PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 177, 257–261 (1984)

Synergism of Antiviral Activity in Cell Cultures Treated with Low Concentrations of Interferon and Interferon-Treated Lymphocytes (41940)

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Abstract. Human T cells treated with low levels of interferon (IFN) (1–10 units/ml), and washed to remove the IFN, transferred the same level of antiviral activity to recipient WISH cells as an equivalent IFN treatment alone could induce in WISH cells. Further, when T cells pretreated with IFN (1–10 units/ml) were cocultivated with WISH cells in the presence of IFN (1–10 units/ml), a 2.5- to 5-fold greater level of protection developed than could be expected from the additive effect of each. Antibody to leukocyte, fibroblast, or immune IFN blocked the antiviral effect of the respective IFN types but had no effect on the transfer of antiviral activity initiated by leukocyte, fibroblast, or immune IFN. Also, treatment of T cells with actinomycin D blocked the transfer of antiviral activity of IFN-treated T cells. Taken together, the data suggest that the increased antiviral activity is not merely an additive effect of the IFN, but represents a synergistic amplification of protection most likely due to the combination of the separate effects of IFN and IFN-induced transfer. Such interactions would be expected to play a major role in early protection against virus infections *in vivo* when low levels of interferon are present and lymphocytes are migrating into the area.

We have previously shown that human B lymphocytes cocultured with various xenogeneic and allogeneic cells produced human leukocyte interferon (IFN) and shortly thereafter transferred antiviral activity in xenogeneic or allogeneic cells (9, 13). The transfer process initiated by IFN required cell contact and was shown to be an important amplification system for the antiviral effect of IFN (8). In the present study, we compared the level of antiviral activity transferred to cells by lymphocytes pretreated with IFN to the level of antiviral activity induced in cells by direct treatment with IFN. In addition, we investigated whether combinations of IFN and IFN-treated T cells could induce higher levels of antiviral activity in recipient cells than could be induced by either alone.

Materials and Methods. *Interferon assays.* Human IFN was quantitated in human amnion WISH cells using a previously described cytopathic effect inhibition (CPE) microassay (10). Sindbis virus was used as challenge at an input multiplicity of 50. Titers are expressed as the reciprocal of the IFN dilution that inhibits 50% of the CPE. One unit of IFN activity in our assay equals one unit of NIH standard IFN activity.

Leukocyte preparation. Leukocytes were prepared from human peripheral blood by the Ficoll–Hypaque gradient separation method (3). Plastic nonadherent cells were obtained by incubating 5 ml of a 10×10^6/ml leukocyte suspension in plastic tissue culture dishes for 1 hr at 37°C. The enriched T-cell populations were obtained from plastic nonadherent cell suspensions by E-rosette formation (4). These cells were suspended at a final concentration of $1–2 \times 10^6$ cells/ml in Eagle's minimum essential medium (EMEM) supplemented with 2% fetal bovine serum (FBS). Greater than 95% cell viability was observed in these fractions. The population of cells was judged to be mostly T cells by (1) their lack of esterase staining; (2) their lack of surface immunoglobulin; (3) their absence of spontaneous NK activity, and (4) their high response to the T-cell mitogen, staphylococcal enterotoxin A.

IFN and antibody treatment. For IFN treatment, 0.3 ml of a leukocyte suspension (1×10^7 cells/ml) was added to dilutions of the various IFNs and incubated for 4 hr at 37°C. The leukocytes were washed three times, suspended in EMEM, and cocultured with recipient human amnion WISH cells as described below. Control lymphocytes were incubated in EMEM without IFN and cocultured identically with recipient cells. For IFN treatment of WISH cells 0.1 ml of

0037-9727/84 $1.50

dilutions of the various IFNs was added to the confluent cells and incubated for 4 hr at 37°C. The cells were washed three times before virus challenge as described below. To neutralize endogenous IFN or IFN carried over from IFN treatment of T cells, WISH cells were treated for 4 hr with an amount of antibody to leukocyte (α), fibroblast (β), or immune (γ) IFN capable of neutralizing 1000 units of IFN-α, IFN-β, or IFN-γ. Control WISH cells were treated identically with mock antibody. One unit of antibody is the amount of antibody required to neutralize one unit of IFN.

Actinomycin D treatment. WISH cell monolayers or enriched human T-cell populations were treated with actinomycin D (5 μg/ml) diluted in EMEM for 1 hr at 37°C. The cells were washed three times and cultured as described below. Actinomycin D treated and control cell cultures were pulsed for 1 hr with [^{3}H]uridine (Amersham Intl. LTD) at 1.0 μCi/ml. Uridine incorporation was measured from TCA-precipitable [^{3}H]uridine collected on glass fiber filters.

Transfer of antiviral activity. Human lymphocytes and WISH cells were cocultivated at a ratio of 1:1 in Eagle's medium in 96-well microtiter plates (Falcon Plastics, Oxnard, Calif.). After 4 hr incubation at 37°C, the supernatant fluids were decanted, and the cell cultures were washed and then challenged with 0.025 ml Sindbis virus at an input multiplicity of 50. After 1 hr at 37°C, the inoculum was decanted and the cell sheets were washed and replenished with fresh medium. Virus yields from pooled triplicate cultures were determined 18 hr post-challenge by methods previously described (14). Sindbis virus used in these studies does not replicate in lymphocytes (13). Fold differences in antiviral activity were compared using the Student t test (7).

Assay of natural killing activity. Human WISH cells were propagated in 96-well microtiter plates (Falcon Plastics, Oxnard, Calif.) in EMEM with antibiotics and 2% fetal bovine serum and prelabeled with sodium chromate (^{51}Cr) at a concentration of 10 μCi/2 $\times$ 10^5 cells per 4 hr at 37°C. After incubation, the target cells were washed three times with EMEM, and human or mouse lymphocytes were added at an effector-to-target-cell ratio of 10:1. Specific ^{51}Cr release was determined from triplicate cultures after incubation at 37°C for 4 hr. The percentage of specific ^{51}Cr release was calculated as $R = (E - S/M - S) \times 100$ where E is the counts per minute in the experimental wells, S is spontaneous release, and M is the maximal release in the presence of 1% saponin.

Reagents. Human fibroblast IFN (IFN-β), 1.7×10^6 units/mg protein, was obtained from HEM Research, Inc. (Rockville, Md.). Human leukocyte IFN (IFN-α), 10^6 units/mg protein was kindly supplied by Dr. K. Cantell. Human immune IFN (IFN-γ), $10^{5.2}$ units/mg protein was prepared as previously described (5). Sheep anti-human IFN-α and anti-human IFN-β were generously provided by Dr. Barbara Dalton and Dr. C. A. Ogburn (Medical College of Pennsylvania, Philadelphia, Pa.). Rabbit anti-human IFN-γ was prepared in our laboratory as previously described (6).

Results. *IFN-treated T cells induce the same levels of antiviral activity as an equivalent amount of IFN alone.* To determine if low levels of human IFN-α could induce T-enriched lymphocytes to transfer high levels of antiviral activity to recipient cells, T-cell populations were treated with low levels of IFN, washed, and then added to confluent cultures of human WISH cells. At the same time, other cultures of WISH cells were treated with the same concentrations of IFN alone. In order to assess the role of the transfer of antiviral activity alone, it was necessary to include antibody to IFN-α in the cocultures to neutralize the effects of any carryover of endogenously produced IFN. The results are shown in Table I. The data (four left columns) indicate that the mean level of antiviral activity transferred by T lymphocytes treated with low levels (1–10 units/ml) of any type of IFN was on the average the same as the level of activity induced by any IFN alone. Thus, it appears that T cells treated with IFN transfer the same level of antiviral activity as direct treatment with IFN alone. The data in Fig. 1 demonstrate that 1000 neutralizing units of antibody specific for IFN-α was not able to block the development of antiviral activity transferred from T cells pretreated with IFN-α. However, the antibody did effectively block

TABLE I. SYNERGISTIC TRANSFER OF ANTIVIRAL ACTIVITY BY IFN AND IFN-TREATED T CELLS[a]

Treatment	Fold reduction in virus yields: Dilution of IFN[b]	IFN alone[c]	IFN-treated T cells alone[d]	Fold difference[e]	IFN + IFN-treated T cells combined: Expected	Observed	Fold synergism observed/expected
IFN-α	1:30	2	3	1.7	5	16	3.2
	1:10	8	9	1.1	17	55	3.2
	1:3	25	15	−1.7	40	100	2.5
IFN-β	1:30	5	3	1.4	8	30	3.8
	1:10	9	7	1.3	16	80	5.0
	1:3	40	20	−2.0	60	150	2.5
IFN-γ	1:30	2	4	2.0	6	18	3.0
	1:10	6	9	1.6	15	53	3.5
	1:3	10	15	1.5	25	62	2.5
Mean				1.6			3.2

[a] Human T cells (E-rosette positive) were treated for 4 hr with IFN, washed three times, and added to WISH cell monolayers at a ratio of one T cell per WISH cell. IFN was added to the WISH cells at the same time. T cells and IFN were removed 4 hr later by washing and the cultures were challenged with Sindbis virus at a multiplicity of 50 to 1. The yield of virus was measured 18 hr later by plaque titration. The data shown are the results from a single experiment performed three times.

[b] Stock preparations of IFN of approximately 30–100 units were diluted by a one-half log dilution scheme.

[c] Fold reduction derived from standard curve.

[d] T cells were treated as described in (a) except they were cocultured with WISH cells in the presence of antibody to IFN.

[e] Fold difference in virus yields between IFN treatment alone and IFN-treated T cells.

the direct antiviral effect of IFN-α. Thus, the data indicate that the IFN-induced transfer of antiviral activity was not due to carryover of IFN or the action of any endogenously produced IFN. Similar results were also obtained with anti-IFN-γ and anti-IFN-β serum treatment of T cells treated with IFN-γ and IFN-β, respectively.

Enhanced antiviral activity by a combination of IFN and IFN-treated T cells. Possible synergistic interactions between IFN and IFN-treated T cells were examined by comparing the additive amounts of antiviral activity induced in WISH cells obtained by direct IFN treatment and IFN-treated T cells to the antiviral activity induced following combined treatment with IFN and IFN-treated T cells. The results of a representative experiment are shown in Table I (three right columns) which shows that an additional 2.5- to 5-fold inhibition in virus yields resulted when combinations of IFN-treated T cells and IFN were used to treat the WISH cells. In this system since antibody to IFN was not present, there was the possibility that carryover of IFN influenced the results. In other experiments, however, we found that pretreatment with IFN was unnecessary since the same level of synergism was observed when IFN and T cells were added to WISH cells at the same time (data not shown). Thus, the data suggest that the inhibition of viral yields induced in WISH cells by IFN and IFN-treated T cells is synergistic, particularly at low levels of IFN.

Inhibition of IFN-induced transfer of antiviral activity by T cells with actinomycin D. To demonstrate that transfer by lymphocytes in this system is an inducible process T cells were treated with actinomycin D before they were treated with IFN and cocultured with WISH cells. The data in Table II show that 5 μg/ml actinomycin D was able to effectively block the transfer of antiviral activity to WISH cells. There was no significant difference in virus yields when WISH cells were cocultured with either nontreated T cells or actinomycin D and IFN-treated T cells. The inhibition of transfer by actinomycin D-treated lymphocytes was not due to transfer

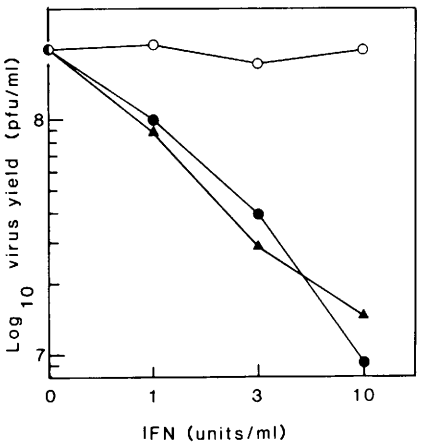

FIG. 1. Inability of antibody to IFN-α to block transfer of antiviral activity by T cells treated with IFN. Human T cells (E-rosette positive) were treated for 4 hr with IFN, washed three times, and then added to WISH cells at a ratio of one T cell per WISH cell. WISH cells were incubated with IFN (●), IFN plus specific neutralizing antibody (○), or IFN-treated T cells plus neutralizing antibody (▲) as described under Materials and Methods. Four hours later T cells, antibody, and IFN were washed out, the WISH cells were challenged with Sindbis virus, and virus titers were determined as described under Materials and Methods. The data shown are the results from a single experiment performed three times.

of actinomycin D from the treated T cells to the WISH cells because incorporation of [^{3}H]uridine by WISH cells was not diminished from control values (data not shown). Thus, the transfer of antiviral activity by T cells in this system is inducible and has the same characteristics as shown in other transfer systems (9, 12).

Cell-mediated cytotoxicity in cocultures of T cells and WISH cells. Another possible explanation for reduced virus yields from WISH cells cocultured with IFN-treated lymphocytes could be that cytotoxicity was mediated by natural killer cells, especially since the natural killer cell activity is enhanced by IFN (11). This possibility was evaluated by measuring ^{51}Cr released from WISH cells cocultured at a 1:1 ratio for 4 hr with IFN-treated T cells. Under these conditions no more than 0.3% specific ^{51}Cr release could be found when the cells were pretreated with up to 3000 units IFN. The data were similar for all three types of IFN. The data show that the transfer of antiviral activity occurs at a much lower ratio of lymphocytes to WISH cells than is commonly used to show cell-mediated cytotoxicity by ^{51}Cr release assay. In addition there was no microscopically observable toxicity in WISH cells cocultured at a 1:1 ratio with T cells for 24 hr. Therefore, cell-mediated cytotoxicity does not appear to mediate the observed reduction in virus yield.

Discussion. This is the first report demonstrating synergism of antiviral activity between IFN and IFN-induced transfer by lymphocytes. Our experimental design utilized T cells because they do not produce detectable endogenous IFN when cocultivated with allogeneic recipient cells. The transfer was effective (1–10 units IFN induces a 2.5- to 5-fold inhibition of virus titer) and efficient (1:1 cell ratio). In addition, antibody to IFN was used to neutralize any potential endogenous IFN and/or any carryover of IFN used to treat the T cells. Specific antibody to all three types of IFN failed to block the IFN-induced transfer of antiviral activity suggesting that the WISH cells were not responding to IFN molecules directly. The transfer of IFN molecules within vesicles is possible but unlikely since a cell producing IFN is not protected in the presence of antibody to IFN (12). The mechanism of transfer by T cells appeared to be similar to others previously described (2, 9) since T cells treated with actinomycin D and IFN induced only a 2-fold increase in antiviral activity while the IFN induced transfer mechanism induced a

TABLE II. INHIBITION OF THE TRANSFER OF ANTIVIRAL ACTIVITY BY ACTINOMYCIN D

WISH cells cocultivated with	Sindbis virus yield (pfu/ml)	Fold inhibition of virus yield
No treatment	2.2×10^8	—
Nontreated T cells	1.6×10^8	1.4
IFN-treated T cells[a]	4.2×10^6	52
Actinomycin D and IFN-treated T cells[b]	1.0×10^8	2.2

[a] T cells were treated with 100 units/ml IFN-α for 4 hr as described under Materials and Methods. The data shown are the results from a single experiment performed two times.

[b] T cells were treated for 1 hr with 5.0 μg/ml actinomycin D and washed 3 times prior to treatment with 100 units/ml IFN as described under Materials and Methods.

52-fold increase. These data suggest that *de novo* RNA synthesis is necessary to allow induction of transfer which appears to be mediated by T cells. The fact that synergism was observed suggests that the antiviral activities induced by IFN and IFN-treated cells may, in part, occur by different mechanisms.

Although the mechanism of transfer is not known, it is known from cloning studies that there is a marked heterogeneity between cells in their sensitivity to IFN and their ability to transfer. Thus a minor fraction of cells determines the rate and level of antiviral activity that develops in cell populations (1, 2). One explanation for the observed synergistic effect is that the transfer mechanism bypasses certain rate limiting steps that occur between the time that IFN binds to its receptor and antiviral activity is established in a cell. Thus, complex cellular interactions could convert the less sensitive slower responding cells to more rapid responders, thereby enhancing the overall rate and degree of IFN action in the WISH cell population. Overall, the data suggest that highly effective levels of antiviral activity may be induced in tissues when both lymphocytes and IFN are present. This may be especially important early in viral infections or at the periphery of spreading infections when only low levels of IFN are present and lymphocytes are first migrating into the site.

The authors thank Christine Becker and Donna Clegg for their expert technical assistance. This work was supported in part by National Institutes of Health Grants EY 01715, EY 03348, and N01-A1-02659, and the James W. McLaughlin Foundation.

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Received March 22, 1984, P.S.E.B.M. 1984, Vol. 177.
Accepted June 18, 1984.