CHAPTER V

EFFECT OF WITHANIA SOMNIFERA ON

CYTOKINE AND CYTOTOXIC T

LYMPHOCYTE (CTL) PRODUCTION
1. Introduction

The most consistent strategies to activate the antineoplastic actions of the immune system have used host endogenous immunostimulatory protein called cytokines. Experimental evidences have shown two major uses of cytokines in cancer therapy, as antitumour agents and as adjuncts to standard therapy. Interferons were the first cytokines to be identified and their potential as antitumour agent involve their ability to stimulate natural killer cells (56). Interleukin -2 is secreted predominantly by CD4+ cells that play a central role in T cell activation. The activation of natural killer cells by interleukin -2 has defined and is known as lymphokine activated killer cell activity which is very important in the non-specific cell mediated immunity. Granulocyte - Macrophage colony stimulating factor (GM-CSF) is a 22KD glycoprotein principally known for its ability to stimulate growth and differentiation of haematopoietic progenitor cells. The cytokine tumour necrosis factor (TNF-α) causes haemorrhagic necrosis in some tumours in vivo and lysis of transformed cells in vitro(57).

There is abundant evidence suggesting that lymphocytes play a central role in the host response to tumours (58). CD8+ cells from the immunized animals can produce immunity against syngenic tumours when adoptively transferred to a native host (59). Lymphocytes kill target cells; including tumour cells, by inducing them to undergo programmed cell death (apoptosis). There are two effector pathways that account for T cell mediated cytotoxicity namely granule exocytosis pathway and Fas pathway. Present chapter mainly deals with the effect of Withania somnifera on cytokine and cytotoxic T lymphocyte (CTL) production.
II. Materials and methods

II.1. Animals

Balb/c mice (4-6 weeks old) were used for the studies.

II.2. Reagents, kits and cell line

IFN-γ; IL-2; GM-CSF and TNF α - were analysed using ELISA kits; (CTX); Mitomycin C was used for mitotic arrest. EL4 (thymoma) cells used as target cells for CTL assay was grown in RPMI-1640 medium containing 10% FCS. All other chemicals used were of analytical reagent grade (Chapter II).

II.3 Drug treatment

Balb/c mice were treated with 5 doses of Withania extract (20mg/dose/animal, i.p) in all the experiments.

II.4 Determination of the effect of Withania somnifera on IFN-γ; IL-2, GM-CSF and TNF α - production.

Two groups of Balb/c mice (3 mice/group) were used for this study. Group I animals were kept as normal without any treatment. Group II animals were treated with 5 doses of Withania extract (20mg/dose/animal, i.p). The animals were sacrificed 24h. after drug treatment. Blood was collected by heart puncture and serum was separated IFN-γ; IL-2, GM-CSF and TNF α – were determined using ELISA kits from Endogen USA.
II.5 Determination of the effect of *Withania somnifera* on spleen colony formation.

Balb/c mice (4 weeks old) were divided into three groups (6 animals/group). All animals were received single exposure of whole body radiation (400 rads/animal). Group I animals were treated with bone marrow cells (1x10^6 cells/animal) from the normal mice through caudal vein. Group II & III received bone marrow cells (1x10^6 cells/animals) from *Withania* treated mice (10 daily doses, 20 mg/dose/animal, i.p.) from above two group; one group (group III) animals were continued with the drug administration (5doses; 20 mg / dose/animal, i.p.). All other animals were sacrificed on 7th day and the number of nodular colonies on the surface of spleen (9-14) was counted. Each colony was derived from a single precursor stem cell designated as colony forming unit spleen (CFU-S).

**Production of CTL**

II.6 Alloimmunization

Alloimmunization was carried out by injecting spleen cells (2x10^7) from C57 BL/6 mice, subcutaneously to Balb/c mice.

II.7 Generation of effector cells

Effector cells were produced *in vivo* as well as *in vitro* by three system.

II.7a System A (*In vivo*).

Spleen cells (effector cells) were obtained 7 days after alloimmunization of Balb/c
mice were treated with and without 5 doses of Withania with spleen cells from C57BL/6 mice as described above. Donor mice were treated with and without Withania extract (20mg/dose/animal, i.p.) for 5 days after alloimmunization.

II.7b System B (in vitro)

Effector cells were produced by a 5 day mixed lymphocyte culture (MLC) of spleen cells from Balb/c mice treated with (20mg/dose/animal, i.p) for 5 days or without Withania extract, and Mitomycin C (MMC 50μg/ml) treated spleen cells from C57BL/6 mice.

II.7c System C (in vitro)

Effector cells were produced by a 5 day coculture of spleen cells from normal Balb/c mice and MMC (50μg/ml) treated spleen cells from normal (C57BL/6) mice in the presence (50μg/ml) and absence of Withania extract.

II.8 Winn’s Neutralization Assay

Winn’s neutralization test was carried out according to the method of Kobayashi et al (62). Briefly, 1ml of (1x10^7) alloimmune spleen cells from Balb/c mice (effector cells) was mixed with same volume of complete medium containing (5x10^5) EL4 cells (target cells) at an effector: target ratio of 20:1. The cells were incubated for 1h. at 37°C in 5% CO₂ atmosphere and 0.2 ml of this mixture was injected intraperitoneal to Balb/c mice. The animals were observed daily for 60 days after tumour inoculation to determine the survivors. Increase in the survival
time of treated animals was compared with that of animals receiving tumour cell alone. Increase
in survival or mean survival days of the treated group was considered as the indication of CTL
activity.

II.9 Determination of the effect of Withania extract on the in vivo generation of CTL.

Effect of Withania somnifera on the generation of CTL was determined by the Winn’s
Neutralization assay as described above. Effector cells were generated by system B. Six groups
of Balb/c mice (6 mice/group) were used. Group I animals received EL4 cells alone (5\times10^5/
ml). Group II animals received EL4 cells and 10 doses of Withania (20mg/dose/animal, i.p) for
10 days. Group III animals received EL4 cells incubated with effector cells from normal ani-
mals. Group IV animals received EL4 incubated with effector cells from normal mice and
continued with 10 doses of Withania (20mg/dose animal, i.p) for 10 days. Group V animals
received EL4 cells incubated with effector cells from Withania treated animals and Group VI
animals received EL4 cells incubated with effector cells from Withania treated animals and
treated with 10 doses of Withania extract (20mg/dose/animal, i.p) for 10 days. All the animals
were looked for their survival.

II.10 Determination of the effect of Withania somnifera on CTL production (In vitro).

CTL was generated in vitro by 2 methods (system B and System C) as explained above.
In system B, six groups of animals (6nos/group) were used. Group 1 animals received only EL4
cells; Group II animals received EL4 cells incubated with effector cells generated using normal spleen cells in system B. Group III animals were similar to that of Group II but continued with drug administration (20mg/dose/animal, i.p) for 10 days. Group IV animals were incubated with effector cells generated using effector cells from Withania treated animals in system B. Group V animals were similar to that of group IV but continued with Withania administration (20mg/dose/animal, i.p) for 10 days.

In system C; three groups of (6 mice/group) animals were used for each study. Group I animals received only EL4 cells; Group II animals received both EL4 cells and normal allo immune spleen cells. Group III animals received both EL4 cells and Withania treated spleen cells. The animals were observed for the survival upto 60 days.

III. Results

III.1 Effect of Withania somnifera on IFN-γ, IL-2 and GM-CSF production.

The effect of Withania somnifera on the production of various cytokines is shown in table V. 1. Administration of Withania extract to normal Balb/c mice showed a significant enhancement of IFN-γ level (75.98pg/ml) compared to normal animals (40pg/ml). Administration of Withania extract was found to enhance IL-2 level (14.16pg/ml) compared to the normal animals (7.37 pg/ml) (p<0.001). GM-CSF level in normal animal was found to be 32.66 pg/ml which was significantly enhanced by Withania administration 49.22 pg/ml.
III.2 **Effect of *Withania somnifera* on TNF-α production.**

*Withania somnifera* extract administration could lower the TNF-α production in normal animals. TNF-α level was found to be 10pg/ml in normal animal which was lowered significantly to 5pg/ml by Withania administration (Table V.1).

III.3 **Effect of *Withania somnifera* on spleen colony formation.**

Effect of Withania extract administration on the development of nodular colonies on the spleen of irradiated mice is given in table V.2. Administration of bone marrow cells from Withania extract treated animals to irradiated mice through lateral caudal vein produced an increase in the number of nodular colonies (5.33/spleen) on the surface of spleen compared to the animals which were treated with bone marrow cells from normal animals (3.03/animal). When these two groups of animals were continued Withania extract administration after the bone marrow cell injection, both the groups of animals produced a significant increase in the number of nodular colonies on the spleen of recipient mice (3.69/animal for Withania treated bonemarrow and 8.33/animal for normal bonemarrow treated groups).

III.4 **Effect of *Withania somnifera* on CTL production in vivo.**

Effect of *Withania somnifera* on the generation of CTL in vivo is given in table V.3. The survival rate of animals in the untreated tumour bearing (EL4 alone) was 21 days while that of EL4 and Withania treated group was 45 days with an increase in life span (ILS) of 114.2%. When the animals were injected with EL4 cells incubated with effector cells from normal ani-
mals the survival rate was 33 days (%ILS=57.14%). When these animals were continued with drug administration (20mg/dose/animal, i.p) for 10 days; the survival was increased upto 53 days (p<0.001). When EL4 cells were incubated with effector cells from Withania treated animals the survival rate was 46 days and when these animals were continued with Withania administration; the survival rate was significantly increased to 61 days (P<0.001).

III.5 Effect of Withania somnifera on CTL production in vitro.

The effect of Withania on in vitro generation of CTL by system B is given in Table V.4. The survival rate of animals treated with EL4 cells alone was 22 days. The survival rate of animals treated with EL4 cells incubated with effector cells from normal spleen was 32 days. When these animals were continued with Withania (10doses) the survival rate was increased upto 52 days. When the animals were injected with EL4 cells which were incubated with the effector cells generated by coculturing from Withania treated animals and MMC treated spleen cells from C57BL/6 mice the survival rate was increased upto 48 days. When these animals were continued with 10 doses of Withania the survival rate was increased upto 61 days.

III.6 Effect of Withania somnifera on CTL.

Effect of Withania somnifera on CTL generation by system C is given in table V.5. The survival rate of animals treated with EL4 cells alone was 22 days. When the animals were treated which effector cells produced a 5 day coculture of spleen cells from normal Balb/c mice and C57 BL/6 mice (MMC treated), the survival was increased upto 35 days. But When the EL4
were incubated with the effector cells generated by coculturing spleen cells from Balb/c and MMC treated C57BL/6 mice in the presence of Withania extract the life span of the animals were increased to 60 days giving an ILS of 172.73%.

IV. Discussion

Administration of Withania extract significantly increased the cytokine levels such as IFN-γ, IL-2 and GM-CSF in normal mice. The lowered levels of these cytokines after the administration of cylophosphamide could be normalised by Withania treatment. The extract was found to enhance the spleen colonies in control as well as radiation exposed mice treated with normal bone marrow. Administration of bone marrow cells from Withania treated donor mice could increase the nodular colonies on the surface of spleen in irradiated mice indicating that administration of Withania extract increased the potential of stem cells to colonize at distant sites.

The antitumour effects of cytokines can be due to the direct effects on tumour cells, including induction of tumour cell differentiation and direct cytostatic or cytotoxic effects. Some cytokines can lead to tumour cell killing indirectly by stimulating host responses; for example induction of lymphokine activated killer cells by γ-interferon and macrophage colony stimulating factor. The results of the present study shows activation of host lymphocytes for the production of cytokines by Withania treatment. Withania treatment enhanced the production of IFN-γ which there by activate natural killer cells. The ability of IFN-γ to augment natural killer cell
activity has been well established and substantial evidence to date suggests that most biological agents that enhance natural killer cytotoxicity do so via their ability to induce IFN (61). IL-2 stimulates certain cellular elements such as cytotoxic T lymphocytes or natural killer cells and thereby kill tumour cells (5). GM-CSF factor is enhanced in Withania treated group compared to normal and cyclophosphamide treated group. Tumour necrosis factor-α level was decreased in Withania treated tumour bearing group compared to normal animals. Cytokines such as interleukin-1 and tumour necrosis factor-α have been shown to augment tumour spread.

Lymphocytes kill target cells through apoptosis. The two pathways that account for T cell mediated cytotoxicity are granule exocytosis pathway by the release of lethal hit protein and Fas pathway in which CTLs with an ineffective granule exocytosis pathway exhibits a residual cytotoxicity only against target cells that express for antigen (58).

Administration of Withania somnifera was found to increase the CTL production in both in vivo and in vitro treated group by the Winn’s Neutralization assay. When the animals were injected with thymomas (EL4) cells incubated with effector cells generated in vitro as well as in vivo (alloimmunization) in the presence of Withania extract, there was a significant increase in the survival time. This indicates that Withania somnifera could enhance the production of cytotoxic T lymphocyte which is very important in the cell mediated immune response.
**Table V - 1**

Effect of *Withania somnifera* on cytokine production in normal mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytokine (concentration, pg/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFNγ-</td>
<td>IL-2</td>
<td>GM-CSF</td>
<td>TNF α-</td>
</tr>
<tr>
<td>Normal</td>
<td>40±07</td>
<td>7.37±15</td>
<td>32.6±13</td>
<td>10±11</td>
</tr>
<tr>
<td>Withania</td>
<td>75.87±0.98*</td>
<td>14.16±15*</td>
<td>49.2±15**</td>
<td>5±12**</td>
</tr>
</tbody>
</table>

*P<0.001  
**P<0.01

Withania treated animals received 5 doses of the drug extract (20mg/dose/animal i.p.)
Table V - 2

Effect of *Withania somnifera* on spleen colony forming unit assay in irradiated mice

<table>
<thead>
<tr>
<th>Spleen Treatment</th>
<th>Number of nodular colony</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal bonemarrow</td>
<td>3.03±1.07</td>
<td></td>
</tr>
<tr>
<td>normal bonemarrow + Withania</td>
<td>8.33±1.2*</td>
<td>125.90%</td>
</tr>
<tr>
<td>Withania treated bonemarrow</td>
<td>5.33±.04</td>
<td>75.91%</td>
</tr>
<tr>
<td>Withania treated bonemarrow + 5 dose continued</td>
<td>13.69±1.1*</td>
<td>156.84%</td>
</tr>
</tbody>
</table>

*P<0.001

Treated animals received 10 daily doses of Withania extract (20mg/dose/animal, i.p.).
Table V - 3

Effect of *Withania somnifera (in vivo)* on CTL generation. System A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival rate of animals (days)</th>
<th>% increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL4 alone</td>
<td>21±1.85</td>
<td></td>
</tr>
<tr>
<td>EL4+ 10 doses Withania</td>
<td>45±2.32**</td>
<td>114.28%</td>
</tr>
<tr>
<td>EL4 + normal alloimmunized effector cells</td>
<td>33±1.72</td>
<td>57.14%</td>
</tr>
<tr>
<td>EL4 + normal alloimmunized effector cells + 10 doses Withania</td>
<td>53±1.48*</td>
<td>152.38%</td>
</tr>
<tr>
<td>EL4 + Withania treated alloimmunized spleen cells</td>
<td>46±1.2**</td>
<td>119.04%</td>
</tr>
<tr>
<td>EL4 + Withania treated alloimmunized spleen cells + 10 doses Withania</td>
<td>51±8.9*</td>
<td>190.48%</td>
</tr>
</tbody>
</table>

*P<0.001  
**P<0.01

CTL was assayed by Winn's neutralization assay. All the animals were treated with EL4 cells (5x10^6 cells/1 ml). Drug continued group were treated with Withania (20mg/dose/animals, i.p). Alloimmunized spleen cells from drug treated animals received five doses of Withania same as above and its drug continued group received 10 doses of Withania. The animals were observed for survival.
Table V - 4

Effect of *Withania somnifera* on CTL production (*in vitro*) System B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival rate of animals (days)</th>
<th>% increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL4 alone</td>
<td>22±2.15</td>
<td></td>
</tr>
<tr>
<td>EL4 + normal co-cultured spleen cells</td>
<td>32±2.40</td>
<td>45.45%</td>
</tr>
<tr>
<td>EL4 + normal co-cultured spleen cells + 10 doses Withania</td>
<td>52±1.41*</td>
<td>136.36%</td>
</tr>
<tr>
<td>EL4 + from Withania treated co-cultured spleen cells</td>
<td>48±3.50**</td>
<td>118.18%</td>
</tr>
<tr>
<td>EL4 + from Withania treated co-cultured spleen cells + 10 doses Withania</td>
<td>61±3.65*</td>
<td>177.27%</td>
</tr>
</tbody>
</table>

*P<0.001  
** P<0.01

CTL was assayed by Winn's neutralization assay. All the animals were treated with EL4 cells (5x10⁵ cells/ 1ml). Effector cells from normal and Withania treated Balb/c animals were cultured along with MMC treated spleen cells from C57 BL/6 mice for 5 days at 37°C. The animals were observed for survival.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival rate of animals (days)</th>
<th>% increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL4 alone</td>
<td>22±2.5</td>
<td></td>
</tr>
<tr>
<td>EL4 + normal spleen cells</td>
<td>35±2.3</td>
<td>59.04%</td>
</tr>
<tr>
<td>EL4 + normal spleen cells + Withania</td>
<td>60±3.1*</td>
<td>172.73%</td>
</tr>
</tbody>
</table>

*P<0.001

CTL was assayed by Winn’s neutralization assay. All the animals were treated with EL4 cells (5x10^3 cells/1ml). Spleen cells from normal Balb/c mice cultured along with MMC treated spleen cells from C57 BL/6 mice in the presence and absence of Withania (50 µg/ml) for 5 days at 37°C. The animals were observed for survival.