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Interactions of Human Leukocyte Interferon with Vinca Alkaloids and Other Chemotherapeutic Agents against Human Tumors in Clonogenic Assay*

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Summary. *Purified human leukocyte interferon produced by recombinant techniques (IFN-αA) was tested in vitro with chemotherapeutic drugs, vinblastine (VLB), vincristine (VCR), vindesine (VDS), vinzolidine (VZL), cis-platinum (PLAT), doxorubicin (DOXO), etoposide (VP-16), and melphalan (MEL).*

The activity of these agents alone or in combination was tested against various human tumor cell lines, using a modified soft agar clonogenic assay. Three human tumor cell lines (myeloma, RPMI 8226; breast, MCF-7; and colon, WiDR) showed sensitivity to these agents at clinically achievable drug concentrations. Statistically significant synergistic activity against in vitro colony formation was observed with the combination of VLB and IFN-αA. An additive or sub-additive effect was usually observed with the other agents tested. Continuous exposure of the 8226 myeloma cell line to both IFN-αA and PLAT showed evidence of a more significant potentiation. It is hypothesized that the synergistic effect observed between VLB and IFN-αA is due to some of their common mechanisms of action.

Introduction

Human leukocyte interferon clone A (IFN-αA) is a highly purified interferon produced in *Escherichia coli* by recombinant DNA techniques [19, 28, 38]. It has recently been introduced in clinical trials and preliminary evidence for antitumor activity is available [23, 26]. With standard anticancer drugs, therapy has generally been more effective when drug combinations rather than single agents are used [5]. Whether this will hold true for interferon is unknown, although animal experiments have already suggested some additive or synergistic effects of interferon and chemotherapeutic agents [10, 22].

The soft agar clonogenic assay for human tumor biopsies has been shown to accurately predict tumor response in vivo in tests of standard cytotoxic anticancer drugs [35, 42]. It has also permitted the identification of additive or synergistic drug combinations, confirming and extending what is known by clinical experience [2]. Furthermore, this assay has been shown to be useful in the study of the antiproliferative activity of interferons [7, 13, 14, 34, 43; S. E. Salmon et al. 1982, unpublished work). We have applied the soft agar clonogenic assay to determine the interactions of IFN-αA and various cytotoxic drugs against several human tumor cell lines. We have observed apparent synergism of vinblastine or cisplatin with IFN-αA against three different human tumor cell lines. Lesser effects were observed with combinations of interferon and other cytotoxic drugs thus far tested, but inhibition of interferon action was not observed.

Materials and Methods

Cell Lines. Various human tumor cell lines were studied in an effort to find cell lines with some interferon-induced inhibition of colony formation. Table 1 gives the name, source, tissue type, medium of culture, and method of harvesting used for each of the six different cell lines studied. Cell lines were cultured at 37° C in a 95% humidity, 5% CO_2 room air incubator, and passed serially as needed. RPMI 8226, a human myeloma cell line, is the only one that grows mainly as cells or groups of cells floating in the medium, with some easily removable cells attached to the bottom of the tissue culture flask. All other cell lines grew as monolayers. The cells were harvested while undergoing exponential growth. Harvesting was done manually (RPMI 8226), by incubation of the PBS (phosphate-buffered saline)-washed flasks either with Tyrode's without glucose [31] for 10–15 min (HEC-1A line) or with Trypsin-EDTA 0.025% (Gibco) for 5 min at 37° C. Medium was added and the cells allowed to incubate for 15 more min. The cells were then shaken off the bottom of the flask and washed twice in McCoy's 5A at 150 *g* for 7 min. Adequate dilutions of single cell suspensions were obtained to give final concentrations per agar plate of 50,000 cells (8226), 20,000 cells (MCF-7), and 10,000 cells (WiDR).

Drugs Used

Interferon. Human leukocyte interferon IFN-αA was obtained from Dr Patrick Trown, Hoffmann-La Roche, Nutley, NJ, USA. This preparation was reported to be more than 95% pure and to have a specific antiviral activity of 200 U per nanogram of protein. Appropriate dilutions were made in McCoy's 5A containing 10% heat-inactivated fetal bovine serum and kept frozen at −80° C, until use.

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Table 1. Human cell lines and culture conditions

Cell line	Source[a]	Tissue	Medium[b] (+ 10% FCS, + 1% PS)	Harvesting[c]
RPMI 8226	ATCC	Myeloma	RPMI 1640	Manual
MCF-7	Dr O. Alabaster, Geo. Washington U., Washington, DC	Breast	McCoy's 5A or RPMI 1640	Trypsin-EDTA
WiDR	ATCC	Colon	RPMI 1640	Trypsin-EDTA
HEC-1A	Dr Jorgen Fogh, MSKCC	Endometrial	McCoy's 5A	Tyrodes
T-47-D	Dr O. Alabster, Geo. Washington U., Washington, DC	Breast	McCoy's 5A	Trypsin-EDTA
ZR-75A	ATCC	Breast	RPMI 1640 + 1% NEAA	Trypsin-EDTA

[a] ATCC, American Type Culture Collection, Rockville, MD 20852; MSKCC, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

[b] FCS, fetal bovine serum (Flow); PS penicillin (100 U/ml)-streptomycin (1 mg/ml) (Gibco): NEAA, non-essential amino acids (Gibco)

[c] Cells harvested at exponential growth phase in the tissue culture flasks

Chemotherapeutic Agents. All these agents were the clinical formulations obtained from the University of Arizona Cancer Center's research pharmacy service. Samples were diluted in normal saline to appropriate concentrations and kept at −80° C until use. Standard cytotoxic agents tested included doxorubicin (DOXO), vincristine (VCR), vinblastine (VLB), and cisplatin (PLAT). Cytotoxic agents still classed as investigational included etoposide (VP-16) (Bristol), vindesine (VDS), and vinzolidine (VZL) (Eli Lilly).

Soft Agar Cloning Assay. A double-layer soft agar assay was used as described previously by Hamburger and Salmon [24, 33]. The following modifications were introduced, adapting this technique to the simpler growth requirements of the cell lines. The bottom and upper layer media were the same as the culture medium used for carrying the cell line. The tumor cell concentration was adapted to give between 300 and 800 colonies per control plate. Each experiment included at least six control plates and each drug or drug combination was tested in triplicate. One-hour drug exposures were done in culture medium and continuous exposure was obtained by incorporation of the drugs in the culture medium of the upper 1.0 ml layer at the appropriate final concentration for the 2.0 ml semisolid cultures. The plates were then incubated for 6–14 days (depending on the growth characteristics of each tumor cell line) before counting. Colonies of at least 60 μm in diameter were counted with a Bausch & Lomb Omnicon FAS II automated image analyzer which had been optimized for tumor colony counts. Results were expressed as the percentage of colony growth compared with the controls.

Choice of Drug Concentration. To ensure results with potential clinical relevance, drug concentrations used in the screening phase of the sensitivity of the cell lines to various chemotherapeutic agents were chosen to be within the clinically achievable range [1; unpublished data]. Recently available data [4] show that human leukocyte interferon levels of at least 100–500 U/ml serum can be sustained for up to 24 h with a single injection in cancer patients. When single drug concentrations are reported, these represent one-tenth of the concentration at which at least a 90% colony inhibition was observed.

Definitions Used. Table 2 shows the definitions of synergism, additivity and other effects. These criteria have been discussed by Valeriote and Lin [41] and Momparler [29]. The multiplication of the surviving fractions with either drug alone (expected results for additivity) is compared with the surviving fraction found (observed result) with the combination. For these interpretations to be valid both drugs must exhibit activity at some concentration.

Table 2. Definitions of drug combination effects[a]

Synergistic	$SF_{A+B} < (SF_A) \times (SF_B)$
Additive	$SF_{A+B} = (SF_A) \times (SF_B)$
Subadditive	$SF_{A+B} > (SF_A) \times (SF_B)$ and $< SF_B$ when $SF_A > SF_B$
Antagonistic	$SF_{A+B} > (SF_A) \times (SF_B)$

[a] SF, surviving fraction; A, B, drugs; A + B, drugs used in combination

Table 3. Sensitivity at to IFN-αA (continuous exposure)

Cell line	40 ng/ml (8,000 U/ml)	4 ng/ml (800 U/ml)	0.4 ng/ml (80 U/ml)
RPMI 8226	S	S	S
MCF-7	S	S	R
WiDR	S	S	R
HEC-1A	R	R	R
T-47-D	–	R	R
ZR-75A	–	R	R

[a] Sensitivity: Colony growth reduced to less than 50% of control

Statistical Considerations. The results obtained were analyzed by a *Z*-test as described by Drewinko et al. [11] when single concentrations and their combination were studied. Synergism or inhibition was determined for each experiment individually, the *P* value being 0.05 in comparison for the simple additivity hypothesis. When concentration effect graphs were observed a similar method was used.

Results

Preliminary testing to identify cell lines exhibiting some sensitivity to IFN-αA is summarized in Table 3. The myeloma cell line, RPMI 8226, proved to be exquisitely sensitive to continuous exposure to IFN-αA, whereas the breast (MCF-7) and colon (WiDR) lines exhibited limited sensitivity. Three additional cell lines tested were resistant to clinically achievable doses of interferon and were not subjected to additional testing. In preliminary experiments with the 8226 cell line, we observed that a 1-h exposure to IFN-αA at concentrations 10–100 times higher than those used in the continuous

Table 4. Combined effects of continuous exposure of myeloma 8226 to IFN-αA 0.4 ng/ml and cytotoxic agents

	Drug										
	VLB		VCR		VDS		VZL		PLAT		DOXO
Time of exposure	1 h	CT	1 h	CT	1 h	CT	1 h	CT	1 h	CT	1 h
Concentration (μg/ml)	10^{-2}	$5 \cdot 10^{-5}$	10^{-1}	$5 \cdot 10^{-3}$	10^{-2}	$5 \cdot 10^{-4}$	10^{-1}	$5 \cdot 10^{-3}$	10^{-2}	$5 \cdot 10^{-4}$	10^{-1}
Observed (%)	18	30	1	28	38	8	20	51	30	28	21
Expected (%)[a]	31	67	10	33	52	4	15	64	19	43	27
Experiments	13	6	1	3	3	1	2	1	5	6	3
Synergistic	6	4	1	0	1	0	0	0	0	3	0
Additive	5	2	0	2	1	0	1	1	2	2	3
Sub-additive	1	0	0	1	1	1	1	0	3	1	0
Antagonistic[b]	1	0	0	0	0	0	0	0	0	0	0

[a] Expected for additivity, see Table 2
[b] Synergism or antagonism if $P < 0.05$ between observed-expected, see statistical section

Table 5. Combined effects of continuous exposure of breast MCF-7 to IFN-αA 4ng/ml and cytotoxic agents

	Drug									
	VLB		VCR		VDS		VZL		PLAT	DOXO
Time of exposure	1 h	CT	1 h	CT	1 h	CT	1 h	CT	1 h	1 h
Concentration (μg/ml)	1	$5 \cdot 10^{-2}$	10^{-1}	$5 \cdot 10^{-3}$	10^{-1}	$5 \cdot 10^{-3}$	10^{-1}	$5 \cdot 10^{-2}$	10^{-1}	10^{-1}
Observed (%)	44	29	26	15	30	32	63	53	82	44
Expected (%)	42	43	12	28	34	24	28	45	50	42
Experiments	5	3	1	3	1	1	1	1	2	2
Synergistic	1	2	0	1	0	0	0	0	0	0
Additive	4	1	0	1	1	0	0	1	1	1
Sub-additive	0	0	1	1	0	1	0	0	0	1
Antagonistic	0	0	0	0	0	0	1	0	1	0

See footnote to Table 4

Table 6. Combined effects of continuous exposure of WiDR to IFN-αA 20 ng/ml and cytotoxic agents

	Drug							
	VLB		VCR		VDS		VZL	
Time of exposure	1 h	CT	1 h	CT	1 h	CT	1 h	CT
Concentration (μg/ml)	10^{-1}	10^{-6}	10^{-2}	$5 \cdot 10^{-4}$		$5 \cdot 10^{-4}$		$5 \cdot 10^{-3}$
Observed (%)	14	25	25	35	NA	8	NA	17
Expected (%)	25	36	35	28	NA	12	NA	15
Synergistic	0	2	0	0		1		0
Additive	1	2	1	2		1		2
Sub-additive	0	0	0	1		0		0

See footnote to Table 4
NA, not available

exposure failed to show any consistently significant effect. Additionally, the colony inhibition obtained with cytotoxic agents was not modified by these 1-h exposures to IFN-αA. Tables 4–6 detail the combined effects observed with continuous exposure to IFN-αA and some of the cytotoxic agents tested. Initial experiments with VP-16 and melphalan on the myeloma 8226 line showed sub-additive to additive effects (data not shown). The most striking result was the apparently synergistic effect observed when vinblastine and interferon were both used for continuous exposures. Overall, six of 13 experiments exhibited synergism with 1 h exposure to vinblastine preceding a continuous exposure to interferon in the myeloma 8226 cell line, and four of six experiments manifested synergism with the same cell line when simultaneous continuous exposure to vinblastine and interferon was used. In the MCF-7 cell line, one of five experiments manifested synergism with a 1-h exposure to vinblastine followed by IFN-αA in continuous exposure, and two of three experiments showed synergism with continuous exposure to both agents. In the WiDR colon cell line, two of four

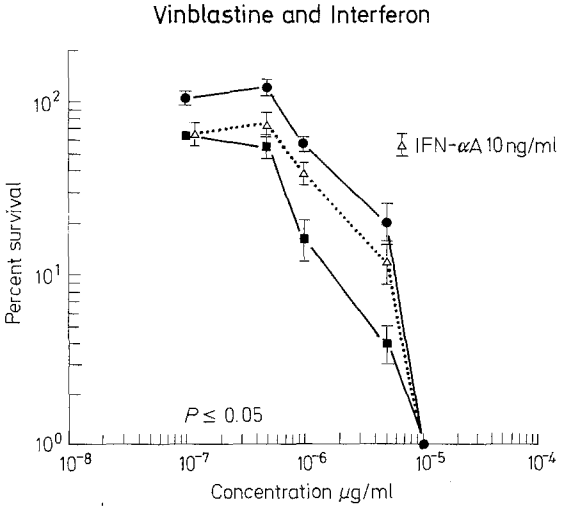

Fig. 1. Continuous exposure of WiDR cells to vinblastine (Velban). The addition of IFN-αA (10 ng/ml) is synergistic ($P < 0.05$). (●) Vinblastine alone; (△) Calculated for additivity; (■) Vinblastine + IFN-αA 10 ng/ml

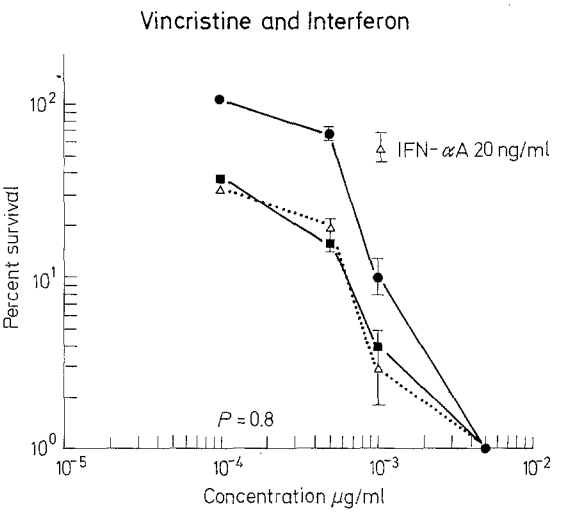

Fig. 2. Continuous exposure of WiDR cells to vincristine. The addition of IFN-αA (20 ng/ml) is simply additive ($P = 0.8$). (●) VCR alone; (△) Calculated for additivity; (■) VCR + IFN-αA 20 ng/ml

experiments using continuous exposure to both agents showed a synergistic interaction, and the one experiment using a 1-h exposure to vinblastine followed by continuous exposure to interferon also showed a more than additive effect, which failed to be statistically significant for synergism.

Synergism of continuous simultaneous exposure to cisplatin and interferon was observed against the 8226 cell line in three of six experiments. One hour's exposure to cisplatin at concentrations 100 times above those used for continuous exposure failed to show significant additivity in the 8226 cell line. Similarly, two experiments with 1-h exposure to platinum followed by interferon showed less than additive activity against MCF-7 cells. The other cytotoxic agents tested (including the other vinca alkaloids) did not show significant potentiation, as the results of most experiments were sub-additive or additive.

The time constraints of the apparent synergistic interaction between vinblastine and IFN-αA was studied in a series of experiments conducted with the 8226 cell line. Pre-exposure to interferon for 6–24 h followed by exposure to vinblastine (1 h or continuous) alone or with interferon did not cause different results than were obtained with no pre-exposure. Simultaneous exposure to interferon and vinblastine followed by IFN-αA in continuous exposure failed to show any better effect than that obtained with the 1-h pre-exposure to vinblastine followed by IFN-αA.

The WiDR cell line was used to further define the extent of this interaction through dose-response curves at various concentrations of continuous exposure to IFN-αA and different concentrations of the vinca alkaloids. Figure 1 shows the synergism observed between vinblastine and interferon. In this case, IFN-αA was used at a concentration of 10 ng/ml, which alone decreased the colony growth to 65% of control. Vinblastine used by continuous exposure at several concentrations modulated colony growth between less than 1% and 125% of the control. Simultaneous exposure to both agents was clearly synergistic ($P < 0.05$). It is important to note that synergistic effects were observed at a concentration of vinblastine which lacked inhibitory effect on colony growth by this drug alone. Conversely, in other experiments (done mainly with the RPMI 8226 cell line), the addition of vinblastine to concentrations of interferon that seemed to be without effect (5–10 times below a concentration showing a more than 50% colony growth inhibition) actually had an additive to synergistic effect. Figure 2 is an example of an additive experiment; in this case, vincristine was combined with interferon 20 ng/ml. At the concentrations studied, the effect of the combination was consistently additive. Similar dose-response curves, each of which represents one experiment, have allowed the confirmation of observations recorded at single concentration points.

Discussion

Using a soft agar tumor colony assay and three different human tumor cell lines (RPMI 8226, MCF-7, and WiDR), we have demonstrated an additive to synergistic effect of the combination of IFN-αA and vinblastine. The other vinca alkaloids (vincristine, vindesine, vinzolidine) and the other chemotherapeutic agents (doxorubicin, VP-16, melphalan, 1 h exposure to cisplatin did not seem to show synergistic action when combined with IFN-αA, although additive effects are observed with these agents. A potentiating effect probably also occurs with cisplatin when it is used at continuous exposure against RPMI 8226. Resistance to clinically achievable doses of IFN-αA was demonstrated in three other cell lines (HEC-1A, T47D, ZR-75A). The method we used to measure additive or synergistic effects is not as definitive as the isobologram approach used in radiobiology, in which 'envelopes of additivity' are determined [39]. The model we have used [29, 41] does not require as many data points but does require that each drug demonstrate some activity. Our data therefore should be reviewed as providing a positive lead (particularly with interferon and vinblastine) which justifies further study both in vitro and in vivo.

Studies of the combination of interferon with other chemotherapeutic drugs were first carried out in animals. The rationale for such combinations has been that when used alone in the presence of a large tumor burden, interferon has been relatively inactive, even though the same tumor may have been sensitive to interferon when the load was minimal. This concept has most recently been discussed with reference to

experiments that showed a cytostatic effect of interferon against mouse P388 leukemia and a survival advantage for mice treated with either platinum or mitomycin C combined with interferon [36]. This is in agreement with our results concerning the in vitro potentiating effect of continuous exposure to both *cis*-platinum and IFN-αA. Other examples include the reduction in tumor load of LSTRA murine leukemia by BCNU, which has been reported to permit a cure of some animals with the combination of interferon and BCNU [10]. The combination of cyclophosphamide and interferon has been found to increase the survival time of mice affected by AKR leukemia by 100% compared with the effect of either drug alone [22]. L1210 murine leukemia has been treated with methotrexate, 6-mercaptopurine, adriamycin, cytosine arabinoside, or cyclophosphamide alone or in combination with murine interferon. Interferon increased the animal survival further only in combination with methotrexate or methotrexate plus 6 mercaptopurine [37]. In the clonogenic assay, we observed simply additive effects when doxorubicin and IFN-αA were combined. The apparent survival advantage conferred by the combination of interferon with a drug shown to have synergistic activity in vitro, and the lack of enhanced survival when the associated drug showed only additive effects in vitro, leads us to speculate that clearly synergistic effects of chemotherapeutic agent-interferon combinations may need to be seen in the clonogenic assay for them to translate into positive results in vivo.

Thus far, we have failed to find any significant antagonism between interferon and chemotherapeutic agents. However, trans-retinoic acid has recently been reported to partially reverse protection conferred by mouse interferon against P388 lymphoid tumors [4]. Trans-retinoic acid alone appeared to enhance tumor growth.

In vitro studies of interferon-based combinations are limited. Observations of the growth inhibition of two human osteosarcoma cell lines and one human lymphoblastoid cell line have demonstrated less than additive to additive effects of the combination of methotrexate and leukocyte interferon [8], while combinations of mouse immune interferon (IFN-gamma) and virus type (IFN-alpha/beta) interferons have shown a mutual synergistic interaction [16].

While our study suggests a synergistic effect between purified recombinant leukocyte interferon and vinblastine, it does not clarify the mechanism of enhanced drug action. Inasmuch as we have tested interferon primarily with the continuous exposure technique, its antiproliferative effect might be cytostatic rather than cytotoxic. In related experiments (data not shown) we have found that when only a 1-h exposure to interferon is used prior to plating, concentrations 2–3 log higher are needed to demonstrate antiproliferative effects. With the 1-h exposure technique, the antiproliferative effect can be considered cytotoxic, although the cellular mechanism of interferon action remains obscure. Inasmuch as our experiments utilized cloned cell lines, the effects of interferon we observed in combination with vinca alkaloids must be considered as direct rather than being mediated by immune or accessory cells. However, the potentially complex mode of action of interferon in vivo [12, 20] makes it difficult to extrapolate from our in vitro experiments. Mechanistic studies will be required to delineate the nature of the potentiating drug interaction. We hypothesize that this might be the result of the known effect of vinblastine on tubulin [40, 44] and the action of interferon on the cytoskeleton [18, 32]. It has also been known that vinca alkaloids can inhibit the release of interferon by vinca-treated cells [25]. Although it is generally accepted that interferon acts through a receptor on the cell surface [12, 17], intracellular interferon receptors similar to those of glucocorticoid hormones might exist, in which case the decreased release of interferon by vinblastine could explain this additive to synergistic effect. Conversely, interferon might slow down the rate of release of vinblastine from tumor cells. It is interesting to note that pyrimido-pyrimidine derivatives can potentiate the effect of interferon [3] and that such a drug can also overcome the resistance of P388 leukemia to vincristine or vinblastine [40]. Whether this indicates a common mechanism of action of vinca alkaloids and interferons that could lead to a synergistic effect remains to be proven. Additionally, studies of this combination on bone marrow stem cell proliferation are also warranted to determine whether this apparently synergistic combination also has an enhanced therapeutic index.

The differential cytotoxic, cytostatic and antiviral effects of interferon have not been systematically studied until recently. These effects have been found to be distinct in various cell lines [15, 27]. The separation between the antiviral and antitumoral effects of interferon has been carefully discussed [20]. In this context, it is of interest that the antiviral effects of interferon are decreased by vinca alkaloids [6, 9]. We have done parallel experiments to evaluate both the antiviral and the antitumoral effect of IFN-αA (as manifested by decreased tumor colony formation) (M. S. Aapro, D. Lucas, unpublished work). Preliminary results suggest that these two effects are divergent, as the antiviral effect of IFN-αA was decreased by vinblastine.

Studies of the in vitro effect of interferon using a colony assay system have been used to determine the activity of various types of interferons against clinical human tumor samples [12–14, 34], and we believe that this system should prove useful to predict response to interferon in phase II trials, as it has done for cytotoxic agents [33, 42].

We feel that further studies of the interaction of recombinant leukocyte interferon A with other drugs should be done with cell lines in the soft agar cloning assay. Additionally, fresh tumor biopsy samples should be tested against the combinations that have shown the most promising results in cell line work. A prospective phase I–II trial of vinblastine plus IFN-αA is warranted. Such prospective trials will help to substantiate the value of this system in predicting tumor response to IFN-αA alone or in combination with cytotoxic drugs.

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