ONCOLOGY REPORTS 1: 439-443, 1994

Natural killer cell and antibody dependent cellular cytotoxicity in gastric carcinoma patients: modulatory effects of IL-2 and 5-fluorouracil

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Received November 3, 1993; Accepted January 21, 1994

Abstract. Non-adherent peripheral blood mononuclear cells (PBMC) from 15 patients with carcinoma of the stomach and 6 normal healthy controls were assessed for natural killer cell mediated cytotoxicity (NKCMC) and antibody dependent cellular cytotoxicity (ADCC) in a 4 hour ^{51}Chromium release assay against target cells, K-562 and sensitized chicken red blood cells (CRBC), respectively. NKCMC and ADCC were significantly reduced in patients with respect to normal controls. However, significant enhancement in NKCMC and ADCC was observed when PBMC from patients were cultured in RPMI-1640 containing 200 IU/ml of Interleukin-2 (IL-2) for 4 days in 5% CO_2 atmosphere at 37°C. Augmentation of NKCMC and ADCC was also detected when a follow-up study of the patients was conducted after one month following initial trial of chemotherapy with 5-fluorouracil (5-Fu). Patients, who had undergone clinical trial with 5-Fu were better responders of IL-2 activation as reflected on NK cell and ADCC potential of these patients. Our investigations highlight the synergistic effect of 5-Fu and IL-2 on effectors of natural immunity in patients with carcinoma of the stomach. Judicious modulation of effectors may be beneficial in killing residual cancer cells *in vivo*.

Introduction

Cancer of gastrointestinal tract (GIT) remains a major cause of death from malignant disorders worldwide. Incidence of carcinoma of the stomach is very high in Central Kerala, India. Approximately 10% of all cancer incidence constitute gastric cancers in this region. Upper GIT cancers are more common than the lower GIT cancers in this region. Almost all the patients have a very poor prognosis, with a survival rate of less than 5 years.

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Key words: NK cell, ADCC, carcinoma stomach, Interleukin-2, 5-fluorouracil.

Two natural defense mechanisms in the body against malignancies and a variety of bacterial infections are natural killer cell mediated cytotoxicity (NKCMC) and antibody dependent cellular cytotoxicity (ADCC). NK cells, a morphological subpopulation of large granular lymphocytes (LGL), are capable of lysing a broad spectrum of syngeneic, allogeneic and xenogeneic tumor cells without prior sensitization and MHC restriction (1,2) NK cells are considered as the first level of defense against spread of tumor *in vivo* (3). The NK activity is mediated by putative NK receptor, while ADCC is mediated by means of IgG Fc receptor in the presence of a target cell specific antibody (4,5). Several studies in cancer patients have documented an inverse relationship between NK activity and metastasis (6,7).

Interleukin-2 (IL-2), a T helper cell derived lymphokine is regulating a variety of cell mediated antitumor immune reactions. It has striking effect on NK cell and K cell stimulation. NK cell immunodeficiency demonstrated in patients with chronic myelogenous leukemia (8-10) has been attributed to defective IL-2 production by T helper cells and NK cells (CD 16$^+$). It has been reported that the impairment in NKCMC observed in advanced malignancies could be restored to normalcy by treatment of lymphocytes with exogenous IL-2 (8,11,12). In many instances the effect of IL-2 on tumor regression has been directly associated with increased NK activity (13,14).

5-fluorouracil is a fluorinated analogue of pyrimidine. It binds thymidylate synthase, preventing the formation of thymine, a basic component of DNA. Like other anti-metabolites it is toxic to bone marrow and gastro-intestinal epithelium (15). The modulatory effects of 5-fluorouracil and IL-2 on ADCC and NK cytotoxic status of patients with carcinoma stomach were also investigated in this study.

Materials and methods

Blood samples. Heparinised peripheral blood samples were collected from patients with carcinoma of the stomach, attending the Radiotherapy unit of the Medical College, Thrissur. Blood samples were collected from both male and female patients of different age groups (23 years to 54 years) before starting the chemotherapy treatment schedule. Blood

Table I. Effect of IL-2 and 5-fluorouracil on NKCMC and ADCC of patients with carcinoma of the stomach.

Groups	No. tested	% specific lysis SD at effector:target ratio NKCMC				ADCC			
		12.5:1	25:1	50:1	100:1	12.5:1	25:1	50:1	100:1
Control PBMC	6	11.46 ± 3.68	16.60 ± 3.39	24.55 ± 3.74	36.68 ± 9.77	14.05 ± 2.38	25.75 ± 10.31	41.68 ± 12.24	57.78 ± 11.87
Patients PBMC	15	2.87 ± 1.46	6.29 ± 2.75	9.256 ± 2.43	13.32 ± 4.11	2.78 ± 1.58	6.07 ± 2.3	10.56 ± 4.04	15.19 ± 4.04
		[a]p <0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Patients PBMC IL-2 activated	8	10.41 ± 2.11	15.15 ± 2.31	17.45 ± 1.62	22.01 ± 6.15	6.22 ± 2.38	16.78 ± 3.35	25.20 ± 2.83	38.45 ± 3.95
		[b]p <0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Patients PBMC after 5-Fu treatment	8	5.30 ± 1.7	9.07 ± 2.24	13.75 ± 3.6	19.19 ± 3.29	4.80 ± 1.83	12.43 ± 3.58	16.50 ± 3.6	30.80 ± 3.46
		[c]p <0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Patients after 5-Fu treatment and IL-2 activation	8	10.12 ± 2.69	14.30 ± 2.19	22.50 ± 3.76	26.75 ± 4.72	14.51 ± 3.2	21.53 ± 3.65	31.27 ± 3.6	49.35 ± 3.78
		[d]p<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a]p = Significance of untreated patients vs. control; [b]p = Significance of untreated patient vs. IL-2 activated; [c]p = Significance of untreated patients vs. 5-Fu treated; [d]p = Significance of untreated patients vs. 5-Fu + IL-2 activation.

samples from healthy volunteers in the laboratory served as control. Peripheral blood mononuclear cells were separated by centrifugation over Ficoll-Hypaque density gradient (Pharmacia, NJ) and used as effector cells for NK cell and ADCC assays.

Culture medium. RPMI-1640 (Flow laboratories, UK) supplemented with 10% fetal bovine serum (Gibco Lab, NY), L-glutamine 2 mM (Sigma), beta-mercapto ethanol (0.05 mM), penicillin (50 µg/ml), streptomycin (50 µgm/ml) were used for culturing cells.

IL-2 activation of PBMC. Peripheral blood mononuclear cells (PBMCs) were depleted of adherent cells by passing through Nylon wool column (Fenwall Laboratories, UK) by the method of Julius *et al* (16) and further incubated in complete medium containing 200 IU/ml of Interleukin-2 (kindly supplied by Dr Jeffrey Rossio, Lymphokine testing laboratory, NCI, Bethesda, MD, USA) at 37°C in 5% CO_2 atmosphere for four days. After incubation, cultures were harvested, washed and assayed for cytotoxicity against radiolabelled K-562 and chicken red blood cell (CRBC), respectively. ^{51}Chromium was obtained in the form of sodium chromate saline injections from Board of Radiation and Isotope Technology (BRIT), Bombay, India.

5-Fu treatment schedule. 5-fluorouracil (5-Fu) was administered at a dose of 500 mg/day, as continuous 2 h intravenous infusion for consecutive 4 days. After one month, blood samples were collected for follow-up studies.

Morphological analysis of PBMC. A total of 5 x 10^4 PBMC were plated on a glass slide. After air drying the slides were stained for 5 minutes with May and Grunwald stain and counterstained for another 5 minutes with Giemsa stain. The morphological distribution was determined by evaluating 300 cells/slide.

NK cell and ADCC. NK cell cytotoxicity was assessed against radiolabelled K-562 target cells in a 4 h ^{51}Chromium release assay as previously reported (11,12). ADCC was assessed using anti chicken RBC antibody sensitized chicken RBC in a 4 h ^{51}Chromium release assay as described (17,18). The standard error for triplicate assays was always less than 10%. Spontaneous release was consistently less than 10% of the total release for a 4 h assay.

Statistical analysis. The data were analysed using the Student's t-test.

Results

NKCMC and ADCC in the PBMC of patients with gastric carcinoma was measured at the time of presentation before starting the chemotherapy treatment schedule. NKCMC and ADCC were tested using radiolabelled K-562 and anti CRBC antibody coated CRBC as respective targets in a 4 h ^{51}Cr release assay. Significant reductions in these activities were seen in patients as compared to the normal controls. The relative reduction in NK and ADCC activities of patients was detectable at all the effector to target ratios (12.5:1, 25:1,

50:1,100:1) (Figs. 1 and 2). The depressed mean cytotoxicity in patients were statistically significant at all ratios ($p < 0.001$, Table I). However, morphological analysis of effector cells revealed no significant changes in the absolute number of NK cells as large granular lymphocytes (LGL) present among the total effector cell population of patients as compared to control.

When PBMC from 8 patients were cultured in RPMI-1640 + 10% fetal calf serum (FCS) containing 200 IU/ml of interleukin-2 for four days, significant enhancement in the killing potential of the effector cells was observed with respect to non activated PBMC from patients. After activation, NK cell and ADCC activities were elevated nearly to the normal levels. Enhancement in cytotoxicity was statistically significant at all the effector to target ratios as compared to non activated PBMC ($p < 0.001$, Table I). ADCC activity in patients was observed to be slightly higher than NKCMC at all the effector to target ratios (Figs. 1 and 2). NK cell and ADCC effectors mediated cytolysis of corresponding targets in a dose-dependent manner, maximum cytolysis being observed at an E:T ratio of 100:1 (22.011 ± 6.5 and 38.45 ± 3.95, respectively) (Figs. 1 and 2).

A follow-up study of 8 patients was carried out one month after the initial administration of 5-fluorouracil. As indicated in Figs. 1 and 2, augmentation of NKCMC and ADCC was detectable in the PBMC of patients in the post-treatment period as compared to pretreatment level. This enhancement was ststistically significant ($p < 0.001$) (Table I).

PBMC of the patients who had undergone treatment with 5-Fu were further expanded in RPMI-1640 and 10% FCS containing IL-2 for four days. The cytotoxicity of these cultured cells was tested against respective targets. As depicted in Figs. 1 and 2, enhancement in the killing potential of NK and ADCC effectors was observed as compared to PBMC of patients after chemotherapy. Similarly augmented natural cytotoxicity was detected with respect to IL-2 activated effectors of patients before chemotherapy. Therefore, the effect of IL-2 on PBMC of patients post chemotherapy was cumulative (Table I). The enhancement in both NKCMC and ADCC was significant at all the effector to target ratios as compared to untreated patients ($p < 0.001$). Enhanced cytolytic activity displayed by the effector lymphocytes following chemotherapy and IL-2 activation was statistically significant with respect to the cytotoxic potential of PBMC post chemotherapy ($p < 0.001$). However, the enhancement in NK activity was not statistically significant as compared to tumoricidal capacity of PBMC of patients following activation with IL-2 but before chemotherapy except for E to T ratios 50:1, 100:1, ($p < 0.001$) (Table I).

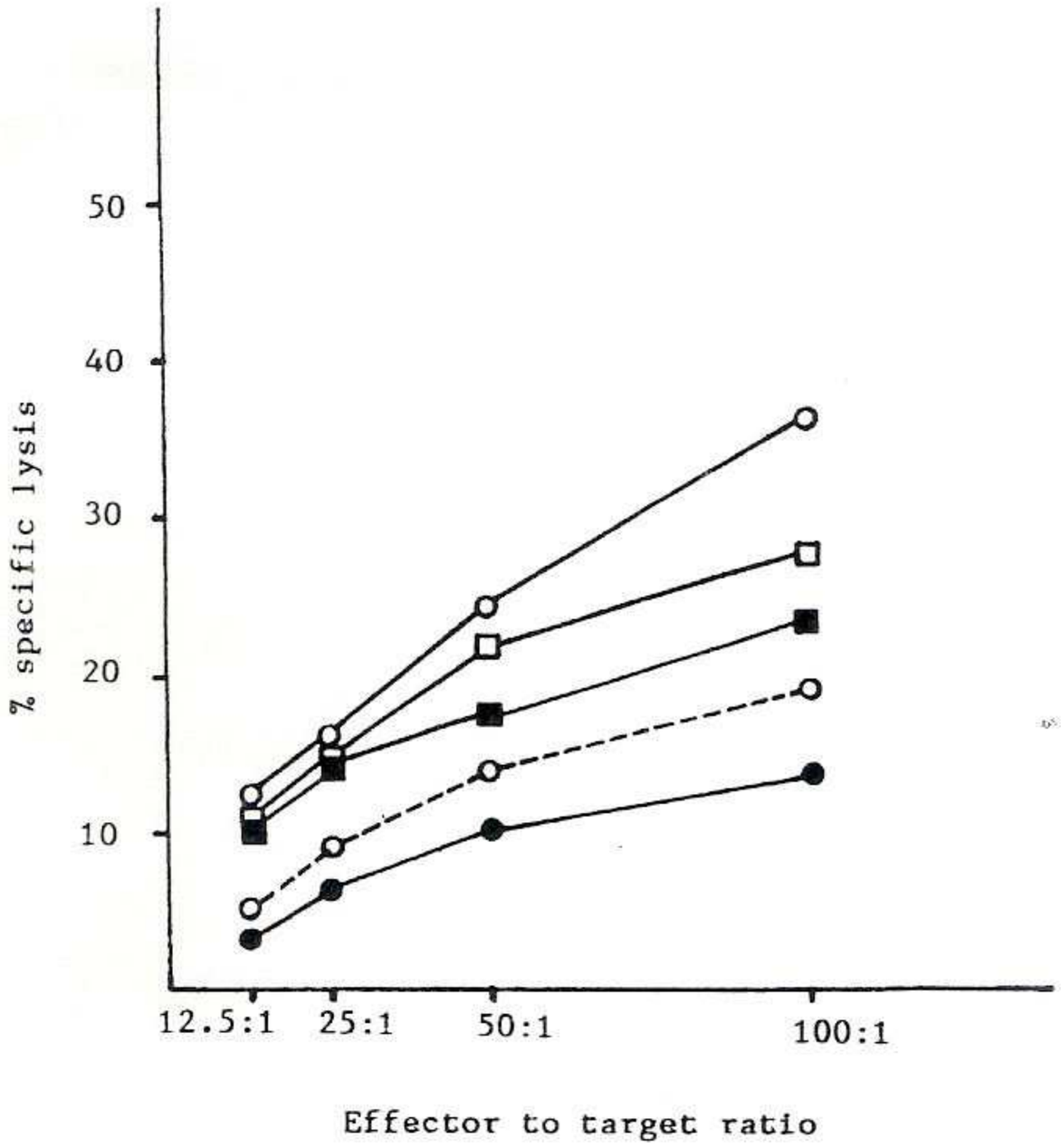

Figure 1. Dose response curves of NKCMC displayed by PBMC of normal control (O——O); patients with carcinoma of the stomach before treatment (●——●); PBMC after expansion in RPMI-1640 containing 200 IU/ml of IL-2 (□——□); PBMC of patients after treatment with 5-Fu (O- - -O), PBMC of patients after treatment with 5-Fu followed by expansion in IL-2 containing medium (■——■). NK cell activity was assessed against radiolabelled K-562 target cells at different effector to target ratios of 12.5:1, 25:1, 50:1 and 100:1 in a 4 h ^{51}Cr release assay.

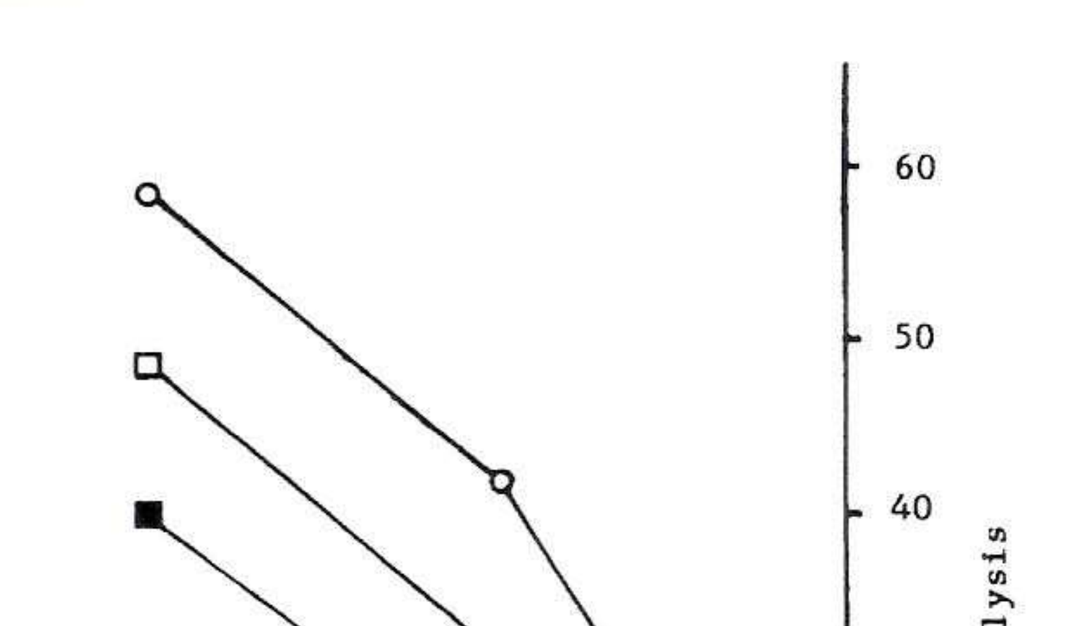

Figure 2. Dose response curves of ADCC displayed by PBMC of normal control (O——O); patients with carcinoma of the stomach before treatment (●——●); PBMC after expansion in RPMI 1640 containing 200 IU/ml of IL-2 (□——□), PBMC of patients after treatment with 5-Fu (O- - -O); PBMC of patients after treatment with 5-Fu followed by expansion in IL-2 containing medium (■——■). ADCC was assessed against radio labelled and sensitized CRBC at different effector to target ratios of 12.5:1, 25:1, 50:1 and 100:1 in a 4 h ^{51}Cr release assay.

Discussion

NKCMC and ADCC in the peripheral blood of cancer patients were assessed against ^{51}Chromium labelled K-562 and sensitized CRBC target cells, respectively. A significant reduction in NKCMC and ADCC was observed in patients as compared to controls ($p < 0.001$). Impairment in NK cell and ADCC was significant at all effector to target ratios (12.5:1, 25:1, 50:1 and 100:1). Compromised or absent natural

immunity as measured *in vitro* by NK activity and/or depressed absolute numbers of circulating NK cells has been associated with development and progression of cancer (18,19). Patients with a variety of solid malignancies and large tumor burdens have been reported to have diminished NK cell activity (20). It has also been reported that, despite extremely low NK cell activity, B cell depleted fraction of PBMC of patients that bound to NK sensitive target falls within the range of normal individuals (7,21,25). Cancer patients are either deficient of naturally occurring cells capable of inducing cytolysis of xenogeneic target cells or they posses naturally occurring cells in an inactive state. This is indicative of an immunologic dysfunction. Morphological analysis of effector cells revealed no significant changes in the absolute number of NK cells (LGL) present among the total effector cell population of patients as compared to normal. Therefore it is likely that defects in the programming for lysis could be the reason for impaired natural immunity in patients. Earlier reports suggest that calcium dependent programming for lysis defect may be the result of alterations in signal transduction across the NK cell surface membrane by molecules such as IL-2 receptor or NKRPI (22,23).

When the PBMC from the patients were assessed for NKCMC and ADCC, following expansion in IL-2 containing medium for 4 days, a significant enhancement in killing potential of the effector lymphocytes was detected. NKCMC and ADCC activities in patients after activation with IL-2 were elevated nearly to the normal level. Activated NK cells are believed to be the major population of anti tumor effectors which *in vivo* are responsible for therapeutic effectiveness of LAK cells (24). ADCC activity in cancer patients were slightly higher than NKCMC following activation with IL-2. Increased expression of Fc receptors in suppressor T lymphocytes on exposure to lymphokines has been reported in cancer patients (21,25). The observed enhancement in ADCC activity of patients with respect to NKCMC could be attributed to the involvement of Fc receptor bearing T cells which can also mediate ADCC. Increased granulation with concomitant increase in lytic activity of NK cells comparable to that of normal has been reported in cancer patients after treatment of the effectors with IL-2 (21,25). Consistent with our earlier report (12), activated killer cells were shown to mediate target cell lysis in a dose dependent manner.

When NKCMC and ADCC were assessed in the peripheral blood of 8 patients in a follow-up study after one month following the first clinical trial with 5-Fu, augmentation of both NKCMC and ADCC was detectable as compared to pretreatment level. According to Balaji and Nayak (Balaji KN and Nayak R: Proc XVIII Annual Conference of Indian Immunol Society and National Symposium on Immunology of Infectious Disease. 108-109, 1991), 5-Fu exerts its immunomodulatory effect through enhanced expression of IL-2 and IL-2R genes. IL-2 and IL-2R mRNA levels and secretory IL-2 protein levels increased linearly up to 2.7 fold and 3.75 fold after 5-Fu administration (Balaji KN and Nayak R: Proc XVIII Annual Conference of Indian Immunol Society and National Symposium on Immunology of Infectious Disease. 108-109, 1991). When PBMC from patients post chemotherapy were cultured in IL-2 containing medium profound enhancement in the cytotoxic potential of effector lymphocytes was observed as compared to patients post chemotherapy and patients after IL-2 activation but before chemotherapy treatment. Synergistic effect of interferon alpha and 5-fluorouracil on cytotoxicity has been reported in cancer patients (27,28). However, 5-Fu in combination with human rIL-2 has been reported to have inhibitory effect on secretory levels of IL-4 protein by mouse thymocytes (Balaji KN and Nayak R: Proc Ind Assoc Cancer Res Sympo. 33-34, 1992). On the contrary, our studies have demonstrated the enhanced NK cell and antibody dependent killing potential displayed by the PBMC of patients post chemotherapy. Measurement of NK activity is also necessary when cancer patients are treated with combinations of cytotoxic drugs and biological response modifiers. Rationale of this approach is to eliminate tumor cells prior to immuno augmentation of residual anti tumor effectors. Serial monitoring of NK activity and ADCC in the patient's blood serves as sensitive and reliable indicators of recovery post chemotherapy and success in generating antitumor killer cells *in vivo*. Synergistic therapeutic effects of cytoreductive therapy and IL-2 are being evaluated with encouraging results and combination chemotherapy with various cytokines is promising and is to be tested in clinical trials. The usefulness of reliable NK cell measurements in screening for such therapy and monitoring their responses is obvious. Natural immunity plays an important role in primary line defense and appears to be a useful indicator of host immune competence. Our study is in progress to delineate molecular events responsible for augmentation of natural immunity followed by IL-2 and 5-fluorouracil treatment in cancer patients.

Acknowledgements

Financial support from Indian Council of Medical Research Grant No. 3/III/17/89-NCD-III and Department of Science and Technology, Government of Kerala Grant No. 28/92/STED are gratefully acknowledged.

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