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Antitumour Potential of a Polysaccharide-rich Substance from the Fruit Juice of *Morinda citrifolia* (Noni) on Sarcoma 180 Ascites Tumour in Mice

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An immunomodulatory polysaccharide-rich substance (Noni-ppt) from the fruit juice of *Morinda citrifolia* has been found to possess both prophylactic and therapeutic potentials against the immunomodulator sensitive Sarcoma 180 tumour system. The antitumour activity of Noni-ppt produced a cure rate of 25%–45% in allogeneic mice and its activity was completely abolished by the concomitant administration of specific inhibitors of macrophages (2-chloroadenosine), T cells (cyclosporine) or natural killer (NK) cells (anti-asialo GM1 antibody). Noni-ppt showed synergistic or additive beneficial effects when combined with a broad spectrum of chemotherapeutic drugs, including cisplatin, adriamycin, mitomycin-C, bleomycin, etoposide, 5-fluorouracil, vincristine or camptothecin. It was not beneficial when combined with paclitaxel, cytosine arabinoside, or immunosuppressive anticancer drugs such as cyclophosphamide, methotrexate or 6-thioguanine. Noni-ppt also demonstrated beneficial effects when combined with the Th1 cytokine, interferon gamma, but its activity was abolished when combined with Th2 cytokines, interleukin-4 or interleukin-10, thereby suggesting that Noni-ppt induces a Th1 dominant immune status *in vivo*. The combination of Noni-ppt with imexon, a synthetic immunomodulator, also demonstrated beneficial effects, but not when combined with the MVE-2 copolymer, a high molecular weight immunomodulator. It was also not effective when combined with interleukin-2 or interleukin-12. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: antitumour; immunomodulator; polysaccharide; Sarcoma 180 ascites tumour; fruit juice; *Morinda citrifolia*.

INTRODUCTION

It was previously shown that the fruit juice of *Morinda citrifolia* (Noni) contains a polysaccharide-rich substance (Noni-ppt) with therapeutic potential on the Lewis lung carcinoma (LLC) in syngeneic mice (Hirazumi *et al.*, 1992, 1994, 1996; Hirazumi and Furusawa, 1999). Noni-ppt is essentially nontoxic and has a potent immunostimulating effect that is abolished by inhibitors of macrophages or T cells. This study continues to characterize the antitumour potential of Noni-ppt against the Sarcoma 180 (S180) ascites tumour in mice, a widely recognized immunomodulator-sensitive allogeneic tumour system that is useful in finding potential agents for the prevention of recurrence of cancer diseases (Bibby, 1999; Kaneda *et al.*, 1998).

MATERIALS AND METHODS

Preparation of agents. The procedure for preparing Noni-ppt from the ripe fruit juice of *Morinda citrifolia* (Noni) collected from the islands of Hawaii has been previously described (Hirazumi and Furusawa, 1999). Briefly, after removing the insoluble parts of the original fruit juice by centrifugation, copious amounts of 95% ethanol were added and the subsequent precipitate (Noni-ppt) was dissolved in distilled water (dH_2O). The ethanol-precipitation cycle was repeated several times according to the methods reported for isolation of polysaccharides (Fujihara *et al.*, 1984). Noni-ppt was also isolated from the commercially available Tahitian Noni juice supplied by Morinda Inc., Utah. The dried Noni-ppt was dissolved in dH_2O and filtered through a 0.2 μm cellulose acetate membrane before use. All 12 chemotherapeutic drugs, adriamycin, cisplatin, mitomycin C, bleomycin, etoposide, 5-fluorouracil (5-FU), vincristine (VCR), paclitaxel, 6-thioguanine (6-TG), methotrexate (MTX), cytosine arabinoside (ara-C) and camptothecin; five recombinant mouse cytokines, interferon gamma (IFN-r), interleukin-2 (IL-2), IL-12, IL-4 and IL-10; and anti-asialo GM1 antibody were purchased from commercial sources. Synthetic chemicals, imexon and the MVE-2 copolymer were kindly supplied by Dr M. A. Chirigos of the National Cancer Institute.

Animal-tumour system. S180 tumour cells, originally obtained from the American Type Culture Collection, have been maintained in this laboratory for several years as the ascites form by serial passages intraperitoneally

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(i.p.) in DBA/2 mice. For antitumour testing, an aliquot (0.15 mL) of a 20× dilution of the ascites in MEM medium (2–6 × 10^5 tumour cells) was inoculated i.p. into young adult (18–20 g) male and female mice of DBA/2, C57BL/6 or BALB/c strain. Treatment with agents, i.p., were performed prophylactically or therapeutically. Daily recording of body weight (B.W.) for each mouse was done for 13–17 days or until the increase in B.W. reached approximately l0 g in the control groups without deterioration of health. The increase in body weight paralleled the growth of the ascites tumour. Treated mice that showed no development of ascites during that time period were further observed for 40–50 days to confirm that the mice had been cured.

Statistical analysis. Experimental results were analysed for their significance by the Student's two-tailed *t*-test and Fisher's exact probability test.

RESULTS AND DISCUSSION

Effect of Noni-ppt on the S180 ascites tumour in mice

In addition to the antitumour potential of Noni-ppt against the syngeneic LLC tumour that originated and can grow only in the C57BL/6 strain of mice previously reported (Hirazumi and Furusawa, 1999), it is now demonstrated that Noni-ppt also exhibits significant antitumour activity against the allogeneic S180 ascites tumour which can grow in any strain of mice. Table 1 shows the summarized results of 45 experiments performed during recent years using the established optimal dose of Noni-ppt (0.5 mg/mouse). This dose is 1/20 of the maximum tolerable dose for multiple daily i.p. injections. It was found that the Noni-ppt, isolated from both the ripe fruit juice of *Morinda citrifolia* from Hawaii and the commercially available Tahitian Noni juice, produced a significant number of cured mice (27%–31%) with therapeutic treatment and a significant number of mice completely resistant to tumour invasion (45%–53%) with prophylactic treatment. The growth of the ascites as measured by the increase in body weight of mice was also significantly reduced (T/C: 42%–57%). There were no significant differences for the growth rate of the tumour and the antitumour activity of Noni-ppt among the three strains (DBA/2, C57BL/6 and BALB/c) used (not itemized in Table 1).

Abrogation of the antitumour potential of Noni-ppt with concomitant treatment of immune cell inhibitors in mice

Our previous study with the LLC system demonstrated that the antitumour potential of Noni-ppt was abolished by the concomitant treatment with specific inhibitors of macrophages and T cells (Hirazumi and Furusawa, 1999). The present prophylactic experiment with the S180 tumour system (Table 1) is also suggestive of the involvement of the innate immune system, possibly including NK cells. Therefore, the study invesigated whether the antitumour activity of Noni-ppt involved the three major components of the cellular immune system, the macrophages, NK cells and T cells, by using the individual inhibitors; 2-chloroadenosine (Schultz *et al.*, 1986), antiasialo GM1 antibody (Schultz *et al.*, 1986) and cyclosporine (DiPadova, 1989; TenHagen *et al.*, 1998), respectively. Table 2 shows the results of experiments performed prophylactically and therapeutically. It was found that both the prophylactic and therapeutic potentials of Noni-ppt were completely abolished by concomitant treatment with these inhibitors. The ascites tumour developed similarly to the untreated control mice. This demonstrates that all three of these immune cells must be concertedly functioning to elicit the antitumour potential of Noni-ppt. Thus, if even one of these immune cells becomes non-functioning, the activity of Noni-ppt is eliminated.

Table 1. Antitumour activity of Noni-ppt on Sarcoma 180 ascites tumour in mice

Agent	Dose/mouse	Increase in body weight on day 14–16 (g)	Number of mice survived/total on day 40–50
Therapeutic treatment			
Control (H_2O, 0.1 mL)	9.5 ± 2.0	T/C	0/94
Noni-ppt (Hawaii) 0.5 mg	4.0 ± 2.1	42%[c]	28/91 (31%)[c]
Control (H_2O, 0.1 mL)	10.6 ± 3.9		0/27
Noni-ppt (Tahiti) 0.5 mg	4.6 ± 2.7	43%[c]	9/33 (27%)[b]
Prophylactic treatment			
Control (H_2O, 0.1 mL)	12.5 ± 1.7	T/C	0/75
Noni-ppt (Hawaii) 0.5 mg	6.5 ± 2.4	52%[c]	39/74 (53%)[c]
Control (H_2O, 0.1 mL)	7.7 ± 1.9		0/20
Noni-ppt (Tahiti) 0.5 mg	4.4 ± 0.3	57%[a]	9/20 (45%)[b]

[a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ compared with controls.
The 2–6 × 10^5 tumour cells were inoculated intraperitoneally (i.p.) into mice (18–21 g) on day 0. Therapeutic treatment with Noni-ppt (0.5 mg dissolved in 0.1 mL H_20) began on day 1 or 2, continued every other day, a total of 4–5 injections, i.p. Prophylactic treatment with Noni-ppt (0.5 mg, i.p.) was done on days 5, 3, 2 and 1 before tumour inoculation, a total of 4 injections. This is the sum of 45 experiments performed over 4 years using three strains of mice (DBA/2, C57BL/6 and BALB/c) and two different sources of Noni-ppt, from Hawaii or Tahiti.

Table 2. Abrogation of the antitumour potential of Noni-ppt on S180 ascites tumour by concomitant treatment of inhibitors of immune cells

Agent and schedule	Dose/mouse	Increase in body weight on day 14–16	Number of mice survived/total on day 40–50
Therapeutic treatment			
Control (H_2O, 0.1 mL) day 1, 3, 5, 7	9.5 ± 2.7	T/C	0/4
Noni-ppt 0.5 mg, day 1, 3, 5, 7	3.3 ± 2.5	35%[a]	2/4
Cl-Ade 0.1 mg, day 1, 3, 5, 7	12.0 ± 2.8	126%	0/4
Noni-ppt + Cl-Ade same as above	8.8 ± 1.3	93%	0/4
Cys-A 2 mg, day 1, 3, 5, 7	11.3 ± 1.0	119%	0/4
Noni-ppt + Cys-A same as above	8.8 ± 4.3	93%	0/4
Prophylactic treatment			
Control (H_2O, 0.1 mL) day –6, –4, –3, –1	11.0 + 2.7	T/C	0/8
Noni-ppt 0.5 mg, day –6, –4, –3, –1	6.2 ± 4.4	55%[a]	4/8[a]
Cl-Ade 0.1 mg, day –6, –4, –3, –1	14.1 ± 4.1	127%	0/8
Noni-ppt + Cl-Ade same as above	13.8 ± 3.7	125%	0/8
Control (H_2O, 0.1 mL) day –5, –3, –2, –1	11.3 ± 3.2		0/10
Noni-ppt 0.5 mg, day –5, –3, –2, –1	7.3 ± 3.9	64%[a]	4/9[a]
Cys-A 2 mg, day –5, –3, –2, –1	13.7 ± 2.1	121%	0/9
Noni-ppt + Cys-A same as above	14.1 ± 2.2	127%	0/10
Therapeutic treatment			
Control (H_2O, 0.1 mL) day 2, 3, 5, 7	7.8 ± 3.0	T/C	0/4
Noni-ppt 0.5 mg, day 2, 3, 5, 7	4.5 ± 1.4	58%[a]	1/4
Anti-asialo GM1 20 μl, as above	7.5 ± 2.0	96%	0/4
Noni-ppt + Anti-asialo, as above	7.3 ± 1.7	94%	0/4
Prophylactic treatment			
Control (H_2O, 0.1 mL) day –9, –7, –4, –2	12.5 ± 2.6	T/C	0/4
Noni-ppt 0.5 mg, day –9, –7, –4, –2	4.0 ± 2.7	32%[a]	2/4
Anti-asialo GM1 20 μl, as above	10.3 ± 3.3	82%	0/4
Noni-ppt + Anti-asialo, as above	11.8 ± 1.5	94%	0/4

[a] $p < 0.05$ compared with controls. Cl-Ade, 2-chloroadenosine; Cys-A, cyclosporine; Anti-asialo; anti-asialo GM1 antibody; day –6, on day 6 prior to tumour inoculation.
The 2–6 × 10^5 tumour cells were inoculated i.p. into mice (DBA/2 and C57BL/6, 18–21 g) on day 0. Treatment with Noni-ppt, the inhibitors of immune cells (Cl-Ade to macrophages, Cys-A to T-lymphocytes, and Anti-asialo GM1 to NK cells), or the combinations was performed therapeutically or prophylactically as shown above. Increase in body weight was monitored for 2 weeks and recorded on day 14. Treated mice that showed no development of ascites were observed for 40–50 days and were considered cured.

Effect of chemoimmunotherapy of Noni-ppt with standard cytotoxic drugs

Our previous study with the LLC system demonstrated that significant beneficial effects of Noni-ppt were obtained when combined with several conventional cytotoxic drugs. In this study, the number of cytotoxic drugs was extended by testing the Noni-ppt with a broader spectrum of standard chemotherapeutic drugs with different mechanisms of action, including DNA-alkylators (cyclophosphamide, cisplatin, mitomycin-C), DNA-intercalators (adriamycin, bleomycin), topoisomerase inhibitors (etoposide, camptothecin), mitotic inhibitors (vincristine, paclitaxel) and antimetabolites (5-fluorouracil, methotrexate, 6-thioguanine, cytosine arabinoside). Cytotoxic drugs were administered at the optimal single dose 1 or 2 days after tumour inoculation (except on day 9 in Exp. 4, day 10 in Exp. 2 or multiple injections in Exp. 13 in Table 3). Treatment with Noni-ppt was given at the optimal dose of 0.5 mg/mouse beginning on day 1 or 2 and continued for a total of 4 or 5 injections (Table 3). It was found that the combination of Noni-ppt with cisplatin (4–50 μg: 1/36–1/3 of the maximum tolerable dose (MTD)), adriamycin (5–20 μg: 1/20–1/5 MTD), mitomycin-C (0.1–0.3 units: 1/10–3/10 MTD), etoposide (0.05–0.4 mg: 1/24–1/3 MTD), 5-fluorouracil (0.2 mg: 1/5 MTD), vincristine (5 mg: 1/4 MTD) or camptothecin (12–50 μg: 1/12–1/3 MTD) demonstrated significantly better inhibition of tumour growth and produced a higher number of long-term tumour-free survivors than those treated with the single agent alone. These relatively lower doses of cytotoxic drugs had been established from our previous papers (Furusawa and Furusawa, 1990; Hirazumi and Furusawa, 1999), our unpublished preliminary data and other reference articles in which tumour growth had been moderately inhibited without any apparent toxicity (Corbett *et al.*, 1996; Kaneda *et al.*, 1990; Jani *et al.*, 1992; Parkins *et al.*, 1993; Li *et al.*, 1987). Preclinical studies have also demonstrated that moderate or low-dose chemotherapy has advantages when combined with immunostimulating agents because of the synergistic or additive antitumour effects without any accompanying toxicity (Verloes *et al.*, 1981; Li *et al.*, 1987). The combnation of Noni-ppt with such lower doses of the aforementioned cytotoxic drugs showed increased antitumour activity without any increase in toxicity. On the other hand, a combination of Noni-ppt with

Table 3. Effect of Noni-ppt in combination with standard cytotoxic drugs on S180 ascites tumour in mice

Agent and schedule	Dose/mouse	Increase in body weight on day 14–16	Number of mice survived/total on day 40–50
Control (Exp. 1)	10.3 ± 1.6	T/C	0/13
Noni-ppt 0.5 mg, day 1, 2, 3, 5, 7	4.3 ± 1.9	42%	8/13
Cisplatin 25, 10 or 4 μg, day 1	2.7 ± 1.2	26%	8/14
Cisplatin + Noni-ppt, same as above	1.5 ± 1.3	15%[a]	14/14[a]
Control (Exp. 2)	10.3 ± 1.6		0/27
Noni-ppt 0.5 mg, day 2, 3, 5, 7, 9	5.2 ± 1.2	50%	4/15
Cisplatin 50 or 20 μg, day 10	5.7 ± 1.7	55%	6/28
Cisplatin + Noni-ppt, same	3.1 ± 1.9	30%[a]	17/29[a]
Control (Exp. 3)	11.3 ± 1.6		0/19
Noni-ppt 0.5 mg, day 2, 3, 5, 7, 9	5.2 ± 1.6	46%	2/16
Adriamycin 20 or 5 μg, day 2	6.6 ± 1.6	58%	7/19
Adriamycin + Noni-ppt, same	3.1 ± 1.8	27%[a]	13/18[a]
Control (Exp. 4)	9.5 ± 1.7		0/10
Noni-ppt 0.5 mg, day 2, 3, 5, 7	5.6 ± 1.2	59%	4/15
Adriamycin 20 μg, day 9	4.7 ± 1.0	49%	1/11
Adriamycin + Noni-ppt, same	3.2 ± 1.0	34% (NS)	4/11 (NS)
Control (Exp. 5)	10.7 ± 2.5		0/9
Noni-ppt 0.5 mg, day 2, 3, 5, 7	4.6 ± 3.9	43%	2/9
Mitomycin-C 25 or 10 μg, day 2	4.7 ± 4.5	44%	3/9
Mitomycin-C + Noni-ppt, same	1.0 ± 2.0	9%[a]	8/9[a]
Control (Exp. 6)	10.4 ± 1.6		0/8
Noni-ppt 0.5 mg, day 2, 3, 5, 7	6.9 ± 2.7	58%	1/8
Bleomycin 0.3 or 0.1 unit, day 2	6.8 ± 2.7	65%	1/8
Bleomycin + Noni-ppt, same	2.0 ± 2.9	19%[a]	4/8[a]
Control (Exp. 7)	9.4 ± 1.1		0/31
Noni-ppt 0.5 mg, day 2, 3, 5, 7	3.8 ± 1.1	40%	12/31
Etoposide 0.4, 0.2, 0.1 or 0.05 mg, day 1	3.7 ± 1.2	39%	17/31
Etoposide + Noni-ppt, same	2.6 ± 1.9	28%[a]	24/31[a]
Control (Exp. 8)	10.0 ± 2.3		0/11
Noni-ppt 0.5 mg, day 2, 3, 5, 7	6.2 ± 2.1	62%	4/13
5-Fluorouracil 0.2 mg, day 2	7.2 ± 3.8	72%	4/11
5-Fluorouracil + Noni-ppt, same	3.1 ± 1.3	31%[a]	8/11[a]
Vincristine 5 μg, day 2	5.9 ± 2.7	59%	3/10
Vincristine + Noni-ppt, same	3.4 ± 1.5	34%[a]	8/11[a]
Control (Exp. 9)	9.9 ± 3.1		0/19
Noni-ppt 0.5 mg, day 2, 3, 5, 7	3.8 ± 2.8	38%	8/17
Cyclophosphamide 3, 2, or 1 mg, day 2	6.4 ± 4.5	65%	6/25
Cyclophosphamide + Noni-ppt, same	5.2 ± 4.0	53%	15/25 (NS)
Control (Exp. 10)	6.9 ± 1.6		0/27
Noni-ppt 0.5 mg, day 2, 3, 5, 7	4.0 ± 0.6	58%	5/28
Paclitaxel 0.5, 0.3, 0.1 or 0.5 mg, day 2	4.5 ± 1.0	65%	8/24
Paclitaxel + Noni-ppt, same as above	4.2 ± 0.9	61%	10/28 (NS)
Control (Exp. 11)	9.3 ± 1.5		0/8
Noni-ppt 0.5 mg, day 2, 3, 4, 6, 8	0.8 ± 1.4	7%	4/8
6-Thiguanine 0.2 mg, day 2	10.2 ± 1.8	110%	0/8
6-Thiguanine + Noni-ppt, same as above	4.9 ± 2.0	53%	1/8 (NS)
Control (Exp. 12)	10.6 ± 2.2		0/7
Noni-ppt 0.5 mg, day 2, 3, 5, 7, 9	2.8 ± 2.1	26%	3/7
Methotrexate 0.6 mg, day 2	5.0 ± 2.5	47%	0/7
Methotrexate + Noni-ppt, same as above	4.5 ± 3.0	42%	0/7 (NS)
Control (Exp. 13)	11.0 ± 2.5		0/9
Noni-ppt 0.5 mg, day 2, 3, 4, 7	7.3 ± 3.3	66%	2/9
Cytosine arabinoside 1 mg, day 2, 3, 4, 7	9.0 ± 2.8	82%	0/9
Cytosine arabino. + Noni-ppt, same	5.8 ± 3.0	53%	2/9 (NS)
Control (Exp. 14)	11.8 ± 4.0		0/11
Noni-ppt 0.5 mg, day 1, 2, 4, 6	5.0 ± 1.4	42%	3/11
Camptothecin 50, 25 or 12 μg, day 1	2.9 ± 2.8	25%	7/13
Camptothecin + Noni-ppt, same as above	0.8 ± 1.2[a]	7%	12/12[a]

[a] $p < 0.05$ compared with combination vs single agent.
NS, not significant compared with single agent.
The 2–6 × 10^5 tumour cells were inoculated i.p. into mice (DBA/2, C57BL/6 or BALB/c: 18–21 g) on day 0. Treatment with cytotoxic drugs was done i.p. on day 1 or 2 only, except on day 10 in Exp. 2 or day 9 in Exp. 4. Treatment with Noni-ppt was done by multiple (4–5 X) i.p. injections starting on day 1 or 2. Control groups were given multiple injections of H_2O (0.1 mL). Each numbered experiment is the sum of several individual experiments with the same cytotoxic drugs with the same or reduced doses. In Exp. 13, cytosine arabinoside was given 4 times. In Exp. 14, Tahitian Noni-ppt was used.

cytotoxic drugs such as cyclophosphamide, paclitaxel, 6-thioguanine, methotrexate or cytosine arabinoside was not more effective than those of the single agents alone. It seems that the combination of Noni-ppt with DNA-binding agents (except cyclophosphamide) enhanced the antitumour effects while the combination with DNA/RNA synthesis inhibitors (except 5-fluorouracil) and agents with immunosuppressive properties (such as cyclophosphamide, methotrexate and 6-thioguanine) were not beneficial. Thus, our data can now help to predict which of the first-line chemotherapeutic drugs would have beneficial effects if combined with Noni-ppt as a supplemental agent for future clinical applications to prevent the recurrence of human cancers.

Effect of Noni-ppt in combination with immune-related cytokines or chemical immunostimulators

Our previous study demonstrated that Noni-ppt was capable of stimulating the release of the cytokines, TNF-a, IL-1b, IL-12, IFN-r and IL-10 from murine effector cells while suppressing IL-4 production and having no effect on IL-2 production (Hirazumi and Furusawa, 1999). This suggested that the Noni-ppt stimulates a Th1 cell-mediated immune response while suppressing the Th2 humoral response in mediating its antitumour effect. Therefore, it is of interest to know whether the antitumour activity of Noni-ppt would be affected *in vivo* when combined with several of these cytokines, specifically the Th1 cytokines, IL-2, IL-12 and IFN-r and the Th2 cytokines, IL-4 and IL-10. Table 4 shows the results. Of the five recombinant mouse cytokines used for combination with Noni-ppt, only IFN-r showed significant beneficial effects therapeutically (Exp. 1) and prophylactically (Exp. 2). Of important interest is that all the mice pretreated with the combination of IFN-r and Noni-ppt were completely resistant to the S180 tumour invasion. The individual cytokines IL-2, IL-12 or IL-4 possessed antitumour activity when administered alone but were not any more effective when combined with Noni-ppt. Additionally, IL-4 almost abolished the antitumour activity of Noni-ppt (Exp. 5). IL-10 exhibited no antitumour activity alone, and also negated the antitumour activity of Noni-ppt (Exp. 6). This suggests that these Th2 cytokines may

Table 4. Effect of Noni-ppt in combination with immunomodulatory cytokines or chemicals on S180 ascites tumour in mice

Agent and schedule	Dose/mouse	Increase in body weight on day 14–16	Number of mice survived/total on day 40–50
Control (Exp. 1)	11.8 ± 2.0	T/C	0/16
Noni-ppt 0.5 mg, day 2, 3, 5, 7	3.6 ± 1.8	31%	4/13
Interferon-r 1000 unit, same	5.0 ± 2.1	42	4/13
Interferon-r + Noni-ppt, same	3.4 ± 1.1	29 (NS)	12/17[a]
Control (Exp. 2)	11.2 ± 2.3		0/8
Noni-ppt 0.5 mg, day –5, –3, –2, –1	5.4 ± 2.0	46%	4/8
Interferon-r 1000 unit, same	5.8 ± 2.2	52	5/9
Interferon-r + Noni-ppt, same	1.4 ± 1.0	13[a]	9/9[a]
Control (Exp. 3)	10.3 ± 3.0		0/10
Noni-ppt 0.5 mg, day 2, 3, 5, 7	4.0 ± 3.2	40%	3/9
Interleukin-2 140 unit, same	6.2 ± 4.0	62	3/9
Interleukin-2 + Noni-ppt, same	3.5 ± 3.3	35 (NS)	4/9 (NS)
Control (Exp. 4)	7.6 ± 1.8		0/15
Noni-ppt 0.5 mg, day 1, 2, 4, 6	3.3 ± 2.0	43%	3/12
Interleukin-12 0.1 μg, same	3.8 ± 2.0	50	3/12
Interleukin-12 + Noni-ppt, same	2.7 ± 2.2	36 (NS)	4/12 (NS)
Control (Exp. 5)	10.4 ± 2.5		0/8
Noni-ppt 0.5 mg, day 1, 2, 4, 6	4.6 ± 2.2	44%	2/8
Interleukin-4 0.1 μg, same	5.9 ± 1.8	57	1/8
Interleukin-4 + Noni-ppt, same	7.9 ± 1.5	76[a]	0/8 (NS)
Control (Exp. 6)	9.5 ± 1.9		0/8
Noni-ppt 0.5 mg, day 1, 2, 4, 6	4.5 ± 2.1	47%	2/8
Interleukin-10 0.1 μg, same	9.2 ± 2.1	97	0/8
Interleukin-10 + Noni-ppt, same	8.5 ± 2.6	89 (NS)	0/8 (NS)
Control (Exp. 7)	8.1 ± 1.6		0/34
Noni-ppt 0.5 mg, day 1, 3, 5, 7	3.8 ± 2.9	48%	6/27
MVE-2 1 or 0.5 mg, same	5.6 ± 1.4	70	6/30
MVE-2 + Noni-ppt, same	2.7 ± 1.5	34 (NS)	7/24 (NS)
Control (Exp. 8)	7.9 ± 0.9		0/31
Noni-ppt 0.5 mg, day 1, 3, 4, 5, 7	4.7 ± 1.5	59%	7/27
Imexon 2 mg, same	4.5 ± 1.2	63	3/18
Imexon + Noni-ppt, same	2.9 ± 0.8	36[a]	15/25[a]

[a] $p < 0.05$ compared with combination vs single agent
NS, not significant. r, gamma.
MVE-2, maleic anhydride divinylether copolymer (MW 15 000);
Imexon, 4-imino-1, 3-diazobicycle-(3, 1, 0)-hexan-2-one.
Tahitian Noni-ppt was used in Exp. 4, 5, 6, 7 and 8.

be neutralizing the Th1 biased antitumour reaction induced by the IL-12 endogenous release from peritoneal macrophages by Noni-ppt (Hirazumi and Furusawa, 1999). The optimal doses of each cytokine used has been established from preliminary experiments and other references (Taylor *et al.*, 2000 for IL-4; Kikuchi *et al.*, 1999a; Kimura *et al.*, 2000 for IL-2; Kaufman *et al.*, 1999; Huhn *et al.*, 1999 for IL-10; Iwasaki *et al.*, 2000; Kikuchi *et al.*, 1999b; Kimura *et al.*, 2000; Nakajima *et al.*, 2001 for IL-12; Shiau *et al.*, 2001 for IFN-r). Among the two synthetic immunostimulators used (Exp. 7 and 8), the antitumour activity of imexon (Chirigos *et al.*, 1990; Furusawa *et al.*, 1992) was greater when combined with Noni-ppt, while the activity of MVE-2 (Kaneda *et al.*, 1998; Furusawa and Furusawa, 1988, 1989) was not any more beneficial by combination. Imexon is a low molecular weight agent, while MVE-2 copolymer has a m.w. of 15 000. Both agents were used positive immunostimulator controls in the S180 tumour system. Our hypothesis that the Noni-ppt acts as an immunostimulator comes from our previous *in vitro* study that showed that Noni-ppt initially stimulates macrophages to release several cytokines, including IL-12, which activates NK cells and naive T helper (Th0) cells to differentiate toward the potent antitumour Th1-immune status with enhanced production of IFN-r and decreased production of IL-4 (Hirazumi and Furusawa, 1999). It is generally accepted that antitumour immunity is enhanced by the Th1 cellular immune response but not the Th2 humoral immune response (Song *et al.*, 2000). Our present *in vivo* study (Table 4) has endorsed our hypothesis of Noni-ppt induced Th1-immune reaction. Addition of exogenous IFN-r, a Th1 cytokine, enhanced the antitumour activity of Noni-ppt while exogenous IL-4 or IL-10, both Th2 cytokines, neutralized its activity. IFN-r is the final end product of the Th1 response and it directly activates cytotoxic T lymphocytes (CTL), NK cells and macrophages. Consequently, the activated CTLs and NK cells autocrinically release more IFN-r, which itself is cytotoxic and stimulates tumour cells to express Fas, a transmembrane protein that triggers apoptosis when bound to the Fas ligand that is expressed only on the activated NK and T cells (Xu *et al.*, 1998; Shiau *et al.*, 2001). IL-12 is a NK cell-stimulating factor and an initiator of the Th1 immune response (Kikuchi *et al.*, 1999b; Ohkawa *et al.*, 2001) and IL-2 is a T cell growth factor (Kikuchi *et al.*, 1999b), therefore it is not clear why these Th1-related cytokines did not enhance the antitumour activity of Noni-ppt (Exp. 3, 4) as IFN-r did (Exp. 1, 2). Possibly because these cytokines are intermediate products of the Th1 immune response rather than the final product like IFN-r, they competed with the endogenous production of IL-12, stimulated by Noni-ppt. IL-4 acts in a way opposite to the Th1 response (Trinchieri, 1995) by stimulating the Th2 response characterized by increased IgGl antibody production (Ishii *et al.*, 1999). IL-4 exhibits pleiotropic actions and can potentially cause murine tumour regression via enhancement of CTL activity and/or direct antiproliferative effects, although the phase II clinical trials in patients with lymphoma were not successful (Taylor *et al.*, 2000). The antitumour activity of IL-4 in the S180 tumour system has been confirmed but its activity, as well as that of Noni-ppt, were both suppressed when they were combined (Exp. 5). This effect is likely to be due to the fact that IL-4 induces a Th2 immune response while Noni-ppt induces an opposing Th1 response. IL-10, another Th2 cytokine that is involved in a feedback mechanism to modulate the Th1 pathway by down-regulating the effects of IFN-r (Arulanandam *et al.*, 2000), also eliminated the antitumour activity of Noni-ppt as did IL-4. Although IL-10 has been reported to enhance the cytolytic activity of CTLs and NK cells (Kim *et al.*, 2000) and possess antitumour activity by inducing nitric oxide release from macrophages (Zidek and Frankova, 1999; Sun *et al.*, 2000) and enhance the therapeutic effectiveness of anticancer vaccines in mice (Kaufman *et al.*, 1999), it was not effective in the S180 tumour system when administered alone. In advanced cancer patients, there appear to be higher levels of Th2 cytokines and lower levels of Th1 cytokines, thus indicating an impairment of the cell-mediated immunity (Goto *et al.*, 1999). Therefore, it seems that administration of Th1 cytokines and/or Th1 immunostimulators such as Noni-ppt have a great potential for cancer immunotherapy in cancer patients.

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