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Immunomodulatory and Therapeutic Potential of a Mycelial Lectin from *Aspergillus nidulans*

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Abstract Lectins bind to surface receptors on target cells, and activate a cascade of events, eventually leading to altered immune status of host. The immunomodulatory potential of purified lectin from *Aspergillus nidulans* was evaluated in Swiss albino mice treated intraperitoneally with seven different doses of purified lectin. Lectin prevented BSA-induced Arthus reaction and systemic anaphylaxis. The enhanced functional ability of macrophages was evident from respiratory burst activity and nitric oxide production in splenocyte cultures. Interferon-gamma and interleukin-6 levels were significantly up-regulated in treated groups. Maximum stimulatory effect was observed at the dose of 1.5 mg/kg body weight. Therapeutic potential of *A. nidulans* lectin was assessed against trinitrobenzene sulfonic acid-induced ulcerative colitis in male Wistar rats. Rats pre-treated with 80 mg/kg body weight of purified lectin intraperitoneally prior to colitis induction showed lesser disease severity and recovery within 7 days, while rats post-treated with the same dose showed recovery in 11 days. The results demonstrate immunomodulatory effects of *A. nidulans* lectin in Swiss albino mice, resulting in improved immune status of the animals and unfold its curative effect against ulcerative colitis in rat model. This is the first report on immunomodulatory and therapeutic potential of a lectin from microfungi.

Keywords *Aspergillus nidulans* · Lectin · Immune stimulation · Colitis · Therapeutic potential

Introduction

The biological information within the cells is encoded in a language of carbohydrate moieties and the carbohydrate-binding proteins (lectins) due to their specific binding with

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these ligands are of therapeutic value, e.g. as biomodulators in the immune system [1]. The biological activity of lectins in relation to animal or human cells manifests itself in phenomena such as mitogenic and immunomodulatory properties, suppression of cell proliferation and anti-tumor activity. Owing to their specificity to bind surface receptors, certain lectins are known to activate cascade reactions, culminating in up-regulation of the components of immune system, thereby producing immunomodulatory effects. Few of them are bolesatine from *Boletus satanus* [2], fungal immunomodulatory proteins from *Flammulina velutipes* [3] and *Volvariella volvaceae* [4] and lectins from *Tricholoma mongolicum* [5], *V. volvacea* [6], *Aleuria aurantia* [7], *Agaricus bisporus* [8] and *Fomitella fraxinea* [9]. Recently, the therapeutic potential of mushroom lectins has been reviewed by our group [10]. Thorough analysis of the principles of molecular recognition is the basis for rational development of clinical applications.

Clinically, inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is an immune-related disorder of gastrointestinal tract characterized by inflammatory lesions and ulceration [11]. The cause of IBD is not yet clear, although immune dysregulation and genetic predisposition are known to be involved [12]. Studies have demonstrated that an imbalance of CD4/(T-helper 1) T_H1 cell response or an inadequate (T-helper 2) T_H2 cell response is involved in experimental colitis [13]. Thus immunomodulatory drugs can be potentially useful in IBD therapy and colitis drug research [14]. In IBD, there is an increased sialylation [15], and the expression of oncofetal Thomsen–Friedenreich (TF) antigen (Gal1,3GalNAc) and sialyl 2,6GalNAc is up-regulated [16]. This offers a site for binding of specific lectins. Many lectins including *A. bisporus* lectin [17], peanut lectin [18] and jacalin [19] have been reported to bind to TF antigen, thereby modulating the proliferation of intestinal epithelial cells, but none of them have been studied for therapeutic effect in ulcerative colitis model.

Lectins have been widely reported from *Aspergilli* [20–23] and *Penicilli* [24]. Preliminary investigations on a lectin from *Aspergillus nidulans* have revealed its specific binding interaction with *N*-acetyl-D-galactosamine [20]. Purified lectin exhibited potent mitogenic activity towards mouse splenocytes, indicating its interaction with immune cells [25]. The present work was aimed at investigating the effect of *A. nidulans* lectin on immune system of Swiss albino mice and its therapeutic effectiveness on trinitrobenzene sulfonic acid-induced colitis in rat model. None of the lectin from microfungi has been studied for its therapeutic potential. This is the first report describing the immunomodulatory and therapeutic properties of *A. nidulans* lectin.

Materials and Methods

Fungal Culture and Cultivation Conditions

A. nidulans MTCC 344, procured from Microbial Type Culture Collection, Chandigarh, was maintained and cultivated as described earlier [20]. Briefly, Erlenmeyer's flasks (1 l) containing 500 ml medium were inoculated with five culture discs (10 mm diameter) of 7-day-old culture from agar plates and incubated at 30 °C for 7 days under stationary condition.

Lectin Extraction and Purification

Mycelium harvested from cultures was washed with phosphate-buffered saline (0.1 M, pH 7.2) and homogenized in the same buffer containing 1 mM benzamidine hydrochloride,

followed by grinding in pestle and mortar in the presence of acidified river sand (80–200 mesh EP, SD Fine Chemicals Ltd., India) as described previously [20]. The crude extract was concentrated by ammonium sulfate precipitation and further resolved on DEAE-Sepharose and Sephadex G100 columns as described earlier [25]. Purified lectin having specific activity 354.32 titre/mg was used for evaluating immunomodulatory and therapeutic potential *in vivo*.

Haemagglutination Assay

Human type O blood was collected in Alsever's solution in the ratio 1:2, and erythrocyte suspension (2%, *v*/*v*) was prepared in phosphate-buffered saline (0.1 M, pH 7.2). Haemagglutination assay was carried out as described earlier [20]. Lectin titre was defined as the lowest concentration of lectin capable of visible agglutination of erythrocytes.

Animals

Male Swiss albino mice (18–22 g) were procured from Central Research Institute, Kasauli, India, and male Wistar rats (250–300 g) were procured from Animal House, Panjab University, Chandigarh. The animals were housed under controlled temperature (25±2 °C) with 12 h light/dark cycle and maintained on standard pellet diet (Kissan Feeds Ltd., Mumbai, India). The experimental protocol was approved by Institutional Animals Ethics Committee, and the care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forests, Government of India.

Acute Toxicity

Acute toxicity was studied as described by Mishra and Bhatia [26]. The mice were given 20 to 200 mg of *A. nidulans* lectin/kg body weight orally and 20 to 200 mg/kg body weight, intraperitoneally. Animals were observed for general behaviour and mortality. Haematological parameters (total leukocyte count, differential leukocyte count and haemoglobin content) were tested over a period of 3 weeks. There was no mortality or abnormality detected as compared to the untreated control group (data not shown). There was no abnormal rise in body weight and spleen weight. The extract was thus apparently safe to use.

Immunomodulatory Activity Assay

Immunomodulatory potential of the purified lectin was assessed in Swiss albino mice. Three separate independent experiments were carried out to delineate the specific objectives of the study: experiment I was limited to study the effect of purified *A. nidulans* lectin on systemic anaphylaxis, experiment II was carried out to assess the Arthus reaction in mice and experiment III was aimed to evaluate other immunological parameters described in the proceeding section. Effectiveness of levamisole as an immune modulator is largely known and the present study also compares the immunomodulatory potential of the lectin to that of levamisole [27]. Based on previous work on immunomodulatory effect of levamisole [26, 28], the dose of levamisole used was 2.5 mg/kg per day.

Systemic Anaphylaxis Reaction

Systemic anaphylaxis reaction in mice was assessed as described by Hsu et al. [4]. The dose pattern of lectin was selected as described by the same workers with slight modifications. The animals were divided into five groups (n=10): group I, positive control; group II, negative control; group III, levamisole treated; group IV, test and group V, lectin control. The animals of groups I, II and V were treated with phosphate-buffered saline on days −6, −3, 0, 3, 6, 9 and 12, while groups III and IV animals were treated with levamisole (2.5 mg/kg body weight) and lectin (7.5 mg/kg body weight), respectively, on days −6, −3, 0, 3, 6, 9 and 12. The animals of groups I–IV were sensitized by intraperitoneal administration of 1 mg bovine serum albumin (BSA) in 0.2 ml aluminium hydroxide suspension (15 mg/ml) on day 0, while the animals of groups V were sensitized by intraperitoneal administration of 1.5 mg lectin/kg body weight on day 0. The animals of groups I and III were shocked intravenously by 1 mg BSA in 0.2 ml phosphate-buffered saline on day 17, while group II animals were injected with 1 mg ovalbumin in 0.2 ml phosphate-buffered saline instead of BSA at the time of shocking. Group IV mice were given shocking injection (intravenous) with BSA (1 mg in 0.2 ml phosphate-buffered saline) 60 min after intraperitoneal administration of 150 μg of purified lectin. Group V animals were shocked by intravenous injection of 1.5 mg lectin/kg body weight in 0.2 ml phosphate-buffered saline on day 17. Systemic anaphylaxis reaction was observed within 30 min after the shocking injection. Death or inactivity of the mice was considered as a positive reaction, while reaction was considered negative when no changes were observed and movement of mice remained normal.

Arthus Reaction

Arthus reaction was assessed in mice as described by Hsu et al. [4]. The dose pattern was selected as described by the same workers with slight modifications. Mice were divided into six groups (n=6): group I, untreated control; group II, immunized control; group III, levamisole treated (2.5 mg/kg body weight, intraperitoneal); group IV, test (treated with 0.75 mg of purified lectin/kg body weight, intraperitoneal); group V, test (treated with 1.5 mg of purified lectin/kg body weight, intraperitoneal) and group VI, test (treated with 7.5 mg of purified lectin/kg body weight, intraperitoneal). Group I animals were kept on normal diet and were not given any treatment throughout the experiment. Group II animals were intraperitoneally given 0.2 ml phosphate-buffered saline (0.1 M, pH 7.2) on days −6, −3, 0, 3, 6, 9 and 12. Group III animals were intraperitoneally given indicated dose of levamisole on the same days, while test animals (groups IV–VI) were intraperitoneally treated seven times with indicated doses of the purified lectin on days −6, −3, 0, 3, 6, 9 and 12. Animals of all the groups except group I were sensitized with 1 mg of BSA in 0.2 ml aluminium hydroxide suspension (15 mg/ml) on days 0 and 7, intraperitoneally. On the 14th day, 20 μl of BSA (0.5 mg/ml) was injected intradermally into the left footpad of mice in all the groups, and an equal amount of phosphate-buffered saline was injected into the right footpad as a control. The thickness of footpads was measured using a micro-caliper at 0, 2, 24, 48 and 72 h after injection of BSA and phosphate-buffered saline. The difference in the thickness of left and right footpads was calculated and taken as a measure of Arthus reaction. Results were expressed as mean±standard deviation (S.D.).

Other Immunological Parameters

The animals were divided into six groups (n=6): group I, untreated control; group II, immunized control; group III, levamisole treated (2.5 mg/kg body weight, intraperitoneal);

group IV, test (treated with 0.75 mg of purified lectin/kg body weight, intraperitoneal); group V, test (treated with 1.5 mg of purified lectin/kg body weight, intraperitoneal) and group VI, test (treated with 7.5 mg of purified lectin/kg body weight, intraperitoneal). Group I animals were kept on normal diet and remained untreated. Group II animals were intraperitoneally given 0.2 ml phosphate-buffered saline on days −6, −3, 0, 3, 6, 9 and 12. Group III animals were intraperitoneally treated with indicated dose of levamisole, and groups IV–VI animals were intraperitoneally treated with indicated doses of the purified lectin, on the same days as specified for group II. The dose pattern of test animals was selected as described by Hsu et al. [4]. Immunized control, levamisole-treated and test animals were antigenically challenged with BSA (1 mg/ml in phosphate-buffered saline) intraperitoneally on days 0 and 7 of treatment. All the animals were sacrificed on day 13 by cervical dislocation, and their spleen was excised aseptically. Splenocytes were isolated by teasing the tissue. Cells were centrifuged (400×*g*, 10 min, 4 °C), and erythrocytes were lysed by Gay's haemolytic solution. Cells were washed thrice in phosphate-buffered saline (0.1 M, pH 7.2), counted in a haemocytometer using a microscope and adjusted to 1×10^6 cells/ml in RPMI 1640 medium for further use.

Respiratory Burst Activity

Respiratory burst activity was assessed as described by Csato et al. [29]. Briefly, splenocyte suspension (1 ml) from treated and control groups was incubated with 0.1 ml nitro blue tetrazolium dye (NBT, 2.5 mM) at 37 °C for 20 min. Pellet was washed with phosphate-buffered saline and then with distilled water. Supernatant was incubated with dioxan (5 ml) for 20 min at 70 °C. Reduction in NBT was measured spectrophotometrically at 520 nm, using dioxan as blank. Results were expressed as mean±S.D. of percentage of nitro blue tetrazolium dye reduced to formazan.

Nitric Oxide Production

Nitric oxide in splenocyte suspension was measured as an indicator of macrophage function according to colorimetric Griess reaction [30]. Briefly, splenocyte suspension (100 μl) from each of the groups was seeded in the 96-well flat bottom microtitre plates at 37 °C for 2 h in 5% CO_2 atmosphere. To the cells, 0.8 ml of RPMI 1640 medium and 40 μl of L-arginine solution (0.1 M) was added and incubated at 37 °C for 24 h in the same atmosphere. An equal volume of Griess reagent (0.5% sulfanilamide, 0.05% napthyl ethylene dianiline dihydrochloride and 1.25% phosphoric acid) was added to the supernatant and incubated at room temperature for 10 min. Colour developed was measured spectrophotometrically at 540 nm against RPMI 1640 medium and Griess reagent as blank. Results were expressed as mean±S.D. percentage of nitric oxide released.

Quantification of Cytokine Levels

Splenocyte suspension (100 μl containing 2×10^6 cells/ml in RPMI 1640 medium) from control and treated groups was cultured with filter-sterilized Concanavalin A (ConA, 0.5 μg/ml) in RPMI 1640 medium containing fetal bovine serum (10%) in wells of the 96-well flat bottom microtitre plates at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2. The levels of cytokines interferon-gamma (IFN-γ) and interleukin-6 (IL-6) were quantified in supernatant of splenocyte cultures using OptEIA murine cytokine enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen, USA). Briefly, the 96-well

ELISA plates were coated overnight with 50 μl of capture antibodies in carbonate–bicarbonate buffer (pH 9.6) at 4 °C. After washing, the wells were incubated with 200 μl of blocking buffer for 1 h at room temperature followed by the addition of 100 μl of supernatant obtained. The plates were kept for 2 h at room temperature, and subsequently, the wells were washed with buffer. Biotinylated mouse secondary antibody was added along with avidin horse radish peroxidase. After extensive washing, substrate solutions (tetramethylbenzidine and hydrogen peroxide) were added and incubated at room temperature for colour development. The reaction was stopped by adding 1 M H_3PO_4, and the plates were read at 450 nm on a Microplate reader (Biorad Laboratories Inc., USA). The cytokines were quantified using cytokine standards. Results were expressed as mean± S.D. of cytokines produced in individual groups in pg/ml.

Therapeutic Potential Against Trinitrobenzene Sulfonic Acid-Induced Ulcerative Colitis

Restorative effect of purified lectin on ulcerative colitis was assessed in male Wistar rats.

Experimental Design

Animals were divided into five groups (n=6): group I, normal control; group II, disease control (only colitis induced); group III, test (intraperitoneally pre-treated with 80 mg of purified lectin/kg body weight/day, for 7 days prior to colitis induction); group IV, test (intraperitoneally treated with 80 mg of purified lectin/kg body weight/day, from day 5 of colitis induction); group V, test (rectally treated with 80 mg of purified lectin/kg body weight/day, from day 5 of colitis induction). The test rats were administered optimal dose of 80 mg/kg body weight based on preliminary trials on trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats (data not shown). Group I animals were not submitted to any intervention and were sacrificed after the acclimation period. Group II animals were induced with ulcerative colitis on day 0 and sacrificed on day 5 upon disease establishment. Group III animals were pre-treated intraperitoneally for 7 days with indicated dose of purified lectin before colitis induction. Colitis was induced on day 0, and dosing was continued till the end of the experiment. Animals were sacrificed on days 7 and 11, and their colon was removed aseptically. In groups IV and V animals, colitis was induced on day 0. Animals were intraperitoneally and rectally treated with indicated doses of purified lectin starting from day 5, respectively, for groups IV and V. Animals from each of the two groups were sacrificed after 7 and 11 days of lectin treatment, and their colon was removed aseptically.

Induction of Colitis

On day 0, animals of groups II–V fasted for 24 h, were lightly anaesthetized with diethyl ether and colitis was induced by intracolonic instillation of 2,4,6-trinitrobenzene sulfonic acid (150 mg/kg body weight) in ethanol using a rubber cannula as described by Carvalho et al. [31]. For the assessment of clinical severity of colitis, stool consistency and rectal bleeding were examined daily.

Histological Analysis of Colon

For the surgical procedure, animals were anaesthetized by ether inhalation and submitted to mid-laprotomy under sterile conditions. The distal colon was removed from rats of all the

groups, opened longitudinally and rinsed with sterile saline. The colonic tissue was fixed in 10% formalin, dehydrated in ethanol and embedded in paraffin. Sections (4 μm) were cut, mounted on a glass slide, deparaffinized with xylene and stained with haematoxylin and eosin. The ulcerative area was determined on histological slides using light microscope. For each rat, histological slides from five loci were taken along the intestine (same loci were taken from all dissections). Twenty to thirty microscopic fields were examined from five histological slides for each rat.

Statistical Analysis

All the experiments were repeated thrice with each group. The results were expressed as mean±S.D. Data of tests were statistically analysed using one-way ANOVA followed by Tukey's multiple range test, applied for post hoc analysis. The data was considered statistically significant if probability had a value of 0.05 or less.

Results

Immunomodulatory Activity Assay of *A. nidulans* Lectin

Swiss albino mice were administered seven doses of purified *A. nidulans* lectin at regular time intervals with each dose thrice a week in different experiments. The effect of lectin on systemic anaphylaxis reaction, Arthus reaction, respiratory burst activity, nitric oxide production and levels of cytokines IFN-γ and IL-6 was assessed. The immunomodulatory potential of the lectin was compared with a well-known immunomodulator, levamisole as a positive control.

Systemic Anaphylaxis

Table 1 shows the effect of *A. nidulans* lectin on systemic anaphylaxis. All the mice in the positive control group sensitized with BSA intraperitoneally on day 0 and then shocked

Table 1 Systemic anaphylaxis reaction in mice treated with purified *A. nidulans* lectin

Group (n=10)	Animals showing anaphylactic symptoms (%)	Animals dead (%)
Positive control	100	50
Negative control	0	0
Levamisole treated	0	0
Test	10	0
Lectin control	0	0

All animals, except lectin control mice, were sensitized by intraperitoneal injection of BSA (1 mg/0.2 ml aluminium hydroxide). Positive control animals were given BSA (1 mg/0.2 ml phosphate-buffered saline) on day 17 intravenously. Negative control animals were injected with ovalbumin (1 mg/0.2 ml phosphate-buffered saline) on day 17. Test and levamisole-treated animals were treated with purified *A. nidulans* lectin (7.5 mg/kg body weight) and levamisole (2.5 mg/kg body weight), respectively, on days −6, −3, 0, 3, 6, 9, and 12. On day 17, test animals were given injection of BSA intravenously 60 min after intraperitoneal administration of 150 μg of purified lectin. Levamisole-treated animals were also given BSA injection on day 17 as described for positive control group. Lectin control animals were given PBS on the indicated days, and sentisitized with lectin (1.5 mg/kg body weight) on day 0 and shocked by intravenous injection of 1.5 mg lectin/kg body weight on day 17

with BSA in phosphate-buffered saline intravenously on day 17, displayed symptoms of systemic anaphylaxis. Five out of ten mice died within 25 min of the shocking injection, while others showed considerable discomfort. In the negative control group, BSA in the shocking injection was replaced with ovalbumin and mice showed no anaphylactic reaction. Levamisole treatment also could inhibit the anaphylactic symptoms. The group IV animals immunized with lectin did not show any anaphylactic reaction. After seven times of lectin administration, including two injections before BSA sensitization, only one mouse displayed symptoms of anaphylactic shock, and no mortality was observed in the treated group.

Arthus Reaction

All the animals in test and immunized control groups were sensitized with BSA on days 0 and 7. Arthus reaction was assessed in mice injected with BSA intradermally on day 14. The results of Arthus reaction revealed that repeated administration of lectin prevented BSA-induced paw edema in mice (Table 2). Untreated mice displayed swelling after 2 h of injection, while the animals in immunized control group showed positive foot pad reactions after 2 and 24 h. Animals treated with lowest dose of lectin (0.75 mg/kg body weight) though displayed some swelling after 2 h but no swelling or redness in mice paw was observed after 24 h. Animals treated with higher dose of lectin successfully inhibited the Arthus reaction with no swelling or edema after 2 h of BSA injection. Levamisole treatment inhibited the development of Arthus reaction, though animals displayed paw swelling after 24 h of injection, indicating a delayed type hypersensitivity reaction in levamisole-treated group.

Respiratory Burst Activity

Nitro blue tetrazolium dye reduction, as a function of respiratory burst activity, was assessed in splenocytes of animals treated with varied doses of purified lectin. Our results revealed a

Table 2 Arthus reaction in mice treated with purified *A. nidulans* lectin

Group (n=6)	Dose (mg/kg body weight)	Footpad thickness (mm)				
		Time period after BSA challenge (h)				
		0	2	24	48	72
Untreated control	−	1.68±0.02	1.70±0.02	1.68±0.01	1.68±0.02	1.68±0.01
Immunized control	−	1.67±0.01	1.70±0.02*	1.73±0.04*	1.69±0.02	1.68±0.01
Levamisole treated	2.5	1.70±0.02	1.70±0.03	1.86±0.02*	2.01±0.04*	1.81±0.03*
Test	0.75	1.67±0.01	1.70±0.03*	1.68±0.02	1.67±0.03	1.67±0.02
	1.5	1.67±0.02	1.67±0.03	1.67±0.01	1.67±0.01	1.67±0.02
	7.5	1.69±0.03	1.69±0.01	1.69±0.02	1.69±0.01	1.69±0.02

Control animals were kept on normal diet, and test and levamisole-treated animals were treated respectively with indicated doses of purified *A. nidulans* lectin and levamisole on days −6, −3, 0, 3, 6, 9 and 12. Animals in test, levamisole-treated and immunized control groups were sensitized intraperitoneally with BSA (1 mg/0.2 ml aluminium hydroxide) on the 0 and 7th day. On day 14, 20 μl BSA (0.5 mg/ml) was injected intradermally into the left footpad of all the animals and an equal amount of phosphate-buffered saline into the right footpad. Footpad thickness was measured with micro-caliper. Data is presented as Mean ± S.D. (n=6), *p<0.001 as compared to 0 h reading of the same group

significant ($p<0.05$) enhancement of dye reduction in animals treated with lectin as compared to immunized control group (Fig. 1a), symbolizing the enhanced functional ability of macrophages and neutrophils. Maximum dye reduction (74.77±5.40%) was observed in animals treated with the moderate dose of lectin (1.5 mg/kg body weight) and was found comparable to levamisole-treated group. At higher dose, reduction of nitro blue tetrazolium (66.95±6.13%) was still significantly higher ($p<0.05$) as compared to immunized control animals.

Nitric Oxide Production

The administration of lectin at various doses to Swiss albino mice resulted in considerable ($p<0.05$) production of nitric oxide as compared to immunized control with maximum production (61.9±5.66%) at 1.5 mg/kg body weight (Fig. 1b). The maximum nitric oxide levels attained were lesser than those observed with levamisole treatment (78.61±4.09%). However, at the higher dose of 7.5 mg/kg, percentage nitric oxide production was still higher ($p<0.05$) than immunized control. This was similar to the pattern observed for dye reduction.

Quantification of Cytokine Levels

The levels of cytokines IL-6 and IFN-γ were quantified in splenocytes of all the animals cultured with ConA. Lectin administration resulted in substantial ($p<0.05$) up-regulation of

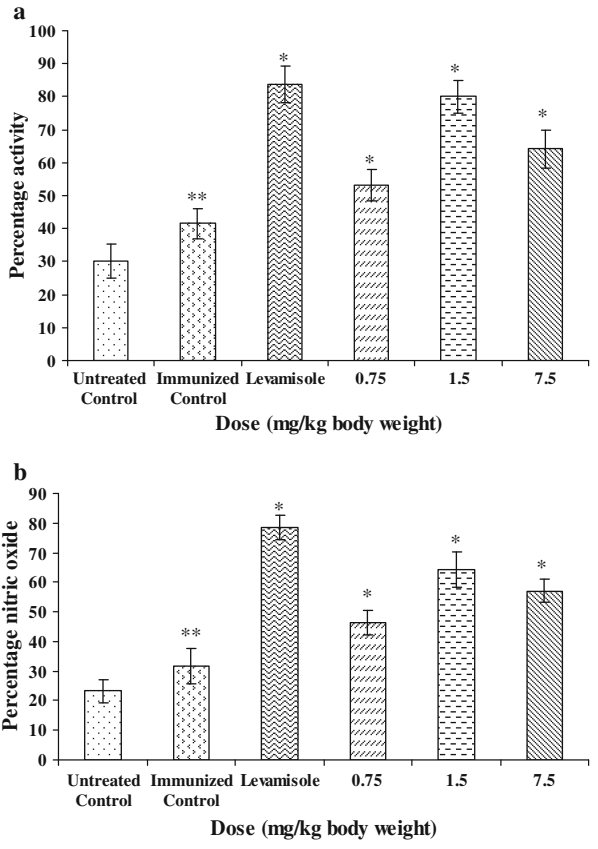

Fig. 1 Percentage activity in splenocytes of mice treated with purified *A. nidulans* lectin. **a** Dye reduction. **b** Nitric oxide production. Data are presented as mean±S.D. ($n=6$); **$p<0.001$ as compared to untreated control; *$p<0.05$ as compared to immunized control

both cytokines in splenocyte cultures of treated animals as compared to immunized control group (Fig. 2a, b). Maximum IFN-γ production (658.5±14.27 pg/ml) and IL-6 peak (446.75±24.7 pg/ml) were attained at the dose of 1.5 mg/kg body weight. IFN-γ levels observed with lectin administration were only slightly lower than the levels attained after levamisole treatment (695.45±12.1 pg/ml). Marginal increase in IL-6 levels (255.13± 13.27 pg/ml) was observed in splenocytes of animals treated with levamisole as compared to immunized control group ($p<0.05$).

Therapeutic Effect of *A. nidulans* Lectin on TNBS-Induced Ulcerative Colitis

The histological features of colon in rats subjected to TNBS enema were characterized by staining with haematoxylin and eosin. Figure 3a shows the histology of normal colon. The four layers of colon wall, i.e. mucosa, submucosa, muscularis externa and serosa are normal. The section shows temporary folds of mucosa and submucosa. Light microscopic examination of distal colon of rats treated with TNBS demonstrated ulcerative inflammation characterized by reddish edematous mucosa (Fig. 3b). Dilapidation of tissue epithelium-induced leukocyte infiltration accompanied with obvious tissue putrescence and acute inflammation. The section showed ulceration, coagulative necrosis and haemorrhage. Erosive lesions were present in the mucosa and extended into the muscularis layer. A narrowing of the lumen of colon adjacent to inflammed sites with proximal dilation of the bowel was also seen in TNBS-treated rats. All rats had persistent diarrhoea and rectal bleeding after TNBS administration.

Treatment of rats with purified lectin resulted in marked decrease in the extent and severity of colonic injury. Treatment with lectin significantly reduced diarrhoea, mortality,

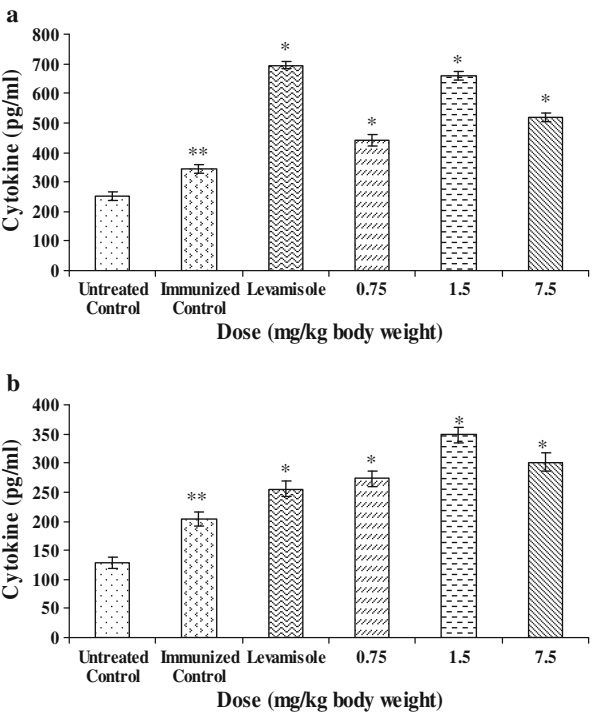

Fig. 2 Cytokine levels in splenocytes of mice treated with purified *A. nidulans* lectin. **a** IFN-γ. **b** IL-6. Data are presented as mean±S.D. ($n=6$); **$p<0.001$ as compared to untreated control; *$p<0.05$ as compared to immunized control

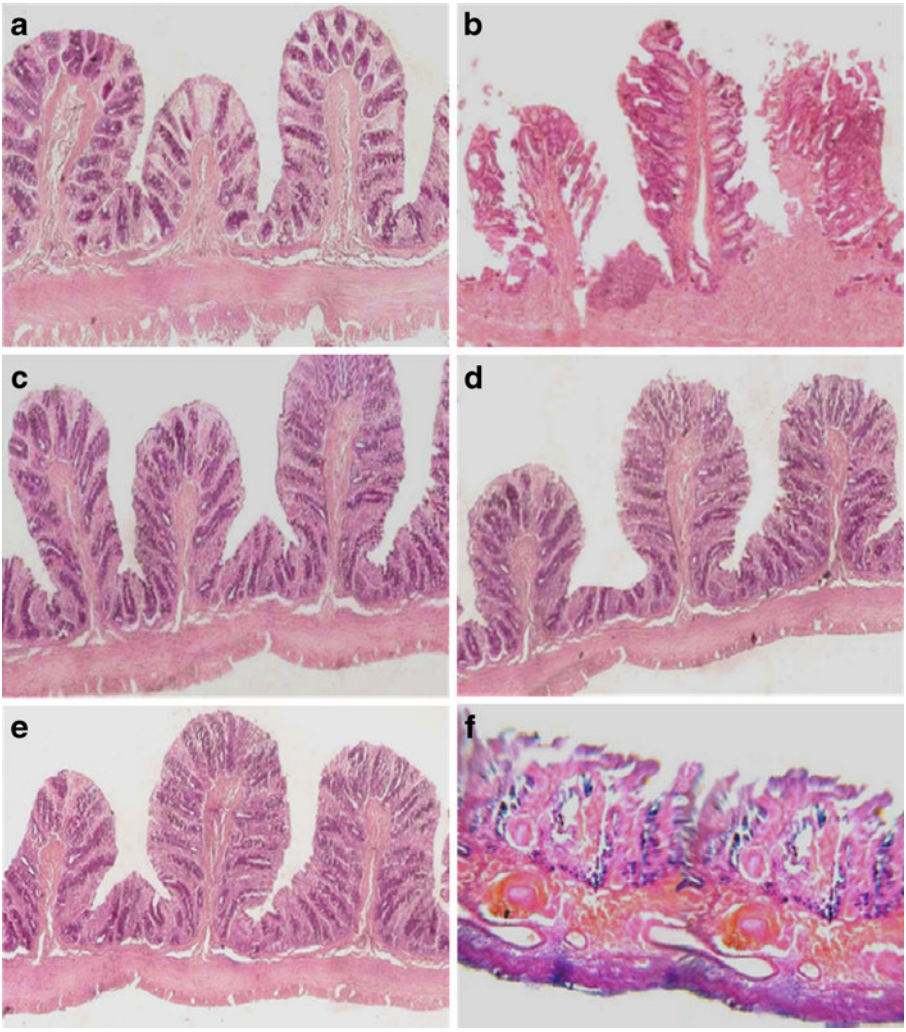

Fig. 3 Microscopic analysis of colon from rats with TNBS-colitis. **a** Normal control. **b** Disease control. **c** Pre-treated intraperitoneally with purified lectin for 7 days prior to TNBS enema and intraperitoneal treatment continued for 7 days after TNBS instillation. **d** Post-treated intraperitoneally with purified lectin for 7 days after TNBS instillation. **e** Post-treated intraperitoneally with purified lectin for 11 days after TNBS instillation. **f** Post-treated rectally for 5 days after TNBS instillation

colon mass and ulcer area. Rats showed no rectal bleeding or diarrhoea after 3 days, and the recovery was evident within 5 days. The animals showed little or no pathological effects. No mucosal erosions were present, and minimal to no neutrophils could be detected in the submucosa. The pre-treated animals showed no signs of inflammation, and normal colon histology was evident (Fig. 3c). The pre-treated group displayed faster recovery than post-treated animals. Histological studies of animals post-treated intraperitoneally with lectin after colitis induction showed low levels of inflammation and partial recovery after 7 days (Fig. 3d). The histopathological features of colon indicated that morphological disturbances associated with TNBS administration were completely corrected after 11 days of lectin

treatment (Fig. 3e). No rectal bleeding or diarrhoea was observed after lectin treatment for 5 days. However, rats administered rectally with purified lectin showed intense neutrophil infiltration, inflammatory infiltrate, marked dilation of blood vessels, extensive haemorrhage, cellular degenerative processes, extensive necrosis and broken epithelial lining, suggesting ineffectiveness of rectal treatment (Fig. 3f). The animals treated rectally had persistent diarrhoea. The damage to colon wall due to repeated insertion of the cannula cannot be ruled out. These animals died within 5–7 days after TNBS administration.

Discussion

Levamisole is a strong immune potentiator that is a well-known stimulant of B cells, T cells, monocytes and macrophages. Hence, the comparative study of levamisole and *A. nidulans* lectin was planned where the effect on anaphylaxis, Arthus reaction, T_H1/T_H2 cytokines and macrophages was studied in BSA-immunized Swiss albino mice.

Based on antigenic stimulus, CD4 T cells differentiate into distinct subsets (T_H1 or T_H2) that can be identified by monitoring their specific cytokines. T_H1 cells mainly produce IFN-γ, IL-2 and TNF-β and act against viral infections, intracellular pathogens and play an important role in disease like AIDS, cancer, etc. T_H2 cells are active against extracellular pathogens and are known to produce IL-4, IL-5, IL-6 and IL-13 [32]. In the present study, elevated levels of IFN-γ as well as IL-6 were observed, which might suggest mixed T_H1/T_H2 activity of *A. nidulans* lectin as reported for *Asparagus racemosus* root extract [33]. Fungal immunomodulatory proteins from *F. velutipes* [3] and *V. volvacea* [4] have also been reported to enhance expression of cytokines elaborated by T_H1 as well as T_H2 subsets. Lectins from *V. volvacea* [6] and *F. fraxinea* [9] modulate T-cell response via IFN-γ production.

The up-regulation of IFN-γ results in activated functional ability of macrophages, which in turn further supports IFN-γ production [34]. The enhanced functional ability of macrophages could also be responsible for the observed high levels of IL-6 [34]. IL-6 has both pro-inflammatory as well as anti-inflammatory properties. On one hand, it induces the synthesis of glucocorticoids and promotes the synthesis of pro-inflammatory mediators [35], while at the same time it inhibits the production of pro-inflammatory cytokines such as GM-CSF [36]. Macrophage activation in lectin-treated animals was well supported by results of other parameters investigated.

Nitro blue tetrazolium is a yellow dye that reduces to formazan by activated macrophages. It is an indirect marker of oxygen-dependent activity of phagocytes and metabolic activity of granulocytes or monocytes [37, 38]. Therefore, enhanced dye reduction in treated group as compared to immunized control group advocates macrophage activation. The functional ability of macrophages was also evident from higher nitric oxide production in splenocyte cultures of lectin-treated mice. Activated macrophages begin to express inducible nitric oxide synthase that oxidizes L-arginine and releases nitric oxide. These findings are in corroboration with enhanced IFN-γ levels in splenocyte cultures. Marked production of nitrite ions is shown in peritoneal macrophages of C57BL/6 mice treated with *T. mongolicum* lectin [5]. *A. bisporus* lectin shows commendable potential to activate RAW 264.7 macrophages producing nitric oxide [8]. Lectin from *Paracoccidioides brasiliensis* has also been reported to induce nitric oxide production in mouse peritoneal macrophages [39].

Anaphylactic reaction, also known as immediate hypersensitivity, is mediated by IgE produced in response to certain antigens. IgE binds to high affinity receptors on mast cells

and basophils and a subsequent exposure to the same antigen cross links membrane-bound IgE which triggers the release of pharmacologically active mediators resulting in a range of symptoms from minor inconvenience to death [34]. *A. nidulans* lectin was found to inhibit systemic anaphylaxis in mice. Anaphylaxis reaction is a hallmark of humoral immune response and is usually mediated by IL-4, a T_H2 cytokine [34]. Although the lectin has been found to up-regulate the levels of IL-6, another T_H2 cytokine, suppression of anaphylaxis suggest that the lectin does not support IL-4 expression by T_H2 cells. This further justifies that the elevated IL-6 levels may be in part due to enhanced functional ability of macrophages [34]. Fungal immunomodulatory proteins from *F. velutipes* [3] and *V. volvacea* [4] are also known to reduce the symptoms of systemic anaphylaxis.

Arthus reaction, a type III hypersensitivity reaction, entails the formation of antigen–antibody complexes ensuing complement activation and release of vasoactive amines that result in increased edema, haemorrhage and neutrophil infiltration at the site of antigen entry. The extent of immune complex deposition is inversely related to the extent of host's knack to clear such complexes [34]. The results of the present investigation reveal that either *A. nidulans* lectin does not support the formation of antigen–antibody complexes in vivo or enhanced functional ability of phagocytes initiates the clearance of such immune complexes formed. In the present study, no foot pad thickness was observed even after 48 h due to unknown reasons. In the focus of inflammation, macrophages counteract the toxic effects of cytokines and oxidative stress by triggering the self-protecting stress response [40, 41]. Fungal immunomodulatory proteins from *Ganoderma lucidum* [42], *F. velutipes* [3] and *V. volvacea* [4] have also been reported to inhibit Arthus reaction in mice.

As ulcerative colitis is an immune-related disorder, the observed immune modulatory potential of *A. nidulans* lectin made it logical to investigate its therapeutic effectiveness on TNBS-induced colon lesions in Wistar rats. TNBS when introduced into the colon via intra-rectal instillation induces mucosal injury leading to massive inflammation characterized by the dense infiltration of leukocytes and macrophages throughout the entire wall of colon. Clinical manifestations include progressive weight loss, bloody diarrhoea, rectal prolapse and the thickening of colonic wall [43]. The results suggested that lectin when administered intraperitoneally has a therapeutic effect against acute mucosal injury.

A. nidulans lectin shows high specificity for GalNAc as reported earlier [25]. It presumably binds GalNAc residues on epithelial cells and might exert therapeutic effect on TNBS-induced colitis via immunomodulation. Wakamto®, a koji-based medicine from *Aspergillus oryzae* used widely in Japan to cure intestinal discomfort, has been reported to be effective in experimental colitis induced by TNBS/ethanol enema in rats. It is most likely to exert its effect by immunomodulation and enhancement of the anti-superoxide activity in colonic tissues [44]. Another drug known to attenuate TNBS-induced colitis is thalidomide which exerts its effect by inhibiting the production of TNF-α and IL-12 [31].

The results of the present investigation demonstrate in vivo effects of *A. nidulans* lectin on macrophage function, resulting in improved immune status of the animals. The immune modulatory potential of the lectin has been found comparable to that of standard immunomodulator levamisole. The purified lectin on one hand significantly up-regulated the expression of IFN-γ and functional ability of macrophages, while on the other hand successfully inhibited Arthus reaction and systemic anaphylaxis. The elevated levels of IL-6 (T_H2 cytokine) could also be due to macrophage activation by the lectin. However, further investigations are required for conclusive correlations. Intraperitoneal administration of lectin to animals with experimental colitis unfolds its curative effect and hence its potential implication in patients with inflammatory bowel disease. The lectin showed no signs of toxicity in animals and is apparently safe to use. The present study is the first report

on therapeutic property of a microfungal lectin on TNBS-induced colitis model. The investigations carried out on *A. nidulans* would provide useful guidelines for evaluation of immunomodulatory and therapeutic potential of lectins from microfungi in general and *Aspergilli* in particular.

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