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Pharmacological Evaluation of Proprietary Preparation from Bacterial Metabolites with Special Reference to its Immunomodulatory Actions

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Abstract – A mixture of several bacterial metabolites (Sterodin®) was used to study its effect on major immunocytes, *in vivo* and *in vitro*. This mixture of bacterial metabolites increased number of macrophages and neutrophils and their phagocytic activity in experimental animals for a transient period. BSA induced antibody production was found to be higher in the drug treated group. These results indicated that the bacterial metabolites probably acted through non-specific defence mechanism against invading organisms and the chance of reinfection was reduced.
Key words – Bacterial metabolites, immunomodulatory actions.

The role of the immune system in the pathogenesis of an ever-increasing number of diseases has inevitably led to attempts to manipulate the various units of the immune machinery for their management. Until recently immunosuppression was the goal of applied immunology for tissue transplantation technology. With the discovery of tumour immunity that excites a set of immune response directed against themselves - the focal point of immunological control has turned to immunopotentiation (Sjorgren, 1961). Use of immunotherapy or use of immunopotentiating agents has become more relevant to patients having immunodeficiencies - congenital or acquired, complicated infectious diseases and malignancy.

It was proved that polyspecific bacterial metabolites elicited an immunopotentiation that is not specific for the bacterial antigens present in the preparation. It has been shown that introduction of a harmless strain of *E. coli* can induce antibody formation of virulent *Haemophilus influenzae*, Type B, the cause of an increasing number of childhood cases of meningitis (Fauve, 1974). *Mycobacterium tuberculosis* (BCG), anaerobic *coryneforms* etc., also stimulated the reticuloendothelial system (Hoffmann, 1971) that plays the key role in immune response.

In the present investigation the pharmacological actions of a mixture of detoxified bacterial metabolities with special emphasis on immunomodulation were studied.

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Experimental

Bacterial Metabolites – Detoxified bacterial metabolites were obtained from a commercially available drug Sterodin® which contained the following detoxified bacterial strains (Cells/ml) - *Streptococcus* (2×10^6), *Micrococcus* (2×10^6), *E. coli* (2×10^6), *Typhoid* (Standard) *O & VI-H* (2×10^6), *Paratyphoid A&B* (2×10^6), *Staphylococcus aureus* (4×10^6), *Staphylococcus albus* (4×10^6), *H. influenzae* (5×10^5), *Bacillus dipthoroid* (5×10^5). The drug also contained bile lipoids (0.148 mg/ml) and preservatives including glycerine (0.3% v/v), alcohol (8.0% v/v), phenol (0.5% v/v) and distilled water (I.P).

Protein estimation – This was done after Lowry (1951).

Lethality – To avoid the interference of preservatives the drug was evaporated to dryness in vacuum lyophilizer and the residual material was dissolved in phosphate buffer (0.01M, pH 7.2) up to the initial volume and was injected intraperitoneally in male albino mice (approximate body weight 20 g) and lethality was observed upto 48 hours.

Total and Differential WBC Count – Normal laboratory acclimatized male albino mice were injected with measured amount of Sterodin® intraperitoneally. The blood was withdrawn from the tail vein on 0 day,

3rd day, 6th day, 15th day and 30th day of injection. The total WBC per cmm were counted under oil immersion microscope and neutrophil and lymphocyte counts were particularly noted. A group of saline injected mice were taken as control.

Total Peritoneal Macrophage Count – Peritoneal exudate cells were obtained by injecting phosphate buffer (0.01 M, pH 7.2) intraperitoneally (40 ml/100 g body wt.) in male albino mice. The peritoneal fluid was collected and centrifuged at 500g. The pellet was resuspended in phosphate buffer and stained with 1% neutral red in saline. The suspension containing macrophages was counted in a haemocytometer chamber. Peritoneal exudate cells contain nearly 90% macrophages with few monocytes and other cells.

Macrophage Phagocytic Activity – This was judged by the rate of removal of injected carbon particle from the blood stream which is a measure of reticuloendothelial/macrophage phagocytic activity (Wagner, 1991). Both control and treated mice received an intravenous injection of 0.2 ml/20 g of Indian ink (Rottering, Germany). Finally blood samples were taken at intervals of 30 sec., 2 min., 5 min., and 15 min., from the retro-orbital plexus (20 μl blood in 3 ml distilled water to lyse the erythrocytes). At the end the absorbance was measured spectrophotometrically at 650 nm. The clearance of carbon particle was inversely proportional with the absorbance at 650 nm.

Neutrophil Phagocytic Activity – The stimulated neutrophil phagocyted the NBT dye (Loba, India) and converted the dye to an insoluble blue deposit. By this experiment percentage of activated neutrophils in treated or control animals' blood can be calculated (Segal, 1974). Neutrophils from the blood of both groups of animals were isolated in Percol (Sigma, USA) density gradient medium. After mixing the cells and dye, the mixture was incubated for 15 min at 37 °C. Then a film was made with the suspension on a microscopic slide and counter stained with dilute carbolfuchsin (15 seconds), washed in tap water, dried and observed under oil immersion microscope and the NBT positive cells were counted per hundred neutrophils.

Activity of T-lymphocyte by SRBC Rosette Formation – T subclass of lymphocyte population possesses surface receptors for sheep RBC which is detrimental for its activity. Isolated lymphocyte suspension and sheep RBC was mixed in RPMI 1640 (Sigma, USA) culture medium and centrifuged at 100 g and incubated overnight at 4 °C. The mixture was fixed in a glass slide and stained with Giemsa (Stanbo, India). The number of rosettes were counted per hundred lymphocytes (Dale, 1994).

Measurement of Antibody Titre Developed Against BSA in Presence and Absence of Bacterial Metabolites – Antiserum was prepared in rabbits against BSA (Sigma, USA) both in presence and absence of bacterial metabolites present in Sterodin® by standard six weeks hyperimmunization schedule. By single radial immunodiffusion the zone of precipitation at different dilutions of serum was measured.

Results and Discussion

1 ml bacterial metabolites present in Sterodin® contains 1.88 μg protein. Different doses of phosphate buffer dissolved Sterodin® were injected intraperitoneally in male albino mice and upto 1 ml of Sterodin® no lethality was observed. This indicated that during the process of detoxification and extraction of bacteial metabolites eventually all the pathogenecity of the virulent bacteria were lost.

At a single dose of bacterial metabolites (0.1 μg/20 g) in male albino mice the mean total WBC count was increased by 59% ($p<0.001$) within 6th day of injection which came to nearly 0 day level/control value within 30th day of injection (Table 1, Fig. 1a & b). Differential count from the same blood sample showed significant increase in neutrophil count (26%, $p<0.001$) on the 6th day of injection (Fig. b). However the normal or the 0 day ratio of neutrophil (N): lymphocyte (L) was nearly attained within 30th day of observation (Table 2).

Neutrophils, non-specific phagocytes, are the most numerous among WBCs and the best studied. In their brief life span (ten hours or so) they primarily migrate

Table 1. Effect of bacterial metabolites on total WBC Count

No. of animals	Dose of bacterial metabolites	Total WBC count/cmm				
		0 day	3rd day	6th day[a]	15th day	30th day
6	0.1 μg/20 g (i.p)	5882±181	6630±198	9345±805	7535±416	6875±190

[a] Maximum increase (59%, $p<0.001$, compared to 0 day value) in total WBC count observed on 6th day

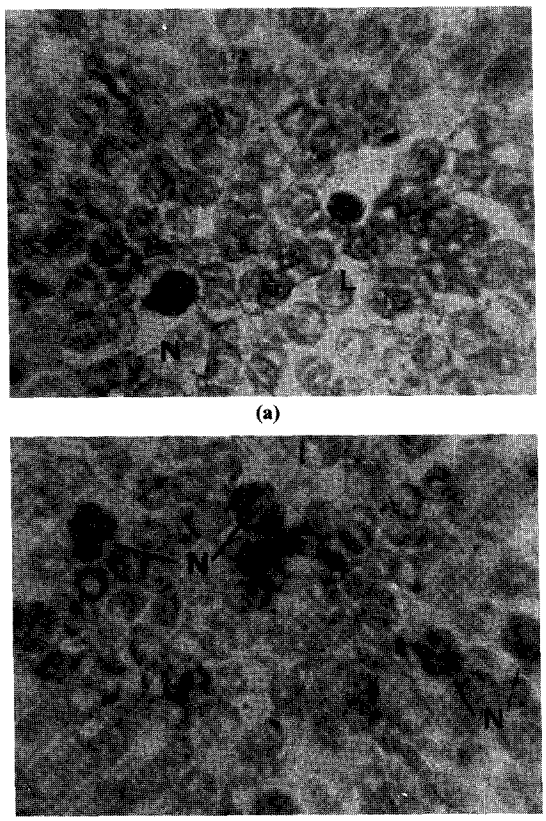

Fig. 1. (a) Differential Count of Control Animals. N=Neutrophil, L=Lymphocyte. (b) Differential Count of Bacterial Metabolites Treated Animals (6th day of treatment) N=Neutrophil, L=Lymphocyte

to the site of infection or inflammatory lesion, performing their phagocytic functions and probably exhausting their metabolic reserves in doing so (Old, 1961). Neutrophil induced phagocytosis usually occurs in cooperation with opsonizing antibodies and complements. Thus a higher neutrophil titre will not only perform their bactericidal function but also reduce the chance of reinfection in association with opsonizing antibodies. Bacterial metabolites in Sterodin® not only increased the number of neutrophils but also increased the phagocytic activity *in vivo*. Neutrophils isolated from bacterial metabolites treated (0.1 μg/20 g) male albino mice showed 3 fold increase (45±5.8 phagocyted cell per 100 neutrophils, significant level is at $p<0.001$) in phagocytic activity than neutrophils isolated from untreated animals (15±2.8 phagocyted cell per 100 neutrophils, Fig. 2). Thus increased number of neutrophils with increased activity can definitely phagocyte and kill the large number of invading organisms and prevent the spread of infection and reinfection (Wilkinson, 1974). These changes of neutrophil count and activity are reflected in the way individuals cope with bacterial or viral infection.

A single dose of bacterial metabolites (0.1 μg/20 g, i.p) in male albino mice produced significant increase in total peritoneal macrophage count. The maximum

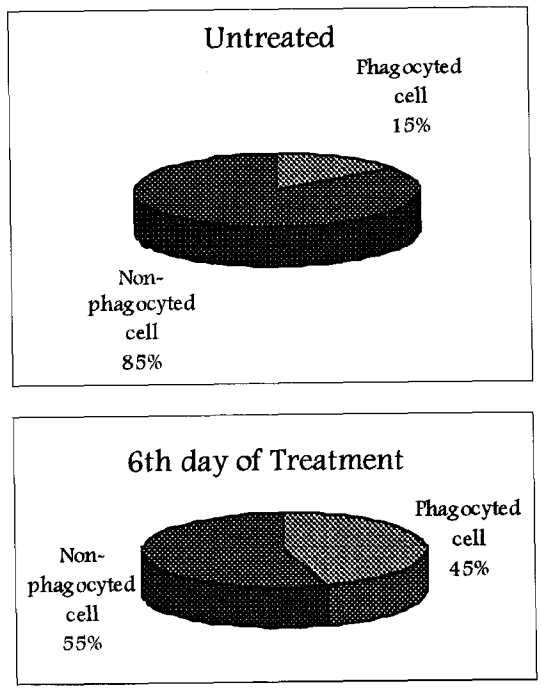

Fig. 2. Effect of Bacterial Metabolites on Neutrophil Phagocytic Activity as judged by NBT-Test.

Table 2. Effect of bacterial metabolites on differential count of WBC

No. of animals	Dose of bacterial metabolites	Differential Count (%)					
		0 day		6th day[a]		30th day	
		N[b]	L[c]	N	L	N	L
6	0.1 μg/20 g (i.p)	61±1.5	39±1.5	77±1.6	23±1.6	67±2.4	33±2.04

[a]Significant increase in neutrophil count (26%, $p<0.001$, compared to 0 day value) observed on 6th day. [b]Neutrophil. [c]Lymphocyte

Table 3. Effect of bacterial metabolites on total peritoneal macrophage count on male albino mice

No. of animals	Dose of bacterial metabolites	Total peritoneal macrophage count ($\times 10^6$)				
		0 day	3rd day	6th day[a]	15th day	30th day
6	0.1 μg/20 g (i.p)	4.51±0.3	5.21±0.1	8.1±0.8	5.63±0.32	4.8±0.21

[a]Maximum increase in total peritoneal macrophage count (80%, $p<0.002$, compared to 0 day value) observed on 6th day.

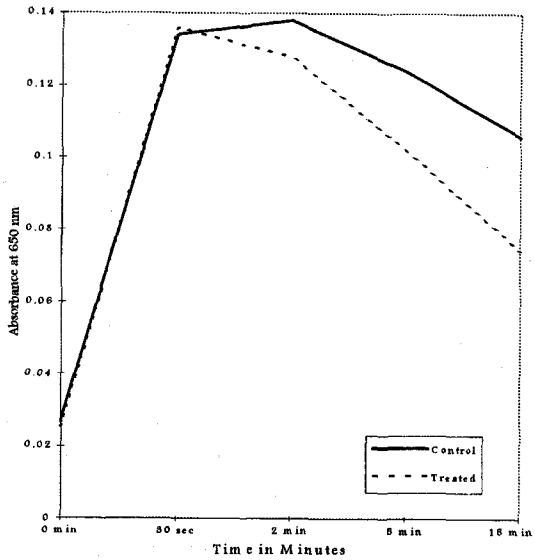

Fig. 3. Effect of Bacterial Metabolites on Macrophage Activity as judged by Carbon Clearance Test.

increase in number (80%, $p<0.002$) was observed on 6th day of injection that nearly came to 0 day level within 30th day of injection (Table 3). The macrophage activity was also found to be higher in the 6th day of injection as judged by carbon clearance test (Fig. 3).

There is overwhelming evidence that immunostimulants in general and bacterial metabolites like *BCG, mycobacteria, E. coli,* anaerobic *coryneforms,* etc., stimulate both macrophage cell population and activity included in the reticuloendothelial system (Hoffman, 1971; Biozzi, 1979).

These macrophages not only carry antigen and interact with lymphocytes but also non-specifically kill the invading organisms. Apart from this unlike neutrophils, macrophages are capable of protein synthesis, cell division and upon stimulation with agents like bacterial metabolites may divide rapidly and form a cell line with a long life, perhaps months. This long life-span of macrophages gives the chance to migrate from one tissue to another and transport foreign or antigenic materials, bringing these into close contact with lymphocytes. This cooperation with lymphocytes induces immune response and reduces the chance of reinfection.

Bacterial metabolites in the present preparation decreased the number of T-lymphocyte-SRBC rosettes depicting the fact that T-lymphocyte reactivity was inhibited. Thus the surface reactivity with SRBC was decreased and incomplete rosettes were formed. This suppression was probably due to the influence of activated macrophages. In an *in vitro* study it has been found that normal responsiveness of the T-lymphocytes reappeared after removal of macrophages from the mixture medium by magnetic elemination (Scott, 1972b). Bacterial metabolites probably, did not directly inhibit T-lymphocytes reactivity and the suppression of T-lymphocytes was probably not due to the factors present in it (Howard, 1973). However current knowledge about the regulatory influence of macrophages over lymphocytes function is highly fragmentary and awaits further study.

Bacterial metabolites contained in Sterodin® along with BSA during hyperimmunization schedule produced an increase in antibody production. In single radial immunogel-diffusion experiment serum of Sterodin® treated animals produced precipitation ring of 15 mm diameter at 1 : 5 dilution whereas the serum of the control animals (that received only BSA) produced precipitation ring of 7 mm diameter at the same dilution (Table 4). Similar effect was observed

Table 4. Effect of bacterial metabolites on antibody titre as judged by diameter of precipitation ring in single radial immunodiffusion test

Conditions	Diameter of precipitation ring in mm (n=6)		
Serum dilution	Undiluted	1 : 2	1 : 5[a]
Serum from animal receiving bacterial metabolites + BSA	20±1.7	17±1.2	15±1.2[a]
Serum from animal receiving only BSA	17±0.9	14±0.7	7±0.3

[a]$p<0.001$ when compared with diameter of precipitation ring obtained by the same dilution of serum from animal receiving only BSA.

with the treatment of *C. parvum* before immunization (Halpern, 1971).

The interaction between macrophages and T-lymphocytes was crucial for antibody production and this process was likely to be reciprocal, governed by a certain quantitative equilibrium. However, the reactivity of B-lymphocytes was not suppressed like T-lymphocytes when they were exposed to bacterial metabolites induced changes (Scott, 1973). In fact the adjuvant effect of bacterial metabolites on B-cells might involve a T-cell bypass mechanism for immune response (Howard, 1973). Apart from these, bacterial metabolites were capable of stimulating antibody synthesis to T-independent antigens (Del Guercio, 1972).

Conclusion

Immunopotentiation is still at its beginning considering its clinical applications. From the present investigation it can be concluded that bacterial metabolites present in Sterodin® possess the capability to stimulate some aspects of immune response. Its actions over neutrophils and macrophages facilitate phagocytosis of the invading organism. For antibody production these activated macrophages occupy a central position and influence the activities to T & B lymphocytes.

Further investigation regarding the mechanism of action of bacterial metabolites on major immunocytes is going on in our laboratory.

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