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Augmentation of Natural Killer Cell Activity by CORYNEBACTERIUM PARVUM: Involvement of Lipoxygenase Pathway

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AUGMENTATION OF NATURAL KILLER CELL ACTIVITY BY *CORYNEBACTERIUM PARVUM*: INVOLVEMENT OF LIPOXYGENASE PATHWAY

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Abstract

AUGMENTATION OF NATURAL KILLER CELL ACTIVITY BY CORYNEBACTERIUM PARVUM: INVOLVEMENT OF LIPOXYGENASE PATHWAY

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The ^{51}Chromium release assay was used to measure natural killer (NK) cell activity associated with Corynebacterium parvum (CP) injection against YAC-1 and MBT-2 targets. The peritoneal exudate cells from intraperitoneal treatment of CP at different dosages consistently gave higher cytotoxicity than their respective spleen cell fractions. In contrast, CP given intravenously showed the reversed effect. Our results demonstrate that the route of administration and dosage of CP are two crucial variables for determining the effectiveness of this immunostimulant. In vitro manipulations of nordihydroguaiaretic acid, indomethacin, and prostaglandin E2 on CP-stimulated NK cells suggest that the lipoxygenase pathway is involved in NK lysis.

Each person whose signature appears below certifies that this manuscript in his opinion is adequate, in scope and quality, in lieu of a thesis for the degree Master of Science.

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INTRODUCTION

The immunotherapy of cancer with Corynebacterium parvum (CP) has been studied in both human and animal models with conflicting and sometimes disappointing results (1-3). It has been suggested that some of the inconsistencies may be explained by differences in route of administration and dosage of CP used (4,5).

Although the mechanism of action for CP immunotherapy remains obscure, it has been suggested that its macrophage activating ability is largely responsible for the observed effects (6,7). Other investigators have suggested a role for T-cells in this regard (8).

More recently, it has been reported that natural killer (NK) cells are stimulated by CP to become cytotoxic towards many tumors, including lymphomas, carcinomas and sarcomas, both in vivo (9-11) and in vitro (5,11,12).

Recent evidence suggests that lipoxygenase activity of arachidonic acid (AA) metabolism is required for NK cell lysis. Rossi et al. (13) have shown that NK lysis is suppressed by inhibitors of lipoxygenation including nordihydroguaiaretic acid (NDGA), 5,8,11,14-eicosatetraynoic acid (ETYA), 3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazole (BW755C), and indomethacin. They further observed that NK activity can be restored by the addition of such lipoxygenase products as leukotriene B_4 (LTB4) and its isomers.

In this report, we have extended these findings to provide evidence that CP-stimulated NK cells require lipoxygenation of AA for lysis of

tumor target cells. We have also demonstrated that the route of administration and dosage of CP are two crucial variables for determining the effectiveness of this immunostimulant in vitro.

MATERIALS AND METHODS

Animals. Eight to 12-week-old C3H/He mice were obtained from Simonsen Laboratories, Gilroy, CA, and later housed in the animal care facility of the Loma Linda University Medical Center. The mice were fed Purina laboratory chow and tap water ad libitum.

Medium. RPMI 1640 medium (GIBCO, Grand Island, NY) was supplemented with fetal calf serum (FCS) (10% for culture, 5% for NK assay), glutamine (0.3 mg/ml), 250 U/ml penicillin and 125 μg/ml streptomycin. For spleen cells incubated with or without interleukin-2 (IL-2), RPMI medium was additionally supplemented with 2-mercaptoethanol (50 μM) and Hepes (20 mM).

Chemicals. Prostaglandin E2 (PGE2), nordihydroguaiaretic acid (NDGA), and indomethacin were purchased from Sigma Chemical Co., St. Louis, MO. PGE2 was dissolved in 95% ethanol at a final concentration of 1.4×10^{-3} M, and stored at -25° C until needed. Stock solutions of NDGA and indomethacin were made fresh daily in 95% ethanol to yield final concentrations of 1×10^{-3} M and 1×10^{-2} M, respectively. Further dilutions were made in FCS-free RPMI medium.

CP treatment. CP strain CN 6134 (7 mg/ml formalin-killed vaccine) was obtained from Burroughs Wellcome Co., Research Triangle Park, NC. All dilutions were made in sterile, physiological saline. Mice received

0.1 ml of the appropriate dilution administered either via the intraperitoneal (IP), or intravenous (IV) route.

Interleukin-2 (IL-2). IL-2 was the kind gift of Dr. Chou-Chik Ting from the National Cancer Institute, Bethesda, MD. This IL-2 was produced by an EL-4 subline (a T-cell leukemia) pretreated with phorbol myristic acetate, washed and incubated for an additional 40 hrs at 5 X 10^6 cells/ml (14). The supernatant was then harvested and stored at -70° C. It contained 10,000 U/ml IL-2.

Chromium release assay (CRA) for NK activity. YAC-1 or MBT-2 (mouse bladder tumor) target cells were labeled by incubating 2-5 X 10^6 cells with 250 μCi/ml $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, MA) for 2 hr at 37° C. Peritoneal exudate cells (PEC) were obtained by peritoneal lavage with Hanks Balanced Salt Solution (HBSS). Cells were centrifuged and resuspended in HBSS. Spleens were finely minced in supplemented RPMI. The resulting cell suspension was filtered on sterile gauze and washed with fresh media following centrifugation. Contaminating red blood cells were removed by treatment with lysing buffer. Cells were washed three times in HBSS and resuspended in RPMI with 5% FCS for use in the CRA. Both PEC and spleen cells were allowed to adhere to sterile plastic petri dishes (VWR Scientific Inc.) for 1 hr prior to collecting the nonadherent cells. All effector cells were counted in a hemacytometer and adjusted to the desired concentrations. They were maintained at 4° C until needed.

^{51}Chromium-labeled target cells were aliquoted into flat-bottom microtiter plates (Corning, NY). To these were added effector cells at varying effector-to-target (E:T) ratios to a final volume of 200 µl per well. The cells were incubated at 37° C in 5% CO_2 for 4 hr and thereafter the supernatant was removed from each well with a Skatron Supernatant Collection System (Skatron Inc., Sterling, VA) and radioactivity determined in a gamma counter.

Percent cytotoxicity was calculated as

$$\% \text{ Cytotoxicity} = \frac{\text{experimental CPM - spontaneous CPM}}{\text{total CPM - spontaneous CPM}} \times 100$$

where spontaneous CPM represents counts recorded from wells containing target cells and medium alone. Total release was obtained by incubating labeled cells with 10% sodium dodecyl sulfate.

Statistics. Statistical analyses were performed by the Student's t Test.

RESULTS

Figure 1 shows time kinetics of cytotoxicity associated with IP administration of different doses of CP. Peak activity was observed on day 4 following injections of 50, 250 or 700 µg CP. For both PEC and spleen cells, these 3 higher dosages declined to lower cytotoxic levels by day 8. However, with the lowest dosage tested, namely 5 µg, significant augmentation was not visible until day 8 for both PEC and splenic cells, despite unusually high activity on day 2 with PEC. It was found that CP treatment led to a significant augmentation ($p < 0.001$) of cytotoxic activity associated with peritoneal cells. This was also true with the 50, 250, and 700 µg dosages using spleen cells ($p < 0.05$).

Figure 2 shows the effects of IP and IV administration of CP on NK activity. Mice were injected with 5, 50, 250, or 700 µg of CP by either the IP or IV route and NK activity measured 4 days later against YAC-1 targets. A 1400 µg dose was also included in the IV treatment. The PEC fractions from IP treatment consistently gave higher cytotoxicity than their respective spleen cell fractions. In contrast, spleen cells from IV injection with 5, 50, and 250 µg dosages gave higher cytotoxicity than their respective PEC fractions. Cytotoxicity attributed to PEC and spleen cells from the 700 and 1400 µg dosages, however, were not statistically different from each other.

Figure 3 shows NK activity associated with IP injection of CP against MBT-2 cells. Significant cytotoxicities were seen with all

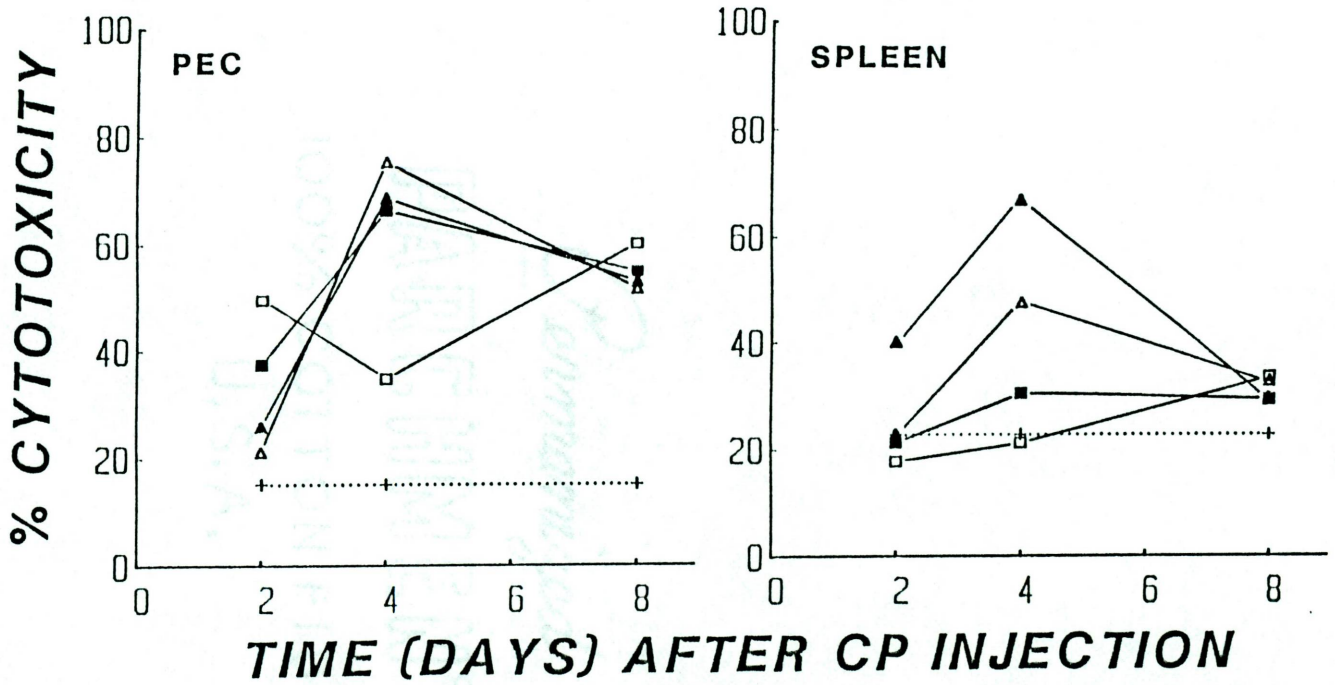

FIGURE 1. Time course of the appearance of cytotoxic NK cells in the peritoneal cavity (PEC) and spleen following IP injection of different CP dosages. Dotted lines represent cytotoxicity cells derived from untreated mice while open boxes, open triangles and closed triangles represent 5, 50, 250, and 700 µg CP respectively. Effector to target ratio = 50:1 for PEC and 100:1 for spleen cells. All assays were performed against chromium-51 labeled YAC-1 tumor cells.

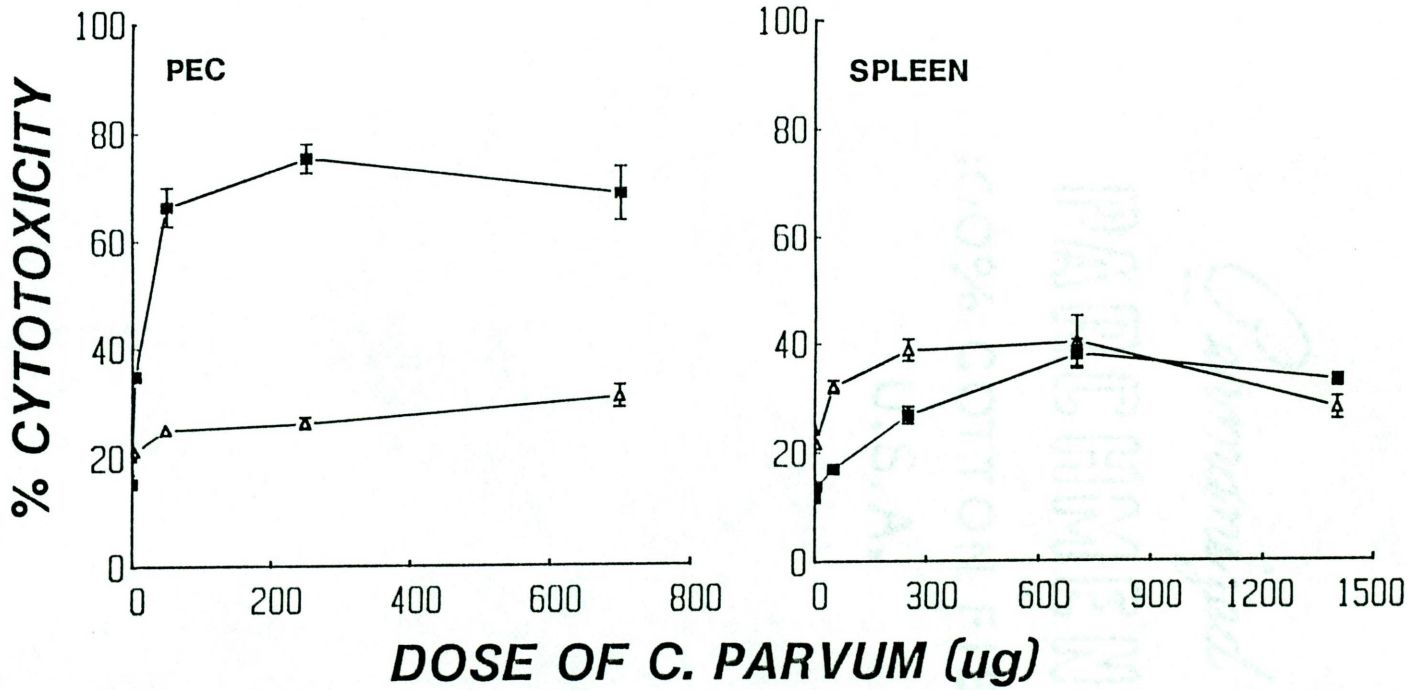

FIGURE 2. Cytotoxicity of PEC (closed boxes) and spleen cells (open triangles) obtained 4 days after intraperitoneal (IP) or intravenous (IV) injection of different dosages of CP. Effector to target ratio = 50:1. Normal PEC and spleen controls are shown as no dose of CP given. All assays were performed against chromium-51 labeled YAC-1 tumor cells.

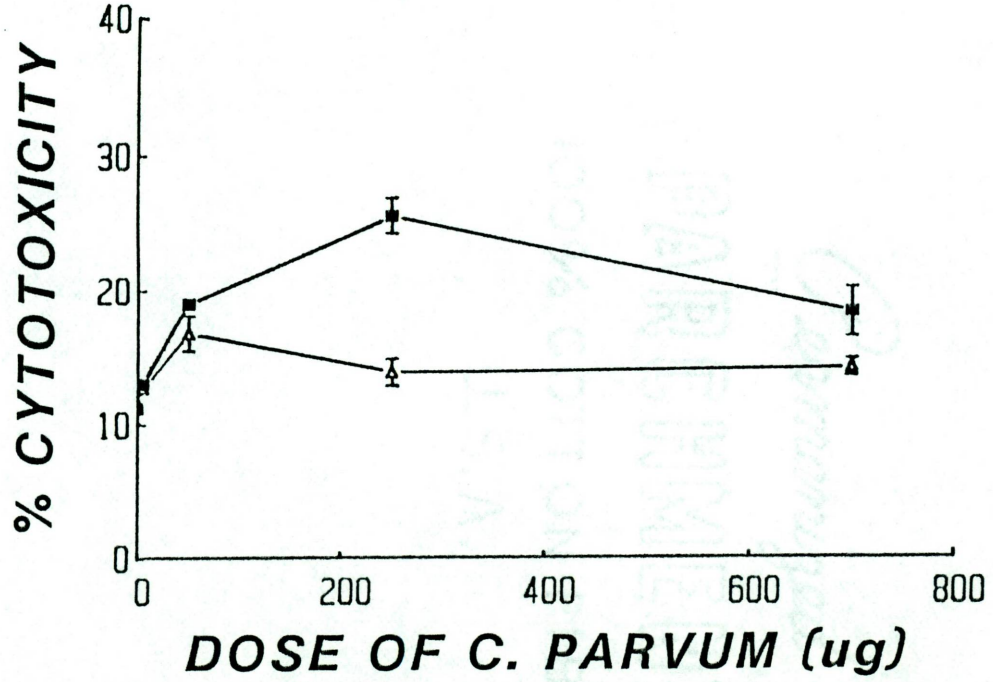

FIGURE 3. Cytotoxicity of PEC (closed boxes) and spleen cells (open triangles) obtained 4 days after intraperitoneal (IP) injection of different dosages of CP. Effector to target ratio = 50:1, and was assayed against chromium-51 labeled MBT-2 cells.

dosages (5, 50, 250, and 700 μg), with the 250 μg dose giving the best activity ($p < 0.001$). The activity of PEC was again greater than splenic cells. IV injections elicited no significant NK cell activity against MBT-2 cells (data not shown).

Figure 4 shows comparative effects of incubating normal untreated and IL-2 treated spleen cells with time against YAC-1 and MBT-2 targets. Augmentation of NK activity was seen with the IL-2 treated spleen cells only. NK activity against YAC-1 targets for the time period tested peaked at day 4 (67% lysis), but against MBT-2 cells it peaked at day 5 (57% lysis).

Table I shows the results of in vitro manipulation of CP-stimulated NK cells with NDGA, indomethacin and PGE2. These chemicals were used to determine whether the lipoxygenase and/or cyclooxygenase pathways were involved with NK activity. A dose of 700 μg CP given IV was used to activate splenic NK cells. Natural killing against YAC-1 target cells was suppressed by NDGA in a dose dependent manner, with significant inhibition between 8.3-33 μM concentrations tested ($p < 0.05$). The addition of a high concentration of indomethacin (300 μM) gave inhibition, whereas lower concentrations (less than 17 μM) showed unaltered activities (same as the controls).

PGE2-induced inhibition was also dose dependent, giving higher suppression of NK activity at more concentrated levels, with significant inhibition at 4.7 and 47 μM tested ($p < 0.05$). The presence of varying concentrations of indomethacin (0.03-300 μM) and 30 μM PGE2 also gave significant suppression ($p < 0.001$) of NK activity.

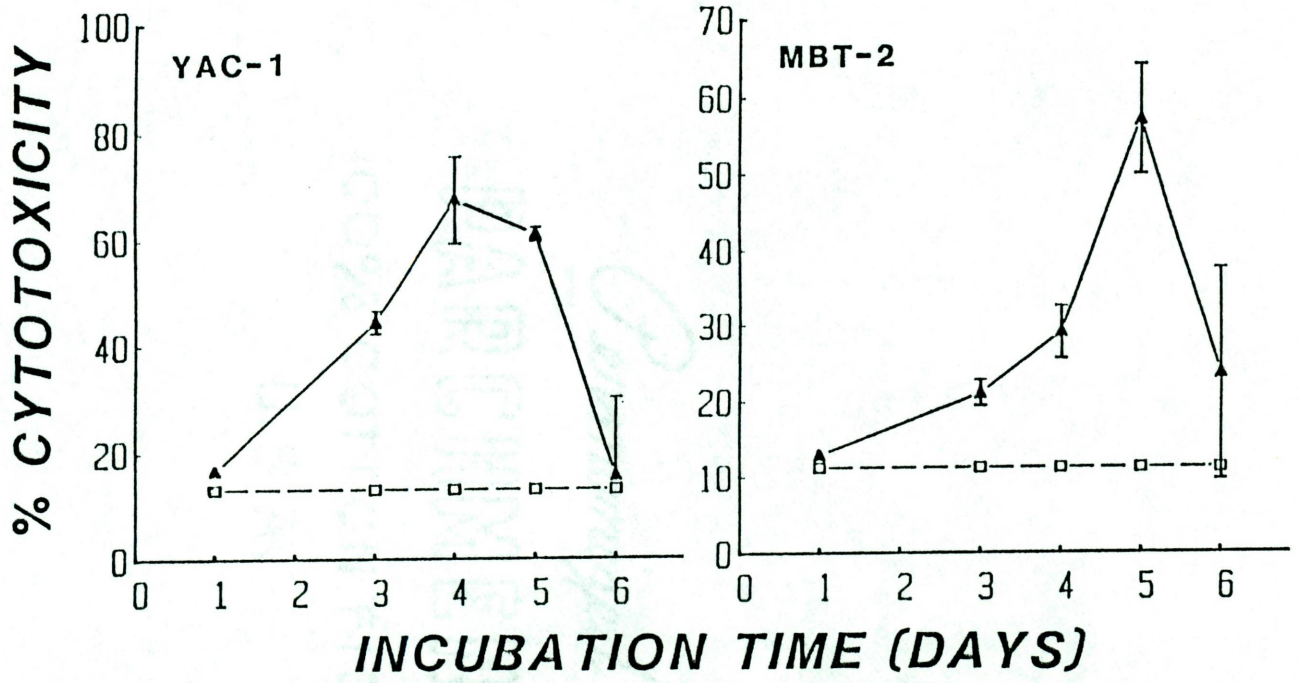

Figure 4. Time course of the in vitro effect of interleukin-2 (IL-2) on murine NK cell activity. 2.0×10^6/ml spleen cells from 10-week-old C3H/He mice were used as effector cells with chromium-51 labeled YAC-1 or MBT-2 target cells in a 4 hr release assay at an effector to target ratio of 100:1. 3% (10,000 U/ml) IL-2 was added to the spleen cells and then incubated at 37°C/CO_2 with time. Closed triangles represent IL-2 stimulated spleen cells, while open squares represent normal, untreated spleen cells.

TABLE I. THE EFFECTS OF NORDIHYDROGUAIARETIC ACID (NDGA), INDOMETHACIN (INDO), AND PROSTAGLANDIN E2 (PGE2) ON NATURAL KILLER CELL ACTIVITY, IN VITRO

% LYSIS WITH[a]							
Concentration (μM)	NDGA	Concentration (μM)	INDO	Concentration (μM)	PGE2	Concentration (μM)	INDO + PGE2
0	36.0±2.4	0	33.3±1.4	0	36.0±2.4	0	31.5±0.4
33	20.6±1.9*	300	17.9±2.1**	47	17.0±0.6*	300 + 30	14.1±1.5**
17	24.7±0.8*	30	29.0±2.0	4.7	22.5±0.8*	30 + 30	15.5±2.1**
8.3	27.8±0.4*	3.0	31.5±1.7	0.47	31.4±1.6	3.0 + 30	17.4±0.6**
4.17	32.9±2.1	0.3	32.6±2.4	0.047	33.0±0.1	0.3 + 30	14.5±0.5**
0.33	36.7±1.6	0.03	31.7±2.1	0.0047	36.3±1.5	0.03 + 30	16.7±1.0**

[a]The % lysis was obtained when CP-activated splenic natural killer cells were added to YAC-1 tumor cells in the presence of varying concentrations of different chemicals. Effector to target ratio = 100:1. All assays were performed 4 days after CP injection (700 μg) in 10 week old C3H/He mice. Values are given as the mean±SD.

*P< 0.05

**P< 0.001

DISCUSSION

This report demonstrates that CP is capable of augmenting NK activity. Both route of administration and dosage of CP were crucial in determining the effectiveness of CP treatment.

CP injected IP resulted in significantly greater augmentation of peritoneal NK activity, against YAC-1 and MBT-2 tumor cells *in vitro*, than that found for spleen cells (Figs. 2 and 3). In contrast, IV injection showed the reversed phenomenon. That is, spleen cells were stimulated more, and in some instances suppressed PEC response. This observation suggests that where CP is given determines how effective it will be in stimulating NK cells. It appears then, that NK cells in the immediate vicinity of the immunostimulant are more activated than distant ones. Exactly how CP is able to stimulate such cells still remains unknown.

At the highest IV dosage tested, 1400 μg, our results suggest suppression rather than augmentation (Fig. 2). This indicates that by considering the route of administration, high dosages of CP do not always give better NK response. In fact, less may be more effective (15). This may prove critical in considering the use of CP in immunotherapy of cancer as seen by Lau *et al.*, (In Press) on a murine bladder tumor model (16).

The lymphokine IL-2 has been shown by many researchers to augment NK activity (17-21). Our results on IL-2 incubated spleen cells against YAC-1 and MBT-2 targets (Fig. 4) also support this observation. How

IL-2 is able to augment NK activity is not fully understood. Chun *et al*. have suggested that IL-2 enhances NK activity by interfering with the negative feedback path of prostaglandin E production, which might otherwise have regulated NK cell activation towards lysis (22). This may suggest that PGE2, a product of the cyclooxygenase pathway of AA metabolism, may be prevented from exerting its inhibitory role on NK activity due to the presence of IL-2.

NDGA, an antioxidant with selectivity for 5-lipoxygenase (23-25), and thus an inhibitor of the lipoxygenase pathway, suppressed CP-activated NK cell activity against YAC-1 tumor cells *in vitro* (Table I). Other investigators (13,26-28) have previously indicated that lipoxygenation products may be necessary for NK lysis of tumor cells. Our data indicate CP-stimulated NK cells may also require the lipoxygenation pathway for their lytic activity, since in the presence of high concentrations of NDGA (8.3-33 μM) suppression was observed.

Inhibition of the lipoxygenation pathway by possibly scavenging hydroxyl radicals with NDGA resulted in marked inhibition of NK activity as demonstrated by Suthanthiran *et al*. (29). Such radicals may also exist for CP-stimulated NK cells and could therefore play a direct role in NK lysis.

To see whether the involvement of the cyclooxygenase pathway exists in our CP-stimulated NK cells, indomethacin and PGE2 were used. Indomethacin at higher concentrations, such as 300 μM, acts as a specific inhibitor of the lipoxygenase pathway. However, at lower concentrations, such as 0.33-17 μM, it acts as a cyclooxygenase

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