Immunotherapeutic Modification by an Ayurvedic Formulation Septilin

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ABSTRACT

Effect of Septilin, an Ayurvedic formulation proven to be effective in the therapy of chronic infections, was investigated on the phagocytic system and humoral response in rats and mice. Septilin exhibited significant protection in E. coli-induced abdominal sepsis in normal mice and in Staphylococcus aureus-induced sepsis in neutropenic mice. It significantly reduced the viable E. coli cells when incubated with neutrophils in rats. Septilin stimulated the phagocytic function of the reticuloendothelial system in mice. In normal rats, Septilin enhanced anti-SRBC hemagglutination antibody titre by 5.7 fold and showed significant protection in cyclophosphamide-induced humoral suppression.

Immune dysfunctions pose a great problem for the physician to treat. Immune deficiencies have been demonstrated following severe thermal injuries, trauma and sepsis. These deficiencies often predispose patients to potentially lethal infections. To correct these deficiencies, immunomodulating agents are increasingly being tested. The initially tested immunomodulators were bacteria and bacterial products, but the complexity of these agents made a complete determination of their mechanism of action difficult to achieve. In addition, these agents had several undesirable effects.

The degree to which a patient becomes abnormally susceptible to infection by this microbial environment depends on the extent of immunosuppression. The suppression of the immune system is associated with cancer, surgery or drugs characterized by a reduction in the number of phagocytic function of neutrophils and macrophages, as well as an impairment of the intracellular bactericidal capacity of these cells. This suppression of the individual elements of the immune system allows opportunistic pathogens to overwhelm the host, so that secondary infections become the most common cause of mortality.

The development of antimicrobials has revolutionized the treatment of bacterial diseases was predicted. But the fact remains, however, that in spite of the availability of myriad antibiotics, patients continue to suffer from serious infections. Some patients are at a greater risk of recurrent bacterial invasions and subsequent infections. In clinical situations with insufficient numbers or deficient neutrophils, or in pathologic conditions in which phagocytic cells cannot reach sequestered organisms (i.e. around foreign bodies), high-dose antibiotic therapy is often incapable of eradicating the infecting microorganisms in spite of the susceptibility of these microbes to the antibiotics used.
An attempt to overcome this problem has been made by introducing the concept of prohost therapy. This approach aims at administering drugs to boost immune defences against infections. Several natural and synthetic substances like B.C.G., Glucans, Levans, Interleukine-1 and colony-stimulating factors have been used to stimulate the non-specific host resistance against infections. But these are too expensive and not without side effects. Hence, it is better to treat these patients by nutritional support, immunization or by using immunoadjuvants.

Medicinal plants commonly used as herbal drugs, are known to have immunomodulatory properties. These may act by stimulating both non-specific and specific immunity. In specific immunity it may stimulate either humoral immunity and cell-mediated immunity or only the humoral response while suppressing the cell-mediated component of the immune system.

Septilin, a herbal formulation containing immunoactive and anti-inflammatory plant principles, has been reported to potentiate the non-specific defence mechanism of the body and thus helps to overcome infective and inflammatory processes. Septilin not only builds up the resistance to disease but also has a capacity to neutralize the causative factors. In our present study we have attempted to evaluate non-specific and specific defence mechanisms by using different animal models, viz. 1. E. coli-induced abdominal sepsis in mice, 2. Bactericidal activity of polymorphonuclear cells in rats, 3. Staphylococcus aureus-induced sepsis in cyclophosphamide-induced neutropenic mice, 4. Carbon clearance by the reticuloendothelial system, in mice. 5. Humoral response using SRBC as an antigen in normal and immune deficient rats.

**MATERIALS AND METHODS**

**Animals** – Swiss albino mice and Wistar rats of inbred colony were used in the study. All animals were given synthetic diet (Hindustan Lever Pellets, Bangalore), clean tap water ad libitum, and maintained at 22º ± 1ºC with 60% relative humidity, and day and night cycle of 12 hr each.

**E.coli-induced abdominal sepsis** – This study was carried out in two parts. In the first part, the strength of E.coli was standardized to induce 100% mortality and in the second part, the effect of E.coli injection in mice and protection by Septilin, if any, was evaluated.

In the first part, the pathogenic strain of E.coli was subcultured in the nutrient broth using a shaker water bath at 37ºC at 50 rpm for 18-24 hr and absorbency of the culture was measured at 540 nm. The viability of the culture was confirmed by serial dilution and surface spreading techniques. After 24 hr of incubation at 37ºC, the total viable cell count was observed from the MacConkey agar plates.

Based on the standard graph (viable cell count vs absorbency), different doses of viable E.coli cells were administered (ip) to different groups of mice, and the optimum dose of viable
**E. coli** cells required to cause abdominal sepsis with 100% mortality up to 48 hr was evaluated.

In the second part of the study 33 Swiss albino mice of either sex weighing between 25 and 35 g were selected. They were housed in a pathogen-free environment and given free access to water and food. The mice were divided into two groups: group I (n=18) served as control and received tap water, while group II (n=15) received Septilin syrup in the dose of 20 ml/kg body weight, orally once a day for 15 days.

On day 15, 3 hr after the last dose of Septilin, blood was collected for total and differential leucocyte counts. *E. coli* (5x10^8) cells were then injected intraperitoneally to mice of both the groups and percentage mortality was observed after 24 hr. The survivors, if any, in both the groups were further observed for 5 days. Throughout the study, blood of dead and surviving animals was cultured on MacConkey agar to confirm the intensity of *E.coli*-induced sepsis. Colonies from MacConkey agar were isolated and identified by Gram staining, motility and biochemical tests.

*Bactericidal activity of polymorphonuclear cells in rats – in vitro study – E. coli* strain subcultured in nutrient broth was centrifuged and the supernatant was discarded. The *E.coli* cells were washed three times, suspended in a known volume of phosphate buffered saline and the turbidity of the suspension was measured spectrophotometrically at 540 nm. The viable cell count was determined by the method of serial dilution and surface spreading technique, and, standard graph obtained with different concentrations. The require viable *E.coli* cells (1.6 x 10^7) were determined.

Wistar rats (28) of either sex were divided into 2 groups. The first group served as control and received water (n=16) in the dose of 10 ml/kg body weight while the second group received Septilin syrup (n=12) in the dose of 10 ml/kg body weight, once a day orally for 15 days.

On day 16, 8 ml of venous blood was collected from each rat in both the groups, in heparinized sterile culture tubes for the determination of total and differential leucocyte counts. The blood samples were centrifuged (at 100 g for 15 min) and the plasma carefully transferred into culture tubes for inactivation. The cells were washed thrice with K.R.P. buffer and suspended to their original volume with K.R.P. buffer and suspended to their original volume with K.R.P. buffer. Polymorphonuclear leucocytes (0.8 x 10^7), *E. coli* cells (1.6 x 10^7), K.R.P. buffer (1.5 ml) and 80% heat inactivated plasma were taken in a sterile culture tube and incubated in a water bath at 37ºC for 60 minutes. Incubated mixtures (0.6 ml) was mixed with 2.4 ml of 5% Saponin to lyse the leucocytes and the number of viable *E.coli* cells were determined by the method of serial dilution and surface spreading technique using MacConkey agar plates. These plates were incubated at 37ºC for 24 hr and the colony forming units were counted by using colony counter.
Staphylococcus aureus-induced sepsis in neutropenic mice – Submaximal dose of Staphylococcus aureus cells required to induce 75% mortality was standardized as per the procedure mentioned earlier for E.coli and was found to be $5 \times 10^8$ cells.

Albino mice (27) of either sex were selected and randomized into two groups. Mice of group I (n=15) received water 20 ml/kg body weight and group II (n=12) received Septilin syrup 20 ml/kg body weight, once a day orally for 15 days. On day 15, 3 hr after the last dose, neutropenia was induced in both the groups with cyclophosphamide, 200 mg/kg by (sc) administration. Mice of both the groups were given respective treatment for 4 days after inducing neutropenia and on day 4, $5 \times 10^8$ viable Staphylococcus aureus cells were injected (iv) to induce sepsis. Mortality and survivors, if any, in both the groups were observed for 120 hr$^{24}$.

Carbon clearance assay in mice – Swiss albino mice (15) weighing between 25 and 30 g were divided into two groups. Mice of group I (n=9) received 10 ml/kg body weight of tap water and group II (n=6) received 10 ml/kg body weight of Septilin syrup, orally once a day for 15 days. On day 15 three hours after the last dose, mice in both the groups were administered 0.1 ml of colloidal carbon (iv) after a warm up period of 15-20 min at 37ºC. One drop of blood was collected at 2, 5, 10, 15, 20, 30, 45, 60 and 90 min by tail nipping. Blood sample (10 µl) was then lysed in 2 ml of 1% glacial acetic acid and the optical density of lysed samples measured spectrophotometrically at 625 nm using the pre-injection blood sample as a blank. The graph of absorbance against time was plotted and the area under curve (AUC) determined$^{25}$.

Humoral response in normal and immunodeficient rats – Wistar rats (106) of either sex were divided into four groups. Group I rats (n=35) were administered 10 ml/kg body weight of water once a day orally from day (-9) to day (+4). Group II (n=26) rats were administered 10 ml/kg body weight of water once a day orally from day (-9) to day (+4) and on day (+2) 400 mg/kg body weight of cyclophosphamide was administered orally in addition to water treatment. Group III rats (n=36) were administered 3 gm/kg body weight of Septilin once a day orally from day (-9) to day (+4) and on day (+2) 400 mg/kg body weight of cyclophosphamide was administered orally in addition to Septilin treatment. On day 0 rats in all groups were immunized (ip) with $5 \times 10^8$ sheep red blood cells (SRBC).

On day (+4), blood samples were collected from each rat after 3 hr. Serum was inactivated by heating at 56ºC for 30 min. Serial 2-fold dilutions of inactivated serum sample made in 25 µl of normal saline containing 0.1% BSA in V-bottomed microtitration plates and were mixed with 25 µl of 0.1% SRBC suspension in PBS. After mixing, SRBC allowed to stand at 25ºC, until control wells showed an unequivocally negative pattern (a small button). The value of highest serum dilution carrying visible hemagglutination was taken as the antibody titre$^{26}$. 
Statistical analysis - All parameters were statistically analyzed either by unpaired Student’s t test or $\chi^2$ test.

RESULTS

E.coli induced abdominal sepsis – Absorbency was found to be proportional to the series of dilutions made from 18-24 hr old culture. Following intraperitoneal injection of varying doses of E.coli cells to different groups of mice, a dose of $5 \times 10^8$ cells was found to be optimum for 100% mortality due to abdominal sepsis in untreated mice.

Septilin-treated mice showed no mortality as compared to 77.8% in control mice, 24 hr after the administration of E.coli cells. Mortality in control mice was 100% as compared to 33.3% in the Septilin treated group, after 48 hr and no further mortality was observed in the Septilin-treated group even after 120 hr. Blood cultures of dead animals from the control group showed very high E.coli growth (++++) confirming that death was due to E.coli-induced abdominal sepsis (Fig.1).

Total leucocyte, absolute lymphocyte and absolute neutrophil counts were significantly increased following 15 days of Septilin treatment as compared to the control group of mice (Fig.2).

Bactericidal activity of polymorphonuclear cells in rats in vitro study – The total number of viable E.coli cells ($x 10^6$/ml) in the Septilin-treated group was $1.36 \pm 0.39$ (n=12), which was significantly lower as compared to the control group, in which it was $3.50 \pm 0.77$ (n=16) (Table 1).

The absolute neutrophil count ($x 10^3$/ml) in the Septilin-treated group (1786.00±163.66) was significantly more than in the control group (1243.50±155.53) (Table 1).
Table 1: Effect of Septilin on bactericidal activity of polymorphonuclear cells in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Total leucocyte count (x 10^3/ml)</th>
<th>Absolute neutrophil count (x 10^3/ml)</th>
<th>E. coli count (x 10^3/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R x Water</td>
<td>8081.25 ± 821.19 (n=16)</td>
<td>1243.50 ± 155.53 (n=16)</td>
<td>3504.39 ± 771.43 (n=16)</td>
</tr>
<tr>
<td>R x Septilin</td>
<td>8608.33 ± 681.15 (n=12)</td>
<td>1786.00 ± 163.66 (n=12)</td>
<td>1364.00 ± 393.99 (n=12)</td>
</tr>
</tbody>
</table>

*ab p<0.01

**Staphylococcus aureus-induced sepsis** – An intravenous dose of 5x10^8 viable *Staphylococcus aureus* cells was found to be the submaximal dose which caused 75% mortality in the cyclophosphamide-induced neutropenic mice.

After 24 hr of iv administration of *Staphylococcus aureus*, the control animals showed 66.6% mortality (n=15) as compared to 20% mortality (n=12) in the Septilin-treated group. No further mortality was observed after 48, 72, 96 and 120 hr.

*Carbon clearance assay* - The total area under the time-absorbency curve in the Septilin-treated group was 10.06 ± 0.71 (n=6) as compared to 15.46 ± 1.78 (n=9) in the control group of mice.

*Humoral response in normal and immunodeficient rats* - Anti-SRBC hemagglutination antibody titre in the Septilin-treated rats was found to be 6.47±0.26 (n=36) as compared to the control rats. 3.97±0.03 (n=35) (Fig.3).

Anti-SRBC hemagglutination antibody titre in the Septilin-treated rats was found to be 4.44±0.13 (n=9) as compared to cyclophosphamide-induced humoral suppressed rats 1.96 ± 0.18 (n=26) (Fig.3).

**DISCUSSION**
Intra-abdominal sepsis continues to be a major cause of morbidity and mortality following trauma and abdominal surgery for bowel perforation. Treatment of this condition has always focused on appropriate surgery, antibiotics and nutritional support. But in spite of this, fatal complications have been reported. A factor which influences the recovery from such an infective process is the host defence mechanism.
In our present study, Septilin significantly protected the mice against *E.coli*-induced abdominal sepsis. In the Septilin-treated mice, there was only 33% mortality with a significant reduction of bacteraemia as against 100% mortality in the controls after 48 hr (Fig.1). No further mortality was observed in the Septilin-treated group even after 120 hr. This indicates that Septilin might have enhanced the capacity of the monocyte-macrophage system. This is further proved by a significant rise in the total, absolute lymphocyte and absolute neutrophil counts (Fig.2). This is substantiated by a significant rise in carbon clearance, indicating stimulation of the reticuloendothelial system.

The mechanism of phagocytosis and its intracellular sequelae is complicated by factors such as the bactericidal properties of blood and other tissue fluids. The possible participation of two populations of phagocytic cells (i.e., polymorphonuclear and mononuclear phagocytes) and the difficulties involved in quantitating the effects of ingestion and intracellular killing of bacterial population are also present. Hence, the protective effect of Septilin was studied *in vitro*. Since it was practically difficult to obtain sufficient polymorphonuclear cells from mice, rats were selected for this study. There was significant reduction in the number of viable *E.coli* cells (Table1) in the Septilin-treated group as compared to control when *E.coli* cells were incubated with polymorphonuclear cells of the respective groups. This suggests that the phagocytic activity of polymorphonuclear cells may be stimulated by Septilin. Further studies are under progress to determine the type of phagocytic stimulation (oxygen dependent or independent) by Septilin. The above findings are substantiated by a significant rise in absolute polymorphonuclear cell counts in the Septilin-treated group (Table 1).

In cyclophosphamide-induced neutropenic mice, Septilin significantly protected the animals against the *Staphylococcus aureus*-induced sepsis as the Staphylococcus aureus-induced sepsis as compared to controls.

Septilin-treated rats showed a 5.7-fold rise in anti-SRBC chemagglutination antibody titre which is mediated by IgG and IgM\(^2\) (Fig.3). This suggests a significant potentiating action of Septilin on humoral immunity.

Septilin significantly protected cyclophosphamide-induced humoral suppression. This suggests that Septilin counteracts primary IgG and IgM suppression induced by cyclophosphamide (Fig.3).

The leucocytosis which probably occurs due to secretion of interleukin-1 and colony stimulating factors from activated macrophages\(^2\) along with increased number and functional capabilities of peritoneal macrophages\(^3\) appears to be the underlying mechanism of protection offered by Septilin\(^4\). It has already been reported that Septilin stimulates phagocytes to ingest bacteria and inhibit their growth\(^1\). Bhasin\(^5\) and Kothari\(^6\) have demonstrated a marked increase in serum IgG levels, following treatment with Septilin.
The present studies thus distinctly corroborate and clearly demonstrate the following facts that Septilin treatment (a) increases the number of neutrophils in mice (b) increases the bactericidal activity of neutrophils in rats, (c) accelerates the elimination of colloidal carbon (d) prevents mortality from E.coli-induced abdominal sepsis in mice (e) reduces bacteraemia after injection with Staphylococcus aureus in neutropenic mice, (f) potentiates humoral immunity in rats, and (g) counteracts cyclophosphamide-induced humoral suppression in rats.

REFERENCES


