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Adjuvant Treatment with Chemotherapeutic Agents and Polyadenylic–Polyuridylic Acid in Operable Stomach Cancers

I. Enhancement of Natural Killer Cell Activity

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Natural Killer (NK) cell activity of 47 operable stomach cancer patients was sequentially studied before and after chemotherapy in association with polyadenylic. polyuridylic acid [poly(A).poly(U)]. NK activity was determined by an in vitro 4 h chromium release assay using nonadherent mononuclear cells isolated from peripheral bloods as effectors and human myeloblastic cells (K562) as targets. The following results were obtained: 1) The mean NK activity of the 47 patients tested before chemotherapy was significantly lower than that of 14 healthy controls. 2) The patients who received chemotherapy consisting of 5 FU (12 mg/kg) and adriamycin (40 mg/M²) showed an increase in NK activity 5 days after injection as compared to that of the same patients tested before chemotherapy. 3) In these patients, an additional administration of poly(A).poly(U) (100mg) resulted in a further significant increase of NK activity 2 days later, whereas the control patients who received placebo showed no change of NK activity.

Key Words: Stomach cancer, NK activity, chemotherapy, poly(A).poly(U)

Spontaneous cytotoxicity mediated by a subpopulation of lymphocytes, termed natural killer (NK) cells, is becoming of great interest to many researchers, since these cells can exert natural cytotoxic responses against a variety of tumors, hemopoietic stem cells and virus-infected cells (Herberman *et al.*, 1978). Evidence that NK cells play an important role in immune surveillance mechanism against tumors has also been accumulated (Hanna *et al.*, 1981; Riccardi *et al.*, 1980).

The activity of NK cells seems to be regulated by various factors, and it has been proved that interferon plays a major role in enhancing this activity (Gidlund *et al.*, 1978). A variety of biologic and synthetic agents has been found to enhance NK activity probably due to their ability to induce interferon (Djeu *et al.*, 1979; Herberman *et al.*, 1977).

Polyadenylic.polyuridylic acid [poly(A).poly(U)], a nontoxic double-stranded complex of synthetic polyribonucleotides (Ducret *et al.*, 1985), has proved to be a potent immunomodulator for both humoral (Braun *et al.*, 1967) and cell-mediated (Johnson *et al.*, 1979) immune responses. It has been successfully used as an adjunct to surgery in spontaneous mammary tumors of mice as well as in transplantable melanoma of hamsters (Lacour *et al.*, 1972).

In our previous studies, we found that treatment of tumor-bearing mice with poly(A).poly(U) in association with cyclophosphamide resulted in a synergistic tumor inhibition. Furthermore, NK activity of these mice who received such combined treatment was significantly increased (Youn *et al.*, 1982). Recently, Lacour *et al.*, has reported the updated (7 years) results of a randomized trial of poly(A).poly(U) in operable breast cancer patients. They observed that overall sur-

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vival was significantly higher in patients treated with this agent following surgery (Lacour *et al.*, 1984). Subsequently, it has been demonstrated that both NK activity and the level of 2-5A synthetase (an enzyme marker for the production and action of interferon) were significantly increased in these patients (Hovanessian *et al.*, 1985).

In this study, we have evaluated sequentially the NK activity of operable stomach cancer patients along with chemotherapy in association with or without poly(A).poly(U) administration as an adjuvant therapy. A significant enhancement of NK activity was observed in patients receiving chemotherapy plus poly(A). poly(U).

MATERIALS AND METHODS

Patients

Forty-seven patients with a histologically proven carcinoma of the stomach were studied. They included 34 males and 13 females hospitalized in Yonsei Medical Center betwen Oct. 5, 1984, and March 21, 1985 and their ages ranged from 30 to 74 years.

Histological diagnoses were made after systematic examination of tumor tissues and lymphnode involvement by numbering of each resected regional lymphnode and staged by TNM classification. As shown in Table 1, they consist mostly of T_2–T_3 without evidence of distant metastases. Most of the patients received a radical distal subtotal gastrectomy and a radical total gastrectomy in some cases. Ten to 15 days later, they were randomly divided into the following 2 groups:

Group A receiving chemotherapy plus poly(A). poly(U)

Group B receiving chemotherapy plus placebo (0.15M NaCl)

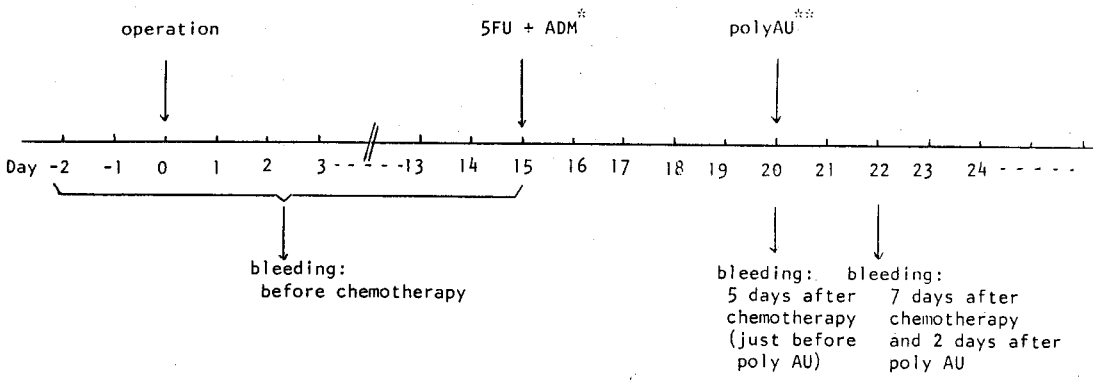
The protocol for the postoperative chemoimmunotherapy as well as the timings of blood samples for in vitro NK assay are illustrated in Figure 1.

Agents used for Chemo-immunotherapy

– Five fluorouracil (5FU) and adriamycin (ADM) were purchased from Choong-Wae Pharmaceutical Co., Seoul and Falmitalis Carlo Erba LTD, Italy, respectively. 5FU was administered intravenously (I.V.) at

Table 1. Histological classification of stomach cancer patients studied

Primary tumor	Regional lymphnode involvement	Number of patients
T1	–	4
	+	1
T2	–	6
	+	20
T3	–	2
	+	11
T4	+	3
		Total 47



* : chemotherapy ; 5FU, 12mg/kg I.V. plus Adriamycin, 40mg/M^2, I.V.

** : poly(A).poly(U), 100mg, I.V.

Fig. 1. *Protocol for postoperative chemo-immunotherapy of stomach cancer patients and the timings of blood samples for in vitro NK assay.*

dose of 12 mg/kg followed immediately by an I.V. injection of 40 mg/m² of ADM.

– Poly(A).poly(U) was prepared by Dr. Michelson (Institut de Biologie Physio-Chimique, Paris) from adenosine diphosphate and uridine diphosphate. The respective monomers were polymerized with polynucleotide phosphorylase and purified polynucleotides mixed in a 1:1 ratio to prepare the complex. The melting temperature of the material was 61°C in 0.15M NaCl and the thermal hyperchromicity (20°C-70°C) at 260 nm was 52%. Melting was cooperative and 100% reversible on cooling to 20°C. The sedimentation value of the complex, Sw 20, was 10.07. Poly(A).poly(U)was dissolved in sterile 0.15M NaCl solution and infused I.V. at a dose of 100 mg in 25 ml per injection.

Preparation of peripheral blood lymphocytes

Venous blood samples were collected from patients and normal healthy volunteers in heparinized plastic syringes. The blood was diluted 1:1.4 with RPMI-1640 medium (RPMI) and peripheral lymphocytes were isolated by centrifugation on a Ficoll-Hypaque density gradient (Histopaque-1077, Sigma Chemical Co., St. Louis, Mo. 63178, USA). Cells were washed twice with RPMI and once with RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 25 mM HEPES buffer (4-2-hydroxiethyl-1-piperazine ethane sulfonic acid, pH 7.6) and antibiotics (complete medium). Washed mononuclear cells were suspended in complete medium and were incubated in plastic dishes at 37°C in a 5% CO_2 humidified incubator for overnight in most cases or for 1 hour in some cases due to technical reasons. Nonadherent mononuclear cells were then collected and used as effector cells in NK assay. In a pilot experiment using peripheral mononuclear cells of a given donor, the incubation of these cells either for overnight or for 1 hour yielded in both cases nonadherent mononuclear cell populations having similar levels of NK activity toward the same target cells (K562).

In vitro NK assay

NK cell activity was determined by a 4h chromium 51 (^{51}Cr)-release assay using in vitro cultured and mycoplasma-free K562 cells derived from human myeloid leukemia (Lozzio *et al.*, 1975) as targets. The target cells were labeled by incubating 10^6 cells in 0.2 ml complete medium with 100 μCi Na_2 $^{51}CrO_4$ (1mCi/ml NEZ-030S, New England Nuclear, Boston, Mass., USA) 37°C in a water bath for 1 hour. After 3 washes, the labeled 10^4 cells in 100 μl were incubated in each well of a round-bottomed microtiter plate (Nunc, Denmark) with 100 μl of effector cells at the ratio of 1:50 for 4 hours at 37°C in a 5% CO_2 incubator. After incubation, 50 μl of the supernatant from each well was carefully removed and its radioactivity was counted by a gammacounter.

Three replicate wells were made for each assay. Target cells were also added to replicate wells containing 100 μl medium alone to determine spontaneous release and to wells containing 100 μl medium plus detergent (0.25% Triton X-100, Sigma Chemical Co.) to determine maximal release. Spontaneous release of ^{51}Cr from K562 targets in medium alone ranged from 7 to 14% of the maximal release.

The NK activity was expressed as:

$$\% \text{ cytotoxicity} = \frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

For simplicity, standard errors, usually less than 5%, were omitted from the tables.

A statistical evaluation of significance was made by paired or unpaired Students' t tests.

RESULTS

NK activity of stomach cancer patients before chemo-immunotherapy

Nonadherent mononuclear cells prepared from peripheral blood of 47 stomach cancer patients, 13 preoperative and 34 postoperative stages before the initiation of chemo-immunotherapy, showed a great variation of NK activity ranging from 0.1 to 96.0% with a mean value of 23.1% cytotoxicity (Table 2). Such individual variation of NK activity was also observed with peripheral lymphocytes similarly prepared from 14 sex and age-matched normal healthy donors: 14.7 to 74.0% with a mean value of 47.3% cytotoxicity. The difference between the mean % cytotoxicities of these 2 groups was highly significant ($p<0.001$).

Further comparisons on NK activity of these patients were made according to their sex, age, operation status and histological staging. As shown in Table 2, no significant differences were observed.

Evolution of NK activity in stomach cancer patients before and after chemo-immunotherapy

The stomach cancer patients were randomly divided into 2 groups after operation: 16 patients in the experimental group (A) and 14 patients in the con-

Table 2. NK activities of normal healthy volunteers and stomach cancer patients before chemo-immunotherapy

Lymphocyte donors	No. of donors	%Cytotoxicity ± SE	p value*
Normal healthy volunteers	14	47.3±6.2	p<0.001
Stomach cancer patients	47	23.1±3.0	
Sex: male	34	23.8±3.7	NS
female	13	21.3±5.2	
Age: over 60	20	23.9±4.2	NS
less than 60	27	23.0±4.3	
Before operation	13	23.3±5.6	NS
After** operation	32	22.7±3.7	
Primary tumor: T_1–T_2	31	23.3±3.3	NS
T_3–T_4	16	22.8±6.1	

*: Student's t tests (unpaired). NS: not significant
**: Tested during 2 to 22 days after operation

Table 3. Sequential NK activities of stomach cancer patients before and after chemo-immunotherapy. (A) Patients received chemotherapy plus poly(A).poly(U)

(A)

No. of Patients	% Cytotoxicity		
	Before CT*	After CT	After CT + poly(A).poly(U)
1	27.4	55.1	159.2
2	71.9	112.3	101.9
7	24.6	40.7	37.6
8	86.2	95.4	145.5
9	31.7	45.9	42.5
10	8.5	38.0	71.1
11	0.1	78.0	23.8
12	7.5	29.2	33.3
14	36.3	37.2	30.5
16	14.1	20.4	28.8
24	49.7	16.6	58.0
34	30.1	76.7	100.9
41	5.5	17.3	4.4
44	8.6	16.4	5.9
45	18.7	27.4	37.4
47	16.7	6.4	5.2
Mean±SE:	27.4±6.0 (16)**	44.6±7.8 (16) P<0.05***	55.4±12.0 (16) P<0.01***

Table 3. Continued (B)
(B) Patients received chemotherapy plus placebo

No. of patients	% Cytotoxicity		
	Before CT	After CT	After CT + placebo
13	75.7	51.6	30.5
15	27.5	17.6	62.4
17	39.9	23.8	47.0
22	22.0	27.7	22.4
23	18.7	23.9	14.3
25	13.6	42.6	60.3
26	7.5	8.1	36.3
33	59.8	119.2	40.5
35	4.4	13.2	0.1
36	6.4	21.1	8.9
37	15.0	28.7	42.7
40	23.4	6.5	12.4
42	18.3	31.8	7.8
46	35.2	125.3	38.7
Mean±SE	26.2±5.5 (14)	38.7±10.0 (14) NS****	29.6±5.2 (14) NS****

* CT = Chemotherapy
** Number of patients
*** Student's t test (paired) vs Before CT.
**** NS = not significant

trol group (B).

Ten to 15 days after operation, they were submitted to the regimen of chemoimmunotherapy according to the protocol as illustrated in Figure 1. The control patients received the same chemotherapy plus physiological saline instead of poly(A).poly(U). The

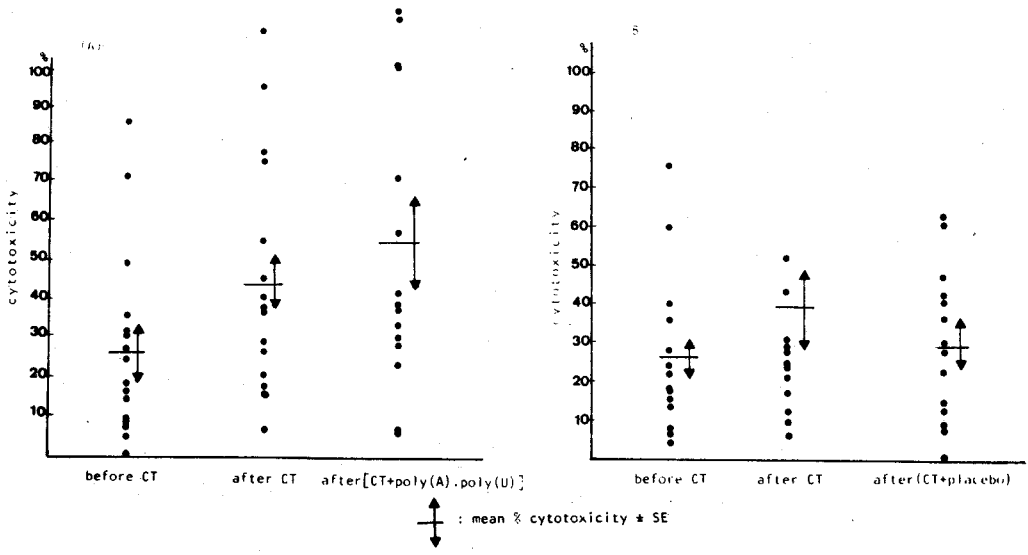


Fig. 2. NK activities of stomach cancer patients before and after chemo-immunotherapy.
(A) Patients received chemotherapy plus poly(A).poly(U) (16 patients)
(B) Patients received chemotherapy plus placebo (14 patients)

Table 4. Changes in NK activity of stomach cancer patients received chemotherapy plus poly(A).poly(U) or placebo.

Lymphocyte donors	No. of patients with changes in % cytotoxicity				
	Total	Increase*	No effect**	Decrease*	
Patients received chemotherapy + poly(A).poly(U)	16	10 (62.5)	6 (37.5%)	0	NS***
Patients received chemotherapy + placebo	14	4 (28.6%)	8 (57.1%)	3 (14.3%)	

* Increase or decrease of more than 15% of cytotoxicity after chemotherapy plus poly(A).poly(U) or placebo
** No change or changes less than 15% of increase or decrease
*** Chi-square test with Yate's correction : $0.1<p<0.05$

venous blood samples were collected 3 times in each patients: before chemotherapy (just before or after operation), 5 days after chemotherapy (just before poly(A).poly(U) injection) and 7 days after chemotherapy or 2 days after poly(A).poly(U) injection. The sequential NK activities of these blood samples from each patient are record in Table 3 and the distribution pattern is shown in Figure 2.

In the experimental group (Table 3-A), the mean % cytotoxicity of 16 patients at 5 days after chemotherapy is significantly ($p<0.05$) increased as compared to that of the "before chemotherapy" and this value was more significantly increased ($p<0.01$) in the "after CT+poly(A).poly(U)". In the control group (Table 2-B), no significant differences of the mean % cytotoxicities were observed in either comparison made between those before and after chemotherapy or those before and after chemotherapy plus placebo.

Further analysis of these results regarding the changes in NK activity of the patients following chemotherapy plus poly(A).poly(U) or placebo is presented in Table 4. In this analysis, an increase or decrease of more than 15% cytotoxicity in each patient after treatment was considered to be significant on the basis of the results obtained from the control patients receiving placebo. The mean value obtained from the difference in % cytotoxicity between the two assays (before and after saline injection) in each patient was 8.3%±4.4. Thus, the changes in % cytotoxicity of more than 15% could be considered to be

significant. As shown in Table 4, 10 patients out of 16 (or 63%) who received chemotherapy plus poly(A). poly(U), showed an increase and 6 patients (37%) showed a decrease in their NK activity. By contrast, in the control group, only 4 patients out of 14 (or 29%) showed an increase and 11 patients (71%) showed no change or a decrease in NK activity. Nevertheless, the statistical analysis by chi-square test revealed little significance probably due to the limited numbers of patients in each group.

DISCUSSION

In this study, NK activity of stomach cancer patients was sequentially evaluated in the course of treatment consisting of operation, chemotherapy and poly(A). poly(U) administration as adjuvant therapy.

The first comparison on NK activity between a group of 47 patients before chemo-immunotherapy and that of 14 sex and age-matched normal healthy controls clearly indicates that the NK activity of the patients was significantly lower than that of the controls. Similar decreases in NK activity in other types of cancer patients, particularly in those with advanced cancers, were reported (Tursz *et al.*, 1982). On the other hand, no such impairment of NK activity in cancer patients was also described (Pross *et al.*, 1976). This discrepency might be explained partly by the fact that the patients studied in most of the works reported represented heterogenous groups in regard to their histopathological diagnosis. The patients studied in our experiments were histologically quite a homogenous group consisting mostly of T_2–T_3 with N_0 or N_1 lymphnode involvement in the immediate vicinity.

Further analysis of our results has revealed that NK activity of stomach cancer patients was not related either to the sex, age, operation status of histological staging. In our study, 32 patients (Table 2) were tested during 2 to 22 days after operation and the mean value of their NK activities was of a similar level to that of 13 tumor-bearing patients. Before drawing any conclusion, further study with a larger number of cases is necessary to determine whether the operation of tumors could influence or restore the lowered NK activity of these patients.

In order to study the effect of chemotherapy on NK activity, 30 operated patients were submitted to NK assay at Day 5 after one injection of 5 FU plus ADM. Unexpectedly, their NK level was not decreased but rather increased as compared to that of the "before chemotherapy" stage. In fact, among these patients receiving such chemotherapy, 23 patients (or 77%) showed no changes or increases in their NK activity. Significant reductions of NK activity were observed only in the remaining 7 patients (or 23%) following chemotherapy.

Depressive effects of NK activity by various chemotherapeutic agents were reported in mice (Santoni *et al.*, 1980) as well as in men (Saijo *et al.*, 1982). However, it is generally admitted that such effects were largely influenced by various factors such as the nature of the agents, the doses, the route of administration and the source of effector cells. Santoni *et al.*, reported that intraperitoneal administration of ADM resulted in a rapid increase of peritoneal NK activity of various mouse strains (Santoni *et al.*, 1980). Thus, in certain conditions, an NK-stimulatory effect could be observed following chemotherapy. Furthermore, ADM has been reported to have little or no inhibitory effect on the cytotoxic activity of macrophages or of NK cells (Mantovani *et al.*, 1977; Mantovani *et al.*, 1978).

The administration of poly(A).poly(U) 2 days after chemotherapy in these patients resulted in a more pronounced enhancement of their NK activity, whereas the control patients treated similarly with placebo showed their NK levels unchanged. These results were consistent with our previous findings in mice (Youn *et al.*, 1982; Youn *et al.*, 1983) as well as in operable breast cancer patients (Hovanessian *et al.*, 1985) treated with poly(A).poly(U).

The mechanism for the NK boosting effect observed in our chemo-immunotherapeutic regimen is obscure. It is well documented that interferon or interferon-inducers enhance NK activity (Gidlund *et al.*, 1978). Thus, in our experimental condition, both injections of the chemotherapeutic agents and poly(A). poly(U) might stimulate the production of interferon which in turn enhances NK activity. Alternatively, it can be postulated that NK suppresor cells (Gerson *et al.*, 1981) which were sensitive to the cytotoxic action of the chemotherapeutic agents might be depressed during the initial step of chemotherapy leading to an unhindered or increased NK activity. Suppressor T cells extremely sensitive to cyclophosphamide were described (Shand *et al.*, 1980).

Considering the role of NK cells is immune surveillance against tumors, a systematic evaluation of NK activity of the patients along with chemo-immunotherapy seems to be one of the important parameters to be used to monitor the clinical application of therapeutic agents. At present, the patients reported in this paper are under continuous chemo-immunotherapy using the same agents. The matching of different in vitro immune responses,

notably of NK activity, to clinical evolution of the patients, should undoubtedly provide more insight into the mechanism involved in immunomodulation of hosts against tumors.

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