CHAPTER IV

EFFECT OF WITHANIA SOMNIFERA ON
CELL MEDIATED IMMUNITY
1. Introduction

Cell mediated immune responses are evoked by several factors. For example, they can be elicited in response to foreign antigens that are presented on allografts. Nonspecific cellular immune responses are mounted primarily by natural killer (NK) cells; macrophages, and granulocytes. These cells appear to respond to a variety of stimuli apparently in a nonspecific manner. In particular, NK cells are peculiar in displaying natural cytotoxicity towards tumor cells (19).

In recent years, there is an increasing interest in the search for potential drugs, especially of plant origin that are capable of modifying immune responses with less side effects (66). In the present chapter the stimulatory activity of Withania somnifera on the cellular effector mechanism is demonstrated.

II. Materials and Methods

II.1 Animals

In all the studies Balb/c mice (4 - 6 weeks) were used.

II.2 Cell lines used

K562 cells were used as target for NK cell activity. Ehrlich ascites tumour (EAT) cells were used for tumour induction.
II.3. Chemicals

$^3$H thymidine and Na$_{51}$CrO$_4$ were used for labelling the cells. RPMI - 1640 - medium, containing FCS; mitogens- PHA; CON A, PWM; and LPS were used in the study. All other chemicals used were of analytical reagent grade (Chapter II).

II.4 Drug treatment

Five doses of *Withania somnifera* extract (20mg/dose/animal, i.p); Withaferin A (500µg/dose/animal, i.p), and Withanolide D (500µg/dose/animal i.p) were given for 5 continuous days.

II.5 Determination of the effect of *Withania somnifera* on Lymphocyte blastogenesis assay.

Four groups of animals (3 mice/group) were used in this study. Group 1 animals were kept as control without any treatment. Group 2 animals were treated with 5 doses of *Withania* extract. Group 3 animals were treated with 5 doses of Withaferin A and group 4 animals were treated with 5 doses of Withanolide D. The animals were sacrificed after 24h. of the last dose of drug treatment and spleen was processed aseptically into single cell suspension by passing through a wire mesh. The spleen cells from treated and untreated animals were cultured (10⁶/ml) in the presence and absence of various concentrations of mitogens such as PHA (2.5 µg/ml); CON A (10µg/ml), PWM (10µg/ml) and LPS (10µg/ml); in RPMI - 1640 containing 10% FCS (final volume 3ml) and incubated in a humidified atmosphere of 5% CO$_2$ at 37°C for 48h. Cells
were labelled by adding 2 μCi of ³H thymidine to each vial and further incubated for 16-18h. in the same conditions. After the incubation, DNA was precipitated twice with 3ml of cold 10% PCA. Pellets were dissolved in 0.5ml of 0.5N NaOH and transferred to 10ml Scintillation fluid and kept in dark overnight and radioactivity was measured using a Rack Beta Liquid Scintillation Counter as explained in chapter II.

II.6 Determination of the effect of *Withania somnifera* on thymocyte blastogenesis assay.

Balb/c mice (3 mice/group) were used in this study. Group 1 animals were kept as control, group 2 animals were treated with 5 doses of Withania extract. Group 3 animals were treated with 5 doses of Withaferin A. Group 4 animals were treated with 5 doses of Withanolide D. Animals were sacrificed after 24h. of the last dose of the drug treatment and thymus was collected and processed into a single cell suspension in medium containing FCS. They were cultured in the presence and absence of mitogens PHA, CON A and PWM and rate of proliferation was checked as above as discussed in chapter II.

II.7 Determination of the effect of *Withania somnifera* on the proliferation of the bonemarrow cells.

Four groups of Balb/c mice (3 mice/group) were used in this study. Group 1 animals were kept as control. Group 2 animals were treated with 5 doses of Withania extract. Group 3 animals were treated with 5 doses of Withaferin A. Group 4 animals were treated with 5 doses of Withanolide D. Animals were sacrificed after 24h. of the last drug treatment and bonemarrow
cells were collected aseptically from the femur into medium containing FCS. Bone marrow cells from treated and untreated animals were cultured (10⁶ cells/ml) in the presence and absence of various concentrations of mitogens such as PHA (2.5µg/ml), CON A (10µg/ml) and PWM (10µg/ml) in RPMI-1640 containing 10% FCS in a final volume was adjusted to 3ml. The cells were incubated at 37°C in 5% CO₂ in a humidified chamber. Rate of Proliferation was checked as in the previous experiment.

II.8 Determination of the effect of Withania somnifera on Natural killer (NK) cell activity.

NK cell activity was determined by 4h chromium release assay (54). Five groups of animals were used in this study. Group I animals were kept as control and they received (1X10⁶) Ehrlich Ascites tumour cells (EAC); Group II animals received 5 doses of Withania extract only group III animals received tumour cells as that of group I and Withania as that of group II; group IV and group V animals pre treated with 5 doses of Withaferin A or Withanolide D and 24h after the last dose of drug treatment, they were injected with tumour cells. Animals were sacrificed and spleen cells were used as effector cells and chromium labelled, K-562 cells were used as targets. The labelled target cells were incubated with effector cells for 4h at 37°C in different effector: target ratios of 100:1; 50:1 and 25:1. Spontaneous and total release was determined by incubating the target cells in the medium alone and in the presence of IN HCL respectively. The released Chromium in the supernatant was counted using a gamma ray Spectrometer. The experiment was set in triplicate and the percentage of specific lysis was calculated as % cell lysis as described in chapter II.
\[
\text{\% cell lysis} = \frac{\text{Experiment release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100
\]

II.9 Determination of the effect of *Withania somnifera* on Antibody dependent cellular cytotoxicity (ADCC).

ADCC activity was determined by 4h. chromium (Cr\(^{3+}\)) release assay (53). Chromium labelled SRBC was used as the target cells and spleen cells from animals as in (11.8) were used as effector cells. AntiSRBC antibody was raised in rabbits and was used as the source of antibody in ADCC assay. The labelled target cells were incubated with effector cells for 4h. at 37°C in different effector: target ratios of 100:1, 50:1 and 25:1 along with 1ml of antibody. The released chromium in the supernatant was counted in a gamma ray spectrometer and the percentage cell lysis was calculated as explained in chapter II.

II.10 Determination of antibody dependent complement mediated cytotoxicity (ACC).

The serum of the animals from the previous experiment (11.8) was separated, heat inactivated at 56°C and was used for the determination of ACC activity. Short term (3h) cytotoxicity assay was performed with the sera samples along with fresh rabbit serum using Ehrlich Ascites tumour cells (1 X 10\(^4\)) as target cells by trypan blue dye exclusion method (42).

III. Results

III.1 Effect of *Withania somnifera* on lymphocytes blastogenesis.

Effect of *Withania somnifera* on lymphocyte proliferation is given in table IV.1. Addi-
tion of PHA significantly enhanced the proliferation up to 6555 cpm in Withania treated group and 6359 in Withanolide D treated group. Similarly addition of CON A showed a significant enhancement in the proliferation (8292 cpm) in Withania treated group. Administration of Withania extract along with PWM mitogen could significantly enhance the proliferation up to 4258 cpm whereas normal group showed only 2899 cpm. Administration of Withaferin A did not significantly enhance the proliferation of splenocytes. There was a six fold increase in the proliferation of withania treated spleen cells in the presence of LPS. For normal group along with the mitogen LPS the cpm was found to be 2342 but for Withania treated group the cpm was found to be significantly enhanced up to 13443 and for Withanolide D treated group there was a significant enhancement in the proliferation up to 12121 cpm.

III.2 Effect of Withania somnifera on thymocyte proliferation.

Administration of Withania somnifera extract showed a significant enhancement in thymocyte proliferation (table IV. 2). In Withania treated group the proliferation of thymus cells enhanced significantly (3095 cpm). Addition of PHA showed a significant increase in the proliferation (8122 cpm) in Withania treated group and in Withanolide D treated group (6359 cpm). Similarly addition of CON A significantly enhanced the $^3$H- thymidine uptake (9246 cpm) in Withania treated group. The mitogenic stimulation by the PWM was significantly increased when the thymocytes from the Withania (6318 cpm) and Withanolide D (6218 cpm) treated animals were used. There was no significant enhancement in the proliferation of thymocyte by Withaferin A administration.
III.3 Effect of *Withania somnifera* on bonemarrow blastogenesis.

Effect of *Withania somnifera* on bonemarrow blastogenesis is given in table IV-3. There was a 3 fold increase in the proliferation of Withania treated group. There was a significant enhancement in the proliferation of bonemarrow cells by the mitogen PHA in Withania treated group (10493 cpm) and Withanolide D treated group (8959 cpm) compared to the normal cells 1263 cpm. Similarly stimulation with CON A also showed a significant increase in the bonemarrow proliferation in Withania treated group (8446 cpm) and Withanolide D treated (8021 cpm). Addition of PWM also showed a significant (P<0.001)enhancement in the proliferation in Withania treated group (6258cpm) and in Withanolide D treated group (6015cpm) when compared with normal (3080 cpm). Administration of Withaferin A did not show any significant enhancement in the proliferation of bonemarrow cells.

III.4 Effect of *Withania somnifera* on Natural killer cell activity (NK).

The effect of *Withania somnifera* on NK cell activity of normal and tumour bearing animals is shown in Fig. IV.1. There was a significant enhancement in the NK cell activity in Withania treated normal (52.77% cell lysis) and tumour bearing animal (48.92%) and the maximum activity was observed on 5th day after tumour induction. Normal animals treated with Withaferin A (cell lysis 36.98%) and Withanolide D (cell lysis 49.35%) showed a maximum NK cell activity on 7th day. In the case of tumour bearing control animals maximum NK cell activity (cell lysis 29.29%) was observed only on 11th day.
III.5 Effect of *Withania somnifera* on ADCC activity.

The effect of *Withania somnifera* on ADCC activity is shown in Fig. IV.2. Maximum lysis of target cells was observed on 9th day (cell lysis 65.25%) in Withania treated normal as well as tumour bearing animals while in the untreated control the maximum activity was observed only on 13th day (28.6% cell lysis) after tumour induction. Administration of Withanolide D could significantly enhance the ADCC activity and the maximum cell lysis of 62.1% was observed on day 9 whereas in Withaferin A treated group maximum lysis was observed only on 11th day (cell lysis 41.62%).

III.6 Effect of *Withania somnifera* on ACC activity.

Effect of *Withania somnifera* on ACC activity is given in Fig.IV.3. Administration of Withania extract could significantly enhance the ACC activity on 13th day (47%) in normal animals and in tumour bearing animals on 15th day (45%). Treatment with Withanolide D could significantly enhance the ACC activity on 13th day (47%). In the Withaferin A treated group maximum lysis of 28% was observed on 17th day while in the tumour bearing control animals the maximum cell lysis was observed only on day 21 (21%) after tumour induction.

IV. Discussion

Present study shows that *Withania somnifera* could stimulate the proliferation of lymphocytes, bone marrow cells and thymocytes. *Withania somnifera* could stimulate NK, ADCC and ACC activities of both normal and tumour bearing animals.
Administration of *Withania somnifera* extract could stimulate both T and B lymphocyte proliferation as it is seen from the increase in the proliferation rate. LPS could stimulate lymphocytes more than six times showing that Withania could stimulate B lymphocytes more than T lymphocytes. Bone marrow and thymocyte proliferation by Withania administration was found to be double than the normal. Administration of Withanolide D could significantly enhance the proliferation of splenocytes, bone marrow and thymocyte but withaferin A did not show any significant increase in proliferation.

NK cells have a great role to play as an effector of non-specific immunity. It has been reported that this activation was caused by interferon induction since both interferon and interferon inducers enhance NK cell activity (15). Withania could significantly enhance NK cell activity in normal and tumour bearing mice. NK cell activity mediated by Withanolide D was found to be significantly enhanced. ADCC activity was found to be significantly enhanced by Withania administration in both normal and tumour bearing animal and in Withanolide D treated mice. ADCC is the cooperative interaction of humoral and cellular immune effectors in the expression of cell mediated cytotoxicity. In this cytotoxicity model; cellular effectors with receptors for the Fc portion of immunoglobulin molecules produce target cell lysis by attachment to the Fc portions of antibodies bound to target cell via their antigen combining sites. ACC activity was also found to be significantly enhanced by Withania and Withanolide D administration but Withaferin-A did not have any significant effect in the NK, ADCC and ACC activities.
These results confirm that *Withania somnifera* could stimulate both humoral and cell-mediated immune responses by enhancing both T and B lymphocyte proliferation and Withanolide-D present in *Withania somnifera* may be responsible for this activity.
Table IV - I

Effect of *Withania somnifera* on lymphocyte proliferation (*in vivo*)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No mitogen</th>
<th>Mitogenic Index (MI)</th>
<th>PHA (2.5μg/ml)</th>
<th>M1 (10μg/ml)</th>
<th>M1 (10μg/ml)</th>
<th>PWM (10μg/ml)</th>
<th>M1 (10μg/ml)</th>
<th>Lps (10μg/ml)</th>
<th>M1 (10μg/ml)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>1635±132</td>
<td></td>
<td>3079±225</td>
<td>0.88</td>
<td>3382±165</td>
<td>1.06</td>
<td>2899±224</td>
<td>0.77</td>
<td>2342±66</td>
</tr>
<tr>
<td>Withania</td>
<td>3488±85*</td>
<td>1.13</td>
<td>5555±66*</td>
<td>2.39</td>
<td>8292±181*</td>
<td>4.07</td>
<td>4258±59*</td>
<td>1.64</td>
<td>13443±95*</td>
</tr>
<tr>
<td>Withaferin A</td>
<td>2135±69</td>
<td>0.01</td>
<td>4154±83</td>
<td>1.54</td>
<td>5579±98</td>
<td>2.41</td>
<td>2997±95</td>
<td>0.83</td>
<td>2389±87</td>
</tr>
<tr>
<td>Withanolide D</td>
<td>3321±48*</td>
<td>1.03</td>
<td>6359±69*</td>
<td>2.88</td>
<td>9051±81*</td>
<td>3.92</td>
<td>4238±59*</td>
<td>1.59</td>
<td>12121±95*</td>
</tr>
</tbody>
</table>

* P<0.001

Treated animals received 5 doses of the corresponding drug.

Withania (20mg/dose/animal, i.p)

Withaferin A (500μg/dose/animal, i.p)

Withanolide D (500μg/dose/animal, i.p)
Table IV - 2

Effect of *Withania somnifera* on thymus proliferation (*in vivo*)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No mitogen</th>
<th>PHA (2.5μg/ml)</th>
<th>M1</th>
<th>CON A (10μg/ml)</th>
<th>M1</th>
<th>PWM (10μg/ml)</th>
<th>M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1264±168</td>
<td>4769±266</td>
<td>2.77</td>
<td>6625±144</td>
<td>4.24</td>
<td>3181±36</td>
<td>1.52</td>
</tr>
<tr>
<td><em>Withania</em></td>
<td>3095±198*</td>
<td>8122±86*</td>
<td>5.43</td>
<td>9246±103*</td>
<td>6.31</td>
<td>6318±51*</td>
<td>3.99</td>
</tr>
<tr>
<td><em>Withaferin A</em></td>
<td>1942±69</td>
<td>5685±92</td>
<td>3.5</td>
<td>7522±195</td>
<td>4.95</td>
<td>4521±59</td>
<td>2.57</td>
</tr>
<tr>
<td><em>Withanolide D</em></td>
<td>3321±48*</td>
<td>6359±69*</td>
<td>4.03</td>
<td>9146±265*</td>
<td>6.3</td>
<td>6218±59*</td>
<td>3.91</td>
</tr>
</tbody>
</table>

* P<0.001

Treated animals received 5 doses of the drug.

*Withania* (20mg/dose/animal, i.p)

*Withaferin A* (500μg/dose/animal, i.p)

*Withanolide D* (500μg/dose/animal, i.p)
Table IV - 3

Effect of *Withania somnifera* on bone marrow proliferation (*in vivo*)

<table>
<thead>
<tr>
<th>Mitogens</th>
<th>Normal</th>
<th>Withania</th>
<th>Withaferin A</th>
<th>Withanolide D</th>
</tr>
</thead>
<tbody>
<tr>
<td>No mitogen</td>
<td>1263±52</td>
<td>3483±85*</td>
<td>1942±69*</td>
<td>3321±48*</td>
</tr>
<tr>
<td>PHA (2.5μg/ml)</td>
<td>5023±63</td>
<td>10493±86*</td>
<td>5685±92</td>
<td>6959±69*</td>
</tr>
<tr>
<td>M1</td>
<td>2.98</td>
<td>7.3</td>
<td>3.50</td>
<td>1.63</td>
</tr>
<tr>
<td>CON A (10μg/ml)</td>
<td>4025±49</td>
<td>8446±103*</td>
<td>6883±63</td>
<td>8321±111*</td>
</tr>
<tr>
<td>M1</td>
<td>2.18</td>
<td>5.68</td>
<td>4.44</td>
<td>5.58</td>
</tr>
<tr>
<td>PWM (10μg/ml)</td>
<td>3080±66</td>
<td>6258±55*</td>
<td>4611±53</td>
<td>6015±59*</td>
</tr>
<tr>
<td>M1</td>
<td>1.44</td>
<td>3.95</td>
<td>2.65</td>
<td>3.76</td>
</tr>
</tbody>
</table>

* P<0.001

Treated animals received 5 doses of the drug.

**Withania** (20mg/dose/animal, i.p)

**Withaferin A** (500μg/dose/animal, i.p)

**Withanolide D** (500μg/dose/animal, i.p)
Figure IV.1 Effect of *Withania somnifera* on natural killer cell activity

Days after tumour induction
Figure IV.2 Effect of Withania somnifera on ADCC activity

% Cell Lysis

Days after tumour induction

Tumour alone — Tumour + Withania — Withania — Withaferin A — Withanolide D
Figure IV.3 Effect of *Withania somnifera* on ACC