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Enhancement by Interferon of the Specific Cytotoxicity of Sensitized Lymphocytes

(target tumor cells/mouse spleen/lymphoid leukemia/^{51}Cr release)

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ABSTRACT **Mouse interferon preparations enhanced the specific cytotoxicity of sensitized lymphocytes for allogeneic target tumor cells. The factor responsible for the enhancement of cytotoxicity could not be dissociated from the antiviral activity of interferon by standard physicochemical means. Thus, in addition to its well-known antiviral activity, and its effect on cell division, interferon also appears to enhance a specialized cellular function. It is suggested that a common mechanism of action underlies these seemingly different biologic phenomena, and that interferon may play a role in the regulation of fundamental cellular processes.**

In addition to its well-known antiviral activity, interferon has been shown to inhibit the multiplication of tumor and normal mammalian cells in culture (1–4). It seems likely, therefore, that the antiviral action of interferon is only one expression of the effect of interferon on cells (3, 4). In the course of our studies on interferon–cell interactions, we observed that prior treatment of sensitized splenic lymphocytes with interferon resulted in an enhancement of the specific cytotoxicity of these cells for target tumor cells. Thus, interferon appears to enhance a specialized cellular function. The results of these experiments and the evidence suggesting that interferon was the factor responsible for enhancement of cytotoxicity of sensitized lymphocytes for target tumor cells are presented herein.

MATERIALS AND METHODS

Immunization of Mice with L 1210 Cells. Mouse lymphoid leukemia L 1210 cells (5) were maintained by serial passage in ascitic form in DBA/2 mice. For immunization, C 57 BL/6 mice were injected intraperitoneally with 5×10^7 L 1210 cells. The spleens of immunized mice were usually harvested 10 days after injection.

Lymphocytes. Suspensions of splenic lymphocytes from immunized or normal C 57 BL/6 mice were obtained according to the method of Brunner *et al.* (6). The washed lymphocytes were suspended in Eagle's medium containing 10% heat-inactivated calf serum at a density of 20×10^6 viable cells per ml.

Target Cells. L 1210 cells, an interferon resistant subline of L 1210 cells (7) (L 1210-R) and Ehrlich ascites cells (7) were grown in nonagitated suspension cultures in nutrient medium RPMI 1640 (Gibco), supplemented with 10% heat-inactivated horse serum, 1% L-glutamine (10 mM/ml), penicillin (200 U/ml), and streptomycin (40 μg/ml). For labeling with radioactive chromium (^{51}Cr), 1 ml of a cell suspension (5×10^6 viable cells per ml) was incubated for 30 min at 37° with 0.1 ml of $Na_2{}^{51}CrO_4$ (200 μCi/ml, specific activity 400 mCi/mg of chromium, Centre d'Energie Atomique, France), according to the method of Brunner *et al.* (6, 8). The cell suspensions were washed three times and readjusted to a density of 2×10^5 viable cells per ml.

Interferon and Control Preparations. Mouse interferon preparations were obtained from the nutrient medium of monolayer cultures of MSV-Ia (9), L-929 (10), and L cells (11, 12) that were inoculated with ultraviolet light-inactivated Newcastle Disease virus (NDV), and from the brains of IC (Institut du Cancer) and C 57 BL/6 mice that were inoculated intracerebrally with West Nile virus (13). Human interferon preparations (generously provided by Dr. C. Chany) were derived from the nutrient medium of leucocyte suspensions inoculated with NDV (14). Rabbit interferon preparations were obtained from the sera of rabbits inoculated with NDV (15). Control preparations consisted of the medium of uninoculated cell cultures or tissues of uninoculated animals. All interferon and control preparations were treated at pH 2 for 18 hr (preparations containing West Nile virus), or 6 days (preparations containing NDV), centrifuged at 30,000 rpm (60,000 $\times$ *g*) for 1 hr in a Martin Christ centrifuge, Omega, and then concentrated 10-fold by forced dialysis. MSV-Ia and mouse brain interferon were purified by filtration through a Sephadex G-75 column (16). L-929 interferon (generously provided by Dr. G. Bodo) was concentrated with zinc acetate, 0.02 M at pH 7.2, dissolved at pH 2.5, chromatographed on sulfoethyl-Sephadex C-25, concentrated, and freeze-dried or further purified by chromatography on DEAE-cellulose and carboxymethyl-Sephadex C-25 (10). L-cell interferon (generously provided by Dr. K. Paucker) was purified by electrophoresis (11, 12).

Mouse, human, and rabbit interferon preparations were assayed on monolayer cultures of mouse L-, monkey BSC-, and rabbit RK13-cells, respectively. One mouse interferon unit (as expressed in the text) equals 4 NIH International Reference Units. The specific activity of semipurified MSV-Ia interferon was 5×10^4 units/mg of protein; the specific activities of the highly purified L-929 and L-cell interferons were 2×10^6 and 7.5×10^5 units/mg of protein, respectively.

Cytotoxicity Tests with Sensitized Lymphocytes. The methods described by Brunner *et al.* (6, 8) were used with slight modi-

Abbreviation: NDV, Newcastle Disease Virus.

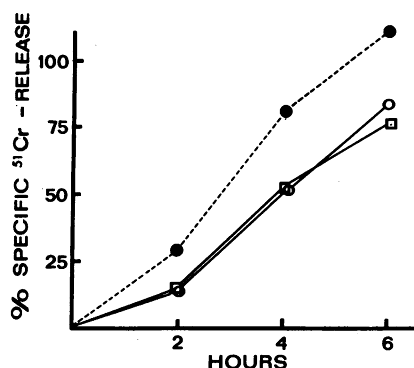

FIG. 1. Effect of interferon on the cytotoxic action of C 57 BL/6 mouse lymphocytes on L 1210 target cells expressed as % specific ^{51}Cr-release (based on data presented in Table 1). □, Sensitized lymphocytes pretreated with medium; ○, sensitized lymphocytes pretreated with control preparation; ●, sensitized lymphocytes pretreated with semipurified C 57 BL/6 brain interferon.

fications. The medium consisted of Eagle's medium supplemented with 10% heat-inactivated calf serum. Spleen-cell suspensions containing 2×10^7 or less cells per ml were mixed with equal volumes of ^{51}Cr-labeled target-cell suspensions containing 2×10^5 cells per ml; 0.25 ml of the reaction mixtures were placed in disposable hemolysis tubes (13 × 75 mm), and incubated at 37° during the indicated period of time. For each reaction mixture, 6 or 10 identical tubes were used. For assay of ^{51}Cr released, 1.75 ml of phosphate buffered saline (PBS) was added to each tube, followed by centrifugation at 1500 rpm (800 × *g*) for 5 min. 1 ml of the supernatant was harvested, and the radioactivity was measured in a well-type scintillation counter (Gammatic, Societé d'Applications Industrielles de la Physique, France). Maximal ^{51}Cr release was determined by incubation of 0.25 ml of the reaction mixtures with 1.75 ml of distilled water at 37° for 6 hr. Percent specific ^{51}Cr release was calculated according to the following formula:

$$100 \times \frac{\left(\begin{array}{l}^{51}\text{Cr release in the}\\ \text{presence of immune lymphocytes}\end{array} - \begin{array}{l}^{51}\text{Cr release in the}\\ \text{presence of normal lymphocytes}\end{array}\right)}{\left(\begin{array}{l}\text{Maximal }^{51}\text{Cr}\\ \text{release}\end{array} - \begin{array}{l}^{51}\text{Cr release in the}\\ \text{presence of normal lymphocytes}\end{array}\right)}.$$

The effect of interferon on the cytotoxicity of sensitized lymphocytes was determined by incubation of lymphocytes with a 1:10 volume of interferon, control preparation, or nutrient medium at 37° for the specified period of time before addition of target cells (see Figs. 1–3). Unless stated otherwise, interferon or control preparations were present in the reaction mixture throughout the experiment.

RESULTS

Effect of interferon on specific cytotoxicity of sensitized lymphocytes

Splenic lymphocytes from C 57 BL/6 mice, immunized with allogeneic L 1210 cells, readily lysed ^{51}Cr-labeled L 1210 target cells within 6 hr of incubation (Table 1). Prior treatment of sensitized lymphocyte suspensions with mouse interferon preparations considerably enhanced this specific cytotoxicity. These results are expressed in Fig. 1 as the percentage of specific chromium release calculated according to the formula described in *Methods*. Enhancement of the cytotoxicity of sensitized lymphocytes by interferon was observed at lymphocyte:target cell ratios of 30:1, 50:1, and 100:1. For convenience, however, a lymphocyte:target cell ratio of 50:1 was used in the following experiments.

Fig. 2 illustrates the kinetics of the development of enhanced cytotoxicity of C 57 BL/6 sensitized lymphocytes incubated with different amounts of interferon. The extent

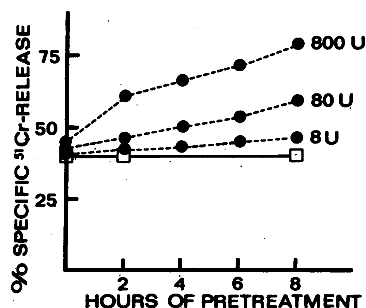

FIG. 2. Kinetics of the development of enhanced cytotoxicity of sensitized lymphocytes incubated with different amounts of interferon.

Lymphocyte suspensions were pretreated with semipurified C 57 BL/6 mouse brain interferon at various concentrations before addition of ^{51}Cr-labeled L 1210 target cells. Lymphocyte: target cell ratio was 50:1. ^{51}Cr-release was measured in all reaction mixtures after 4 hr of incubation. Interferon concentrations are expressed as units per 10^6 lymphocytes. □, Sensitized lymphocytes pretreated with medium only; ●, sensitized lymphocytes pretreated with interferon.

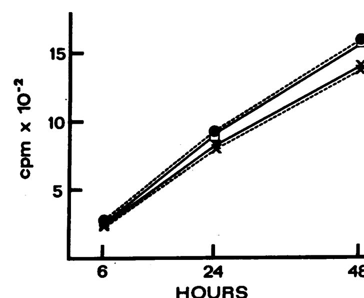

FIG. 3. Effect of interferon on the cytotoxic action of normal (nonsensitized) C 57 BL/6 mouse lymphocytes on L 1210-R target cells.

Suspensions of lymphocytes and target cells (ratio 100:1) were incubated with interferon or nutrient medium without interferon at 37° throughout the experiment. ^{51}Cr-release is expressed as counts per minute. (The release of ^{51}Cr from target cells incubated with *normal* lymphocytes and interferon for 48 hr corresponds to a *specific* ^{51}Cr-release of 5%. See Figs. 1 and 2 for comparison with the percentage of specific release of ^{51}Cr from target cells after incubation with *sensitized* control or interferon-treated lymphocytes.) □——□, Target cells and normal lymphocytes; ●- - -●, target cells and normal lymphocytes treated with semipurified C 57 BL/6 brain interferon (320 U/10^6 lymphocytes); ×——×, target cells only; ×- - -×, target cells treated with interferon.

and the appearance of enhancement was related to the amount of interferon in the test mixture.

Two types of experiments were undertaken to demonstrate that interferon acted on the sensitized lymphocytes and not on the target cells: (a) After treatment of sensitized lymphocytes with interferon for 2 hr, the cells were washed three times and then incubated with L 1210 target cells. Enhancement of activity was as marked when interferon was removed from the medium (by this procedure) as when interferon was left in the medium throughout the experiment. (b) Interferon enhanced the cytotoxicity of sensitized lymphocytes for L 1210-R cells (selected for resistance to interferon) as much as for cells of the parent L 1210 cell line.

Correlation between the antiviral activity and the cytotoxicity enhancing activity of interferon preparations

All mouse interferon preparations tested (whether allogeneic or syngeneic) were found to enhance specific cytotoxicity (Table 2). A greater degree of enhancement was obtained with purified interferon than with crude interferon preparations. Highly purified mouse interferon preparations (purified either by three different chromatography steps or by electrophoresis) were fully active. Control preparations and heterologous interferons did not exert a significant cytotoxic effect.

The factor in the interferon preparations responsible for the enhancement of specific cytotoxicity fulfilled the various physicochemical criteria that characterize interferon. Thus, prior treatment of these preparations with trypsin or sodium periodate (17) destroyed both the antiviral activity of interferon and the cytotoxicity enhancing activity. Heating at 60° for 1 hr markedly decreased both activities. Ribonuclease and deoxyribonuclease had no effect on interferon.

Specificity of the interferon-mediated enhancement of cytotoxicity of sensitized lymphocytes

The lysis of allogeneic target cells by sensitized lymphocytes is an immune-specific reaction (8, 18, 19). The specificity of the interferon-mediated enhancement of cytotoxicity of sensitized lymphocytes was investigated by mixing ^{51}Cr-labeled Ehrlich ascites target cells of BALB/c origin with C 57 BL/6 lymphocytes sensitized against L 1210 cells of DBA/2 origin. Neither control immune lymphocytes nor interferon-treated immune lymphocytes proved to be cytotoxic for Ehrlich ascites target cells during 6 hr of incubation. In a second experiment, interferon-treated sensitized lymphocytes (to L 1210 cells) were first stimulated by incubation with unlabeled L 1210 cells for 2 hr. A second population of either L 1210 cells or Ehrlich ascites cells labeled with ^{51}Cr was then added to the reaction mixture. Enhanced lysis of L 1210 cells was observed, but no lysis of Ehrlich ascites cells occurred.

TABLE 1. *Effect of interferon on the cytotoxic action of C 57 BL/6 mouse lymphocytes on L 1210 target cells*

Target cells incubated with	Time of incubation: 2 hr counts/4 min*	4 hr counts/4 min*	6 hr counts/4 min*
Normal lymphocytes	925 ± 17†	1031 ± 26	1084 ± 18
Sensitized lymphocytes pretreated with medium	1397 ± 31	2611 ± 53	3324 ± 35
Sensitized lymphocytes pretreated with control preparation	1379 ± 34	2570 ± 40	3626 ± 39
Sensitized lymphocytes pretreated with interferon‡	1735 ± 45	3441 ± 39	4261 ± 65

Lymphocyte suspensions were pretreated with interferon, a control preparation, or nutrient medium for 3 hr at 37°, before addition of ^{51}Cr-labeled target cells. Lymphocyte: target cell ratio was 50:1.

* Mean value for 10 tubes per reaction mixture.

† 0.95 Confidence interval.

‡ Semipurified C 57 BL/6 brain interferon (320 U/10^6 lymphocytes).

TABLE 2. *Effect of homologous and heterologous interferon preparations on the cytotoxicity of sensitized lymphocytes**

Preparation	Origin of preparation	% Specific ^{51}Cr-release by target cells	Units of interferon per 10^6 lymphocytes
Homologous			
None	—	40	0
Interferon	MSV-Ia cells (crude)	60	800
Interferon	MSV-Ia cells (semi-purified)	70	360
Interferon	Syngeneic mouse brain (semipurified)	71	360
Interferon	Allogeneic mouse brain (semipurified)	72	360
Interferon	L-929 cells (highly purified)	70	360
Interferon	L-cells (highly purified)	62	200
Control	MSV-Ia cells	45	<10
Control	Syngeneic mouse brain	45	<10
Control	Allogeneic mouse brain	42	<10
Heterologous			
Interferon	Rabbit serum	39	<10†
Interferon	Human leucocytes	36	<10†

* Sensitized lymphocytes were pretreated with interferon preparations for 3 hr. Release of ^{51}Cr from target cells was measured after 5 hr of incubation of lymphocyte–target cell mixtures at a ratio of 50:1.

† Titers of rabbit and human interferon preparations, assayed on rabbit or monkey cells, were 320 and 125 units/10^6 lymphocytes, respectively.

Effect of interferon on the cytotoxicity of normal lymphocytes

Splenic lymphocytes from nonimmunized C 57 BL/6 mice were not cytotoxic for L 1210 target cells, nor did prior treatment of these lymphocytes with interferon render them cytotoxic (tested after incubation of lymphocytes and target cells for 6 hr). To determine whether interferon enhanced the cytotoxicity of nonsensitized lymphocytes for L 1210 cells when lymphocytes and target cells were incubated in the presence of interferon for a longer period of time (24 or 48 hr), it was necessary to use L 1210-R target cells to avoid unrelated effects of interferon on the target cell. As can be seen from Fig. 3, "normal" (nonsensitized) lymphocytes

exhibited a slight cytotoxicity for L 1210 cells after 48 hr of incubation, but this effect was not enhanced by interferon.

DISCUSSION

The experimental results presented herein demonstrate that mouse interferon preparations enhanced the specific cytotoxicity of sensitized lymphocytes for allogeneic target cells. The following findings suggest that interferon was the factor responsible for the enhancement of specific cytotoxicity: (*a*) All preparations of *mouse* interferon, regardless of the tissue of origin and the viral inducer used, were effective. Control preparations and heterologous interferon preparations were ineffective. (*b*) Highly purified interferon preparations (purified by three different chromatography steps or by electrophoresis) proved effective (even more effective than crude interferon preparations of comparable titer). (*c*) The factor responsible for cytotoxic enhancement could not be dissociated from the antiviral activity of interferon by standard physicochemical means.

The L 1210 cells used as target cells in these experiments are sensitive to the antiviral and anticellular activities of interferon (3). However, the enhanced cytotoxicity of sensitized lymphocytes resulted from the effect of interferon on lymphocytes and not from an effect on the target cells: (*a*) The enhancing effect of interferon on sensitized lymphocytes was also observed when interferon was removed from the suspensions (by washing the lymphocytes) prior to addition of the target cells. (*b*) Interferon resistant L 1210 cells were lysed to the same extent as interferon sensitive L 1210 cells when incubated with interferon-treated sensitized lymphocytes. (*c*) Neither interferon alone, nor interferon and normal lymphocytes, were cytotoxic for target cells within the observation period of 6 hr.

Svet-Moldavsky *et al.* (20, 21) and Borecký *et al.* (22) reported that interferon rendered normal lymphocytes (from nonimmunized mice) cytotoxic for syngeneic or allogeneic tumor cells. Since in their experiments, interferon, lymphocytes, and target cells were incubated together for 48 hr or more, interferon may have acted on the target cells themselves. As stated above, this possible source of error was circumvented in our experiments by use of the interferon-resistant line of L 1210 target cells. Under these conditions interferon-treated normal lymphocytes did not exhibit an enhanced cytotoxicity even after 48 hr of incubation of lymphocytes and target cells.

The mechanism of specific target-cell lysis mediated by sensitized lymphocytes remains unknown (18, 19). To our knowledge, there is only one other reported example of an enhancement of cytotoxicity of sensitized lymphocytes. Brunner and his coworkers (19) found that prior treatment of sensitized lymphocytes with specific soluble antigen increased their cytotoxicity. It is of interest that contact between specific antigen and sensitized lymphocytes or target cell and sensitized lymphocytes induces a rapid release of interferon (23, 24). It is thus possible that the enhancement of cytotoxicity observed by Brunner *et al.* (19) was in fact mediated by interferon.

The interest in the results presented herein may be 2-fold. First, if interferon enhances the cytotoxicity of sensitized lymphocytes *in vivo* as it does in our *in vitro* experiments, it may help to explain in part the observed efficacy of interferon in the treatment of animals inoculated with viruses (25), protozoa (26), or transplantable tumors (27, 28). Second, the enhancement of a specialized cellular function by interferon may not be limited to the system of sensitized lymphocytes that we have investigated, but may be a more general phenomenon. In this regard, Stewart and his coworkers have shown that the "priming" of cells in culture by interferon (which enhances subsequent interferon production after inoculation of an inducer) is an activity distinct from the antiviral activity of interferon (29). Likewise, Huang and his coworkers have recently shown (30) that mouse interferon preparations enhanced the phagocytosis of colloidal carbon particles by mouse peritoneal macrophages.

Interferon has now been shown to exert varied and seemingly unrelated effects on cells (i.e., inhibition of viral multiplication, inhibition of cell division, and enhancement of specialized cellular function). It seems reasonable to us to suggest that a common mechanism of action underlies these apparently different biologic phenomena, and that interferon plays a role in the regulation of fundamental cellular processes.

We thank Drs. G. Bodo and K. Paucker for their gifts of purified mouse interferon, C. Chany for his gift of human interferon, and K. T. Brunner and J. C. Cerottini for introducing us to their method for measurement of cell-mediated cytotoxicity on ^{51}Cr-labeled target cells, and for following the development of these experiments with us. I. G. is indebted to Dr. Sidney Farber, Director of the Children's Cancer Research Foundation, Boston, Mass. 02115 for his continued interest and support.

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