

ANTI-VIRAL ACTIVITY INDUCED  
BY CULTURING LYMPHOCYTES WITH TUMOR-DERIVED  
OR VIRUS-TRANSFORMED CELLS

Enhancement of Human Natural Killer Cell Activity by Interferon  
and Antagonistic Inhibition of Susceptibility of Target Cells to Lysis\*

BY GIORGIO TRINCHIERI‡ AND DANIELA SANTOLI

*(From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104)*

Accumulating evidence indicates that interferons may have a regulatory role in several immune functions. Interferons suppress proliferation of lymphocytes in response to mitogens and antigens (1), inhibit antibody formation (2-4), and enhance the generation of specific cytotoxic effector cells (5, 6). The phagocytic activity of mouse macrophages (7, 8) and the suppression of tumor cell growth mediated by mouse spleen cells (9) have been shown to be enhanced by interferon in *in vivo* and *in vitro* experiments.

Lymphocytes from human peripheral blood obtained from any normal donor are spontaneously cytotoxic *in vitro* for target cell lines (10-14). The cytotoxic activity is mediated by natural killer cells that have been identified as Fc-receptor positive, surface immunoglobulin negative lymphocytes (11, 12). Only a small proportion of natural killer cells form rosettes with sheep erythrocytes (11, 12, 15, 16) when very sensitive techniques employing erythrocytes treated with a sulphhydryl reagent (17) or with enzymes (18) are used. The natural killer cells in human peripheral blood cannot be separated from cells involved in antibody-dependent cell-mediated lysis which suggests that the two types of cytotoxicity may be mediated by the same cell population (12-14, 19).

We have shown that cells from certain tumor-derived or virus-transformed lines effectively induce interferon production when cultured together with human or mouse lymphocytes (20). For the present study we investigated the effects that the interferon produced in such mixed cultures exerts on the two cell populations: interferon enhances the spontaneous cytotoxic activity of the lymphocytes and has an antagonistic inhibitory activity on the susceptibility of the target cell lines to cell-mediated lysis.

### Materials and Methods

*Reagents.* Cycloheximide and 2-aminoethylisothio-uronium bromide hydrobromide (AET)<sup>1</sup>

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‡ Present address: Swiss Institute for Experimental Cancer Research, CH 1066 Épalinges, Switzerland.

<sup>1</sup> *Abbreviations used in this paper:* AET: 2-aminoethylisothio-uronium bromide hydrobromide; FBS, fetal bovine serum; TCID<sub>50</sub>, tissue culture infectious dose 50%; HAU, hemagglutination unit; NDV, Newcastle disease virus; PBS, phosphate-buffered saline; PFU, plaque-forming units; PHA, phytohaemagglutinin; RDMC, rhabdomyosarcoma cell line; VSV, vesicular stomatitis virus; BCG, Bacillus Calmette-Guérin.

were obtained from Sigma Chemical Co. (St. Louis, Mo.); actinomycin D from Calbiochem (San Diego, Calif.); fluorescein isothiocyanate-conjugated goat F(ab')<sub>2</sub> fragment anti-human IgG F(ab')<sub>2</sub> fragment from Cappel Laboratories, Inc. (Cochranville, Pa.); and sheep erythrocytes from Flow Laboratories, Inc. (Rockville, MD).

*Cell Lines and Viruses.* The origins of most of the cell lines used in this study are summarized in Table I (20). Adherent cells were subcultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories). The human lymphoid line EB-P8 was obtained by transforming peripheral blood lymphocytes from a multiple sclerosis patient with Epstein-Barr virus. It was subcultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 international U/ml), and streptomycin (100 µg/ml) (RPMI-FBS, Flow Laboratories).

Newcastle disease virus (NDV) was obtained from T. J. Wiktor (Wistar Institute) [allantoic fluid,  $3.3 \times 10^6$  plaque-forming units (PFU)/ml on L929 mouse cells]. The Indiana strain of vesicular stomatitis virus (VSV) was originally obtained from T. J. Wiktor and grown in tissue culture on human rhabdomyosarcoma-derived cells (RDMC) ( $3 \times 10^6$  PFU/ml on L-F2 mouse cells). Vaccinia virus (Lister strain), provided by M. Herlyn (Wistar Institute) was originally obtained from Wyeth Laboratories (Radnor, Pa.) and grown in WI38 cells [ $10^6$  tissue culture infectious doses 50% (TCID<sub>50</sub>)/ml on WI38 cells]. The Hong Kong/107/68 (H3N2) strain of influenza A virus was obtained from W. Gerhard (Wistar Institute) (allantoic fluid, 850 hemagglutination units [HAU]/ml).

*Lymphocyte Separation and Culture.* Mononuclear cells were obtained from heparinized human peripheral blood by separation on a Ficoll-Hypaque gradient. The methods for separating subpopulations of human lymphocytes have been previously described (20). Briefly, lymphocytes were depleted of monocytes and of most B cells by incubation on a nylon column, according to a modification (20) of the method of Julius et al. (21). T-cell-enriched and depleted fractions were obtained by separating lymphocytes rosetting with AET-treated sheep erythrocytes (22) from nonrosetting lymphocytes on a Ficoll-Hypaque gradient. Erythrocytes were eliminated from rosetting cells by distilled water lysis. For culturing lymphocyte preparations in the presence or absence of interferon, cell suspensions at  $5 \times 10^6$  cells/ml in RPMI-FBS were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere, in standing tissue culture flasks (3013, 2-3 ml/flask; 3024, 10 ml/flask; BioQuest, BBL, & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md.). At the end of the incubation, cells were washed three times in phosphate-buffered saline (PBS), pH 7.2, and viable cells were counted with an erythrosin B solution (23) and resuspended in RPMI-FBS.

*NDV Induction of Lymphocyte Interferon.* NDV at a multiplicity of infection (MOI) of 20 was added to a suspension ( $10^7$  cells/ml in RPMI-FBS) of human lymphocytes. After an 18 h incubation (37°C) the cells were eliminated by centrifugation (30 min, 30,000 g); the supernatant was then brought to pH 2.0 with HCl, 1 N, incubated for 5 days at 4°C, neutralized to pH 7.0 with NaOH 1 N, passed through a sterile filter (porosity 0.45 µm, Nalge Co., Nalgene Labware Div., Rochester, N. Y.) and stored at -80°C.

*Interferon Production in Mixed Tumor-Lymphocyte Cultures.* Human lymphocytes were depleted of adherent cells by two passages (1 h each at 37°C) on glass Petri dishes and resuspended at  $10^7$  cells/ml in RPMI-FBS. 8-10 ml of the cell suspension was added to confluent monolayers of RDMC cells in 75 cm<sup>2</sup> tissue culture flasks (3024, BioQuest, BBL, & Falcon Products) and incubated 18 h at 37°C. The supernates were centrifuged at 30,000 g for 30 min, filtered through a sterile 0.45 µm filter, and stored at -80°C.

*Interferon Assay.* Anti-viral titers of human interferon preparations were measured as previously described (20) by inhibition of the cytopathic effect of VSV on a monolayer of human fetal skin fibroblasts (FS1). Anti-viral units are expressed as the reciprocal of highest dilution inhibiting 50% of the cytopathic effect and are equivalent to approximately 1 reference U of the NIH Human Reference Interferon G-023-901-527.

*Cell-Mediated Cytotoxicity on Adherent Target Cells.* Trypsinized target cells were seeded into the wells of flat bottom microtiter plates (3040, BioQuest, BBL, & Falcon Products) at a dose of  $2 \times 10^4$  cells/well; 2 µCi of Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (New England Nuclear Corp., Boston, Mass.) were added to each well, and the plates were incubated overnight at 37°C. The plates were then washed three times and 0.1 ml of RPMI-FBS or interferon diluted in medium was added in each well. Effector cell dilutions of 0.1 ml were added, and the plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere for the indicated times. After centrifugation, 0.1 ml of the supernatant A was then collected from each well and 0.1 ml of 1% Triton X-100 added. The plates were further incubated

for at least 4 h at 37°C and 0.1 ml of supernatant B was again collected from each well. Samples A and B were counted for  $^{51}\text{Cr}$  activity; percent  $^{51}\text{Cr}$  release (E) was calculated as:

$$E = \frac{A}{B + A/2} \times 100.$$

All experimental and control values were the mean of at least three replicates. Where S is the percent  $^{51}\text{Cr}$  release from target cells in the presence of medium alone, the specific percent  $^{51}\text{Cr}$  release (R) was computed as:

$$R = \frac{E - S \times 100}{100 - s}.$$

*Antibody-Dependent Cell-Mediated Cytotoxicity.* The method has been previously described in detail (13, 24, 25). The established lymphoblastoid B-cell line EB-P8, with phenotype HLA-A3, B5, 7 was used as target. [ $^{51}\text{Cr}$ ]labeled target cells were sensitized with a mixture of anti-HLA sera BsSh (anti-B7, diluted 1/20) and ToBU 1.64 (anti-B5, 12, purified IgG, 0.1 mg/ml) both of which were obtained from the Instituto di Genetica Medica, University of Torino (Italy).

*Computation of Lytic Units.* Dose-response curves of specific cytotoxicity (both for antibody-dependent and direct cell-mediated cytotoxicity) were determined by plotting specific  $^{51}\text{Cr}$  release versus the number of effector lymphocytes. The number of lymphocytes necessary to lyse 50% of the target cells in the incubation time was referred to as 1 lytic U. Determination of the 50% lytic units was done graphically or computed on the basis of a modification (24) of the van Krogh equation.

*Mouse Anti-Human Cytotoxic T Cells.* A cell-mediated cytotoxic response against human cells was elicited in C57BL/6J mice by intraperitoneal injection of  $3 \times 10^7$  RDMC cells, as previously described (26). The cytotoxic test, using effector spleen cells on human adherent target cells, was performed as described above, at the peak of the cytotoxic response 11 days after immunization.

## Results

*Additions of Exogenous Interferon to Lymphocyte-Target Cell Cultures.* Interferon preparations induced in human lymphocytes either by culturing with RDMC monolayers or by exposure to NDV were added to a mixture of lymphocytes and target RDMC cells in an assay of cell-mediated cytotoxicity. In the cultures in which interferon was added an enhancement of cytotoxicity, usually from two to fourfold, was observed at 4–8 h of incubation (Fig. 1). The rate of cytotoxicity after 8 h of incubation was usually not higher in cultures to which interferon had been added than in control cultures, high levels of endogenous interferon being present in both cultures (Fig. 1). Some cell lines were unable to induce production of interferon from lymphocytes (Table I); the use of these cells as targets allowed us to detect, in some experiments, an interferon-induced increase in the rate of cytotoxicity at longer incubation times also. However, the antagonistic effect of interferon on the susceptibility to lysis of most target cells (see below), often reduced the observed effect of exogenous interferon preparations on cytotoxicity.

*Effect of Interferon Produced in the Lymphocyte-Target Mixture on Cell-Mediated Cytotoxicity.* Lymphocytes incubated at 37°C for 24 h completely lose their ability to produce interferon when cultured with cells from inducer lines (20). This observation allowed us to evaluate indirectly the effect of interferon produced in mixed cultures on the spontaneous cell-mediated cytotoxicity of human lymphocytes. Lymphocytes that were freshly separated or previously incubated 24 h at 37°C were tested as effector cells in an 18-h cytotoxic test (Fig. 2). No difference was observed in the cytotoxic efficiency of fresh versus preincubated lymphocytes when cells were tested against targets unable to

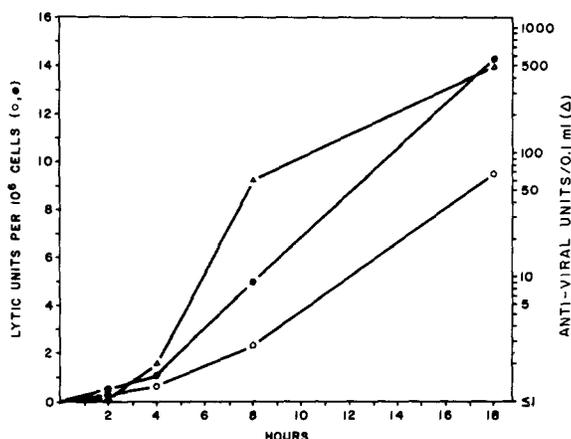


FIG. 1 Effect of exogenous interferon on spontaneous cytotoxicity mediated by human lymphocytes against RDMC target cells. ●—●, 500 anti-viral U of RDMC-induced lymphocyte interferon added to lymphocyte-target cell mixtures at the beginning of the cytotoxic test; ○—○, no exogenous interferon added during the cytotoxic test; Δ—Δ, endogenous anti-viral activity in the supernate of the cultures with no exogenous interferon. At 4, 8, and 18 h of incubation the amounts of  $^{51}\text{Cr}$  release were significantly different ( $P < 0.01$ , Student's  $t$  test) between cultures to which interferon was added and those to which it was not (considering only the dilutions of effector lymphocytes giving cytotoxic values in the linear part of the titration curve, 30–70%).

induce interferon (Fig. 2b). In contrast, when interferon-inducing cells were targets, the cytotoxicity of the preincubated lymphocytes was reproducibly decreased to only 10–30% of that observed with freshly separated cells (Fig. 2a). This observation, reproducible when cell lines other than those in Fig. 2 were used as targets, suggests that the interferon released in the culture mediates an enhancement of cytotoxicity that represents up to 70–90% of the total spontaneous cell-mediated cytotoxicity observed with fresh lymphocytes in an 18 h test.

*Pretreatment of Lymphocytes with Interferon.* When lymphocytes were pretreated for 18 h with interferon preparations then washed and tested as effector cells against several different target cell lines, they displayed a cytotoxic efficiency up to 10-fold higher than that of untreated lymphocytes (Fig. 3). The increase in the nonspecific cytotoxicity of human lymphocytes cultured in the presence of interferon is quantitatively comparable to or higher than that observed upon stimulation of lymphocytes with optimal concentrations of phytohaemagglutinin (PHA) (Fig. 3). In some experiments interferon-treated cytotoxic lymphocytes induced more than a 50%  $^{51}\text{Cr}$  release in a 4-h test (RDMC target) at a ratio of effector to target of 1:1. These levels of cytotoxicity were maintained or increased after lymphocytes were treated for 48 h with interferon; beginning on the 3rd day cytotoxicity declined to levels close to those of untreated lymphocytes (Fig. 2). At 5–7 days in culture, a spontaneous increase in the cytotoxicity of the lymphocytes was usually observed: the presence of interferon in the culture depressed this late increase (Fig. 2).

The enhancement of cytotoxicity was dependent on the dose of interferon added to the culture; a twofold increase was obtained with about 10 anti-viral U

TABLE I

*Human Cell Lines: Ability to Induce Interferon When Cultured with Lymphocytes and Susceptibility to the Interferon-Induced Resistance to Cytotoxic Lymphocytes and Inhibition of Viral Replication*

Cell line	Origin	Passage	Units Interferon Induced* (mean $\pm$ Standard Error)	Inhibition of cytotoxicity‡ %	Inhibition of viral replication§
FS1	Fetal skin fibroblasts	14	<1	92	4.0
Pa	Skin fibroblasts	8	<1	86	3.6
LR-1	Newborn brain	4	<1	82	3.3
WI38	Fetal lung fibroblasts	42	<1	62	3.0
MRC5	Fetal lung fibroblasts	45	<1	40	1.8
LN-SV	Skin, SV40-transformed	253	<1	77	3.0
W18.VA2	Lung, SV40-transformed	245	<1	75	2.1
S1054TR	Brain, SV40-transformed	32	193 $\pm$ 137	20	<0.3
SW690	Melanoma	90	850 $\pm$ 552	75	2.4
SW691	Melanoma	85	6,000 $\pm$ 3,000	13	<0.3
SW480	Colorectal carcinoma	112	125 $\pm$ 0	24	<0.3
D98 (HeLa)	Cervical carcinoma	>60	312 $\pm$ 165	30	<0.3
HT1080	Fibrosarcoma	117	0	15	0.6
RDMC	Rhabdomyosarcoma	203	3,494 $\pm$ 1,008	18	<0.3

\* Anti-viral units in the supernate (24 h incubation of mixed cultures of monolayer of the cell line with human lymphocytes; four or more different lymphocyte preparations tested for each cell line).

‡ Average percent inhibition of cytotoxicity. After treatment with interferon (18 h,  $10^3$  anti-viral U), cells from the various lines were tested as target against two different preparations of effector lymphocytes (stimulated by  $10^3$  anti-viral U of NDV-induced lymphocyte interferon, 18 h).

§  $\text{Log}_{10}$  of the reciprocal of the dilution of a lymphocyte interferon preparation (NDV-induced,  $10^4$  anti-viral reference U) inhibiting 50% of the cytopathic effect of VSV or monolayer of the cell line.

of interferon administered for 18 h (Fig. 4 and 5b). The same enhancement of cytotoxicity was obtained, at equivalent anti-viral doses, with interferon preparations obtained by stimulation of human lymphocytes with either virus (NDV) or an inducer cell line (RDMC). When the anti-viral activity and the cytotoxicity-enhancing activity of 14 RDMC-induced interferon preparations from various lymphocyte donors were compared, a significant positive correlation was observed ( $P < 0.01$ , Fig. 5b). No enhancement of cytotoxicity was obtained with supernate from lymphocytes or inducer cell lines cultured alone. Supernates from mixed cultures of allogeneic lymphocytes (24 h incubation) and from cultures of lymphocytes with noninducing cell lines neither displayed activity nor enhanced cytotoxicity.

The cytotoxicity-enhancing activity in the human interferon preparation was destroyed by trypsin treatment (1 mg/ml, 1 h at 37°C, neutralized with soybean trypsin inhibitor bound to agarose beads) and was resistant to 5 days treatment at pH 2.0. The cytotoxicity-enhancing activity in the RDMC-induced preparations was eluted from a Sephadex G100 column in a peak of approximate mol wt

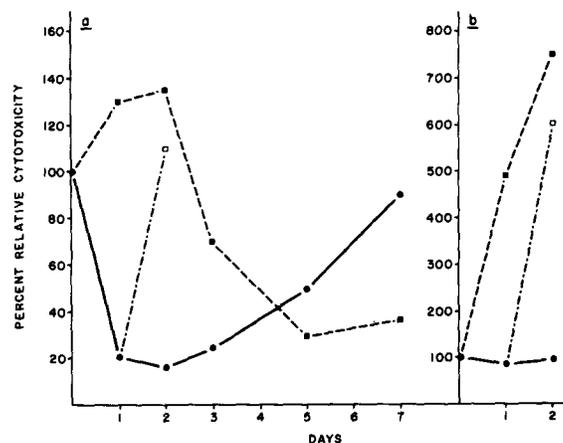


FIG. 2. Effect of interferon on the cytotoxic efficiency of human lymphocytes in culture. The lymphocytes were cultured for the number of days indicated in the presence or absence of  $10^3$  anti-viral U of RDMC-induced interferon. Their cytotoxic activity was then tested in an 18-h test against an interferon-inducing target, RDMC (Fig. 2a), and against a noninducing target, FS1 (Fig. 2b). The relative cytotoxicity is expressed as the ratio of the lytic units of the cultured lymphocytes over the lytic units of the freshly separated ones. ●—●, no interferon added; ■----■, interferon added at time 0; □----□, interferon added after 24 h of culture at 37°C.

of 25,000 daltons, corresponding to the major peak of anti-viral activity (Fig. 6a). Some activity was observed in correspondence with the minor peak of anti-viral activity of approximate mol wt 45,000 (20). A variable amount of anti-viral activity and concordantly of cytotoxicity-enhancing activity, were also eluted in different experiments with the major peak of protein close to the front of the column; these results are presumably due to the ability of the interferon molecules to bind to albumin and other proteins (27).

Medium from lymphocytes stimulated with PHA (HA15, Wellcome Research Lab., Beckenham, England, final dilution 1:100, 24 h incubation at 37°C) contained some anti-viral (5–50 anti-viral U) and was able to enhance lymphocyte cytotoxicity. After gel filtration of the supernate from PHA-stimulated lymphocytes both the anti-viral activity and the cytotoxicity-enhancing activity were recovered in a peak discrete from the higher molecular weight activity associated with the lectin molecules.

Mouse interferon preparations obtained from spleen cells and from L cells were almost ineffective in enhancing human lymphocyte cytotoxicity.

*Characterization of the Cytotoxic Lymphocytes Affected by Interferon.* The cytotoxic lymphocytes showing activity enhanced by interferon were characterized by two techniques: adherence to a nylon fiber column and gradient separation of cells rosetted with AET-treated sheep erythrocytes. With these separation methods the cytotoxic activities of spontaneous (natural) killer cells, antibody-dependent killer cells and the cytotoxic cells with activity enhanced by interferon were always associated with the same fractions (Table III). The effector cells responsible for the three activities were non-phagocytic, did not adhere to nylon fiber columns, had no detectable surface immunoglobulins, and

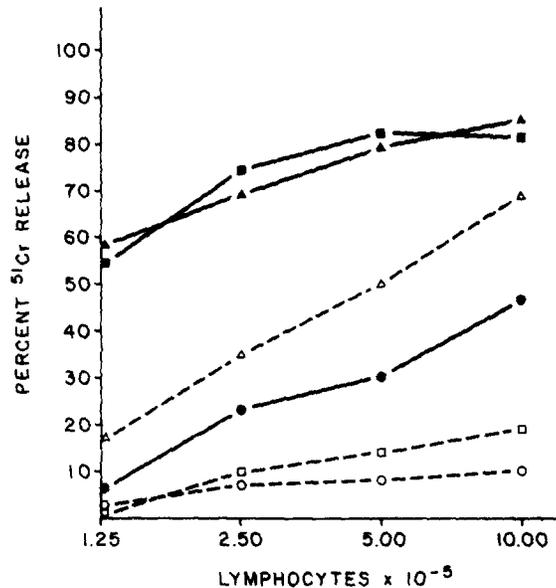


FIG. 3. Effect on cell-mediated cytotoxicity of pretreatment of lymphocytes and target cells with interferon preparations. Human lymphocytes were culture for 18 h at 37°C in the presence of 10<sup>3</sup> anti-viral units of RDMC-induced interferon, PHA (HA15 Wellcome, final dilution 1/100) or normal RPMI-FBS medium, washed three times, and then tested as effector cells (18-h test) on FS1 target cells pretreated or not pretreated with 10<sup>3</sup> anti-viral U of interferon. The SD of each experimental point was less than 2%. ●—●, untreated lymphocytes, untreated target cells; ○---○, untreated lymphocytes, interferon-treated target cells; ■—■, interferon-treated lymphocytes, untreated target cells; □----□, interferon-treated lymphocytes, interferon-treated target cells; ▲—▲, PHA-stimulated lymphocytes, untreated target cells; △----△, PHA-stimulated lymphocytes, interferon-treated cells.

exhibited very low affinity for sheep erythrocytes (even when the extremely sensitive rosetting technique with AET-treated sheep erythrocytes was used) (28) (Table II).

When the effect of interferon pretreatment on the antibody-dependent cytotoxic activity of human lymphocytes was tested, no significant increase of the antibody-dependent activity was observed in repeated experiments although interferon strongly increased the cytotoxicity of the pretreated lymphocytes against the target lymphoid line in the absence of anti-target antibodies (Table III).

*Inhibition of Target Cell Susceptibility to Lysis by Pretreatment with Interferon.* Incubation of FS1 cells with 10<sup>3</sup> anti-viral U of RDMC-induced interferon for 18 h had no toxic effects. The spontaneous release of <sup>51</sup>Cr in 18 h was 27.8 ± 2.1 (mean ± SD, six experiments) from untreated target cells and 28.3 ± 3.3 from pretreated cells: the difference was not significant (Student's test for paired data, *t* = 0.42, NS). The preincubation of FS1 target cells with interferon induced up to 98% protection against the cytotoxicity mediated either by fresh or by interferon-stimulated human lymphocytes (Fig. 3). To reach the maximal level of inhibition it was necessary to preincubate the target cells for several

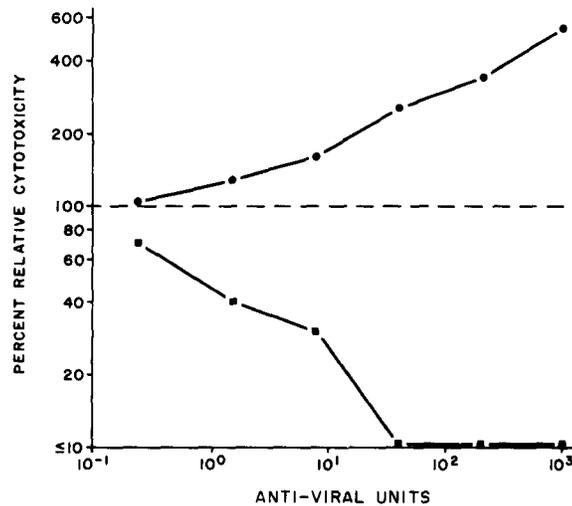


FIG. 4. Effect on lymphocytes and FS1 target cells of pretreatment with different doses of interferon. Lymphocytes, ●-----●, and FS1 target cells, ■——■, were incubated for 18 h in the presence of the indicated anti-viral units of RDMC-induced interferon and then tested reciprocally against untreated FS1 target cells or against lymphocytes pretreated with 10<sup>3</sup> anti-viral U of interferon. The relative cytotoxicity is expressed as the ratio between lytic units observed with interferon-pretreated and not pretreated lymphocytes or target cells.

hours (Fig. 7). The presence of interferon was not required for the entire period. Treating of FS1 cells for 30 min with interferon, then incubating them for 24 h without interferon induced an 85% inhibition of cytotoxicity, whereas pretreatment for 30 min immediately before the test induced only 35% inhibition (Fig. 7). The induction of resistance to lysis upon pretreatment with interferon (Fig. 8a) was prevented by concurrent treatment of FS1 cells with an inhibitor of RNA synthesis (actinomycin D, Fig. 8b) or with an inhibitor of protein synthesis (cycloheximide, Fig. 8c). Virus infection of the target cells (influenza A and vaccinia viruses) also prevented the induction of most of the resistance in FS1 cells (Fig. 8d and e).

14 different cell lines have been tested for interferon-mediated induction of resistance to the cytotoxic effect of lymphocytes (Table I). Fibroblasts are susceptible to both the anti-viral and target protective activities of interferon. The fetal lung fibroblasts WI38 and MRC5, tested in the experiment shown in Table I, were at very high passage and were relatively less susceptible to both interferon activities; other experiments with lower passages of these same cells showed a higher susceptibility to the two activities. When various SV40-transformed or tumor-derived cell lines were tested, a variable susceptibility to interferon was observed in different lines. The tumor-derived lines tested, however, were not susceptible to either the anti-viral or the target protective activity of interferon, with the exception of the melanoma-derived SW690 cell line. A significant positive correlation was observed between cell line susceptibility to the anti-viral and to the target protective activity of interferon ( $r = 0.954$ ,  $P < 0.001$ ).

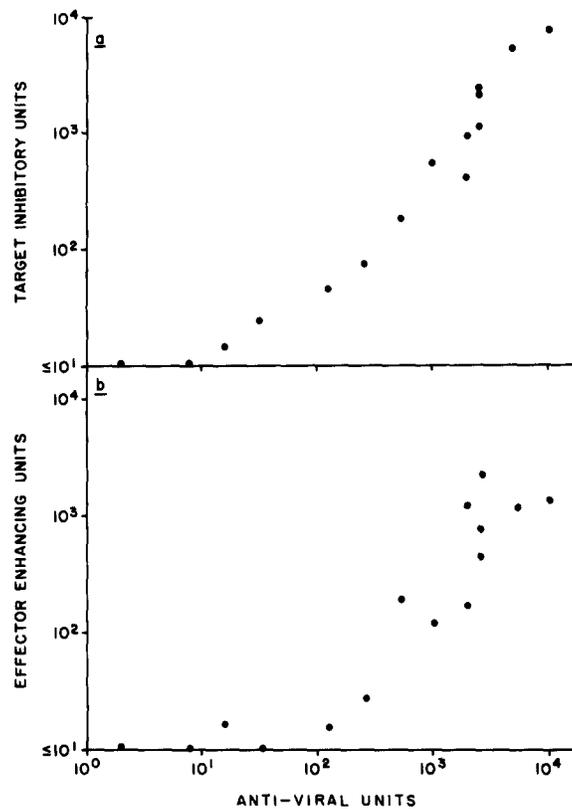


FIG. 5. Correlation between anti-viral activity and (a) protective effect on target cells or (b) enhancing activity on cytotoxic lymphocytes in 15 different preparations of RDMC-induced interferon. The different preparations were titrated for inhibition of target cell (FS1) susceptibility to lysis and enhancement of lymphocyte cytotoxicity as shown in Fig. 4. Target inhibitory units are defined as the reciprocal of the titer inhibiting 50% of the cytotoxicity on FS1 target cells mediated by lymphocytes stimulated with 10<sup>8</sup> anti-viral U of interferon (data shown are the average of two determinations with lymphocyte preparations from different donors). Effector-enhancing units are defined as the reciprocal of the titer inducing a twofold increase in the cytotoxicity of pretreated lymphocytes on FS1 target cells. Correlation between anti-viral activity and target inhibitory activity:  $r = 0.945$ ,  $P < 0.001$ ; correlation between anti-viral activity and effector enhancing activity:  $r = 0.628$ ,  $P < 0.01$ .

The interferon-mediated inhibition of target cell susceptibility to lysis was dose dependent: 1-2 anti-viral U of interferon were required for 50% inhibition of cytotoxicity on FS1 target cells (Fig. 4 and 5a). Inhibition of cytotoxicity was observed with both NDV- and RDMC-induced lymphocyte interferon. The anti-viral activity and the target protective activity were tested in 15 RDMC-induced interferon preparations obtained with lymphocytes from different donors: a significant positive correlation was observed between the two activities ( $r = 0.945$ ,  $P < 0.001$ ; Fig. 5a). None of the control preparations described for effector-enhancing activity contained anti-viral activity, and neither they nor mouse interferon preparations were able to induce resistance in human target cells.

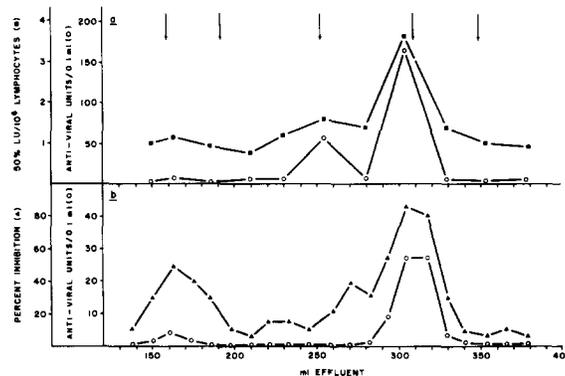


FIG. 6. Gel filtration on Sephadex G100 fine column of (a) RDMC-induced and (b) NDV-induced interferon preparations. 30 ml of preparation was concentrated ten times to 3 ml by vacuum dialysis. Column bed  $25 \times 960$  mm ( $471 \text{ cm}^3$ ), void vol 160 ml. Arrows indicate molecular weight markers, from left to right: blue dextran 2,000 (mol wt 2,000,000), aldolase (158,000), ovalbumin (45,000), chymotrypsinogen A (25,000), ribonuclease A (13,700). The collected fractions were dialyzed against medium, sterilized by filtration, and tested for anti-viral activity, enhancing activity on cytotoxic lymphocytes (RDMC target cells, 8-h test, cytotoxicity of untreated lymphocytes: 1.04 lytic U/ $10^6$  cells) and inhibitory activity on FS1 target cells (18-h pretreatment, 18-h test, effector lymphocytes stimulated with  $10^3$  anti-viral units of interferon).

TABLE II  
*Effect of Interferon on Lymphocyte Subpopulations*

Fraction	ANAE*	S-Ig†	E-RFC‡	Ab-CMC	Sp-CMC¶	If-CMC**	If-CMC
							Sp-CMC
<b>Exp. A</b>							
Ficoll-Hypaque separated cells	17.9	22.7	62.9	250	555	1,224	2.2
Nylon column nonadherent	1.4	2.4	86.4	909	1,111	3,571	3.2
Nylon column adherent	30.4	55.8	24.2	95	83	250	3.0
<b>Exp. B</b>							
Nylon column non-adherent	0.9	1.7	88.4	192	313	714	2.3
AET-SRBC Non-rosetting	11.4	32.0	0	526	1,562	4,348	2.8
AET-SRBC Rosetting	0	0	97.6	50	59	153	2.6

\* Staining for nonspecific acid esterase (20), strongly positive cells (monocytes).

† Positive fluorescence for surface immunoglobulin with fluorescein-tagged rabbit F(ab')<sub>2</sub> anti-human light chains.

‡ Cells forming rosettes with AET-treated sheep erythrocytes (18 h incubation at 4°C) (22).

|| Lytic U/ $10^6$  cells in antibody-dependent cell-mediated cytotoxicity.

¶ Lytic U/ $10^6$  cells, 8 h test, RDMC target cells, effector cells preincubated 18 h in RPMI-FBS.

\*\* Lytic U/ $10^6$  cells, 8 h test, RDMC target cells, effector cells preincubated 18 h with 500 anti-viral U of RDMC-induced interferon.

The protective activity was resistant to pH 2.0 and destroyed by treatment with trypsin. Gel filtration on a Sephadex G100 column of NDV-induced interferon gave a peak of anti-viral and target protective activity with an approximate mol wt of 25,000 daltons; in addition, as discussed above, a variable proportion of the activity was eluted with the major protein peak (Fig. 6b).

*Characteristics of Interferon-Mediated Target Cell Resistance to Cytotoxic Lymphocytes.* FS1 target cells treated with  $10^3$  anti-viral U of interferon for 18 h were almost completely resistant to the cytotoxic effect of spontaneous

TABLE III  
*Effect of Interferon on Antibody-Dependent Cell-Mediated Cytotoxicity*

Time in culture	Interferon	EB-P8 Target*	Increase‡	EB-P8 Target + Anti-HLA*	Increase‡
<i>h</i>					
0	0	30		2,564	
24	0	28	3.3	1,050	1.18
24	500	93		1,250	
48	0	24	12.6	952	1.31
48	500	303		1,250	

Lymphocytes were cultured in presence or absence of 500 anti-viral units of RDMC-induced interferon and tested at the different times in a 4-h assay against EB-P8 cells sensitized or not sensitized with a mixture of appropriate anti-HLA sera.

\* Lytic U/10<sup>6</sup> cells.

‡ Ratio between lytic units of lymphocytes incubated in the presence and absence of interferon.

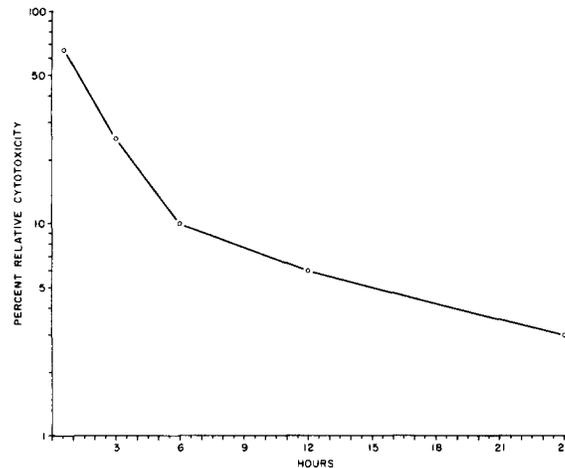


FIG. 7. Induction of target cell (FS1) resistance to lysis after pretreatment with interferon for different time periods. FS1 target cells were pretreated with 10<sup>3</sup> anti-viral U of RDMC-induced interferon for the times indicated, washed three times, and tested against interferon-stimulated (10<sup>3</sup> anti-viral U, 18-h pretreatment) cytotoxic lymphocytes. The solid dot indicates the cytotoxic effect on FS1 cells incubated for 30 min in the presence of interferon, washed and incubated without interferon for 24 h before testing. The relative cytotoxicity is expressed as the ratio between lytic units obtained against interferon-pretreated and untreated target cells.

(natural) cytotoxic lymphocytes and interferon-stimulated lymphocytes, but were only partially protected against PHA-induced cytotoxic human lymphocytes (Fig. 3). Moreover, in repeated experiments treated and untreated FS1 cells were equally susceptible to mouse anti-human cytotoxic T cells.

Interferon-treated and untreated unlabeled FS1 cells (cold targets) were tested for their ability to compete for the killing of [<sup>51</sup>Cr]labeled FS1 cells, using interferon-stimulated human lymphocytes as effector cells. 5 × 10<sup>4</sup> cold target cells treated or not treated with interferon were added to the wells of microtiter

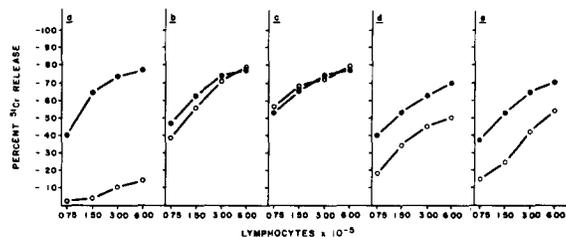


FIG. 8. Effects of metabolic inhibitors or virus infection on interferon-mediated inhibition of target cell (FS1) susceptibility to lysis. ●—●, FS1 cells not treated with interferon; ○—○, FS1 cells treated with interferon. a: FS1 cells were pretreated for 18 h with  $10^8$  anti-viral U of RDMC-induced interferon and then tested against different dilutions of interferon-stimulated ( $10^8$  anti-viral units, 18 h pretreatment) cytotoxic lymphocytes. b, c: 1 h before and during interferon treatment  $1 \mu\text{g/ml}$  of actinomycin D (b) or  $50 \mu\text{g/ml}$  of cycloheximide (c) were added. d, e: 1 h before interferon treatment FS1 cells were infected with  $0.1 \text{ ml}$  ( $850 \text{ HAU/ml}$ ) of the HK strain of influenza A virus (d) or with  $0.1 \text{ ml}$  ( $10^6 \text{ TCID}_{50/\text{ml}}$ ) of the Lister strain of vaccinia virus (e). The SD of each experimental point was less than 2%.

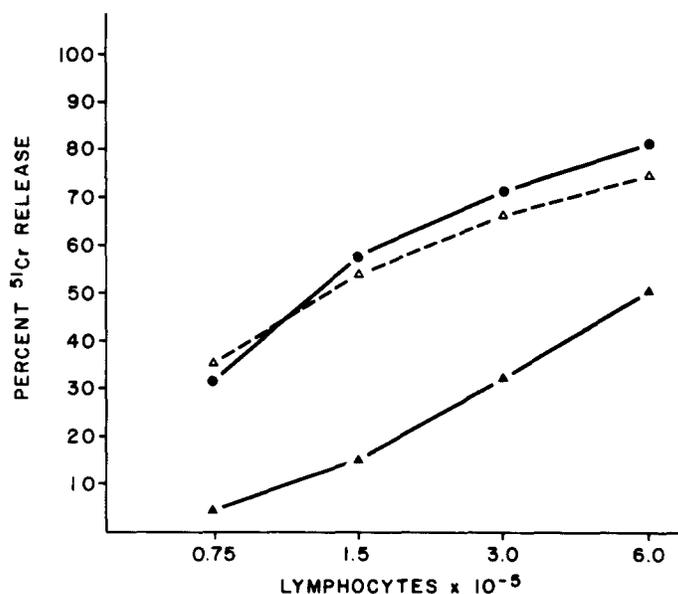


FIG. 9. Cold target competition experiment with interferon-treated or untreated competitor cells.  $5 \times 10^4$  treated ( $10^8$  anti-viral U, 18 h) or untreated FS1 cells were added to  $2 \times 10^4$  adherent [ $^{51}\text{Cr}$ ]labeled FS1 target cells. Effector cells were interferon-stimulated human lymphocytes; the incubation time of the cytotoxic test was 11 h. The SD of each experimental point was less than 2%. ●—●, no cold target cells;  $\Delta$ — $\Delta$ , interferon-treated FS1 cold target cells;  $\blacktriangle$ — $\blacktriangle$ , untreated FS1 cold target cells.

plates containing a monolayer of about  $2 \times 10^4$  target cells labeled with  $^{51}\text{Cr}$ . Different dilutions of effector lymphocytes were then added: as depicted in Fig. 9 about 75% inhibition of cytotoxicity was observed with untreated cold target cells, whereas no inhibition was obtained with interferon-treated cells.

### Discussion

Since its discovery in 1957 by Isaacs and Lindenmann (29) as "an anti-viral substance produced by cells in response to virus infection", interferon has been shown to affect various cellular functions not directly connected with viral replication and to be induced by a variety of non-viral stimuli. In vivo interferon may regulate several cellular functions and defense mechanisms, and its anti-viral activity may represent only one aspect of its physiological role. Interferon is induced in immunological reactions, and it may either stimulate or depress several immune functions (1-9).

Interferon probably enhances a specific cell-mediated cytotoxic response against tumor antigens, in analogy to what has been demonstrated for alloreactive response (5, 6), and also stimulates nonspecific defense mechanisms such as phagocytosis (7, 8) and nonspecific cell-mediated cytotoxicity (9). Stimulation of the host defense mechanisms to more efficiently oppose tumor growth, and a direct effect on tumor cell DNA synthesis and replication, which decreases tumor growth rate and invasiveness, are probably responsible for the anti-tumor effect in vivo of interferon and interferon inducers (30). We have demonstrated that most tumor-derived cell cultures have the ability to induce human and mouse lymphocytes to produce interferon in vitro (20). This observation suggests that in vivo transplantable tumors used in experimental tumor immunology, and possibly spontaneous tumors, induce interferon, which, in the first phases of tumor growth, might stimulate host defense mechanisms.

The present study demonstrates that induction of lymphocyte interferon by tumor-derived cells affects the natural cytotoxic activity of human lymphocytes present in the cultures. Interferon preparations increase several fold the nonspecific cytotoxicity of human lymphocytes: the effector cells are cytotoxic for any target cell line tested, regardless of the cell line (or virus) used to induce interferon. Cell separation experiments showed that the activity of spontaneous (natural) killer lymphocytes could not be separated from the cytotoxic activity induced by interferon preparations. This observation suggests that interferon preparations do not generate a new population of effector cells, but rather enhance the activity of the natural killer cells or increase their number by recruiting quiescent cells. All the evidence indicates that in the interferon preparations it is the interferon itself that mediates the cytotoxicity-enhancing activity (31, 32). A very good correlation was found in both tumor cell-induced and virus-induced interferon preparations between anti-viral and cytotoxicity-enhancing activities. All control preparations were negative for both activities; moreover, the cytotoxicity-enhancing activity showed the species preference and the physicochemical characteristics of interferon.

The mechanism by which interferon enhances lymphocyte cytotoxicity is still obscure. Interferon does not act directly on target cells: interferon preparations are not toxic for target cells and the enhancement of cytotoxicity is present when lymphocytes are pretreated with interferon and washed before being tested as effector cells. The effect of interferon on cytotoxic lymphocytes is not immediate, but reaches a maximum after several hours; addition of interferon to a lymphocyte target cell mixture is less efficient than pretreating lymphocytes with interferon. Interferon therefore does not act by providing a recogni-

tion-binding system, as, for example, IgG molecules do in inducing antibody-dependent cell-mediated cytotoxicity, but directly stimulates effector cells. It is not possible to determine whether this stimulation requires active protein synthesis in cells, because although protein synthesis in the lymphocytes is not required for immediate cytotoxicity, pretreatment of lymphocytes with protein synthesis inhibitors determines a decrease of the cytotoxic efficiency (33 and our unpublished observation). This observation however suggests that cytotoxic activity of lymphocytes requires protein factors with a rapid turnover; interferon, which has been shown, for example, to increase the expression of H-2 antigens on murine cells (34), might affect the synthesis of such factors.

It has been suggested that human natural killer cell activity is partly mediated by IgG antibodies adsorbed to the Fc-receptor of antibody-dependent cytotoxic lymphocytes (19); in this case two mechanisms that might increase cytotoxicity would be an enhancement of the efficiency of antibody-dependent killer cells or an induction of *in vitro* synthesis of IgG able to arm killer cells. Both of these mechanisms seem to be excluded by the following observations (a) the activity of antibody-dependent killer cells is not enhanced by interferon (Table III); (b) interferon inhibits antibody secretion in several *in vitro* systems (4); (c) the cytotoxicity and the generation of interferon-induced killer cells is not affected by concentrations of rabbit f(ab')<sub>2</sub> fragment anti-human IgG able to completely suppress antibody-dependent cell-mediated cytotoxicity (25 and our unpublished observation).

The interferon-mediated enhancement of cytotoxicity *in vitro* lasted for 2-3 days. After 6-8 days of incubation the cytotoxicity of the untreated lymphocytes, which reached its lowest level at 4-5 days, increased again to levels close to or higher than those observed with freshly separated lymphocytes. This nonspecific increase of cytotoxicity, which parallels a simultaneous increase in [<sup>3</sup>H]thymidine incorporation in the lymphocytes, is depressed by interferon. This phenomenon might be due to a polyclonal T-cell stimulation induced by mitogenic factors present in the culture medium or serum, however the nature of the effector cells involved has not been analyzed.

Various phenomena have been described which may be related to the cytotoxicity-enhancing activity of interferon. Peter et al. (35) reported that in mixed cultures of human lymphocytes and tumor-derived cell lines, a factor is released that inhibits DNA synthesis and enhances the spontaneous cell-mediated cytotoxicity of lymphocytes. That factor could be identical to the cell-induced lymphocyte interferon that we have described. Although Peter et al. considered the factor a lymphotoxin, no evidence of a direct toxic effect was presented. Several studies have shown that human lymphocytes kill virus-infected cells more efficiently than uninfected target cells (36-41): although anti-viral antibodies adsorbed on the membranes of the lymphocytes or secreted in the culture play a role in some systems (40, 41), lymphocytes cultured with virus-infected cells, or directly with virus, produce high level of interferon, which is the main factor responsible for the observed increase in cytotoxicity.<sup>2</sup> It

<sup>2</sup> D. Santoli, G. Trinchieri, and H. Koprowski. Cell-mediated cytotoxicity in humans against virus-infected target cells. II. Interferon induction and activation of natural killer cells. Manuscript submitted for publication.

has been reported that cytotoxic effector cells are generated by culturing human lymphocytes with tumor-derived cell lines (42-44). Although no direct analysis has been reported in these studies, we assume that in some of these systems the effector cells are probably induced by interferon released in the culture system. Porzsolt et al. (44) have described the generation of cytotoxic cells in a mixed culture of human lymphocytes and cultured melanoma cells with kinetics resembling that obtained with interferon stimulation (Fig. 2). Rapid induction of human cytotoxic lymphocytes *in vitro* has also been described with interferon inducers, such as synthetic polyribonucleotides (45) and *Bacillus Calmette-Guérin* (BCG) (46), and variously interpreted. *In vivo* it has been reported that injection of viruses (47) or BCG (47, 48) into mice increases the natural killer cell activity with a peak at day 3. Interestingly, the same phenomenon has been described after injection of transplantable tumor cell lines (47); some of these same cell lines have been tested in our laboratory and found to be able to induce interferon *in vitro* when cultured with human or mouse lymphocytes (20).

When target cells are treated with interferon, they are less susceptible to the cytotoxicity of effector lymphocytes. Interferon-treated fibroblasts are almost completely resistant to nonspecific cytotoxic lymphocytes. The refractory status of the target cells appears specific for the cytotoxicity mediated by natural killer cells; the spontaneous or interferon-enhanced cytotoxicity of human lymphocytes is almost completely suppressed, whereas the activity of mouse cytotoxic T cells is not affected. Human cytotoxic lymphocytes induced with PHA, which probably stimulated both cytotoxic T cells and natural killer cells (49), are only partially inhibited. Interferon therefore does not induce a general resistance in target cells: the inability of interferon-treated cold target cells to competitively inhibit the cytotoxicity of natural killer cells suggests that in the treated cells some surface characteristics responsible for recognition by cytotoxic lymphocytes are lost or masked. Although the phenomenon of the specific inhibition of the target cells cannot yet be interpreted, we feel that this observation may provide a clue for the specificity of the natural killer cells. Two considerations suggest that interferon does not act by affecting the expression of antigens coded by endogenous viruses present in the target cells and recognized by the cytotoxic cells (50): no evidence of the presence of virus or viral antigens in normal human fibroblasts have ever been obtained and interferon increases rather than decreases the expression of C-type virus antigens or particles on the surface of murine cells (51).

The identification of the molecules mediating the protection of target cells as interferon is based on the same characteristics already mentioned for the cytotoxicity-enhancing effect. Further, these traits meet the criteria established by Lockart (31) and Oxman (32) for the identification of interferon. The protection of target cells by interferon is not direct. Rather, the experiments with metabolic inhibitors suggest that interferon acts through an intracellular mechanism that involves synthesis by the cells of both RNA and protein. Infection with influenza A or vaccinia virus decreased or, in some experiments, abolished the induction of resistance to lysis in the target cells; this phenomenon, which has also been observed for the antiviral activity of interferon, may

be due to the inhibition of host cell protein synthesis after infection with the two viruses tested (52, 53). The ability of different cell lines to respond to the anti-viral and to the target inhibitory activities of interferon were strictly correlated. Because cell susceptibility to other effects of interferon (e.g., inhibition of DNA synthesis and of cell growth) is not correlated with susceptibility to anti-viral activity, this correlation might indicate that the anti-viral and the target inhibitory effects of interferon follow a common intracellular pathway. Experiments with human-mouse somatic cell hybrids are in progress to test if products of the human chromosome 21, which presumably codes for the anti-viral protein (54), are responsible for the target inhibitory activity of interferon.

The protective effect of interferon on target cells offers a possible explanation of the paradoxical existence in vivo of natural killer cells, which, in vitro, can efficiently lyse any target cells, including normal autologous cells (13). Normal fibroblasts are more susceptible, in general, to the anti-viral and target cell inhibitory activities of interferon than cultured tumor cells or virus-infected cells. Interferon, by stimulating very efficient nonspecific cytotoxic cells and by simultaneously protecting normal cells from lysis, might render the natural killer cell system an inducible selective defense mechanism against tumor cells or virus-infected cells. Some tumor cell lines however (for example SW690 in Table I) maintained their susceptibility to the interferon target inhibitory activity: in this case interferon, by protecting the cells from lysis, may furnish an efficient escape mechanism to the tumor. A phenomenon of this type may perhaps explain why, in some transplantable tumor systems in mice, tumor cells cultured in vitro are more susceptible to killer cells than those grown in vivo (55, 56).

It is, however, difficult to speculate about the actual role that interferon has in vivo in regulating nonspecific defense mechanisms either in physiological conditions or during growth of spontaneous tumor and during viral infection. Mouse spleen cells from apparently healthy animals and, in some cases, human peripheral blood lymphocytes, spontaneously secrete low levels of interferon, suggesting that the presence of subliminal amounts of interferon are present in vivo even in physiological conditions. It is possible that the activity of the natural killer cells present in vivo is maintained by a continuous stimulation mediated by this low level of interferon. A better understanding of the role of interferon in vivo in modulating natural killer cell activity in physiological, pathological or experimental conditions must await further investigation.

### Summary

Interferon, induced in lymphocytes either with viruses or cell lines, increases severalfold the natural cytotoxicity of human lymphocytes on target cell lines. Cell separation experiments support the hypothesis that interferon enhances the activity of natural killer cells rather than generating a new population of effector cells. In mixed culture of lymphocytes and cell lines in which endogenous interferon is produced, interferon mediates an enhancement of cytotoxicity that represents up to 70-90% of the observed cytotoxicity. The effect of interferon on target cells is antagonistic to the effect on the lymphocytes: the susceptibility

to cell-mediated lysis of various cells upon pretreatment with interferon is decreased and in some cases almost completely suppressed. Interferon renders target cells resistant to natural killer cells acting by an intracellular mechanism which requires RNA and protein synthesis. While normal fibroblasts are protected, virus-infected cells and most tumor cells usually are not protected by interferon. Interferon by stimulating very efficient nonspecific cytotoxic cells and by protecting at the same time normal cells from lysis, might render the natural killer cell system an inducible selective defense mechanism against tumor and virus-infected cells.

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