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Restoration of Adriamycin and Vincristine Dependent Tumoricidal Activity by Interferon in Mice with Implanted Tumor Cells

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The survival of implanted tumor cells in mice which had been treated with interferon in combination with either adriamycin or vincristine was evaluated. While the majority of tumor cells implanted into normal mice failed to survive (52.1 to 63.5%), most of those implanted into mice which had been pretreated with either adriamycin or vincristine survived. If the mice were secondarily treated with interferon, the ability of adriamycin or vincristine to inhibit the survival of implanted tumor cells was restored within 24 hours. Restoration of tumoricidal activity by interferon treatment was more evident in the adriamycin pretreated mice. Peritoneal macrophages isolated from mice pretreated with both interferon and adriamycin had an increased tumoricidal activity, when compared with those isolated from mice treated with adriamycin alone. This interferon dependent enhancement of tumoricidal activity was comparable with that obtained by treating mice with lymphokines a product of Con A treated lymphocytes isolated from BCG treated mice. These results suggested that both adriamycin and vincristine may damage the macrophages required for the natural host defense mechanism and allow the implanted tumor cells to survive. Interferon may, however, protect the macrophages from drug induced damage.

Key Words: Anti-cancer chemotherapeutics, interferon, macrophage, natural host defense, lymphokine, implantation.

After several decades of antitumor drug treatments for the palliation of advanced cancer, the role of chemotherapy is now being reassessed. Various therapeutic agents employed for tumor therapy can suppress both humoral and cellular immunocompetence (Brenbaum 1974; Harris *et al.* 1976). An anticancer chemotherapeutic agent can prevent the proliferation of lymphocytes and monocytes (Harris *et al.* 1976; Harris and Sinkovics 1976; Haskell 1977; Leventhal *et al.* 1974; Makinodan *et al.* 1959; Spreafico and Anaclerio 1977) and can inhibit their functions. Both T and B lymphocytes appear to be equally sensitive to the damaging effect of short treatment courses of the multiple agents used for cancer therapy (Harris *et al.* 1976). For induction of both T and B cell mediated immunity, uptake and processing of antigen by macrophages are required (Rosenthal *et al.* 1975). Studies with the *in vitro* experimental system suggested that the uptake of antigen by the reticuloendothelial cell system is sensitive to a number of widely used anticancer drugs including the glucocorticoides, cyclophophamide, chlorambucil, and methotrexate (Rosenthal *et al.* 1975; Hersh 1973). Therefore, it will be necessary to develop ways to restore the immune functions impaired by the use of anticancer drugs. In this experiment, the ability of interferon to suppress the damaging effects of adriamycin (ADR) and vincristine (VCN) on tumoricidal activity of macrophages has been studied in mice with implanted tumor cells.

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MATERIALS AND METHODS

Tumor cell line (S-180YS)

S-180 tumor cell line, clonned from the original S-180 (ATCC, Rockvile, Md., U.S.A.), has been maintained in this institute for more than 5 years in McCoy's 5A medium (Flow Lab., Australia). The cells have been

routinely maintained both *in vitro* as well as *in vivo* within the inbred strain of ICR mice. Cultures were grown in glass (Corning Co., Pyrex Co., U.S.A.) and plastic (Costar, U.S.A.) flasks as necessitated by the specific experiment. Changes in the stem cell lines were frequently monitored by studying their karyologic patterns.

Animals

ICR mice weighing approximately 25 g were randomly selected for these experiments. The mice were fed a commercial diet throughout the experiments.

Therapeutic agents

Adriamycin (ADR) and vincristine sulfate (VCN) were obtained from Kyowa Hakko Kyogo Ltd. Japan and Lilly Co., U.S.A. respectively, while interferon (α/β IFN) was supplied by the Interferon Co., U.S.A. The concentrations of ADR and VCN were diluted for the *in vitro* experiments, and for the *in vivo* experiments, were adjusted to be equal to those used in clinical applications. Tumor cells ($1\times10^5-10^6$ cells/animal) were injected intraperitoneally prior to drug treatment. A single dose of IFN (2.2×10^5 IRU per mouse) was then injected intravenously or intraperitoneally.

Lymphokine production

ICR mice were immunized weekly with viable BCG for 4 weeks, and then the animals were randomly killed 3 to 6 weeks following the final immunization. The spleens were aseptically removed, and the splenocytes were collected through repeated injection of the spleen with serum free cell culture media (RPMI, Flow Lab., Australia). Splenocytes suspension were layered onto lymphocyte seperation media (LSM, density 1.077-1.080, Bionetics, U.S.A.) at 20°C, and centrifuged at 250×G for 30 minutes at 4°C. The lymphocytes were resuspended in a concentration of 5×10^7 viable cells/ml in RPMI medium containing 10% inactivated fetal bovine serum (FBS, Flow Lab., Australia).

Twenty ml of the lymphocyte suspension were mixed with Con A (3-5 μg/ml, Flow Lab., Australia) in a 75 mm^3 flask (No. 3203 Biotech, U.S.A.), and incubated for 48 hours at 37°C. Supernatant fluid from the replicate plates was pooled, centrifuged at 450×G for 15 min at 4°C and divided into aliquots. These were stored at 4°C until needed (Nettesheim and Hammon 1970; Meltzer 1976).

Peritoneal cells (PC)

Following injection of 6 ml of RPMI medium into the peritoneum, peritoneal exudate cells were withdrawn with a No. 19 gauge needle and suspended in medium supplemented with 2g of $NaHCO_3$/Liter, 10% FBS, penicillin and streptomycin (100 u and 100μg/ml, Flow Lab, U.S.A.). Fluid from 3 to 10 mice was pooled, put into 15 ml roller tubes, and incubated for 6 hours. Those macrophages which adhered to the glass walls of the tubes were then exposed to lymphokine dilutions and mouse IFN (Ruco and Meltzer 1977; 1978). The PC suspensions ($5-8\times10^5$/0.5 ml) were dropped in 24 wells of plastic culture plates (Costar, Australia) and were incubated at 37°C in moist 5% CO_2 atmosphere for 2-3 hours. The non-adherent PCs were incubated for an additional 4 hours in a diluted solution of lymphokine (Ruco *et al.* 1978).

Macrophage induced tumor cell cytotoxicity

The S-180YS cells (4×10^4 cells/well) were incubated in 0.5 ml of RPMI medium supplemented with sodium bicarbonate buffer, 5% FBS, and 0.5 μci/ml of methyl-^{3}H thymidine (^{3}HTdR, sp. act. 1.9 ci/mM, Amersham Internat. Ltd., UK) in plastic culture wells for 18 to 24 hrs (Meltzer *et al.* 1975b). The resulting tritiated S-180YS cells were used as target cells. The target cells were then incubated with the washed adherent PC effector cells for an additional 3 hrs. The ratio between target and effector cells was 20:1. Following this incubation the S-180YS target cells were separated from the non-adherent effector cells by thorough washing to minimize the contamination of effector PCs to the target cell population. Washed target cells were incubated for an additional 48 hrs in the growth medium. The cytotoxicity of tumor cells was estimated by measuring the amount of radioactivity in the cell free culture supernatants, and it was expressed as a percentage of specific release to total radioactive counts (Ruco and Meltzer 1978; Meltzer *et al.* 1975b) as measured by a beta scintillation counter. The target cells were frequently examined using an inverted phase contrast microscope to confirm the results of cytotoxicity.

RESULTS

1. Decreased tumoricidal activity in mice treated with ADR.

The ability to eliminate implanted tumor cells was

evaluated by estimating the percent of recovered viable tumor cells following treatment with ADR. The recovered cells were stained with the vital dye, trypan blue, and the number of stained cells were counted by using a hemocytometer. The remaining cells were immediately explanted in plastic culture flasks to confirm the cell count result. The number of viable tumor cells from control animals (without ADR treatment) was significantly lower than those from the ADR treated mice (24 hrs to 36 hrs after the initial implantation). The percent of viable tumor cells recovered from mice treated with ADR was almost twice as high as that found in the control animals (Table 1). The higher recovery rate in ADR treated mice was approximately steady for 84 hrs. In contrast, the recovery rate of viable tumor cells from control mice remained low (38.8-47.9%) until 76 hrs. At 84 hrs, the percent of viable tumor cells from the control animals was even greater (96.8%) than that of ADR treated mice (73.9%), (Fig. 1). The high increase in tumor cell viability in ADR treated mice was maintained for 36 hours following drug treatment; then began to decrease.

Table 1. Percent of viable S-180YS tumor cells recovered from mice following chemotherapy

Treatments	Percent of recovered tumor cells at given intervals			
	24 hour	36 hour	72 hour	84 hour
None	47.9	36.5	–	96.8
ADR	92.6	82.8	–	73.9
VCN	88.5	46.4	20.0	–

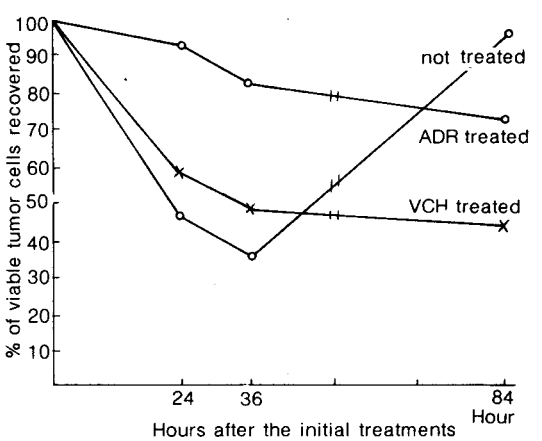

Fig. 1. Percent of viable tumor cells recovered from mice treated with ADR and VCN.

When the mice were treated with VCN instead of ADR, a decrease in tumoricidal activity was evident only at 24 hours. Thereafter, the tumoricidal activity was even greater than that manifested by the control.

2. Cytotoxic effect of ADR and VCN on S-180YS *in vitro*.

Target tumor cells (1×10^6 cells/plate) were placed in 15 ml flasks. Following administration of drug, tumor cell death was estimated by noting the number of surviving cells in 1 mm^3 of area on the bottom of the flasks. It was clearly demonstrated that ADR was cytotoxic within 48 hrs (Table 2).

In contrast, VCN produced a significant cytotoxic effect after 48 hrs and continued to exert additional toxic effects. This delayed action is indicative of the known mechanism of the drug. It was also noted that both drugs did not eliminate all available target cells. When the target cells were washed 24 hrs after initial exposure to the drugs, cell death percentages remained unchanged.

Table 2. Cytotoxic effect of ADR and VCR on S-180YS *in vitro*

drug con. μg/ml		Percent of tumor cells lysed following exposure to the drug at given intervals			
		24 hours	48 hours	72 hours	84 hours
2.5	ADR	69.9	60.8	–	78.6
	VCN	10.0	70.2	94.8	92.9
1.25	ADR	77.0	24.0	–	75.6
	VCN	21.4	79.8	95.0	94.1
0.625	ADR	74.0	21.7	–	72.7
	VCN	0.0	77.5	94.6	91.6

Target cells were partially synchronized by explanting cells from the stock culture into 15 ml plastic flasks (1×10^6 cells/plate). The average number of cells in 1 mm^2 of the flask was about 50.

Table 3. Results of experiment using ^{3}H-thymidine to measure the cytotoxic effects of ADR, VCN, and IFN *in vitro*

Time (hour)	Control (cpm)	% of tumor cell elimination following treatment			
		ADR	VCN	IFN(α/β)	None
D+24	3121.80	79.8	51.9	97.0	51.1
D+48	557.38	64.3	86.9	79.4	63.5

3. Percent of cytotoxicity of ADR, VCN, and IFN as measured by tritiated thymidine uptake.

Tumor cells (1×10^6 cells/animal) labeled with ^{3}H-thymidine were implanted into the peritoneum of the mice. Subsequently ADR and VCN (2.5 mg/kg BW) and mouse IFN (α/β, 2.2×10^5 IRU/animal) were injected via tail veins to compare their independent effects on the *in vivo* survival of the implanted tumor cells. It was again demonstrable that within 24 hrs of ADR treatment tumor cell cytotoxicity was lost due to the suppression of tumoricidal activities of the host animal (Table 3). However, VCN treatment did not appear to diminish the tumoricidal activity of the host, instead, VCN induced tumoricidal activity had gradually increased by 48 hrs of observation. Twenty four hrs after treatment with IFN alone tumoricidal activity was significantly enhanced, and this effect gradually returned to normal levels by 48 hrs.

4. Cytotoxic effects of ADR and VCN in combination with IFN.

Tumor bearing mice were exposed to the drugs under study in a variety of combinations, with the second treatment following the first by one day. When chemotherapy with ADR was combined with IFN, the ability of the mice to eliminate tumor cells was significantly enhanced as compared with that produced by ADR treatment alone or by ADR-ADR combination treatment (Table 4).

IFN treatment was also found to be highly effective in helping the host eliminate the implanted tumor cells. The ADR treatment followed by IFN appeared to be more effective (93.3%) than that observed by the initial IFN treatment followed by ADR (85.7%). On the contrary, when the initial IFN treatment was combined with the secondary VCN treatment, the ability of the host to eliminate tumor cells was significantly suppressed. It was also noticed that if two different chemotherapeutic agents were used in combination, the desired cytotoxic effect could be enhanced significantly as compared with that observed by treating the host with a single or repeated treatment with the same drug. This was particularly true in the case with ADR.

5. Effects of IFN before and after chemotherapy.

Since the combination of chemotherapy and IFN greatly enhanced the host's ability to eliminate the implanted tumor cells, these results were further analysed to define the best sequence of treatment.

Table 4. Cytotoxic effects of chemotherapy combined with interferon

Primary treatment	Percent of tumor cells eliminated		
	Secondary treatment		
	IFN	ADR	VCN
None	73.6%	64.3%	86.9%
IFN	91.6%	93.3%	77.9%
ADR	85.7%	57.6%	93.1%
VCN	34.6%	74.6%	87.5%

Table 5. Comparison of cytotoxic effects and sequence of drug administration

IFN	Percent of tumor cells eliminated Drug		
	IFN	ADR	VCN
Pretreatment	91.6%	85.7	34.6
Posttreatment	*n.t.	93.3	77.9

*n.t.: not tested

Table 6. Tumoricidal activity of peritoneal macrophages (PMØ) on S-180YS *in vitro*

Target cells	Effector cells	Treatment	cpm(tritium)	Enhancement (in percent)
S-180YS	PMØ	None	812.00	–
S-180YS	PMØ	IFN	1028.04	26.6
S-180YS	PMØ	Lymphokine	1035.30	27.5

In both cases, chemotherapy followed by IFN treatment rendered a significantly enhanced ability of the host to defend against the implanted tumor cells (Table 6). However, it should be remembered that combination of IFN with VCN had no enhancing effect on the host defense upto 84 hours of incubation (Table 4).

6. Enhanced tumoricidal activity of macrophages isolated from IFN treated mice.

The tumoricidal activities of peritoneal macrophages from mice were evaluated to investigate whether the enhanced tumoricidal effect observed in the *in vivo* experiment could be supported in the *in vitro* experiment. Thus, the effect of an *in vivo* treatment with IFN on the macrophage mediated cytotoxic

action for the tumor cells was examined *in vitro*. The IFN treatment alone significantly enhanced the macrophage induced tumoricidal activity for the target tumor cells. The percent of increased cytotoxicity of the macrophages from IFN treated mice was 27.5% when compared with the tumoricidal effect of macrophages from control animals.

Macrophages isolated from mice which had been treated with lymphokines prepared from the spleen cells of BCG treated mice (see materials and methods) also demonstrated an enhanced cytotoxic activity. The enhanced activity was comparable with the effect produced by the IFN treatment. None of the mice had been previously sensitized with S-180YS in these experiments.

DISCUSSION

Although it is generally accepted that chemotherapeutic agents are immunosuppressive to the host, it has been also reported that ADR has immunomodulating activity. Depending upon the conditions, ADR could selectively affect particular immune functions, thus ultimately resulting in inhibition or augmentation of the immune reponse in humans and animals (Santoni *et al.* 1980; Tomazic *et al.* 1980; Tomazic *et al.* 1981; Orsini *et al.* 1977; Ehrke *et al.* 1983; Arinage *et al.* 1985; Arinnage *et al.* 1986). Arinaga *et al.* (1986) reported that peripheral blood monocytes were converted to cytotoxic cells to the B-lymphoblastoid cell line, Raji, by ADR treatments and these were related to the imbalance of T-cell subsets and the increased production of interleukin 2 in the patients receiving ADR treatments. The changes in immunologic function reported in those studies were evaluated using *in vitro* methods rather than *in vivo* tumor cell implantation experiments. In this study, the immediate and direct effect of ADR and VCN on the survival of implanted tumor cells were studied. Both chemotherapeutic agents impaired the host defense mechanism(s). The results suggest that the overall direct cytotoxic effects of the drugs on the cells involved in the natural host defense appeared to be greater than those on target tumor cells, especially in the early phase of tumor initiation. This phenomenon may only be discerned in implantation experiments, as it is clinically impossible to recognize and score such events occuring in the initial phase of tumor cell survival following implantation. Almost all cases of human tumors are advanced far beyond the initial selective phase of tumor initiation *in vivo*. Therefore, it may be a convenient indicator for monitoring any changes in host defense mechanisms induced by the agents employed in cancer research.

The drug induced decrease in tumoricidal activity in mice was especially evident in the ADR cases. This reduction in activity was observed as early as 24 hours following inital chemotherapy. This suggested that the suppression for the host's defenses was mainly due to damage(s) in the non-specific natural defense system of the host. Among the natural defenses against tumor cells, cell mediated tumor cell cytotoxicity is considered to be the most responsive one in humans and animals. Among the cells involved in the cell mediated defense system, the roles of macrophages and natural killer (NK) cells have been studied intensively along with lymphokines.

Macrophages, especially dendritic macrophages, provide a large surface area for the fixation of antigens, and thus permit lymphoid cells to interact with these antigens. This one of the extremely important functions in the tumor bearing host and one in which both soluble tumor antigens and antibody complexed tumor antigens can block lymphocyte mediated cytotoxocity.

Phagocytosis of tumor cells by macrophages is also a well documented event. Mouse sarcoma cells have been destroyed by a process in which the macrophage pinched off tumor cell extensions which were interdigitated with the macrophage's cytoplasm (Chambers and Weiser 1973). In an *in vitro* study, the presence of an opsonizing isoantibody was found to be necessary for the phagocytosis of viable tumor cells by activated macrophages; without the antibody, even immune macrophages failed to engulf and destroy the tumor cells (Bennet *et al.* 1964). Therefore, it is tentatively assumed that the tumoricidal activity of the macrophages observed in this experiment was due to direct cell killing activities rather than a phagocytic process.

Macrophages appear to express a cytotoxicity to the surface configuration of the neoplastic cell. Activated macrophages are not cytotoxic to normal syngeneic mouse fibroblasts or mouse kidney cells but do destroy the fibroblasts of neoplastic phenotypes (Hibbs 1973; Krahenbuhl and Remington 1974). The activation of these macrophages which distinguished neoplastic cells from their normal counterparts was not tumor-specific.

Non-activated macrophages were not cytotoxic at all. It was assumed that this might be one of the most important phenomena in tumor elimination, especially in the initiation phase which was the focus of this study.

It has been shown that macrophage activity can be readily increased by a variety of stimulatory agents: IFN, MIF, IFN inducers, zymosan, lipopolysaccharides, etc. (Alexander and Evans 1976). In general, macrophage activators suppress tumor growth *in vivo*, while tumors grow faster in animals treated with macrophage inhibitors (Levy and Wheelock 1974). In this aspect, ADR and VCN could be considered to be strong macrophage inhibitors as it was found that tumor growth was not impaired in mice treated with the chemotherapeutics in this experiment.

From the harvested peritoneal exudate cells from the mice treated with the drugs, it was almost impossible to obtain proper number of cells required for the *in vitro* assay.

In the *in vitro* experiments, the tumoricidal activity of mouse peritoneal macrophages was appreciably enhanced by IFN treatment, as was the tumor elimination ability of the mice treated with IFN following ADR. This might suggest that ADR which is active for a very short time could not affect the entire macrophage population and the other cells in the host's line of defense. In contrast, when the mice received VCN, which is active for relatively long time, tumor growth was not suppressed by IFN injection. It is also possible that ADR and VCN can have different *in vivo* effects as their pharmacological actions are quite different. The timing of IFN treatment in the combination therapy also affected the results. Administration of IFN following ADR within 24 hours appeared to be the most effective.

SUMMARY

It has been demonstrated that although adriamycin and vincristine are beneficial chemotherapeutics, there is a disadvantage. These experiments show that initially these drugs impair the host's defense system by damaging the macrophages and thus limiting immediate tumoricidal activity. Although the mechanism is not known, the cytotoxic effects and resulting decrease in tumoricidal activity have been well defined.

However, the studies discussed above have shown that when using these two drugs, normal levels of tumoricidal activity can be maintained or enhanced by incorporating interferon into the drug regimen. This is most effective when interferon is administered within 24 hrs of the initial drug treatment. The macrophages may be protected from drug-induced damage by interferon.

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