

ANTI-ARTHRITIC ACTIVITY OF BOSWELLIC ACIDS IN BOVINE SERUM ALBUMIN (BSA)-INDUCED ARTHRITIS

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Abstract — The effect of boswellic acids on bovine serum albumin (BSA)-induced arthritis in rabbits was studied. Oral administration of boswellic acids (25, 50 and 100 mg/kg/day) significantly reduced the population of leucocytes in a BSA-injected knee and changed the electrophoretic pattern of the synovial fluid proteins. The local injection of boswellic acids (5, 10 and 20 mg) into the knee 15 min prior to BSA challenge also significantly reduced the infiltration of leucocytes into the knee joint, reduced the infiltration of leucocytes into the pleural cavity and inhibited the migration of PMN *in vitro*. The leucocyte-inhibitory activity of boswellic acids was not due to its cytotoxic effect. The boswellic acids did not show any detergent or surfactant properties.

The alcohol-extracted fraction of salai guggal, the oleo gum resin obtained from *Boswellia serrata* Roxb., has been reported to possess marked anti-inflammatory and anti-arthritic activities in a variety of experimental models of inflammation and arthritis (Singh & Atal, 1986). It was also found to inhibit the antibody synthesis and delayed-type hypersensitivity responses to sheep red blood cells in mice and the migration of leucocytes into the pleural fluid in carrageenan-induced pleurisy in rats (Sharma, Khajuria, Kaul, Singh, Singh & Atal, 1988). An attempt made to identify the pure principles responsible for these activities yielded a mixture of four pentacyclic triterpene acids (boswellic acids).

Although rheumatoid arthritis is a chronic inflammatory disorder and is believed to result from an immune reaction (Zvaifler, 1973), the research on anti-inflammatory activity evaluation in animal pharmacology has been mainly based on non-immune acute inflammatory tests with Freund's adjuvant polyarthritis of rats being the only immunological model in current use (Tarayre & Laouressergues, 1979). In the present investigation, the effect of boswellic acids has been studied in bovine serum albumin (BSA)-induced arthritis in rabbits. This test model of immune-mediated arthritis first proposed

by Dumonde & Glynn (1962) and described by Gall & Gall (1980) is acknowledged to have resemblance with many facets of human arthritis.

EXPERIMENTAL PROCEDURES

Materials

Boswellic acids were obtained from phytochemistry discipline of our Laboratory, bovine serum albumin (BSA, Cohn Fraction V), Freund's complete adjuvant (FCA) and silicon grease were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.), carrageenan from Marine Colloids Div. (Springfield, U.S.A.), phenylbutazone from SG Pharmaceuticals (Baroda, India), oester glycogen from Biochemical (England), minimum essential medium (MEM) from HiMedia (Bombay, India), heparin, Microlab (Bombay, India), penicillin from Alembic Chemical Works Co. Ltd (Baroda, India), streptomycin from Sarabhai Chemicals (India), haematocrit capillaries from Top Syringe Manufacturing Co. (Bombay, India), microtitration plates and migration chambers (MCFB) from Laxbro (Pune, India), and dimethyl sulphoxide (DMSO) from E. Merck (A.G. Darmstadt, Germany).

Animals

Himalayan strain rabbits (1.5–2.50 kg) and Charles Foster rats (120–160 g) reared in our laboratory animal house were used.

Arthritis

Arthritis was produced according to the procedures proposed by Dumonde & Glynn (1962) as described by Gall & Gall (1980). Rabbits were immunized by injecting 3.0 ml of sterile emulsion containing 1.5 ml BSA (5 mg/ml in saline) and 1.5 ml FCA into the foot pads, gluteal and neck muscles on day -17. Seventeen days after immunization (day 0) the animals were challenged by injecting 0.5 ml of sterile 30% BSA in saline into the right patellofemoral joint. An equal amount of sterile saline was introduced into the left knee which served as control. The synovial fluid was collected at 2, 4, 6, 12 and 24 h and then daily for 32 days, by rinsing the joint with 0.5 ml sterile normal saline, for total and differential leucocyte counts and electrophoretic studies.

The rabbits were weighed at regular intervals after immunization and challenge and mortality was recorded. The animals were divided into groups of four each. In one set of experiments boswellic acids (BA 25, 50 and 100 mg/kg) were administered orally as fine homogenised suspension in gum acacia (1% w/v) daily for 32 days starting from day 0, and in another 5, 10 and 20 mg were injected into the knee joint 15 min before the challenge and synovial fluid was collected at various intervals.

Carrageenan-induced pleurisy

Pleurisy was induced in rats by the method of Meacock & Kitchen (1979). One hour after the oral administration of test drug or vehicle the animals were given an intrapleural injection of carrageenan (0.5 ml of 1% w/v suspension in normal saline). The animals were killed 4 h later for the collection of pleural fluid to determine the total and differential leucocyte counts.

Polymorphonuclear leucocyte (PMNL) migration in vitro

Essentially, the procedure described by Geczy & Hopper (1981) was employed with slight modification. Granulocytes (PMNLs) were collected aseptically from the pleural cavity of rats 24 h after an intrapleural injection of 0.5 ml of a 1% (w/v) suspension of carrageenan in normal saline by lavaging the pleural cavity with 5 ml Hank's minimum essential medium (MEM) containing 50

units of preservative-free heparin. The cells were washed twice in MEM and finally suspended (2×10^7 cell/ml) in MEM containing 10% autologous serum, 1 mM/ml sodium pyruvate, 100 µg/ml streptomycin and 100 units/ml penicillin. Heparinized micro haematocrit capillaries were filled with the cells, sealed with silicone grease and centrifuged at 500 rotations/min for 3 min. Capillaries were cut at the interface and placed in leucocyte migration chambers filled with MEM containing 25, 50 and 100 µg/ml of boswellic acids solution in DMSO. The control wells contained MEM alone and an equal volume of DMSO in MEM. The plates were incubated for 18 h at 37°C in 5% CO₂ and 100% humidity in a CO₂ incubator (Brunswick, Scientific, NJ, U.S.A.). The tests were carried out in triplicate. Cell migration was determined by weighing the paper cutouts of the area of the image projected by camera lucida under stereomicroscope. The average percentage migration inhibition was calculated as:

$$1 - \frac{\text{mass of area of cell migration (mg) with BA}}{\text{mass of area of cell migration (mg) without BA}} \times 100.$$

Polymorphonuclear leucocyte (PMNL) viability

PMNLs were collected from the peritoneal cavity of a rabbit stimulated with 100 ml of Oester glycogen (0.1% w/v) 24 h earlier (Rosenthal, Santilli, Scotese, Bell & Rubin, 1980). The cell viability was examined by eosin dye exclusion test after incubating the cells (2×10^6 /ml) with different concentrations of BA dissolved in dimethylsulphoxide (Sharma *et al.*, 1988).

Haemolysing activity

The *in vitro* haemolysing effect of BA was determined microscopically as described by Nissim (1954). To 50 µl of serially diluted solutions of BA in DMSO and aqueous suspension in normal saline in microtitration plates, 50 µl of 0.5% suspension of three times washed SRBC in normal saline were added. The parallel controls were run with serial two-fold dilutions of DMSO in normal saline and saline alone. The plates were incubated at 37°C for 30 min and degree of haemolysis was observed under a microscope.

Local irritant activity

For the assessment of local irritant effect, various concentrations of BA (10–100 µg/ml), in gum acacia – normal saline suspension were filled in the

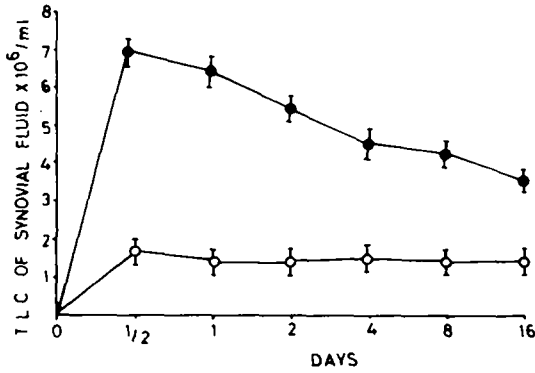


Fig. 1. Total leucocyte counts (TLC) of synovial fluid obtained at various intervals from experimental (BSA-treated ●) and control (saline-treated ○) knees in BSA-induced arthritis in rabbits. The animals were immunized on day -17 and challenged on day 0. Each point represents the mean of four values ± S.E.M.

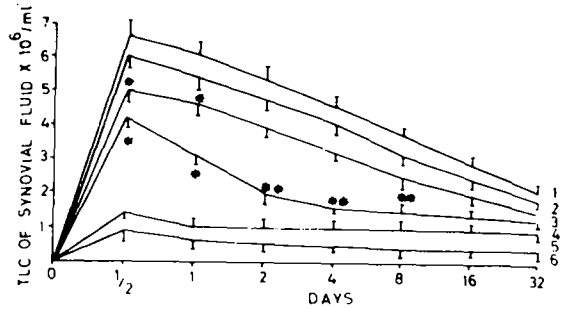


Fig. 3. Effect of various doses of boswellic acids (BA) on synovial fluid leucocyte counts in BSA-induced arthritis. The animals were immunized on day -17 and challenged on day 0. BA (25, 50 and 100 mg/kg) was administered orally on days 0-32. The patellofemoral joints were rinsed with 0.5 ml sterile normal saline at various intervals. (1) Control BSA knee, (2-4) BSA knee of BA-treated animals (doses of 25, 50 and 100 mg/kg p.o.), (5) control saline knee, (6) BA 50 mg/kg p.o. treated saline knee. Each point represents the mean of four values ± S.E.M. * $P < 0.05$, ** $P < 0.01$ as compared to control.

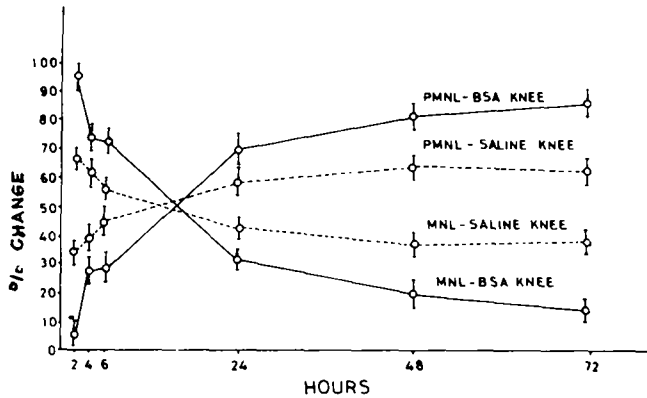


Fig. 2. Differential leucocyte counts of synovial fluid obtained at various intervals from experimental (BSA-treated) and control (saline-treated) knees in BSA-induced arthritis in rabbits. MNL, mononuclear leucocytes; PMNL, polymorphonuclear leucocytes. Each point represents the mean of four values ± S.E.M.

cupped lower lids of the rabbits. After 30 s contact time the eyes were observed for hyperemia and swelling.

Statistical analysis

Data were analysed statistically by Student's *t*-test.

RESULTS

BSA-induced arthritis

The synovial fluid collected at various intervals after challenging the knee joint with BSA, demonstrated an increase in the total leucocyte count

(TLC) during the first few hours after challenge, followed by a gradual decline to low level (Fig. 1). No such increase in cell counts was observed in saline-treated knee, nor in the synovial fluid collected prior to challenge. Differential leucocyte counts carried out on synovial fluid 2-6 h after challenge demonstrated an initial increase in mononuclear leucocyte (MNL) in the BSA-injected knee, while the fluid collected at 24-72 h showed the predominance of polymorphonuclear leucocytes (PMNL) (Fig. 2). The samples collected later on demonstrated the mixed nature of the contents of the synovial exudate. The oral administration of BA produced a significant dose-related reduction of leucocytes in the synovial fluid. The effect appeared

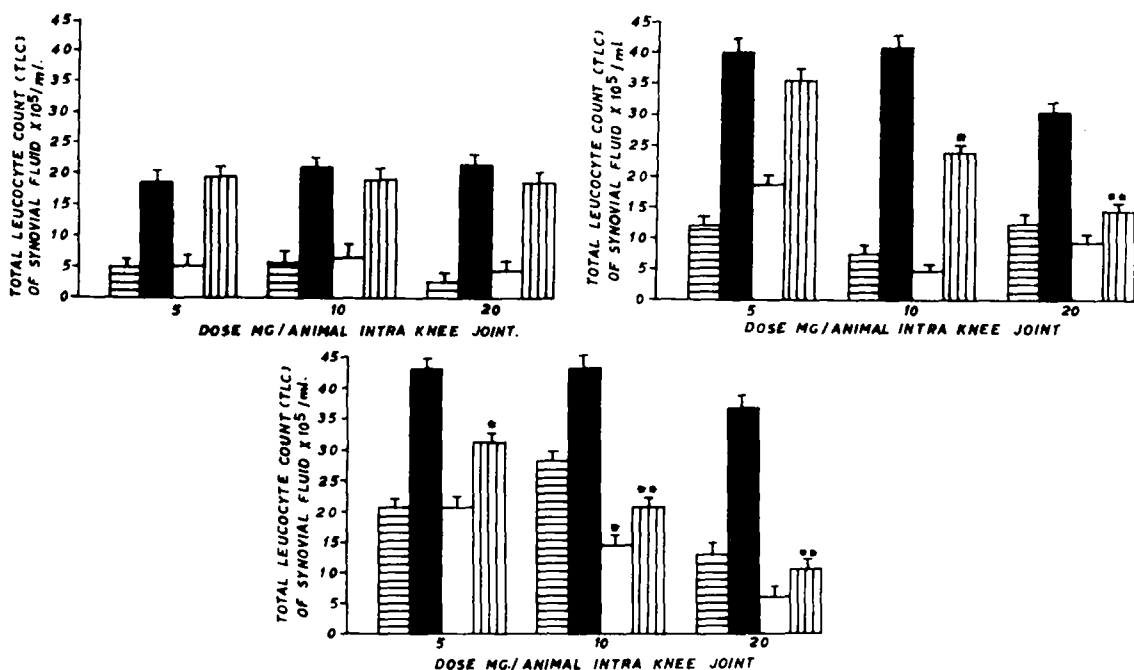


Fig. 4. Effect of local (intra-knee joint) injection of boswellic acids. BA (5–20 mg/knee) was injected into the knee joint 15 min prior to challenge and synovial fluid was collected at various intervals following the challenge. (a) Day 1, (b) day 14, and (c) day 28. The cell counts of the synovial fluid of control saline knee (□), BSA-knee (■), BA-saline knee (▨) and BA-BSA knee (▤). * $P<0.01$ and ** $P<0.001$ as compared to respective controls.

Table 1. Effect of boswellic acids (BA) and phenylbutazone (PBZ) on cell count in carrageenan-induced pleurisy in groups of eight rats at 4 h

Treatment	Dose mg/kg p.o.	Total leucocyte counts $\times 10^3/\text{cm}^2 \pm \text{S.E.M.}$	Absolute no. of leucocytes $\times 10^3/\text{cm}^2 \pm \text{S.E.M.}$	
			Polymorph	Mononuclear
Control	—	60.88 ± 3.13	55.09 ± 2.25	5.78 ± 1.35
BA	25.00	58.25 ± 2.69 (–4.33)	51.55 ± 1.90 (–6.42)	6.69 ± 0.95 (+15.74)
BA	50.00	$44.75 \pm 5.00^\dagger$ (–26.50)	$38.03 \pm 2.09^\dagger$ (–30.96)	6.71 ± 1.03 (+16.08)
BA	100.00	$33.00 \pm 1.58^\dagger$ (–45.00)	$23.59 \pm 4.29^\ddagger$ (–57.17)	$9.40 \pm 2.30^\ddagger$ (+62.62)
PBZ	100.00	$49.00 \pm 2.68^*$ (–19.28)	43.97 ± 2.00 (–20.18)	5.82 ± 0.83 (–13.14)

* $P<0.05$; $^\dagger P<0.01$; $^\ddagger P<0.001$.

% Change is depicted in parentheses.

within 6–12 h after treatment and persisted through day 32 (Fig. 3). In the experiments in which BA were injected into the knee joint 15 min prior to challenge, a significant dose-related reduction in TLC was observed on days 14 and 28 post challenge (Fig. 4). Electrophoretic studies carried out on synovial fluid obtained 28 days post challenge also revealed a slight reduction in the synovial fluid protein concentration of the experimental knee in BA-treated rabbits.

No change in general behaviour or food intake of the animals was observed after immunization, while they became sluggish and lost appetite following challenge. There was on the whole a 6–15% reduction in body weight during the first fortnight after challenge, which recovered to original levels within the next fortnight. In control groups, immunization with 3 ml of emulsion and subsequent challenge with 0.5 ml of 30% BSA resulted in 25% mortality within 8 days after challenge, whereas very

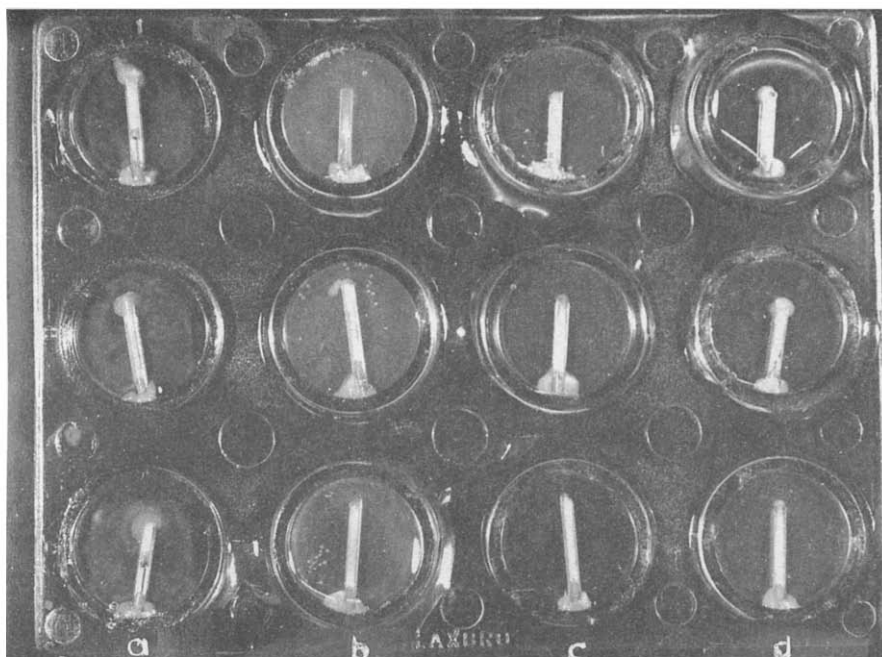


Fig. 5. Effect of BA on PMNL migration. (a) DMSO control; (b) BA 100 $\mu\text{g/ml}$; (c) BA 50 $\mu\text{g/ml}$; (d) BA 25 $\mu\text{g/ml}$. A marked inhibition of migration was seen at 100 and 50 $\mu\text{g/ml}$ concentrations.

low (8%) mortality was observed in BA-treated groups.

Carrageenan-induced pleurisy

BA (25–100 mg/kg) given orally 1 h before the intrapleural injection of carrageenan reduced significantly the population of leucocytes in the pleural fluid and inhibited the infiltration of PMNLs at the higher dose level (Table 1).

PMNL migration

BA in concentrations of 25–100 $\mu\text{g/ml}$ in the migration chambers caused a significant and dose-related inhibition (33.7–60.6%) of migration of PMNLs. DMSO showed no effect on cell migration as compared to the medium control (Fig. 5).

Haemolysing activity

BA did not cause haemolysis of SRBC on incubation at 37°C for 30 min in as high a concentration as 500 $\mu\text{g/ml}$. However, severe crenation of the cells was observed at higher concentration (1000 $\mu\text{g/ml}$). No change in cell contour was seen in the DMSO and normal saline controls.

Local irritation

The application of aqueous suspension of BA in increasing concentrations (100–1000 $\mu\text{g/ml}$) to conjunctival surface of rabbit elicited no irritant effect.

PMNL viability

BA in concentrations of 1×10^{-6} to 1×10^{-3} g/ml exhibited no cytotoxic effect on PMNLs, as revealed by dye exclusion test.

DISCUSSION

A chronic synovitis has been produced in rabbits by means of an initial systemic immunization followed by the injection of the same antigen into a knee joint. The condition was associated with an initial increase in synovial fluid cell count, which later fell to levels consistent with low grade inflammation (Fig. 1). In the experimental knee, the replacement of initial predominance of MNLs by the increased population of PMNLs within 24 h (Fig. 2) is a phenomenon similar to that observed in initial synovitis of rheumatoid arthritis (Gall & Gall, 1980).

In view of the fact that rheumatoid synovitis is associated with a perivascular lymphocytic infiltration (Zaiflar, 1979) and that the interference with

migration of leucocytes into the inflammatory site is an important mechanism of action of the non-steroidal anti-inflammatory agents (NSAI) (Walker, Smith & Ford-Hutchinson, 1976), this study of the effect of boswellic acids, on the infiltration of leucocytes into the inflammatory site is of great significance. In both the multiple dose oral schedule, as well as with a single intrapatellar injection, a significant dose-related reduction in the leucocyte count was observed in BSA-induced arthritis in rabbits. The results are consistent with our previous observation on salai guggal which inhibits the migration of leucocytes into the pleural cavity of rats in the carrageenan pleurisy model (Sharma *et al.*, 1988), and has also been demonstrated in *in vitro* experiments (Fig. 5). The effect on migration of leucocytes is not due to the cytotoxic activity of BA and may be attributed to inhibition of release or formation of chemotactic factors which manifest the initial Arthus reaction immediately after challenge in the immunized animals (Sell, 1980). Although BA are a mixture of pentacyclic triterpenes, their anti-inflammatory activity was not due to counter irritant

phenomena, as these were found to be equiactive on intraperitoneal and oral administration. Moreover, they were found to be devoid of the surfactant and local irritant activities associated with some triterpenes, as evidenced by lack of their haemolytic and local irritant effects.

A pre-clinical acute toxicity study in rats and mice, sub-acute toxicity in rats for 1 month and chronic toxicity in primates for 6 months revealed boswellic acids to be safe (unpublished observations). Clinical trials conducted in Patiala Medical College*, on patients of arthropathies fitting into the American rheumatoid arthritis criteria, showed that administration of BA orally in a fixed dose of 200 mg three times a day over 8 weeks not only significantly improved the stiffness, tenderness and swelling of various joints and restored their functional activity, but also improved the clinical parameters, i.e. erythrocyte sedimentation and rheumatoid factor. These results indicate that BA is a promising anti-arthritis drug of a disease modifying category with high tolerance and freedom from adverse effect.

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