially on SA, Con A-Sepharose, and DEAE-Sephaloc. RecIFN-gamma (●), IFN-alpha (▲), and IFN-beta (■) are described in Materials and Methods. Activities of IFN-alpha and -beta are expressed in international units, while the potency of IFN-gamma is expressed in laboratory units.

TABLE 1

| Source of PBL cells    | Treatment          | Ifn-gamma U/ml | LT U/ml | Nonadherent cells | Ifn-gamma U/ml | LT U/ml | Adherent cells | Ifn-gamma U/ml | LT U/ml |
|------------------------|--------------------|----------------|---------|-------------------|----------------|---------|----------------|----------------|---------|
| Buffy coat             | None               | <4            | <2      | <4                | <2            | <2      | <4             | <2            | <2      |
|                        | PHA 5 µg/ml        | 12            | 16      | 64                | 96            | <4      | 16             | <4            | <2      |
|                        | TPA 20 ng/ml       | <4            | 4       | 24                | 8             | <4      | 24             | <4            | 128     |
|                        | TPA and PHA        | 320           | 96      | 4,096             | 1,024         | 64      | 96             | 64            | 96      |
| Plateletpheresis residue | None               | <4            | 4       | <4                | <2            | <4      | <4             | <2            | <2      |
|                        | PHA 5 µg/ml        | 16            | 4       | 512               | 56            | <4      | 56             | <4            | 12     |
|                        | TPA 20 ng/ml       | 192           | 32      | 96                | 24            | <4      | 24             | <4            | 512     |
|                        | TPA and PHA        | 10,240        | 640     | 7,680             | 800           | 512     | 512            | 512           | 512     |

* Details of procedure are described in Materials and Methods.

which cell population was producing LT activity in these cultures, plateletpheresis residue or buffy coat-derived mononuclear leukocytes were separated into nonadherent and adherent cell subpopulations. Cultures seeded with cells from the interface of Ficoll-Hypaque gradients (total cells), and cultures seeded with similar numbers of nonadherent or adherent cells, were stimulated with either TPA (20 ng/ml) or PHA (5 µg/ml) alone, or by combined treatment with TPA and PHA. IFN-gamma and LT activities were determined in culture supernatants. The results of two representative experiments with cultures of PBL derived from pooled buffy coats or a plateletpheresis residue are shown in Table 1.

In agreement with earlier findings (30), unseparated PBL cells stimulated with PHA or TPA alone produced low yields of IFN-gamma and LT. Combined treatment with TPA and PHA produced a synergistic enhancement in the production of both lymphokine activities which was particularly marked in total
cells derived from the plateletpheresis residue. It was noted earlier that cultures from buffy coats generally produce lower yields of IFN-gamma than cells from plateletpheresis residues (32). Nonadherent cells from buffy coats were more responsive to all inducing treatments; nonadherent cells from plateletpheresis residues were more responsive to PHA and about equally responsive to the other inducing treatments as total cells. These results suggest that adherent cells in some instances suppress IFN-gamma and LT induction, but this conclusion and the exact nature of the putative suppressor cell will have to be clarified in reconstitution experiments.

A clear dissociation between the appearance of IFN-gamma and LT activities occurred in the adherent cells. IFN-gamma production was detectable only after combined stimulation with TPA and PHA; since the yields were relatively low, it is possible that IFN-gamma in these cultures was produced by residual nonadherent cells that were not removed during the cell separation procedure. In contrast, LT activity was induced in the adherent cells with TPA alone and no further increase in this activity occurred after the addition of PHA. These results suggested that two types of cytotoxic factors, indistinguishable in the LT assay, might be produced in PBL cultures.

This conclusion was also supported by immunological evidence. A polyclonal rabbit antiserum prepared against highly purified LT from a human B lymphoblastoid cell line, RPMI 1788 (40), neutralized LT activity produced by nonadherent cells in response to PHA treatment whereas cytotoxic activity produced by adherent cells or total cells in response to TPA treatment alone was barely neutralized. Supernatants from total cells treated with TPA and PHA were only partially neutralized by this antiserum (results not shown).

Attempts to Separate IFN-gamma from Cytotoxins by Physicochemical Methods. A procedure designed for the partial purification of IFN-gamma (35, 36) was used in an attempt to separate IFN-gamma and LT activities present in the supernatants of unseparated (total) PBL cultures stimulated by combined treatment with TPA and PHA. The two activities could not be separated in the first two steps of this procedure consisting of sequential adsorption and elution on SA and Con A-Sepharose (data not shown). Both activities adsorbed completely to SA and were quantitatively recovered in fractions eluted with 50% ethylene glycol. 80 and 60% of IFN-gamma and LT activities, respectively, were recovered in the fractions eluted from Con A-Sepharose with alpha-methyl-D-mannopyranoside.

IFN-gamma and LT activity-containing fractions, eluted from Con A-Sepharose and equilibrated by dialysis against 20 mM sodium phosphate buffer, pH 8.2, were then applied to DEAE-Sephascel ion exchange columns and eluted with an NaCl gradient. Both LT<sup>+</sup> and IFN-gamma activities were recovered in the unbound fraction and neither one of the two activities was recovered upon elution of the column with salt gradients ranging from 0.1 to 0.5 M NaCl (data not shown). To determine the isoelectric points of the proteins responsible for the two activities more accurately, we applied concentrated, partially purified preparations into the middle of an isoelectric focusing gel with a pH gradient ranging from 3.5 to 10.0 and analyzed the resulting fractions for both IFN-gamma and LT activities. Both lymphokines were heterogeneous with respect to charge. The major component of LT activity focused at a pH of 7.6, with minor
components ranging in pI from 6.8 to 8.8. The two major peaks of IFN-gamma activity focused at pH 9.2 and 8.5, with minor components overlapping the pH range of LT activity (data not shown). Even the high resolving power of FPLC could not separate the IFN-gamma and LT activities. Elution of the Mono S cationic exchanger column resulted in the separation of several distinct protein peaks. Although the ratios of the two biological activities present in the eluted fractions varied, IFN-gamma and LT activities overlapped (data not shown). Thus, we were unable to separate these two activities completely on the basis of charge either by isoelectric focusing or by FPLC.

To determine whether IFN-gamma and LT activities could be separated by molecular size, a sample of partially purified conditioned medium was subjected to molecular sieve chromatography using a Biogel P200 column (Fig. 2). LT activity chromatographed in one major peak at 58,000 mol wt, while IFN-gamma activity chromatographed at a molecular weight corresponding to 50,000. However, when the same material was treated with 0.1% NaDodSO4 and subjected to SDS-PAGE, LT activity eluted in two peaks (Fig. 3), a major peak at 60,000 mol wt and a minor peak at 20,000 mol wt. This 20,000 mol wt activity, not seen in the absence of denaturing treatment on Biogel P200, coeluted from the same 1-mm gel slice with the 20,000 mol wt monomer of IFN-gamma activity. However, the 25,000 mol wt monomer and undissociated dimer of IFN-gamma could be separated from the 60,000 mol wt form of LT, which might represent an oligomeric form (40). Data summarized in the legend of Fig. 3 also reveal that LT activity is more stable than IFN-gamma upon NaDodSO4 treatment.

**Immunological Characterization and Separation of LT and IFN-gamma Activities.** Cytotoxic activity present in unprocessed conditioned medium could not be completely neutralized by antiserum to highly purified LT derived from RPMI 1788 cells (not shown). However, after sequential purification on SA, Con A-Sepharose, and DEAE-Sepharose, all cytotoxic activity was neutralized by this antiserum as well as by sera prepared against LT from human tonsillar tissue (25). These results (Table II) imply that a non-LT cytotoxin present in the original crude preparations was separated from LT during the purification procedure. None of the anti-LT sera neutralized IFN-gamma activity, and LT
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Figure 3. Profile of LT (---) and IFN-gamma (----) activities on SDS-PAGE. A 300-μl sample of conditioned medium processed through the Biogel P200 step was adjusted to contain 0.1% NaDodSO₄ and incubated for 10 min at room temperature before application to the gel. This sample originally contained a total of 3,840 U of LT activity and 336,000 U of IFN-gamma activity. After NaDodSO₄ treatment, the total LT activity was reduced by 20% to 3,072 U while IFN-gamma activity was reduced by 81.7% to 61,440 U. The final recoveries of LT and IFN-gamma activities after SDS-PAGE were 53.5 and 7.6% of the amounts applied onto the gels, respectively.

Table II

Effect of Antisera Against IFN-gamma or LT on Antiviral or Cytotoxic Activities of Partially Purified Lymphokine Preparations

| Lymphokine preparation* | Antisera added* | Units/ml | IFN-gamma | LT |
|-------------------------|----------------|----------|-----------|----|
| 1 None                  | 77             | 51       | <4        | <2 |
| Rabbit anti-IFN-gamma<sub>partially pure</sub> | <4 | <2 |
| 2 None                  | 1,024          | 64       | 768       | <2 |
| Rabbit anti-LT<sub>1788</sub> | <4 | 64 |
| Rabbit anti-IFN-gamma<sub>pure</sub> | <4 | 64 |
| 3 None                  | 2,048          | 64       | 2,048     | <2 |
| Rabbit anti-LT<sub>WS</sub> | 2,048 | <2 |
| Goat anti-LT<sub>alpha-2</sub> | 1,024 | <2 |

* Lymphokine preparations were obtained from TPA-PHA-stimulated supernatants of unfractionated PBL cultures derived from plateletpheresis residues. Preparations 1 and 2 were partially purified by sequential processing on SA, Con A-Sepharose, and DEAE-Sephacel. Preparation 3 was purified in the same way as preparations 1 and 2 except that the Biogel P200 step was added.

* Neutralization assays were performed as described in Materials and Methods.
Separation of LT and IFN-gamma by Immunoprecipitation with Monoclonal Antibodies to IFN-gamma

| Monoclonal antibody added* | Staphylococcus aureus cells | Units/ml | IFN-gamma | LT |
|---------------------------|-----------------------------|----------|-----------|----|
| None                      | -                           | 512      | 160       |
| B3                        | -                           | <4       | 128       |
| B1                        | -                           | 512      | 192       |
| None                      | +                           | 512      | 256       |
| B3                        | +                           | <4       | 256       |
| B1                        | +                           | <4       | 256       |

* 100-μl aliquots of HAT medium (RPMI 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine; for formula, see 39), IFN-gamma-neutralizing MAb B3, or IFN-gamma-binding MAb B1 were incubated for 90 min at 37°C with 100 μl of concentrated, partially purified, TPA-PHA-induced conditioned medium (processed sequentially through the DEAE-Sephacel step). A 100-μl aliquot of rabbit anti-mouse IgG (24 μg/ml) was added and the mixture was further incubated for 30 min. Finally, 100 μl of washed Staphylococcus aureus Cowan strain 1 (Pansorbin) was added as indicated and the mixtures were incubated for another 50 min at 37°C. PBS was added instead of Pansorbin to the appropriate groups. The immunoprecipitates were collected by centrifugation in a Beckman microcentrifuge for 2 min. The supernatants were then assayed for LT and IFN-gamma activities.

Selective Immunoprecipitation of IFN-gamma with MAb. Isolation of several mouse MAb specific for human IFN-gamma was recently described (38, 39). One of these, MAb B3, neutralizes IFN-gamma activity, while another one, MAb B1, binds with high affinity to IFN-gamma without neutralizing its antiviral activity. However, the addition of protein A-containing Staphylococcus aureus cells (Pansorbin) to IFN-gamma that had been reacted with MAb B1 was shown to result in the formation of a precipitate (39).

In an attempt to selectively remove IFN-gamma, an aliquot of a preparation containing IFN-gamma and LT was incubated with either MAb B1 or B3, with or without subsequent immunoprecipitation with the aid of Pansorbin. MAb B3 neutralized all IFN-gamma activity without significantly affecting LT activity (Table III). Addition of MAb B1 did not neutralize either IFN-gamma or LT activities but did result in the removal of all IFN-gamma activity after immunoprecipitation.

Synergistic Inhibitory Effect of IFN-gamma and LT on HeLa Cell Clonal Survival. Separation of IFN-gamma and LT with the aid of the MAb to IFN-
TABLE IV
Synergistic Inhibitory Effect of Purified recIFN-gamma and LT on HeLa Cell Clonal Survival*

| RecIFN-gamma (units/ml) | Percent control survival ± SD after addition of LT units/ml |
|-------------------------|----------------------------------------------------------|
| None                    | 100.0                                                   |
| 16,384                  | 90.1 ± 8.3                                              |
|                         | 95.4 ± 12.6                                             |
|                         | 75.2 ± 11.0                                             |
|                         | 33.7 ± 5.8                                              |
|                         | 2.2 ± 1.9                                               |

* HeLa cells were seeded in triplicate into a 24-well Linbro plate at a concentration of 100 cells/well. After cell attachment (4 h), a dilution of recIFN-gamma was added alone or in combination with different dilutions of purified LT. LT was purified by sequential processing of TPA-PHA-induced supernatants of PBL cultures on SA and Con A-Sepharose. IFN-gamma was removed by binding to an IFN-gamma antibody affinity column consisting of a neutralizing monoclonal IFN-gamma antibody bound to AH-Sepharose (see Materials and Methods). The flow-through material from this affinity column, concentrated 10-fold, contained LT but no IFN-gamma activity. The average number of colonies in untreated control cultures (100% clonal survival) was 60.3 ± 3.7.

Discussion

Earlier we observed (31) that crude IFN-gamma preparations produced by human PBL stimulated with TPA and PHA exert a greater growth-inhibiting and cytotoxic effect on HeLa cells than highly purified IFN-gamma preparations (31). In another report (30) we demonstrated that high levels of both LT and IFN-gamma activities were induced in human PBL cultures after stimulation with TPA and PHA. These observations prompted us to determine what role LT (and, possibly, some other cytotoxic proteins present in the crude IFN-gamma preparations) might have in the cytotoxic activity.

Experiments with fractionated PBL showed that the LT activity produced by combined treatment with TPA and PHA is a mixture of at least two cytotoxic factors. One factor resembles classic LT in that it is produced primarily by lymphocytes (nonadherent cells) in response to PHA treatment, and it is neutralized by antiserum to a highly purified preparation of human RPMI 1788 cell line-derived LT and by antiserum to LT from tonsillar cells. The other factor is produced by adherent cells in response to TPA; its production is not enhanced by the addition of PHA and it is not neutralized by antiserum to LT from RPMI.
1788 cells. This cytotoxin from adherent cells might be related to tumor necrosis factor (TNF) (26, 27) and/or macrophage cytolytic factor (28). Crude supernatants from TPA-PHA-induced unseparated PBL cultures were only partially neutralized by anti-LT \textsubscript{1788}, while supernatants processed through the three initial steps of the IFN-gamma purification scheme (SA, Con A-Sepharose, and DEAE-Sephaloc) were totally neutralized by this antiserum, indicating that the adherent cell cytotoxic activity present in the original supernatant was separated from classic LT during the purification. It has been reported that TNF binds to Con A-Sepharose (27), while an endotoxin-induced monocyte cytolytic factor does not (29). Therefore, the cytotoxin(s) produced by adherent cells may have been separated from classic LT by Con A-Sepharose chromatography.

Classic LT activity shares many physicochemical properties with IFN-gamma and therefore separation of these two lymphokine activities by physicochemical methods proved difficult. Other investigators (24, 25, 41) have reported a separation of distinct molecular weight classes of LT from mitogen-induced PBL or tonsillar lymphocytes by gel filtration. However, whether these various LT classes are aggregates derived from a single species has yet to be determined. In contrast, we find only one form of LT activity on gel filtration with a 58,000 mol wt. Similarly, the isolation of only one form of RPMI 1788 cell LT, with a 64,000 mol wt determined by gel filtration, was reported (40). Although LT activity produced by TPA-PHA induction had an apparent 58,000 mol wt upon gel filtration, two peaks were demonstrated on SDS-PAGE with apparent molecular weights of 60,000 and 20,000. A similar dissociation of the 64,000 mol wt LT from RPMI 1788 cells into 25,000 and 20,000 mol wt monomers after SDS-PAGE treatment was described (42). It is, therefore, conceivable that native LT, like native IFN-gamma (36), exist as aggregated glycoproteins which, after SDS treatment, partially dissociate into smaller molecular weight monomers.

Since we were unable to separate LT and IFN-gamma activities, we were initially uncertain whether the two activities are due to distinct proteins. However, immunologic studies in which an MAb to IFN-gamma removed the IFN-gamma activity but did not diminish the amount of LT activity present in the supernatant proved that these activities are due to two different proteins. This result gave us a means to physically separate these two lymphokines and to study their interactions with respect to HeLa cell cytotoxicity. We showed a potent synergistic effect on cytotoxicity when purified LT and recIFN-gamma were added together to a culture of HeLa cells. As little as 16 U/ml of LT added to 16,384 U/ml of recIFN-gamma resulted in a 97.8% reduction in survival over that observed with either lymphokine alone. Recent reports from other laboratories also demonstrated a synergistic enhancement of antiproliferative activity when recombinant IFN-alpha (43) as well as recombinant IFN-gamma (42) were used in combination with LT.

It was recently reported (38) that HeLa cell cytotoxicity induced by preparations of IFN-gamma was completely neutralized by an MAb to IFN-gamma. This finding is not contradictory to our results, since it is clear that in some cell lines IFN-gamma alone can be cytotoxic. For instance, a recent study\textsuperscript{2} demonstrated

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\textsuperscript{2} Le, J., Y. K. Yip, and J. Viček. Cytolytic activity of interferon-gamma and its synergism with 5-fluorouracil. Submitted for publication.
that purified human recIFN-gamma (which contained no LT activity demonstrable in the L 929 assay) was cytotoxic for two out of six human cell lines tested (44). This cytotoxicity of recIFN-gamma was abolished by the addition of an MAb that neutralizes IFN-gamma activity.

Summary

Crude preparations of interferon (IFN)-gamma derived from human peripheral blood leukocyte (PBL) cultures induced with 12-O-tetradecanoylphorbol-13-acetate (TPA) and phytohemagglutinin (PHA) were more cytotoxic to HeLa cells than partially purified native or highly purified recombinant human IFN-gamma preparations. Conditioned media from PBL cultures contained, in addition to IFN-gamma, a mixture of cytotoxins, including classic lymphocyte-derived lymphotoxin (LT), and a TPA-induced cytotoxic activity produced by the adherent cell population (presumably monocytes). These two types of cytotoxins, indistinguishable in the mouse L929 cell LT assay, could be differentiated by an antiserum prepared against LT derived from the B lymphoblastoid cell line RPMI 1788. This antiserum neutralized lymphocyte-derived classic LT but failed to neutralize the activity of the monocyte-derived cytokinin. Processing of conditioned media by sequential chromatography on silicic acid, Con A-Sepharose, and DEAE-Sepharose failed to separate IFN-gamma from the LT activity. However, this procedure did remove the monocyte-derived cytotoxic activity present in the original starting material, leaving predominantly classic LT. This LT showed a slightly basic isoelectric point (pI 7.6) which partially overlapped the more basic pI range of IFN-gamma. The two lymphokine activities also could not be completely separated by fast protein liquid chromatography or molecular sieve chromatography. LT in these partially purified preparations was associated with a protein having an apparent molecular weight of 58,000 on gel filtration. This form dissociated partially into a 20,000 mol wt species after denaturation with 0.1% NaDodSO4. IFN-gamma could be selectively removed from preparations containing both IFN-gamma and LT with the aid of monoclonal antibody to IFN-gamma. The addition of purified LT to purified E. coli-derived recombinant human IFN-gamma resulted in a marked synergistic enhancement of cytotoxicity for HeLa cells.

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