Immunopotentiating Activity of Septilin

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ABSTRACT
Oral administration of Septilin (100 mg/animal/dose; five doses) was found to enhance natural killer cell mediated cytotoxicity and antibody-dependent cellular cytotoxicity in normal mice as well as tumor-bearing mice. Septilin treatment also activated the peritoneal macrophages, which produced cytotoxicity to L929 cells. Septilin increased proliferation of bone-marrow cells and there was an increase in the number of $\alpha$-naphthyl acetate esterase staining cells in the bone marrow. In addition to the activation of cellular immunity, Septilin was found to increase the number of antibody producing cells in the spleen and activation of antibody-dependent complement-mediated cell lysis. These studies justify the use of this herbal preparation in improving immunocompetence in disease states.

Septilin is a proprietary Ayurvedic product of The Himalayan Drug Company, Bombay, containing various herbs and minerals. It is extensively used in the treatment of several acute/chronic infections. It has been demonstrated that Septilin stimulated phagocytosis and thereby helped in controlling infections. Recently we have shown that Septilin could increase the total count of leukocytes and the percentage of polymorphs in the peripheral blood. It could also protect mice from cyclophosphamide-induced myelosuppression and subsequent leukopenia. In the present study the effect of Septilin on the cell-mediated and humoral immune responses in mice has been investigated.

MATERIALS AND METHODS
Drug-Pure Septilin powder was supplied by The Himalayan Drug Co., Bombay. Its main ingredients are (mg): Balsamodendron mukul, 162; Shank bhasma, 32; Maharasnadi quath, 65; Tinospora cordifolia, 49; Rubia cordifolia, 32; Emblica officinalis, 16; Moringa pterygosperma, 16; Glycyrrhiza glabra, 6.

Drug extraction –Septilin powder (6 g) was boiled in 600 ml of distilled water for 30 min. It was centrifuged and filtrate evaporated to dryness on a water bath and made up to 45 ml. This extract (0.75 ml) was used for oral administration and it contained the extract from 100 mg of Septilin. For all the experiments a total of 5 doses of this extract was given orally on alternate days unless mentioned. This concentration was found to be non-toxic. The control animals received an equal volume of saline.

Reagents and chemicals –Eagles minimum essential medium (MEM) Rosewell Park Memorial Institute medium (RPMI)-1640 and Dulbeco’s minimum essential medium (DMEM) were purchased from Hi-media Laboratories, Bombay. Concanavalin-A (CON-A) was purchased from Sigma Chemicals, St. Louis, MO, USA,) and phytohemagglutinin (PHA) from Difco Laboratories, USA. $^3$H-thymidine (Sp. activity 10,000-25,000 mCi/mmole) and sodium chromate ($\text{Na}_2\text{CrO}_4$)}
(sp. activity-5mCi/mg) were brought from Board of Radiation and Isotope Technology, BARC, Bombay. Alpha-naphthyl acetate and pararosaniline were obtained from Loba chemie, Bombay, agarose from SRL Bombay. All other reagents and chemicals were of Analytical grade.

Cell lines – L-929 (mouse lung fibroblast) cells and YAC-1 (mouse lymphoma) cells were obtained from National Facility for Animal Tissue and Cell Culture, Pune. Ehrlich ascites tumor (EAT) cells (spontaneous tumor of mammary gland of mice) were initially obtained from Cancer Institute, Bombay and were maintained as ascites tumor in Swiss albino mice. Sheep red blood cells (SRBC) were freshly collected in Alsever’s solution.

Animals – Female Balb/C mice 20-22 g (6-8 weeks old) were purchased from National Institute of Nutrition, Hyderabad. They were housed in ventilated cages in air controlled rooms.

Balb/C mice were divided into four groups (20 animals/group). Group I, normal controls. Group II, Septilin treated animals. Group III, tumour-bearing controls and group IV, tumour-bearing animals treated with Septilin.

Five doses of 0.75 ml of Septilin (equal to 100 mg powder) was given orally daily to groups II and IV. Group I received only saline. Along with the last dose, EAC \(10^6\) were injected to groups III and IV. The animals were sacrificed on various days and the spleens were processed into single cell suspension in RPMI-1640 containing 10% FCS and used as effector cells. Blood was collected from these animals by heart-puncture, sera separated and heat inactivated at 56°C for 30 min. Sera was used as anti-EAC antibody for ACC estimation.

YAC-1 cells \(10^6\) cells or SRBC \(10^7\) cells were incubated along with \(\text{Na}_2\text{CrO}_4\) \(100 \mu\text{Ci}\) at \(37°C\) for 1 hr. They were washed, suspended at a concentration of \(10^5\) cells/ml and used as targets for NK cells (YAC-1) and ADCC (SRBC) assay as given below:

The labelled target YAC-1 cells \(10^4\) cells/tube were incubated with spleen cells at an effector target ratios of 100:1, 50:1, and 25:1 at \(37°C\) for 4 hr. After the incubation 100 \(\mu\)l of the supernatant was counted for radioactivity released after cell death using a gamma ray spectrometer. The percent lysis due to NK-cell activity was calculated by the formula:

\[
\% \text{Lysis} = \frac{\text{ER} - \text{SR}}{\text{TR} - \text{SR}} \times 100
\]

Where, ER is the experimental release, SR is the spontaneous release and TR is the total release. SR and TR were determined by incubating the target cells alone or with HCl (IN, 100 \(\mu\)l) respectively.

Determination of antibody-dependent cellular cytotoxic activity – ADCC activity was determined using a chromium release assay described by Kim et al. Labelled SRBC \(1x10^4\) cells was incubated with effector cells at different effector: target ratios of 100:1, 50:1 and 25:1 along with 0.1 ml of anti-SRBC antibody. An aliquot of the supernatant was counted for released radioactivity as above.
Determination of antibody-dependent complement-mediated lysis – Ehrlich ascites tumor cells (1x10^4 cells) were incubated with serially diluted sera sample (100 µl), and 50 µl of 1:10 diluted fresh rabbit serum as a source of complement at 37°C for 3 hr. The cytotoxicity was assessed by trypan blue exclusion method⁹.

Lymphocyte blastogenesis assay- Lymphocyte blastogenesis assay was done as described previously¹⁰. 24 hr after the drug treatment the animals were sacrificed and spleen cells were used for lymphocytes. Controls receivedonly saline. Spleen cells (10^⁶ cells/ml) were incubated with various concentrations of mitogens (CON A – 10 and 5 µg/ml or PHA – 6 and 3 µg/ml) at 37°C for 48 hr in 3 ml or RPMI-1640 supplemented with 10% FCS after incubation ³H-thymidine (2 μCi/vial) was added to each vial and incubated for 18 hr. DNA was precipitated with 10% PCA and the amount of radioactive thymidine incorporated into DNA were counted using liquid scintillation counter (Rack Beta).

Bone-marrow cell proliferation assay– The assay was done as described for lymphocyte proliferation assay. Instead of spleen cells bone marrow cells (10⁶ cells/ml) from treated and untreated animals were incubated in presence and absence of mitogens. The amount of radioactive thymidine incorporated into the cells were used as a measure of cell proliferation.

Determination of macrophage-mediated cytotoxicity – Peritoneal macrophages were induced by injecting (ip) 0.2 ml of sodium caseinate (5%) on the last day of drug treatment. After 5 days the macrophages were collected in Hank’s Balanced Salt Solution (HBSS). It was washed and suspended in DMEM containing 10% FCS at a concentration of 2.5x10⁵ cells. Target L929 cells (5x10^³ cells/well) were incubated with macrophages (100 µl) from treated and untreated animals for 48 hr. in a final volume of 200 µl at 37°C in a humidified atmosphere having 5% CO₂. After incubation cells were fixed and stained with crystal violet and cellular cytotoxicity was assessed morphologically¹¹.

Determination of antibody producing cells – All animals of both treated and untreated groups (10 per/group) received SRBC (2.5x10⁸ cells) along with the last dose of Septilin. The animals from each group were sacrificed on various days and the spleen cells were used to perform Jerne’s plaque assay¹².

Quantitation of circulating antibody titre – Along with the last dose of Septilin treatment all animals (6/group) received 0.1ml of 20% SRBC. Blood was collected from the tail vein prior to SRBC injection and on every third day thereafter. Sera separated and heat inactivated at 56°C for 30 min. The titre was determined by hemagglutination method¹³.
RESULTS

**Effect of Septilin on natural killer cell activity** (Fig.1A) – the NK-cell activity was found to increase progressively from 48 hr after drug treatment in both Septilin treated normal and tumour-bearing mice. The maximum NK-cell activity was observed on day 7 with a percentage cell lysis of 55 and 58 respectively. In the case of control tumour-bearing animals the maximum NK-cell activity was observed on day 9 of tumour inoculation with a target cell lysis of 48%.

**Effect of Septilin on antibody-dependent cellular cytotoxicity activity** – ADCC was found to be enhanced in normal and tumour-bearing animals after Septilin treatment (Fig.1B) Maximum ADCC activity was observed on day 5 in both groups with percent target lysis of 63 and 69. In tumour-bearing controls the maximum ADCC was observed on day 7 after tumour inoculation with a target, cell lysis of 47%.

**Effect of Septilin on antibody-dependent complement-mediated lysis activity in mice** – Administration of Septilin produced only a marginal increase in ACC activity in tumour-bearing animals (data not shown). The maximum cytotoxicity (24%) was found on day 15 in Septilin treated animals and on 21st day in control animals (19%).

**Effect of Septilin on macrophage activation** – Administration of Septilin was found to activate the macrophages and was cytotoxic towards L929 cells (Fig.2) as seen from the loss of tumour cell morphology. Macrophages isolated from untreated animals did not show any cytotoxic effect.

**Effect of Septilin on bone marrow and spleen cell proliferation** – Septilin treatment significantly enhanced the proliferation of bone-marrow cells in culture (Table 1). Degree of enhancement of proliferation was nearly 10 times in animals treated with Septilin, as seen from the increased

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Fig. 2: Effect of Septilin on macrophage activation. (A) Normal L929 cell grown in monolayer. (B) L929 cells treated with peritoneal macrophages from Septilin treated mice.
incorporation of $^3$H-thymidine when compared to controls. Addition of mitogens did not alter the rate of proliferation of bone-marrow cells in the treated animals. The proliferation of spleen cells in presence or absence of mitogens were not affected by the treatment of Septilin (data not shown).

In a similar experiment $\alpha$-naphthyl acetate esterase positive bone marrow cells were found to be enhanced by Septilin treatment (804 cells/4000 bone marrow cells) compared to controls (648/4000 bone-marrow cells).

### Table 1: Effect of Septilin on bone-marrow cell proliferation in mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thymidine incorporation (CPM)</th>
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<tbody>
<tr>
<td></td>
<td>No mitogens</td>
</tr>
<tr>
<td>Control</td>
<td>909 ± 154</td>
</tr>
<tr>
<td>Septilin</td>
<td>10951 ± 154*</td>
</tr>
</tbody>
</table>

Five doses of Septilin equivalent to 100 mg of extract was given orally, and 24 hr after the drug treatment bone-marrow cells were cultured to assess the proliferation. The values are mean ± SD of triplicates of two experiments. *$p$<0.001.

### Table 2: Effect of Septilin on the number of antibody producing cells in mice spleen (Values are mean ± SD of 6 animals/group done in triplicates)

<table>
<thead>
<tr>
<th>Antibody producing cells (PFCs)/10$^6$ spleen cells</th>
<th>Days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Control (SRBC alone)</td>
<td>150 ± 5</td>
</tr>
<tr>
<td>Septilin + SRBC</td>
<td>110 ± 8</td>
</tr>
</tbody>
</table>

*$p$<0.001.

**DISCUSSION**

The data presented here demonstrate the effect of Septilin on both cellular and humoral immune response. Null cells such as NK and killer cells are lymphoid cells lacking both T- and B-cell markers. They have a great role to play in reducing the development of cancer and its metastasis$^{14}$. Oral administration of Septilin significantly enhanced NK-cell activity, ADCC and ACC in both normal as well as tumour-bearing animals. Moreover, Septilin activated peritoneal macrophages were highly cytotoxic to L-929 cells. It has been reported that L-929 cells are highly sensitive to cytotoxicity mediated by tumor necrosis factor (TNF)$^{15}$. Thus the mechanism of tumour cell kill by the macrophages isolated from Septilin treated animals may be mediated by TNF. We have already shown that Septilin could increase the life span of tumour-bearing animals$^5$. Septilin was not directly cytotoxic to tumour cells. Hence, the tumour reducing activity of Septilin may be by the activation of macrophages.

Septilin administration significantly enhanced proliferation of bone-marrow cells from the treated animals in culture. There was also an increase in the number of cells positive for esterase (data not shown). In our previous study$^6$ Septilin was found to protect mice from cyclophosphamide induced myelosuppression. This may be due to the stimulation of bone-marrow stem cells either directly or indirectly through augmented secretion of growth factors.
In addition to the augmentation of cellular immune responses, Septilin was found to activate humoral responses. Septilin treatment enhanced the number of plaque forming cells in the spleen and marginally activated the secretion of antibodies into the circulation and ACC in mice.

Septilin contains several herbomineral principles and at present we do not know the active material involved in the stimulation of the immune system. Septilin was shown to contain polysaccharides which activate properdin system, and increase chemotaxis of polymorphs at the site of infection, phagocytosis and subsequent destruction of microorganisms\textsuperscript{17}. At present we do not know the component in Septilin responsible for this action.

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REFERENCES