Modulation of humoral and cell-mediated immune responses by *Azadirachta indica* (Neem) in mice

A Ray, B D Banerjee & P Sen

Department of Pharmacology & Biochemistry
University College of Medical Sciences
(University of Delhi), Shahdara, Delhi 110 095, India

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The effects of *A. indica* (AI, Neem) were evaluated on tests of humoral and cell-mediated immune responses after 3 weeks of oral AI (leaf extract) treatment in ovalbumin immunized mice. At the dose levels tested, AI (10, 30 or 100 mg/kg), had no appreciable influence on different organ (liver, spleen, thymus)/body weight indices, when compared to controls. In tests for humoral immune responses, AI (100 mg/kg) treated mice had higher (a) IgM and IgG levels, and (b) anti-ovalbumin antibody titres, when compared to the vehicle treated group. In tests for cell-mediated immune responses, there was an enhancement (0/0) of (a) macrophage migration inhibition, and (b) footpad thickness after AI (100 mg/kg) treatment. These results are discussed in light of the possible immunopotentiating effects of AI.

*Azadirachta indica* (AI, Neem) is a widely prevalent and highly esteemed wonder tree of the Indian subcontinent and several of its beneficial properties are reported\(^1\)\(^,\)2\(^,\)4. It forms an inseparable component of the ecosystem and its mammalian and environmental safety is well recognized. The use of neem products has been reported in ancient medicine and modern medical applications are receiving widespread attention. The environmental compatibility of AI products, the lack of resistance development to them, their harmless nature against non-target organisms, and lack of residual/cumulative effects or toxicity—all have significantly enhanced the integrated use of AI in biology and medicine\(^3\). Several of the behavioural, physiological and biochemical properties are known and biomedical research has shown that AI possesses antihyperglycemic, contraceptive and anti-fertility properties, and more recently, anti-infective and insecticidal effect have been reported\(^1\)\(^,\)2\(^,\)4. The immune system plays a crucial role in the regulation of health and disease and the present study thus critically evaluated the effects of AI on some non-specific and specific aspects of immunity in mice.

Albino mice (25-30 g) of either sex were used for the study. They were housed under standard laboratory conditions, viz. 12 hr light - 12 hr dark schedule and a temperature of 22±2°С, with free access to food and water.

Extracts of leaves of AI (Dabur India Ltd), containing 100 mg of the drug/capsule, were prepared by dissolving appropriate amount in groundnut oil. The experimental groups were fed orally with 10, 30 and 100 mg/kg of AI per day. The control group received equal volume of groundnut oil in an identical manner.

The mice were immunized (sc) in the back (above tail) with 3 mg of ovalbumin dissolved in 0.1 ml of normal saline emulsified with equal volume of Freund's complete adjuvant (Difco). These immunized mice were injected intraperitoneally with 2 ml sterile liquid paraffin (0.88 g/ml BDH, England) 48 hr before use.

The animals were anesthetized with chloroform, and blood was collected by cardiac puncture following thoracotomy without opening the abdomen after 3 weeks of ovalbumin immunization. The serum was separated from individual samples and kept at -20°С until analysed. Peritoneal macrophages were collected later from these animals for the macrophage migration inhibition (MMI) test. Peritoneal exudate cells were obtained by washing peritoneal cavity with tissue culture medium RPMI 1640. Media (3-5 ml) was injected into peritoneal cavity, agitated well for 5 min and the peritoneal exudate cells were drawn into a syringe under aseptic conditions. Another 3-5 ml medium was added twice to the peritoneal cavity and the peritoneal washings were collected in siliconized centrifuge tubes.
The liver, spleen and thymus were removed immediately and weighed, for calculation of organ-body weight index.

Quantitation of serum IgM and IgG were carried out by single radial immuno-diffusion in 0.9% agarose slides containing respective anti-IgM and anti-IgG as mentioned earlier. The serum antibody titre to ovalbumin was estimated by enzyme-linked immunosorbent assay (ELISA) according to the detail procedure described earlier. In brief, flat bottomed polystere plates were coated with 12.5 µg of ovalbumin dissolved in 100 µl of 0.1 M sodium carbonate buffer (pH 9.6) containing 0.02% NaN3 at 4°C for 12 hr. The coated plates were washed 3 times with phosphate-buffer saline (0.15 M, pH 7.2) containing 0.05% Tween-20 (PBS-Tw). The wells were incubated with 100 µl of 1% BSA in sodium carbonate buffer at 37°C for 1 hr. Serial dilutions of sera in PBS-Tw were prepared and 100 µl was incubated with the coated wells for 1 hr at 37°C. After washing, diluted (1:1000) anti-mouse IgG conjugated with peroxidase (100 µl) was added and plates were incubated at 37°C for 1 hr. The enzyme activity was determined by addition of 100 µl of o-phenylenediamine (400 µg/ml) in sodium citrate buffer (0.1 M, pH 4.9, containing 1.5 µl of 30% H2O2/ml). The enzyme reaction was stopped with 8 N H2SO4 (50 µl) after 30 min, the absorbance was measured at 490 nm. The antibody titres were expressed as log2 of the reciprocal of the highest dilution of the test serum showing three times or more absorbance as compared with normal mice serum. For the determination of delayed type hypersensitivity (DTH) reaction, the mice were challenged (sc) with 50 µg ovalbumin in 0.05 ml PBS in the left hind footpad. The increase in footpad thickness was measured 24 hr after the challenge with the help of a dial caliper (Mitutoyo, Japan). The right hind footpad was injected with 0.05 ml vehicle and this served as the control. The degree of DTH reaction was expressed as the percentage increase in footpad thickness (L-R) over the control value.

For the macrophage migration inhibition (MMI) test, peritoneal exudate cells from ovalbumin immunized mice were centrifuged at 400 g for 10 min. The cell pellete thus obtained was washed three times with tissue culture medium RPMI-1640, pH 7.2 and a final suspension containing 15 x 10^5 cells/ml was prepared. The cells were used in the direct migration inhibition assay as described by us earlier. The concentration of ovalbumin was adjusted to 125 µg/ml in antigenic chambers.

The data were analysed using the Mann-Whitney U test (two-tailed). A P value of at least 0.05 was used as the level of significance in all statistical tests.

AI (10, 30 and 100 mg/kg) treatment for 3 weeks, per se, did not produce/induce any overt signs of neurobehavioural toxicity and no morbidity or mortality was seen. There was also no significant changes in the organ/body weight index with respect to liver, spleen or thymus (data not shown). Immunoglobulin (IgM and IgG) levels were measured in both unstimulated and ovalbumin stimulated mice after AI treatment. No significant changes in IgM or IgG levels were seen after 10, 30 or 100 mg/kg of AI treatment, when compared to the vehicle treated group. The basal IgM and IgG levels in the groundnut oil (vehicle) treated group were 0.95±0.16 mg/ml and 11.75±2.50 mg/ml, respectively (data not shown). However, in the ovalbumin-stimulated group, AI (100 mg/kg) elevated both IgM and IgG levels, when compared to control values. As shown in Table 1, the IgG values were increased by approximately 25% (P < 0.05). Similarly, the anti-ovalbumin antibody titres were also higher in the AI (100 mg/kg) and to a marginal extent on the AI-30 mg/kg treated group. The results of AI treatment on CMI are summarized in Table 2. In the macrophage migration inhibition test, the effects of AI (10, 30 and 100 mg/kg) ap-

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Table 1—Effect of *Azadirachta indica* (AI, Neem) on humoral immune responses in mice, immunized with ovalbumin

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Immunoglobulin levels (mg/ml)</th>
<th>Antibody titre (-log2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>Controls</td>
<td>1.15±0.18</td>
<td>12.20±1.50</td>
</tr>
<tr>
<td>AI (10)</td>
<td>1.08±0.10</td>
<td>12.13±1.58</td>
</tr>
<tr>
<td>AI (30)</td>
<td>1.12±0.20</td>
<td>12.33±1.40</td>
</tr>
<tr>
<td>AI (100)</td>
<td>1.26±0.24</td>
<td>15.13±1.38</td>
</tr>
</tbody>
</table>

aP < 0.05 (Compared to respective controls)
Table 2—Effects of *Azadirachta indica* (AI) on cell-mediated immune response in mice  
[Values are mean ± SD of 8 to 10 animals per group]

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Macrophage migration inhibition test (% inhibition)</th>
<th>Footpad thickness test (% increase in paw vol.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>36.55±6.25</td>
<td>20.20±3.50</td>
</tr>
<tr>
<td>AI (10)</td>
<td>35.20±5.75</td>
<td>19.68±2.10</td>
</tr>
<tr>
<td>AI (30)</td>
<td>44.28±8.90</td>
<td>22.70±4.18</td>
</tr>
<tr>
<td>AI (100)</td>
<td>54.55±7.00(^a)</td>
<td>26.40±4.40(^a)</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.05 (compared to respective controls)

appeared to be dose-related. Whereas little or no significant changes in per cent macrophage migration inhibition was seen with the lower doses (an approximate 25% increase after AI, 30 mg/kg; P < 0.05), the higher dose 100 mg/kg clearly induced a marked increase in the per cent inhibition in macrophage migration. Similarly, in the footpad thickness test, only AI (100 mg/kg) was found to enhance the paw volume (and the per cent increase) by a significant extent (by 33%, P < 0.05) when compared to vehicle controls.

The results of the present study show that AI (aqueous leaf extract) modulates different aspects of humoral and cell-mediated immune (CMI) responses in *in vitro* and *in vivo* situations in mice. The observations corroborate the findings of an earlier pilot study with AI in which reversal of stress-induced immune suppression was shown. However, the differences between the two studies are as follows—the antigen was ovalbumin (SRBC, in the earlier study), the mode and duration of AI administration was oral and for 21 days, (single, ip injection in the earlier study) and the species was mice (rat, in the earlier study). Each or a combination of the above biological factors could well have influenced the tests for immune function in a differential manner. The fact, that this was not evident and a basically consistent pattern was seen in the nature of AI effects on immune regulation, confirms the general immunopotentiating role of this plant extract. Further, whereas the earlier study emphasized the role of AI in immunocompromised situations, the present study highlights the effect of this agent on immunocyte functions during basal condition.

Long term administration of AI did not influence the organ/body weight indices by any appreciable extent suggesting—(a) the probable non-involvement of AI in the catabolic/anabolic functions, and (b) no clearcut temporal relationship between immunomodulation and spleen/thymus weight changes. In tests for humoral immunity, there was a significant enhancement in IgM and IgG levels and also the serum antibody titres against ovalbumin were raised. These changes were seen only at the highest dose level of the drug (100 mg/kg) used and in agreement with an earlier study and the potentiation in the humoral immune response may be mediated by the T-lymphocyte (since ovalbumin is a T-dependent antigen). Release of cytokines from sensitized T-lymphocytes in response to antigenic challenge is an important event/marker of the CMI, and plays a crucial role in several immunologically mediated pathophysiological states. Macrophage migration inhibition (MMI) and footpad thickness (FPT) tests are sensitive tests for CMI and in the present study AI (a) increased the extent of MMI and (b) enhanced paw volume changes in the FPT test. These are suggestive of AI-induced, lymphokine mediated, augmentation of the CMI response. Recent biomedical research with AI ingredients have shown its wide spectrum of beneficial effects. This has lead to the increase in the medicinal/commercial use of AI products in humans. Further, the increasingly popular role of AI as an insecticide is also becoming evident. The proposed immunostimulant/immunoprotective role of AI may have immense environmental significance, particularly in view of the fact that some commonly used insecticides (viz DDT, Lindane) are markedly immunotoxic.

References


