Natural Product Sciences
16(3) : 159-163 (2010)

Anti-inflammatory Activity of Detoxified Bacterial Strains in Wistar Rats

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Abstract – A mixture of several detoxified bacterial strains (Sterodin®) has been studied for anti-inflammatory effect in Wistar rats on carrageenin, dextran and prostaglandin E_1 (PGE_1) induced edema in acute model and cotton pellet and carrageenin induced sub-acute model, while, Freund's adjuvant induced chronic model. The bacterial strains showed strong inhibitory activity in acute, sub-acute and chronic models of inflammation. Further, it reduced α_1 acid glycoprotein and α_2 macroglobulin levels in serum and prostaglandin E_2 in inflamed paw. These results indicated that the bacterial strains probably act through prostaglandin mediatory pathways and may be useful in treatment of inflammation.
Keywords – Bacteria, Inflammation, Prostaglandins, *Freund's complete adjuvant*, Glycoprotein, Macroglobulin

Introduction

Infectious agents, parasitic diseases or cellular pathologies are becoming more resistant to conventional methods of protection or treatment. These methods include vaccines, antibiotics, or immunomodulating drugs. In controlling disease, one field that is gaining momentum is the development of compounds that stimulate the body's natural defenses (Schoenborn and Wilson, 2007). Polysaccharides located on bacterial cell membranes have been found to enhance the general or cell mediated immunity of animals to various diseases and preventing and treating various infections as well as in treating carcinomas. D'Hinterland *et al*. (1988) isolated a membrane polysaccharide (comprises a chain of galactofuranose and galactopyranose units) from gram negative bacteria which was found to have an immunomodulating effect on natural killer cells to destroy Maloney's lymphoma. 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 increasing number of childhood cases of meningitis (Fauve, 1974). *Mycobacterium tuberculosis* (BCG), anaerobic *corniforms* etc., also stimulated the reticuloendothelial system (Hoffmann, 1971) that plays the key role in immune response. Recently, BCG immunotherapy showed helpful in the treatment of bladder cancer (Suttmann *et al*., 2004).

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Earlier, we reported that mixture of bacterial strains (Sterodin®) increased the number of macrophages and their phagocytic activity non-specifically (Auddy *et al*., 1999). Cell mediated immunostimulating action of bacterial strains has also been reported (Dey Ray *et al*., 2009). Recently, we observed that bacterial strains reduced the pro-inflammatory responses induced by lipopolysaccharides (data not shown). In the present investigation the anti-inflammatory actions of a mixture of detoxified bacterial strains with its mode of action were studied.

Experimental

Detoxified bacterial mixture – Detoxified bacterial mixture were obtained from a commercially available drug Sterodin® which contained the following detoxified bacterial strains (cells/ml)-*β-Streptococcus* (2×10^6), *N. caterrhalis* (2×10^6), *E. coli* (2×10^6), *S. typhi* (standard O & H) (2×10^6), *S. paratyphi* A & B (2×10^6), *Staphylococcus aureus* (4×10^6), *Staphylococcus albus* (4×10^6), *H. influenza* (5×10^5), and *Diptheroid bacilli* (5×10^5). The drug also contained bile lipoids (0.148 mg/ml) and preservatives including glycerin (0.3%v/v), alcohol (8.0%v/v), phenol (0.5%v/v) and distilled water (I.P.).

Chemicals – All the chemicals and reagents were of analytical grade. Carrageenin, dextran, prostaglandin E_1, prostaglandin E_2, bovine serum albumin, l-hydroxyproline, *Freund's complete adjuvant,* were obtained from Sigma (St. Louis, MO, USA). The serum α_1-acid glycoprotein

and α_2-macroglobulin levels were determined using highly sensitive two site enzyme linked immunoassay kits (Immunological Consultants Laboratory, Inc., Newberg, Oregon, USA).

Animals – Inbred male Wistar rats (150 ± 5 g) were used in this study. The animals were kept in colony cages under identical housing conditions, *i.e.,* 12 h light : 12 h dark cycle, 50 - 60% humidity and 22 - 25 ºC temperature. They were fed with commercial pellet diet made for rat and water *ad libitum*. Food was restricted to the animal overnight before they were used for experimentation. The care and maintenance of the animals were as per approved guidelines (Committee for the Purpose of Control and Supervision of Experiments on Animals, India). The Institutional Ethics Committee (*Registration No. 507*) approved the study.

Safety evaluation/Lethality – To avoid the interference of preservatives the bacterial mixture (Sterodin®) was evaporated to dryness by nitrogen gas and the residual material was dissolved in phosphate buffer saline (PBS, 0.01 M, pH 7.2) up to the initial volume and was injected intramuscularly in rats and the lethality was observed up to 48 h (Sur *et al*., 2004).

Selection of doses – The effective dose for anti-inflammatory studies of the bacterial mixture was selected to be 100 µl/150 g rat intramuscularly, on the basis of the previous study (Auddy *et al*., 1999) and the dose was close to one tenth of LD_{50} dose. The animals were divided into three groups (n = 8) and treated as follows: group-I was pretreated with PBS (100 µl/150 g rat), group-II with Sterodin® (100 µl/150 g rat), while group-III with diclofenac sodium (10 mg/kg). All drugs were given intramuscularly to rats. The animals were pretreated three doses of Sterodin® for every alternative day (–4, –2 and 0 day) prior study to boost its effect and continued till the end of experimentation (in case of sub-acute and chronic studies).

Acute inflammation – Acute paw edema was induced in groups of eight rats, each using three different experimental models, viz., carrageenin-induced paw edema, dextran-induced paw edema and prostaglandin-induced paw edema.

Carrageenin-induced paw edema – Acute inflammation was induced by carrageenin according to the model of Winter *et al*. (1962) and slightly modified in our laboratory (Sur *et al*., 2002). For this purpose, 0.1 ml of 1% suspension of carrageenin in 0.9% saline was injected into the sub-planter tissues of right hind paw in rats. The right paw volume was measured plethysmographically (Techno, Lucknow, India) at 0 h and 3 h after carrageenin injection. The treated drugs were administered intramuscularly 1 h prior to carrageenin injection.

Dextran-induced paw edema – In this experiment, 0.1 ml of 1% dextran in sterile saline was injected into the sub-planter tissue of the right hind paw of rats (Arunachalam *et al*., 2002). The paw volume was measured plethysmographically before and after 30 min of the dextran injection.

Prostaglandin-induced paw edema – In accordance with the method of Willis and Cornelsen (1973) and slightly modified in our laboratory (Sur *et al*., 2003), Prostaglandin E_1 at the dose of 1 mg/kg was administered into the sub-planter region of the right hind paw of rats. The paw volume was measured before and after 30 min of the prostaglandin E_1 injection.

Sub-acute inflammation – Sub-acute inflammation was induced in rats using two different experimental models: cotton pellet induced granuloma and repeated administration of carrageenin in paw.

Cotton pellet induced granuloma – Wistar rats were anesthetized by light ether and wound was made by implantation of two sterile cotton (15 ± 1 g), one on either side in the lumber region on the dorsal surface (Banerjee *et al*., 2000). The treated drugs were administered in alternate days for 10 days in the same dose as mentioned (100 µl/150 g rat). The animals were sacrificed on day 10. One of the granulation tissue formed on implanted cotton was weighed and further, dried at 60 ºC overnight and then the dry weight was taken. Moreover, the other part of granuloma tissue was homogenized in phosphate buffer (0.1M, pH 7.2) and protein was estimated spectrophotometrically according to the method of Lowry *et al*. (1951) and hydroxyproline as described by Woessner (1961).

Carrageenin-induced sub-acute model – In this method, 0.1 ml of 1% carrageenin was injected into the sub-planter region of right hind paw in rats for three consecutive days (Winter *et al*., 1962). The test drugs were administered intramuscularly 1h prior to carrageenin injection for 3 days. Then the animals were lightly anesthetized, blood was collected from heart, serum was separated and preserved for Immuno-absorbent assay. Thereafter, the animals were sacrificed under deep anesthesia and the inflammed paw was excised from ankle joints and preserved in –80 ºC for prostaglandinE_2 estimation. Serum a_1-acid glycoprotein and serum α_2-macroglobulin concentration was assessed using commercially available ELISA kit (Immunological Consultants Laboratory, Inc., Newberg, Oregon, USA). Prostaglandin E_2 in inflamed paw tissue was possessed (Harada *et al*., 1996) and estimated by HPTLC.

Chronic inflammation – Chronic inflammation was induced in rats using *Freund's complete adjuvant* in paw.

Adjuvant arthritic induced inflammation – Adjuvant arthritis was induced by subcutaneous injection of 0.1 ml of *Freund's complete adjuvant* into sub-plantar tissue of the right hind paw of each rat (Sudaroli and Chatterjee, 2007). The swelling in the injected and contra-lateral hind paws of the rats were monitored at day 15 and day 28 using plethysmograph (Techno, Lucknow, India).

Statistical analysis – The results were expressed as Mean ± SEM of two independent experiments. The statistical significance was determined by One-Way Analysis of Variance (ANOVA) followed by paired t-test. $P < 0.05$ was considered to be statistically significant.

Results

Safety/Lethality evaluation – Different doses of PBS dissolved Sterodin® were injected intramuscularly in rats and up to 1.0 ml of Sterodin® no lethality was observed.

Carrageenin-induced paw edema – Bacterial detoxified mixture, Sterodin® (100 µl/150 g rat) inhibited paw edema caused by carrageenin significantly ($P < 0.001$) in rats similar to standard diclofenac sodium. Sterodin® reduced paw volume 47% within 3h, while diclofenac sodium reduced paw volume 59% than normal saline treated control (Table 1).

Dextran-induced paw edema – Sterodin® and diclofenac sodium significantly ($P < 0.001$) reduced the paw volume in dextran induced inflammation. Paw edema was reduced to 45% by Sterodin® and 56% by diclofenac sodium when compared to control (Table 1).

Prostaglandin-induced paw edema – Sterodin® exhibited mild inhibition (26%) while, diclofenac sodium showed maximum reduction (44%) in paw edema formation during PGE_1-induced inflammation within 30 min, though both of them were significant ($P < 0.001$) in comparison to control (Table 1).

Cotton pellet induced granuloma – The continuous treatment of bacterial strains, Sterodin® reduced the formation of granuloma tissue and thereby its wet weight (30%) and dry weight (33%) also lowered. Further, Sterodin® enhanced the protein concentration (29%) and hydroxyproline concentration (57%) in granuloma tissues when estimated spectrophotometrically. Diclofenac sodium also exhibited the positive results (Table 2).

Carrageenin-induced sub-acute model – Sterodin® did not show any significant change in serum α_1-acid glycoprotein level but it reduced the formation of serum α_2-macroglobulin (30%). Sterodin® also significantly

Table 1. Effect of detoxified bacterial strains, Sterodin® on acute inflammation in Wistar rats

	Paw volume increased (ml)		
	Control	Sterodin®	Diclofenac sodium
Carrageenin induced inflammation	1.218 ± 0.14	0.643 ± 0.09* (47)	0.493 ± 0.06* (59)
Dextran induced inflammation	0.843 ± 0.09	0.456 ± 0.07* (45)	0.368 ± 0.06* (56)
PGE_1 induced inflammation	0.645 ± 0.12	0.472 ± 0.08* (26)	0.361 ± 0.05* (44)

N = 8 in each group; Mean ± SEM; the values are compared statistically with respective control by using one way analysis of variance followed by paired t-test (spss v-12 package); * indicates $p < 0.05$; parenthesis indicates % change/inhibition in comparison with control.

Table 2. Effect of detoxified bacterial strains, Sterodin® on sub-acute inflammation in Wistar rats

	Control	Sterodin®	Diclofenac sodium
Cotton pellet granuloma formation			
Wet weight of granuloma tissue (g)	0.241 ± 0.02	0.168 ± 0.02* (30)	0.121 ± 0.01* (49)
Dry weight granuloma tissue (g)	0.078 ± 0.004	0.052 ± 0.003* (33)	0.036 ± 0.003* (53)
Protein in granuloma tissue (mg/g tissue)	26.41 ± 1.08	34.11 ± 1.96* (29)	40.52 ± 1.74* (53)
Hydroxyproline in granuloma tissue (µg/g tissue)	41.62 ± 2.70	65.37 ± 3.48* (57)	74.5 ± 3.02* (73)
Carrageenin-induced sub-acute inflammation			
Serum α_1- acid glycoprotein (ng/ml)	71.0 ± 9.9	56.4 ± 6.1 (20)	52.8 ± 4.8 (25)
Serum α_2-macroglobulin (ng/ml)	329.0 ± 30.5	228.7 ± 34.1* (30)	206.4 ± 26.8* (37)
Tissue PGE_2 (ng/gm tissue)	3.29 ± 0.64	2.35 ± 0.56 (28)	1.90 ± 0.36* (42)

N = 8 in each group; Mean ± SEM; the values are compared statistically with respective control by using one way analysis of variance followed by paired t-test (spss v-12 package); * indicates $p < 0.05$; parenthesis indicates % change/inhibition in comparison with control.

Table 3. Effect of detoxified bacterial strains, Sterodin® on *Freund's complete adjuvant* induced arthritis in rats

	Swelling paw volume increased (ml)	
	Day 15	Day 28
Control	0.98 ± 0.07	1.57 ± 0.09
Sterodin®	0.53 ± 0.04* (-45)	0.49 ± 0.02* (68)
Diclofenac sodium	0.48 ± 0.03* (-51)	0.46 ± 0.02* (70)

N = 8 in each group; Mean ± SEM; the values are compared statistically with respective control by using one way analysis of variance followed by paired t-test (spss v-12 package); * indicates $p < 0.05$; parenthesis indicates % change/inhibition in comparison with control.

reduced the concentration of PGE_2 (28%) in inflammed paw tissues in rats. Diclofenac sodium showed similar results (Table 2).

Adjuvant arthritic induced inflammation – Prolonged treatment with Sterodin® showed 68% inhibition in swelling of paw induced by FCA within 28 days. Diclofenac sodium showed similar type (70%) of reduction in swelling paw (Table 3).

Discussion

Most of all investigators have reported that inhibition of carrageenin-induced inflammation in rats is one of the most suitable test procedures to searching anti-inflammatory agents (Mondal *et al*., 2003). The development of carrageenin-induced edema is bi-phasic, the first phase is attributed to the release of histamine, 5-HT, and kinins, while, the second phase is related to the release of prostaglandins and different macroglobulins (Sur *et al*., 2003). From the present study it is noted that, in carrageenin induced acute model, there was significant inhibition of paw edema in the animals treated with detoxified bacterial strains in Sterodin®. Further, it reduced the formation of edema when in dextran was used.

Cotton pellet granuloma is a model of non-immunological type of inflammation mediated by the activation of the chemical mediators of inflammation, mostly kinins. The action of kinin involves the activation of two membrane receptors, B_1 and B_2. B_1-receptor plays an important role in inflammatory processes (Farmer and Burch, 1992). This method has also been used to evaluate the transudative and proliferative components of the subacute inflammation (Chattopadhyay *et al*., 2002). The wet weight of the cotton pellets correlates with transudes while the dry weight of the cotton pellets correlates with the amount of granuloma tissue formation (Banerjee *et al*., 2000). In this study, it was reported that both the wet and dry weight of granuloma tissue were reduced in bacterial strains treated rats, indicating that Sterodin® may act by the way of inhibiting the B_1-receptor. Further, it enhanced the concentration of protein and hydroxyprolin that indicated its role in the formation of higher level of fibroblasts and myofibroblasts. The fibroblasts are responsible for production of the mucopolysaccharide ground substances (Xing *et al*., 2000). There is also formation of granuloma tissue in which collagen turn over increases. Hydroxyproline is one of marker of collagen formation (Woessner, 1961). This study indicates that bacterial strains in Sterodin® may be effective in the treatment of sub-acute inflammation, particularly during surgical condition.

Prostaglandin biosynthetic pathway or the cyclooxygenase (COX) activity of the enzyme is the site of action of non-steroidal anti-inflammatory drugs (NSAIDS). Diclofenac sodium is a widely used potent NSAIDS (Tonussi and Ferreira, 1994). It has been recognized recently that mammalian cells express two forms of COX. COX-1 is the major form present in platelets, while COX-2 is only found in elevated levels in inflammatory exudates (Willoughby, 1998). Increased production of nitric oxide in the inflammed tissue activates COX-1 and COX-2 resulting in an increase in production of PG (Vane and Booting, 1995). Therefore, the inhibitory action of Sterodin® on PGE_1 induced edema and PGE_2 on inflammed paw tissues explains that it may modulate PGs production by inhibition of COX.

Further, bacterial strains lowered the concentrations of α_1-acid glycoprotein and α_2-macroglobulin in serum during inflammation. α_2-Macroglobulin is not only a protease inhibitor, but it is also a specific cytokine carrier that binds pro-inflammatory and anti-inflammatory cytokines implicated in inflammations (Gourine *et al*., 2002). Protease inhibitors are often considered nonspecific defense molecules, with the function of protecting tissues from unwanted proteases released by pathogenic microorganisms or from the dying host (Harada *et al*., 1996; Schoenborn and Wilson, 2007).

One of the reasons for the wide utilization of Freund's adjuvant induced arthritis is due to the strong correlation between the efficacy of therapeutic agents in this model and in rheumatoid arthritis in humans and it is characterized by very rapid erosive disease. Adjuvant arthritis is associated with increased production of prostaglandins and pro-inflammatory cytokines (William *et al*., 2007). After FCA injection on the rat hind paw, a pronounced swelling and hyperalgesia appeared. In the present context, more pronounced and reliable anti-inflammatory

activity was observed in Sterodin®, which significantly inhibited the development phase of chronic joint swelling induced by FCA on both the paws.

It is, therefore, now unequivocally established that the mixture of detoxified bacterial strains (Sterodin®) has mild anti-inflammatory action. More detailed experiments should be done to strongly suggest the anti-inflammatory activity of the test material. Further investigation regarding the mechanism of action of detoxified bacterial strains on cytokines is going on in our laboratory.

Acknowledgement

The present study was supported by funds received from M/s. Union Drug Company Limited, Kolkata, India.

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Received March 16, 2010
Revised August 26, 2010
Accepted August 27, 2010