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Enhancement of Immune Responses to Neem Leaf Extract (*Azadirachta indica*) Correlates with Antineoplastic Activity in BALB/c-mice

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Abstract. *An aqueous plant extract from Azadirachta indica and its chromatographic fraction F1 (neem extract) exerted immunomodulating and antimetastatic activities in BALB/c-mice. Regular subcutaneous administration of neem extract yielded significantly increased spleen weight and significant enhancement of peritoneal macrophage activity in the chemiluminescence assay, and activation marker CD-44 expression. The thymus weight and thymocyte counts did not show significant differences in the control and neem extract-treated groups, however, determination of peripheral blood cells revealed significant up-regulations of leukocyte subsets, the lymphocytes and monocytes. Flow cytometric analaysis of lymphocyte supopulations documented increased counts of CD-4 and CD-8 cells and an inreased activation marker expression on lymphocytes (CD-25) and monocytes (MAC-3) in neem-treated mice compared to the control animals. To evaluate the antimetastatic activity of neem extract, sarcoma L-1 cells and lymphosarcoma RAW cells were intravenously inoculated into BALB/c-mice. In these model systems the number of experimental lung and liver metastases decreased relevantly, however, biometrically non-significantly in neem extract-treated animals, as compared to the control mice which received injections of saline solutions. Neem extract can be regarded as an immunomodulating and antimetastatic substance which holds promise for further experimental and clinical investigation.*

The indigenous Indian plant *Azadirachta indica* (commonly known as neem; a large evergreen tree) has been used in traditional medicine since antiquity. Different parts of the plant have been reported to possess medicinal properties (1).

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Neem leaf has been shown to be non-toxic, non-mutagenic and it was found to possess anti-inflammatory and immunostimulatory activities (2, 3). Distinct neem preparations have been documented to have anticarcinogenic properties, however, their mode of action remains to be evaluated (4).

Cancer development can be divided into several phases, each consisting of distinct steps. It has been clearly demonstrated that carcinogenesis requires progression from a premalignant to an autonomous malignant state; defined molecular changes; and additional events such as angiogenesis, leading to the final phase of cancer, metastasis (5). Although advances in surgery, radiation and clinical oncology have led to very effective management of primary cancers, innovative approaches to cancer treatment are warranted.

Intrathymic T-cell differentiation is a process in which immature thymocytes expand and develop by undergoing complicated maturational events, leading to the acquisition of immunocompetence and subsequent emigration to the periphery (6, 7).

The objective of this investigation was to evaluate the influence of neem leaf extract (*Azadirachta indica*) on thymocyte proliferation and there emigration into peripheral blood, on the activity of mononuclear cells and on metastatic organ colonization in BALB/c-mice.

Materials and Methods

Plant extract preparation. Dried neem leaves of *Azadirachta indica* were ground to powder. Ten g of the neem powder were boiled in 100 mL distilled water for 5 min, kept at room temperature for 30 min, and centrifuged (15,000 rpm; 25 min; 4˚C). The supernatant was freeze-dried to powder form (crude extract) and the dry extract was maintained at –20˚C.

Five hundred mg of the crude extract was dissolved in 2 mL distilled water and applied to 1.5 x 80 cm columns of sephadex G-25 (8). The columns were eluted with distilled water at a flow rate of 0.5 mL/min and the fluid was collected. Absorbance of the fractions was measured at 280 nm and the fractions were collected, freeze-dried and stored at –20˚C until use.

Animals. Inbred male BALB/c-mice (Charles River Wiga Breeding Co., Sulzfeld, Germany), 8-12 weeks old, weighing 20-22 g, were used for these investigations. The animals were kept in plastic cages and allowed free access to food and water.

Tumor. L-1 sarcoma (syngeneic murine tumor, spontaneously grown in the lung of a BALB/c-mouse) and RAW lymphosarcoma (Abelson virus-derived tumor) were maintained in cultures, as described elsewhere (9,10).

Experimental design. Groups of 5 BALB/c-mice each were subcutaneously (*s.c.*) injected with neem F (crude extract) and sepadex G-25 chromatography fraction F1 (0.2 and 0.1 mg/mouse/day) for 3 subsequent days. Control groups of mice were treated with equivalent volumes of physiological saline (PBS; phosphate-buffered saline) following the timing scheme of the neem extract. On day 4, 0.1 mL of tumor cell suspensions ($5x10^4$ L-1 cells; $1x10^4$ RAW cells/mL medium) were intravenously inoculated into the tail veins of BALB/c-mice followed by 3 further neem applications on days 7, 8 and 9. The tumor cell density and neem treatment scheme had proved to be optimal in preceding investigations. Lung (L-1 sarcoma) and liver (RAW-lympho-sarcoma) tumor nodules were counted under a dissecting microscope by 2 independent observers 14 days after tumor cell inoculation, as previously described (11).

To investigate its immunological activities, neem leaf extract preparations F and F1 (0.2 and 0.1 mg/mouse/day) were *s.c.* administered on days 1, 2, 3, 7, 8 and 9. On day 10, the following experimental procedures were initiated:

i) to measure spleen weight, experimental animals were killed, autopsied and the organ weight was determined and calculated on the basis of 20 g body weight (bw);

ii) peritoneal macrophages (PMs) were obtained immediately after sacrificing the mice. Following laparotomy, the peritoneal cavity was rinsed with Hanks Balanced Salt Solution (HBSS, Invitrogen) containing 10 IU of heparin (Sigma Chemicals Co.). The fluid was collected, centrifuged at 400xg for 10 min and the cells were resuspended in HBSS supplemented with 10% inactivated fetal bovine serum (FBS). The cell density was adjusted to $5x10^6$ cells/mL and 2 mL of this suspension was placed into sterile Petri dishes (Falcon, Becton Dickinson,Heidelberg, Germany) which were kept in an incubator (37˚C, 5% CO_2) for 2 h. After removal of the supernatant, the adherent cells were washed twice with HBSS, gently removed from the Petri dish and resuspended in minimum essential medium (MEM, Biochrom AG Seromed, Berlin, Germany), supplemented with 1% FBS for the chemiluminescence experiments. After counting in a hemocytometer, the cell viability was determined and the macrophages diluted to a density of $5x10^6$ viable cells/mL. Chemiluminescence experiments were performed with the Luminoskan 1251 Carousel (Thermo, Turku, Finland) according to the method of Williams *et al.* (12). The PM responses of neem extract-treated and control BALB/c-mice were measured in the presence of lucigenine (Sigma Chemicals Co.) after activation with zymosan containing fresh mouse serum as stimulus; the activity of PMs was further investigated by FACScan analysis with anti-CD-44 monoclonal antibodies (Becton Dickinson), as described below.

iii) the cell suspension procedure for BALB/c-mice thymus has been described extensively (13, 14);

Table I. *Spleen and thymus weight of BALB/c-mice after subcutanous administration of neem extract.*

Experimental groups of BALB/c-mice treated with	Organ weight (mg per 20 g of b.w.)		Average number of thymocytes x 10^6 (% of control)
	spleen (+/– SD)	thymus (+/– SD)	
Control (physiol. saline)	70 (+/– 4.5)	38 (+/– 4.4)	6.6 (100%)
Experimental group			
F (0.2 mg crude extract)	78 (+/– 5.3)**	41 (+/– 8.1)	7.5 (114%)
F1 (0.1 mg)	85 (+/– 6.4)*	45 (+/– 4.7)	10 (152%)

*$p<0.01$, **$p<0.05$: statistically significant as compared to control. Each experimental group consisted of 5 BALB/c-mice.

iv) peripheral blood was drawn from the BALB/c-mice for analysis of the white blood cell count and differentiation, in accordance with standard routine methods. The cell suspending medium for FACScan-analysis of lymphatic cells was Cellwash (Becton Dickinson). Immunofluorescence staining of mouse lymphoid cells was performed using fluorescein-conjugated monoclonal antibodies (CD-4: murine helper/inducer T-cells; CD-8: murine suppressor/cytotoxic T-cells; CD-25: interleukin-2 receptor; MAC-3: monocyte/macrophage activation marker, all obtained from Becton Dickinson). FACScan (Becton Dickinson) was used for the differentiation of peripheral blood and thymic lymphatic subsets as recently described (13, 14).

Statistics. The Student's-*t*-test was used for statistical analysis of the data. All experiments were performed twice and yielded reproducible results.

Results

The immunomodulatory and antimetastatic activities of aqueous neem extract (crude extract F and chromato-graphically-separated fraction F1) were measured in BALB/c-mice on day 10 after *s.c.* administration of optimized dosages (0.2 and 0.1 mg/day/mouse) and following optimized timing schedule (days 1, 2, 3, 7, 8 and 9). This experimental design had proved to be optimal in preceding investigations.

The immunoactivating potency of neem extract was investigated in BALB/c-mice by spleen and thymus enlargement test and by determination of thymocyte counts. The data are presented in Table I and suggested a strong immunomodulatory effect of neem extracts F and F1, which resulted in a significant spleen weight gain. The thymus weight and thymocyte counts, however, increased moderately without statistical significance in response to neem extract administration.

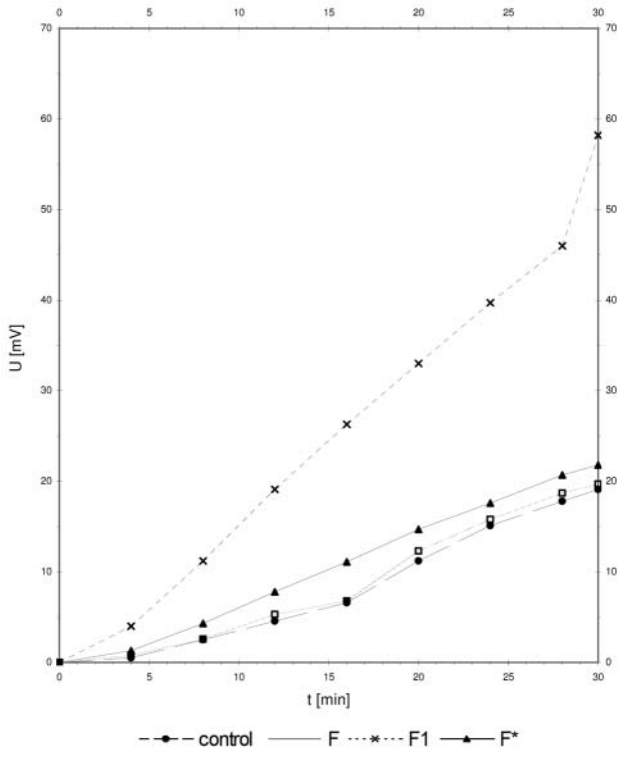

Figure 1. *Chemiluminescence response of peritoneal macrophages from BALB/c-mice using zymosan-activated mouse serum as a stimulus. Control: PBS; F: 0.5mg crude s.c.; F*: 0.25 mg crude s.c.; F1: 0.1 mg.*

Since the spleen represents the main organ of the macrophage-phagocyte system (MPS), the activity of PMs of BALB/c-mice was investigated after neem extract administration. In Figure 1, the chemiluminescence response of PMs after zymosan stimulation is shown. In comparison to non-treated animals, the macrophages of neem extract-treated mice yielded a significantly enhanced chemiluminescence response ($p<0.05$), correlating with activity.

The enhancement of PM counts and activity after neem extract administration to BALB/c-mice was confirmed in FACScan experiments, demonstrating significantly increased CD-44 expression correlating with PM activity (Table II).

Peripheral blood leukocyte counts and their differentiation were established in BALB/c-mice to evaluate the influence of neem extract (F; 0.2 mg/mouse/day and F1; 0.1 mg/mouse/day) on the absolute numbers of these cells. As shown in Table III, the absolute counts of leukocytes, lymphocytes and monocytes were significantly enhanced ($p<0.01/0.05$) after neem extract administration. Granulocytes, however, presented non-significant fluctuations.

Directly fluorescence-conjugated murine monoclonal antibodies (anti-CD-4; anti CD-8; anti-CD-25; anti-MAC-3) were used in the FACScan staining experiments to evaluate T-helper / T-suppressor distribution and T-cell (CD-25/IL-2 receptor) and monocyte (MAC-3) activities. As shown in Table IV, *s.c.* administration of neem extract F induced biometrically-significant increased counts (peripheral blood helper and suppressor T-lymphocytes) and activities (T-lymphocytes and exerts). Neem extract F1, induced comparable and statistically relevant enhancements of immune cell counts and activities.

Table II. *Peritoneal macrophages per μl and activated macrophages per μl (CD-44).*

	Macrophages per μl	CD-44 pos.
PBS (control)	3500 (+/– 2829)	1054 (+/– 158)
neem F	4900 (+/– 141)**	1806 (+/– 197)*
neem F1	5300 (+/– 282)*	1416 (+/– 98)**

*$p<0.01$, **$p<0.05$: statistically significant as compared to control. Each experimental group consisted of 5 BALB/c-mice.

The reproducible demonstration of the distinct immunoactivating capacity of neem extracts F and F1 encouraged investigations into the development of experimental lung (L-1 sarcoma) and liver (RAW lymphosarcoma) metastases after treatment. Compared to a control group of BALB/c-mice (treated with equivalent volumes of physiological saline), the numbers of experimental lung and liver metastases were relevantly lower on day 14 after tumor cell inoculation in neem extract F-, and F1-treated animals. In Table V, data on the extent of the experimental lung and liver metastases are summarized for the treatment and control groups.

Discussion

Azadirachta indica (commonly named neem) is an indigenous plant/tree widely found in India and Burma. Different parts of this plant have been reported to possess medical properites such as hypoglycemic, antiseptic, anti-inflammatory and immunostimulatory activities (1). Aqueous extracts of neem leaf are well known for their distinct medical properites in Ayurvedic medicine (15). The juice of fresh green leaves is still consumed by sanyasees (ascetics) to suppress “kam vasana” (sexual desire).

The increasing evidence that the macrophage phagocyte system (MPS) is of prime importance in host defence mechanisms has stimulated interest in agents which can enhance MPS-mediated destruction of tumor cells. Recent results indicate that neem extract exerts anti-inflammatory and immonostimulatory activities (2, 3).

Table III. *Average number of BALB/c-mouse peripheral blood leukocytes, lymphocytes, monocytes and granulocytes per μl after neem treatment*

BALB/c-mice injected with	Peripheral blood [cells/μl] (+/– SD)			
	Leukocytes	Lymphocytes	Monocytes	Granulocytes
PBS (control)	3300 (+/– 132.28)	1980 (+/– 206.63)	170 (+/– 20.0)	1160 (+/– 105.35)
neem F	4300 (+/– 236.78)**	2900 (+/– 144.91)**	320 (+/– 18.25)*	1320 (+/– 42.42)
neem F1	4600 (+/– 294.39)**	3330 (+/– 197.82)*	290 (+/– 8.16)*	980 (+/– 88.31)

*$p<0.01$, **$p<0.05$: statistically significant as compared to control. Each experimental group consisted of 5 BALB/c-mice.

Table IV. *Quantitave cellular receptor expression of T-cells and monocytes in BALB/c-mouse peripheral blood after neem treatment.*

BALB/c-mice injected with	Peripheral blood [cells/μl] (+/– SD)			
	CD-8	CD-4	MAC-3	CD-25
PBS (control)	162 (+/– 11.53)	509 (+/– 8.18)	7 (+/– 2)	82 (+/– 3.60)
neem F	238 (+/– 30.64)**	575 (+/– 32.36)**	15 (+/– 2.44)	111 (+/– 7.68)**
neem F1	227 (+/– 13.66)**	523 (+/– 14.62)	9 (+/– 0.81)	154 (+/– 3.16)*

*$p<0.01$, **$p<0.05$: statistically significant as compared to control. Each experimental group consisted of 5 BALB/c-mice.

Lucigenine-dependent chemiluminescence may be considered a sensitive method for measuring the release of oxygen radicals which are known to possess tumoricidal properties (12). Because of their toxicity, they obviously cause cytotoxic effects on transformed cells and tissue damage during inflammation (16). Here, it was shown that treatment of BALB/c-mice with aqueous neem extract (chromatographically-separated fraction F1) significantly enhanced the chemiluminescence response of PMs as compared to phagocytes from non-treated animals. Data on the effect of the neem extract on the spleen and thymus weights confirmed the results of other investigations, indicating a close correlation between spleen/thymus weight gain and immunomodulatory/antineoplastic activity (14). Thus, especially the spleen enlargement test may be considered a simple method for comparative studies on the effect of immunostimulating agents on lymphoreticular characteristics, whereas weight gain of the thymus apparently correlates with the proliferation and activity of lymphatic cells (14).

Investigations on lymphocyte recirculation have shown that most mature lymphocytes recirculate continuously between the blood and lymphoid organs (17). To gain information on the absolute number and activity of peripheral blood lymphocytes (PBL), adequate parameters were determined (including FACScan-analysis to define subpopulations and activities) and confirmed the immunomodulating potency of neem extract. Both the absolute counts and activities of PBL were evidently increased (statistically significantly for PBL and their subsets CD-4 and CD-8 cells and for CD-25 and MAC-3 expression) after regular neem extract administration. Since activation markers (CD-25/IL-2 receptor on T-cells and MAC-3 on monocytes/macrophages) are induced when appropriately presented antigens or antibodies interact with the receptor complex, neem extract can be suggested to be a potent stimulus.

Table V. *Mean number of L-1 sarcoma lung colonies and liver RAW-P colonies in BALB/c-mice inoculated i.v. with tumor cells. Experimental animals were s.c. treated with neem. Control-mice were treated with PBS.*

Groups of BALB/c-mice (n=5) s.c. treated with	Mean number of lung L-1 colonies (+/– SD)	%	Mean number of liver RAW-P colonies (+/– SD)	%
PBS (control)	114 (+/– 26.70)	100	90 (+/– 24.47)	100
F	82 (+/– 20.369)	72	51 (+/– 9.11)	57
F1	67 (+/– 19.0)	59	44 (+/– 15.92)	49

To investigate the antimetastatic activity of neem extracts F and F1 *in vivo*, a well established experimental model was chosen (BALB/c-mouse/sarcoma L-1 sarcoma, respectively, RAW lymphosarcoma). As compared to the control animals

(treated with equivalent volumes of physiological saline), neem extract treatment of BALB/c-mice resulted in significantly fewer experimental lung and liver metastases. This antimetastatic effect of neem extract may be attributed to its immunomodulating properties, such as augmented macrophage/monocyte and lymphocyte activation.

In conclusion, these results provide evidence for the immunomodulating and antineoplastic activities of aqueous neem leaf extract. Further experimental and clinical studies are now warranted to investigate the benefit of neem extract treatment for cancer patients.

References

1 Chopra RN, Nayer SL and Chopra IC: Glossary of Indian Medical Plants, CSIR Press New Dehli, 1956.

2 Chattopadhyay RR and Chattopadhyay SM: Possible mechanisms of antiinflammatory activity of *Azadirachta indica* leaf extract. Indian J Pharmacol *25*: 99-100, 1993.

3 Ray A, Banerjee BD and Sen P: Modulation of humoral and cell-mediated immune responses by *Azadirachta indica* (Neem) in mice. Indian J Exp Med *34*: 698-701, 1996.

4 Subapriya R, Bhuvaneswari V, Ramesh V and Nagini S: Ethanolif leaf extract of neem (*Azadirachta indica*) inhibits buccal pouch carcinogenesis in hamsters. Indian J Med Res *2*: 63, 2005.

5 Nicolson GL: Cell surface molecules and tumor metastasis. Exp Cell Res *150*: 3-22, 1984.

6 Scollay R: Thymus cell emigration: cells emigrating from the thymus to peripheral lymphoid organs have a "mature" phenotype. J Immunol *128*: 1566-1570, 1982.

7 Scollay R, Chen WF and Shortman K: The functional capabilities of cells leaving the thymus. J Immunol *132*: 3521-3528, 1986.

8 Pulverer G, Ko HL, Roszkowski W, Beuth J, Yassin A and Jeljaszewicz J: Digestive tract microflora liberates low molecular weight peptides with immunotriggering activity. Zbl Bakt Hyg *272*: 318-327, 1990.

9 Beuth J, Ko HL, Gabius H-J and Pulverer G: Influence of treatment with the immunomodulatory effective dose of β-galactoside-specific lectin from mistletoe on tumor colonization in BALB/c-mice for two experimental model systems. In Vivo *5*: 29-32, 1991.

10 Beuth J, Ko HL, Schirrmacher V, Uhlenbruck G and Pulverer G: Inhibition of liver tumor cell colonization in two animal tumor models by lectin blocking with D-galactose and arabinogalactan Clin Expl Metastasis *6*: 115-120, 1988.

11 Roszkowski W, Beuth J, Ko HL, Uhlenbruck G and Pulverer G: Blocking of lectin-like molecules on pulmonary cells inhibits lung sarcoma L-1 colonization in BALB/c-mice. Experientia *45*: 584-588, 1989.

12 Williams AJ, Hastings MJG, Easmon CSF and Cole PJ: Factors effecting the *in vivo* opsonization: a study of the kinetics of opsonization using the technique of phagocytic chemi-luminescence. Immunology *41*: 903-911, 1980.

13 Beuth J, Ko HL, Gabius H-J and Pulverer G: Determination of lectin dependent alterations of cellular parameters by immunophenotyping during adjuvant lectin application. *In*: Lectins and Glycobiology. Gabius H-J and Gabius S (eds.). Springer Verlag Berlin, 1993.

14 Beuth J, Ko HL, Tunggal L, Steuer MK, Geisel J, Jeljaszewicz J and Pulverer G: Thymocyte proliferation and maturation in response to galactoside-specific mistletoe lectin-1. In Vivo *7*: 407-410, 1993.

15 Parshad O, Gardner MT, The TL, Williams LAD and Fletcher CK: Antifertility effects of aqueous and steroidal extracts of neem leaf (*Azadirachta indica*) in male Wistar rats. Phytother Res *11*: 168-170, 1997.

16 Ohshima Y, Beuth J, Yassin A, Ko HL and Pulverer G: Stimulation of human monocyte chemiluminescence by staphylococcal lipoteichoic acid. Med Microbiol Immunol *177*: 115-121, 1988.

17 Fowlkes BJ, Zuniga-Pflücker JC, Bolen JB and Chused TM: Early T-lymphocytes: differentiation *in vivo* of adult intrathymic precursor cells. J Exp Med *162*: 802-822, 1985.

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