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Inositol-phosphate-induced enhancement of natural killer cell activity correlates with tumor suppression

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In recent studies, we have demonstrated that inositol hexaphosphate ($InsP_6$) inhibits experimental colon carcinogenesis. Since natural killer (NK) cells are involved in tumor cell destruction, we investigated the effect of $InsP_6$ on murine NK cell activity. We show that; (i) 1,2-dimethylhydrazine (DMH), a colon carcinogen, depresses NK activity; (ii) *in vivo* treatment of mice with $InsP_6$ enhances baseline NK activity and reverses DMH-induced depressed NK activity with an inverse correlation ($r = -0.9811$) with tumor incidence, (iii) short-term *in vitro* treatment of spleen cells and NK-enriched fraction with $InsP_6$ also enhances NK cytotoxicity in a dose-dependent manner, (iv) inositol potentiates the action of $InsP_6$. Our data suggest yet another important role of inositol phosphates in the regulation of cellular activity.

Introduction

Inositol (Ins*) is a common constituent of plant foodstuffs, such as seeds, cereal, grains, fruits and vegetables where a considerable portion of it is present as inositol hexaphosphate ($InsP_6$) (1,2). It has been reported that intestinal lipodystrophy can be prevented by Ins treatment (3,4). Recent studies in our laboratory demonstrate that $InsP_6$ inhibits experimental colon carcinogenesis not only in rats but also in mice (5,6). There is a correlation between neoplastic diseases and depressed natural killer (NK) cell activity, and there are reports that carcinogens inhibit NK cell activity (7–9). Most of the evidence suggests that NK cells are involved in the destruction and growth inhibition of tumor cells *in vivo* (10,11). Transfer of large granular lymphocytes (LGLs) into NK-depressed animals partially or fully restores the ability to inhibit metastases: evidence that LGLs high NK activity are involved in *in vivo* resistance to tumor (12). Transplanted NK-sensitive tumors in general grow better in mouse strains with relatively low levels of NK activity and vice versa (13). Chemical agents that increase NK activity directly (interferon) or indirectly by induction of interferon (poly-I-C, BCG, *C.parvum*) have been shown to have antitumor effects *in vivo* (14,15). Since in our earlier studies we have observed (i) an antineoplastic effect of $InsP_6$ 2 weeks or 5 months after carcinogen administration and (ii) that Ins potentiates the antineoplastic action of $InsP_6$, we investigated the effect of $InsP_6$ with or without Ins on NK cell activity following induction of experimental colon tumor in CD-1 mice.

Abbreviations: NK, natural killer; Ins, inositol; CF, cytotoxicity factor; HIFCS, heat-inactivated fetal calf serum; AIDS, acquired immunodeficiency syndrome; IL-2, interleukin-2; DMH, 1,2-dimethylhydrazine; $InsP_6$, inositol hexaphosphate; LGL, large granular lymphocyte; E:T, effector–target ratio

Materials and methods

Male CD-1 mice were given weekly s.c. injection of 1,2-dimethylhydrazine (DMH) at 15 mg/kg body wt for 13 weeks as described before (6). Control animals received tap water alone. Treatment groups consisted of supplementation of drinking water with 2% Ins or 2% $InsP_6$ or a mixture of 1% Ins + 1% $InsP_6$. The number of animals for each group at the beginning of experiment were as follows: DMH only = 30; DMH + 2% $InsP_6$ = 30; DMH + 2% Ins = 20; DMH + 1% Ins + 1% $InsP_6$ = 20; control: five animals each, receiving Ins, $InsP_6$, $InsP_6$ + Ins or tap water.

Rationale for dosage. In our previous studies (5,6), we had reported that the rats tend not to consume the water when the concentration of Na-$InsP_6$ is >2%; this is, however, within the range of availability in natural sources. Since a significant *in vivo* effect was observed with 1–2%, we rationalized that the concentration needed for *in vitro* effect could be much less. We arbitrarily chose 1/10th the concentration *in vivo*.

For NK-cell isolation, spleens from CD-1 and C3H/JSed mice were removed aseptically from the animals, gently teased by means of dissecting forceps and allowed to sediment in RPMI-1640. The cell suspension was passed through three layers of sterile gauze to remove debris. The cells were then washed with RPMI-1640. NK-enriched fractions of spleen cells were purified by Percoll-discontinuous density centrifugation (16). Cytolysis of targets by spleen cells or NK-enriched fraction was assayed by the 4-h ^{51}Cr-release method (17,18). Briefly, following the labelling of 2×10^7 target cells in 1 ml RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (HIFCS) with 200 μCi sodium chromate ^{51}Cr (ICN, Irvine, CA) for 1 h at 37°C, the cells were washed three times with RPMI-1640, resuspended in RPMI + 10% HIFCS and then exposed to NK cells at three different effector–target (E:T) ratios (50:1, 100:1, 150:1) in a total volume of 0.2 ml in 96-well, round-bottom microtitre plates in triplicate. After 4 h incubation at 37°C, 100 μl of supernatant was removed and the radioactivity was measured in a gamma counter. Maximum release was measured by incubation of the targets in 0.1% Triton X. Spontaneous release was measured by incubation of the targets in RPMI-1640 alone. The percentage of specific lysis was calculated as follows:

$$\text{Percentage cytotoxicity} = \frac{\text{experimental c.p.m.} - \text{spontaneous c.p.m.}}{\text{maximum c.p.m.} - \text{spontaneous c.p.m.}}$$

YAC-1, a cell line of a Moloney virus-induced lymphoma of A/Sn mice origin, sensitive to lysis by NK cells, originally from the Amercian-type culture collection, was used as target cell.

In vitro assay for NK activity

Splenic NK cells from untreated mice were either assayed directly (control) for cytotoxicity or were first treated *in vitro* with 0.05% Ins + 0.05% $InsP_6$ for 1 h at 37°C, washed three times with HBSS and then assayed for cytotoxicity using YAC-1 cells and diluted in RPMI-1640 + 10% HIFCS in different E:T ratios and ^{51}Cr release was measured in a gamma counter. This concentration of Ins + $InsP_6$ does not affect the cell viability as determined by trypan blue exclusion. A dose–response study was subsequently performed on splenic cells isolated from C3H/JSed mice (10 weeks of age). Three different concentrations (final concentration in the media: 0.05, 0.1, 0.5%) of $InsP_6$, Ins and $InsP_6$ + Ins were tested for percentage cytotoxicity. Following 1 h treatment, the splenic cells were washed and the cytotoxicity was evaluated in a 4-h ^{51}Cr release assay using YAC-1 target cells at 12:1, 25:1, 50:1 and 100:1 E:T ratios.

Results

NK-enriched spleen cell fraction from control mice without DMH treatment showed 31.32 ± 1.23% baseline NK activity. *In vivo* treatment with 1% $InsP_6$ + 1% Ins supplemented in drinking water increased NK activity by 58% (Table I). DMH treatment

Table I. Cytotoxicity of murine NK cells against YAC-1 target cells following *in vivo* treatment

Subcutaneous injection of mice	Supplementation in drinking water			
	None	$InsP_6$	Ins	$InsP_6$ + Ins
Saline (control)	31.32 ± 1.23	43.88 ± 1.97	44.88 ± 7.42	49.43 ± 2.91
DMH	19.38 ± 0.99	31.12 ± 1.87	32.10 ± 1.65	39.82 ± 1.62
Significance	$P < 0.005$	$P < 0.005$	$P < 0.01$	$P < 0.005$

Values are mean ± SEM of percentage cytotoxicity. Effector–target ratio = 150:1. The experiments were done in triplicate. The cytotoxicity was done with NK-enriched fractions of spleen cells from each experimental group: 19 spleens from 19 mice of DMH; 21 from DMH + $InsP_6$; 20 from DMH + Ins; 16 from DMH + $InsP_6$ + Ins. Five spleens from (from five mice) each control group provided cells from control (saline treatment).

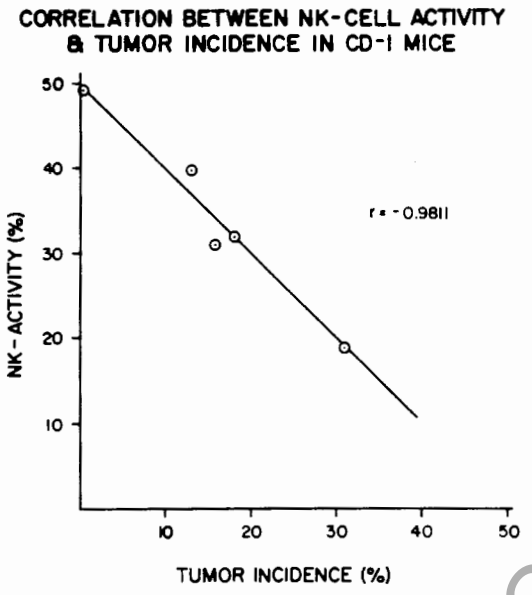

Fig. 1. Effect of Ins, $InsP_6$ on experimental animals. When NK activity is high, tumor incidence is reduced. The incidence of gross tumors for each experimental group was as follows (6): DMH, 31.6%; DMH + $InsP_6$, 19.0%; DMH + Ins, 15.0%; DMH + Ins + $InsP_6$, 12.5%. The NK cell activity of the corresponding groups are from Table I. There is a significant correlation between tumor incidence and NK activity ($r = -0.9811$, $P < 0.0005$).

alone significantly ($P < 0.005$) reduced the baseline NK activity by 38.1%. This reduction of NK activity may be connected with the immunosuppressive effects of chemical carcinogens (19). Treatment of mice with $InsP_6$ or Ins caused 40 and 43% increase of NK activity respectively over the baseline, and 61 and 66% respectively in DMH-treated animals. Treatment of mice with $InsP_6$ + Ins doubles (105% increase) the NK activity of DMH-injected mice. $InsP_6$ + Ins not only enhances the baseline NK activity of normal mice (by 58%), but also reverses the depressed NK activity in all groups as a result of carcinogen administration (Table I). Table I shows that the effects of Ins and $InsP_6$ were similar. However, the mixture of $InsP_6$ + Ins resulted in even further enhancement of NK activity in the control as well as DMH-injected mice. The consistency of depressed NK activity by DMH and enhanced activity by $InsP_6$ ± Ins is confirmed since the results are similar for all the three E:T ratios (data not shown). This enhancement in NK activity is inversely correlated ($r = -0.9811$, $P < 0.0005$) with tumor incidence

Table II. Cytotoxicity of NK cells from untreated mice following *in vitro* treatment with Ins + $InsP_6$

In vitro treatment	Effector–target ratio		
	150:1	100:1	50:1
None (control)	31.32 ± 1.23	22.56 ± 2.55	13.96 ± 1.71
Ins + $InsP_6$	52.03 ± 1.59	38.29 ± 1.36	27.16 ± 1.76
Significance	$P < 0.005$	$P < 0.005$	$P < 0.005$

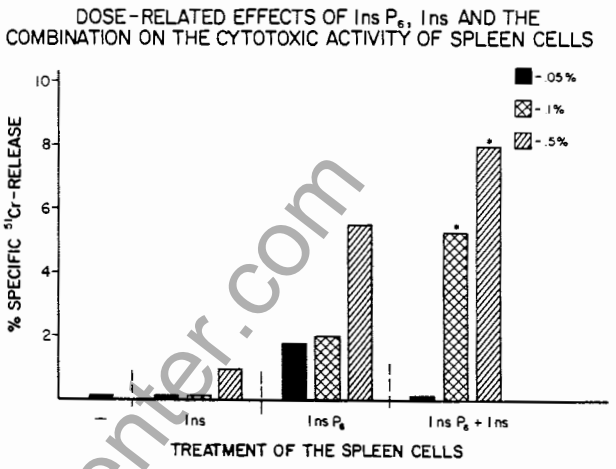

Fig. 2. Dose-related response of splenic cell cytotoxicity following *in vitro* treatment with $InsP_6$, Ins and $InsP_6$ + Ins. Splenic cells from 10-week-old C3H/JSed mice were either untreated control (−), or treated with the respective compounds at 0.05, 0.1 and 0.5% concentration for 1 h followed by 4-h ^{51}Cr release assay of YAC-1 target cells. Note that maximum effect is seen with the highest concentration of $InsP_6$ + Ins. *Denotes statistically significant differences ($P < 0.05$ or better) between $InsP_6$ and $InsP_6$ + Ins at 0.1 and 0.5%. Data points are means of percentage cytotoxicity of quintuplicates, 50:1 E:T ratio.

(Figure 1) (6). It is to be noted that *in vivo* treatment of mice with $InsP_6$ + Ins maximally induced NK activity. Similar induction in NK activity was also demonstrated when the NK-enriched fraction from normal untreated mice was exposed to $InsP_6$ + Ins. In order to see whether the *in vivo* results correlated with *in vitro* NK activity, we treated the splenic NK cells with 0.05% Ins + 0.05% $InsP_6$ and the NK activity was measured following washing. In all three E:T ratios Ins + $InsP_6$ increased the NK activity by 66–95% (Table II). The results of a dose–response study (Figure 2) demonstrate that $InsP_6$ ± Ins enhances the cytotoxicity in a dose-related manner. While Ins alone does not significantly enhance the cytotoxicity, $InsP_6$ and mixture of $InsP_6$ + Ins demonstrate this phenomenon. In keeping with the *in vivo* results, $InsP_6$ + Ins at maximum dose (0.5 %) induced the strongest enhancement of cytotoxicity. In addition to the fact that, compared to the untreated control, $InsP_6$ enhanced cytotoxicity, a combination of $InsP_6$ + Ins also significantly enhanced the cytotoxic compared to $InsP_6$ alone($P < 0.05$ for 0.1% concentrations and $P < 0.01$ for 0.5% concentrations).

Discussion

It is not clear how NK cells select their targets and by what mechanism they kill the target. However, physical contact between the effector and target is very essential. It has been suggested that NK cell granules containing perforin may kill the

targets, while others contend that serine proteases activate pore-forming complex (20,21). In addition, the translocation of acid hydrolases and free oxygen radical generation have been involved in cytotoxicity (23,24). It has also been suggested that NK cytotoxicity factor (NKCF) is important for cytotoxicity (24). The target cell binds NKCF and is subsequently lysed. Intimate contact between the plasma membrane of both cells affects the cytotoxic reaction. Little is known, however, about the initial events that take place after target cells bind to NK cells. Others have shown that 12-*O*-tetradecanoylphorbol-13-acetate and calcium ionophore, which could mimic phosphoinositide hydrolysis products by activating protein kinase and raising intracellular calcium levels, can induce proliferation of NK cells as well as the release of NKCF (25). Likewise, interleukin-2 (IL-2) has also been reported to induce cytolytic activity and enhance NK activity (26,27). Acquired immunodeficiency syndrome (AIDS) virus appears to depress NK activity and IL-2 enhances the depressed NK activity of AIDS patients (28,29). It appears that $InsP_3$ is generated in activated NK cells (30). The specific role of $InsP_3$ and its appearance during NK cytotoxicity is far from clear. We show that delivery of Ins + $InsP_6$ enhances NK activity. Since phytases and phosphatases in the food items as well as in the intestine dephosphorylate $InsP_6$ to the lower-phosphorylated forms and commercial $InsP_6$ also contains lower-phosphorylated Ins (31), we hypothesized that addition of Ins would allow ready reaction with free phosphates to produce more $InsP_3$ which could exert a negative feedback, inhibition and decreased cell proliferation (5,6). But it is not known how the addition of Ins potentiates the $InsP_6$-mediated enhanced cytotoxicity. Certainly, the hypothetical enhanced $InsP_3$ could affect the membrane phosphatidyl inositol proteins which may be important in attachment and subsequent fusion with the target cell. We have observed that the NK cells fuse with the target cells followed by accumulation of secondary lysosomes in the target cell prior to cell death (data not shown). However, it does not rule out the other proposed mechanisms.

We demonstrate that DMH depresses the NK activity and inositol compounds significantly increase the NK activity and reduce the incidence of tumor. In support of our observation (5,6,32), others have reported that $InsP_6$ significantly lowers tumor growth rate (33). These findings suggest that changes in NK activity are related to progressive cancer growth. Suppression of tumor incidence in experimental animals may at least, in part, be mediated by NK cells. Since commercial $InsP_6$ contains lower-phosphorylated forms of Ins, and the phosphatases and phytases present on the cell surface can dephosphorylate $InsP_6$ (31), the relative importance of each of the inositol phosphates in mediating the observed effect is not known at this time. Further studies are certainly needed to answer these questions. Since inositol compounds are effective in enhancing the NK activity both *in vivo* and *in vitro*, they may have potential application in therapy of cancer and other diseases that are associated with depressed NK cytotoxicity.

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