


Improvement of liver function in rats subjected to hepatotoxin by a crude protein derived from leaves of Cajanus indicus

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Introduction

Cajanus indicus is an edible herb. Its fruit is used as pulses throughout the Indian subcontinent. In rural India, especially in the Ganges plains, water extract of the leaves of Cajanus indicus has been extensively used for the treatment of jaundice and hepatomegaly for many years but the active principle is not known.

Hepatic dysfunctions due to ingestion of hepatotoxins are increasing worldwide. As there is no confirmed remedy to this dangerous situation, which is often irreversible and fatal, we have undertaken a systematic search to identify the active principle in the water extract of leaves of Cajanus indicus.

Method

The proteins of the water extract of leaves were precipitated by 90% Ammonium Sulphate, centrifuged and reconstituted in Tris-HCl buffer pH 7.3. Sephadex G-10 column chromatography and 12% SDS-PAGE showed two proteins (m.wt approx 40 and 42 Kd). Rats (male, b.wt 90 ± 10 g) were treated with a hepatotoxic agent—Carbon tetrachloride (0.3 ml, two doses at an interval of 7 days oralK) and the crude protein fraction were injected (0.5 ml/conc 42 μg/ml) ten days after the first CCl₄ dose when the rats showed changes in stool and urine colour (whitish brown and deep yellow that stained the abdominal wall near the genitals, respectively).

The experimental rats were divided into two groups, keeping suitable controls. Rats treated with CCl₄ were injected with (1) only one dose of the protein—group 1 or (2) 7 doses (1 dose per day for 7 days) consecutively—group 2. Twenty four hours after the last injection at group 2 all the rats were sacrificed (groups 1 and 2). Heart blood and liver impression smears were taken. The serum was tested for bilirubin, GOT, and GPT. The liver impression smear was fixed in methanol, stained with Giemsa stain and viewed under microscope.

Conclusion

Results (Table 1) showed that serum bilirubin and GPT were reduced significantly in the group that was treated with the crude protein for 7 days. No appreciable change was observed with GOT and histological examinations showed slight improvement in the liver architecture in the 7 day treatment group. Thus, it seems that the proteins present in the water extract of leaves of Cajanus indicus play an important role in improving liver function of animals suffering from jaundice.

Table 1. Effect of leaf protein of Cajanus indicus on liver function of rats

<table>
<thead>
<tr>
<th>Test</th>
<th>Bilirubin mg%</th>
<th>GOT U/L</th>
<th>GPT U/L</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>7 ± 2</td>
<td>10 ± 5</td>
<td></td>
</tr>
<tr>
<td>CCl₄</td>
<td>7 ± 2</td>
<td>18 ± 3</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>CCl₄ + Ci(1)</td>
<td>4 ± 1</td>
<td>18 ± 1</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>CCl₄ + Ci(7)</td>
<td>1 ± 0*</td>
<td>15 ± 4</td>
<td>14 ± 2*</td>
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</tbody>
</table>

Results (Table 1) showed the mean ± SD of 10 sets of experiments in each group (p < 0.001 CCl₄ + Ci(7) vs Ci(1) and for 1 or 7 days with crude protein.

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Improvement of liver function in rats subject to hepatotoxin by a crude protein derived from leaves of Cajanus indicus

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Cajanus Indicus

GPT (p < 0.001)

Reference
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Hepatoprotective effect of a protein isolated from *Cajanus indicus* (Spreng) on carbon tetrachloride induced hepatotoxicity in mice

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Treatment with hepatotoxin namely carbon tetrachloride (CCl₄) (0.1 ml / 100 g of body weight; twice a week) induced acute hepatic necrosis in Swiss albino mice (male; body weight 30 g ± 2), with significant alteration in the activities of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT); alkaline phosphatase (AP) and serum bilirubin. Administration of a protein fraction isolated from the leaves of *C. indicus* counteracted the action of CCl₄ on transaminase, phosphatase showing hepatoprotection. Daily treatment with a purified protein fraction 'X' from the above plant (0.5 mouse ml; 50-60 μg/ml) for a period of 7, 14, 21 days respectively showed decreased activities of serum transaminases alkaline phosphatase and decreased levels of serum bilirubin. These findings were further confirmed by histopathological study of liver.

Hepatic dysfunctions due to ingestion or inhalation of hepatotoxins (acetaminophen; cadmium chloride; ethanol; carbon tetrachloride, allyl alcohol) are increasing worldwide. In this study, CCl₄ has been chosen, as its immense use in industry leads to severe exposure to mankind, resulting in acute liver disorder. Mehendale has reviewed the mechanism of liver damage by CCl₄ (ref. 3) CCl₄ induces successive hepatic changes consisting of hepatic steatosis, fibrosis, massive infiltration and cirrhosis.

In modern medicine there is hardly any drugs that stimulate liver function, offer protection to liver from damage or help regeneration of hepatic cells. However, there are various hepatoprotective substances, effective in improving hepatic function. Many of these formulations containing herbal extracts are sold in Indian market for the treatment of various types of liver disorder. *Cajanus indicus* (Spreng) is one such edible herb. Its fruit is used as pulses throughout Indian subcontinent. In rural India, specially Gangetic plains, aqueous extract of the leaves of this plant has been extensively used for the treatment of jaundice and hepatomegaly for many years, but the active principle remains unknown.

Considering the above facts it was proposed to study the effect of the above plant on CCl₄ induced hepatic damage. Materials and Methods

**Purification of protein**—Aqueous extract of the dried leaves of *Cajanus indicus* (Spreng) were precipitated by 90% saturation of (NH₄)₂SO₄ (ref. 15), centrifuged at 4°C at 10,000 rpm for 25 min to obtain a solid precipitate. The precipitate was then reconstituted in 50 mM Tris HC1 buffer at pH 7.2. It was further taken for ice-cold acetone precipitation at 4°C in order to remove the excess chlorophyll. The acetone powder was again reconstituted in 50 mM Tris HCl buffer, pH 7.2, dialysed against distilled water and protein concentration was estimated by Bradford's Method.

The crude protein at a dose of (0.5 ml mouse, ip; 42 μg/ml) was injected in mice for testing its bioactivity. Sephadex G-50 fine (Sigma, USA) column chromatography of the crude protein showed 3 main peaks. All the 3 fractions of the protein were tested separately at a dose of 0.5 ml r.p.; 50-60 μg/ml. Fraction 2 showing hepatoprotective activity was further purified. It was further taken for ion exchange chromatography in DEAE cellulose (Sigma, USA) 12%, 10% and 8% SDS PAGE electrophoresis was done, showing two
main bands (mol. wt approximately 40-42 kd).

**Animal experiment**—Swiss albino mice (male, bodyweight 30 g ± 2) were maintained under laboratory condition and provided with standard pellet diet (Lipton India Ltd.) and water ad libitum. The animals were divided into several groups of six (n = 6 in each group).

Group 1: Served as control without any treatment.

Group 2: Control treated: Animals were treated with Bovine serum albumin (BSA) (0.2 ml ip, 1 mg/ml).

Group 3: CC14 treated (spectroscopic grade; SD Fine Chemicals Pvt Ltd) at a dose of ip. 0.1 ml/100 g of body weight, twice a week along with liquid paraffin as vehicle.

In both pre and post treated group, the animals were intoxicated with CC14 twice in a week. 36 hr after the first dose of CC14 injection the second dose was given.

Group 4: CC14 treated, mice were injected with BSA (0.2 ml ip, 1 mg/ml).

Group 5: Pretreated group: The animals were treated with protein fractions 1, 2 and 3 separately (0.5 ml, ip. 50-60 μg/ml) prior to CC14 administration, daily for 7, 14 and 21 days respectively in three different sets of experiment. 24 hr after the final dose of drug (protein fractions), the animals were intoxicated with CC14, twice a week. 36-48 hr after the second dose of CC14 administration the animals were sacrificed.

Group 6: Post treated group: The animals were first subjected to CC14 (ip. 0.1 ml/100g of body weight; twice a week) 36-48 hr after the second dose of CC14 administration, the animals were sacrificed.

Biochemical studies—The mice were sacrificed at -20°C for biochemical estimation of GOT and GPT.

**Histopathological examination**—Small pieces of liver were quickly removed after autopsy to Bouin's fluid. The tissues were processed and kept in cedar wood oil for clearing. Paraffin sections 5 μm to 10 μm were prepared, stained in hematoxylin and eosin and mounted in neutral DPX medium.

**Results**

Changes in activities of GOT, GPT, AP and bilirubin in serum, and GOT and GPT in liver tissue—As evident from Figs. 1and 2, the levels of all the marker enzymes of serum GOT, GPT, AP and bilirubin were found to be significantly increased in CC14 and CC14 with BSA treated mice. Whereas, treatment with a partially purified protein fraction 2 isolated from *C. indica* showed decreased activities of serum transaminases (P < 0.001); serum AP (P < 0.001) (Fig 1) and decreased levels of serum bilirubin (P < 0.001) (Fig. 2).

The activities of GOT and GPT in liver tissue increased significantly in CC14 treated mice. Treatment with the purified protein fraction 2 from the above plant resulted in decreased levels of GOT (P < 0.001) and GPT (P < 0.001) when compared to CC14 treated group. It was found that in both pre and post treated groups protein fraction 2 showed significant decrease in activities of the liver transaminases (Fig. 2).

It was further observed that, both protein fraction 1 and 3 isolated from the above plant showed no significant decrease in the elevated serum GOT, GPT, AP and bilirubin levels when compared to the protein fraction 2 treated mice.

Histopathological evaluation—Liver samples from the control and experimental animals were taken for histological studies on liver damage. Normal histology of the mouse liver showing sinusoidal architecture of hepatocytes having no sign of necrosis or degeneration (fig. 3A). The liver section of the mouse treated with CC14 showed cellular degeneration, hydropic changes more around the central veins, fatty changes, widespread hepatocellular necrosis, Kupffer cells
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Fig. 1— Alteration in activities of serum glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and alkaline phosphatase (AP) following treatment with protein fractions of the leaves of C. indicus in mice intoxicated with CCI4.

Values are mean ± S.E. (n=6 in each group) ** P<0.001

I—Alteration in activities of serum glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and alkaline phosphatase (AP) following treatment with protein fractions of the leaves of C. indicus in mice intoxicated with CCI4.
Fig 2—Alteration in activities of serum total bilirubin, and liver glutamate-oxaloacetate transaminase (GOT), and glutamate-pyruvate transaminase (GPT), following treatment with protein fractions of the leaves of C. indicus in mice intoxicated with CCl₄.
Fig. 3—(A) Normal section of mouse liver (B) Section of CCl₄ treated mouse liver showing cellular destruction after 36-48 hr of CCl₄ toxicity (C) Section of CCl₄ treated mouse liver after 7 days of CCl₄ toxicity showing widespread hepatic cellular necrosis (•), fatty changes (•%), kupffer cell hyperplasia (•), hepatitis and centrilobular necrosis and atrophy (•) (D) Section of post-treated Fnu 2 mouse liver showing no significant hepato cellular damage (E) Section of pretreated Fnu 2 mouse liver showing micro fatty changes and small areas of focal degeneration [H & E x 100]
hyperplasia, centrolobular necrosis and steatosis as seen in Fig. 3B & 3C. Section of post and pre treatment 2 treated mouse liver (Fig. 3D & 3E) showing microfatty changes with dense collection of lymphoid cells suggesting evidence of very little necrosis or degeneration. There is no significant hepatocellular damage. Only small areas of focal degeneration and sinusoidal dilation are observed in Fig. 3E.

Discussion

The presentation of CCl₄ hepatotoxicity is supposed to be produced by enhanced production of active metabolites by the ethanol activated mixed function oxidation system. This eventually leads to hepatocellular necrosis and is reflected in our experiment by marked changes in various enzymatic and non-enzymatic parameters of CCl₄ treated mice. The most serious delayed effects of CCl₄ result from its hepatotoxic and nephrotoxic action. Liver dysfunction begins soon after ingestion of CCl₄ and maximum malfunction occurs within 48-72 hr. Ceylanus indicus, protein fraction 2 treatment, was effective in counteracting the toxic effects of CCl₄, as shown by reversed levels of the altered parameters, both in serum as well as liver.

The exact mechanism by which the herbal protein provides the protection is not known. However, injury produced by CCl₄ seems to be mediated by a reactive metabolite - trichloromethyl free radical (CCl₃), formed by the homolytic cleavage of CCl₄, or by an even more reactive species - trichlor methyl peroxo free radical (Cl₃COO⁻) formed by the reaction of CCl₄ and O₂⁻. The hepatoprotective properties of the protein fraction 2 derived from C. indicus might be related to scavenging of free radicals generated by microsomal reductase of CCl₄.

It is also known that CCl₄ induces hepatic damage and tumor necrotic factor (TNF) was detected in the serum of these animals. It was hypothesized that TNF is the mediator of liver damage, and other markers of liver function are released from the liver as a result of the damage. Therefore, it is necessary to see whether this herbal protein fraction 2 may directly or indirectly affect the production of TNF from the Kupffer cells of the liver.

The present study shows that protein fraction 2 (both pre-treated and post-treated) could offer protection to the experimental animals from deleterious effects of CCl₄ induced hepatotoxicity. This herbal protein does bring about modulation in both pre- and post-exposure condition. It alleviates the symptoms of liver damage as evident from biochemical assays and by histopathological study of liver.

Acknowledgement

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Role of bone marrow and thymus secretory protein in maintaining immune homeostasis and haemopoiesis in control and malnourished mice

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Injection of Salmonella typhi 'H' antigen was observed to produce a differential effect on bone marrow and thymus secretory profile depending upon the nutritional status of the host. The paracrine effect of Thy F1 (thymus fraction 1) was more significant \( P < 0.01 \) than the autocrine effect of BIM (Bone marrow immunomodulator) in malnourished mice. BIM moreover, also had a paracrine effect on thymus irrespective of the nutritional status of the host. An improvement in neutrophil population \( P < 0.01 \) and phagocytic myeloperoxidase activity \( P < 0.01 \) was observed in BIM treated malnourished immunosuppressed mice, whereas no appreciable change was observed by Thy F1. However, Thy F1 irrespective of the nutritional status of the host improved large lymphocyte population in circulation \( P < 0.01 \). These findings indicate that both bone marrow and thymus play a major role in haemopoietic microenvironment of BDF (basal diet fed) control and malnourished mice.

Host defence to infection is the result of complex interaction between specific defence mechanisms. The immune homeostasis is maintained by both the central and peripheral lymphoid organs and it seems that cytokines play a major role, which is achieved by modulating neuroendocrinal or neurometabolic state. During steady state haemopoiesis only a few cytokines are demonstrated but as soon as an antigen is introduced, a finely orchestrated manoeuvre by the immune system elaborates a plethora of cytokines with diverse biological functions in control basal diet fed (BDF) animals. In contrast, malnourished immunosuppressed animals show suppressed bone marrow and thymus secretory protein secretion.

In recent years an increasingly important role is being attributed to hematopoietic cytokines in the organism’s response to infection. Constant increase in antibiotics resistance in the microbes and various side effects caused by drugs has prompted the use of immunomodulators to immunomodulate immunosuppressed conditions by some secretory proteins, mainly in malnourished individuals.

In the present study we have considered the possible interdependence of bone marrow and thymus microenvironment in maintaining immune homeostasis and the role of bone marrow and thymus secretory protein in neutro and lymphopoiesis in control and malnourished mice.

Materials and Methods

Animal experiment

Swiss albino mice (male, body weight 25 gms ±2) were maintained under laboratory condition and provided with standard pellet diet (Lipton India Ltd.) and water ad libitum. The animals were divided into several groups of five.

Group 1: Basal diet fed (BDF) control mice

Group 2: Mice were made malnourished of vitamin B complex, keeping suitable controls

Group 3:

(a) BDF control mice were immunized once with Salmonella typhi 'H' antigen (Ag) (0.5 ml ip, Span India Ltd.) keeping unimmunized control

(b) Similarly, malnourished mice were immunized once with Salmonella typhi 'H' antigen (Ag) (0.5 ml ip, Span India Ltd.) keeping unimmunized control

After 24 hr of Salmonella typhi 'H' antigen injection, BDF control and malnourished mice were sacrificed by ether anaesthesia and cervical
dislocation and bone marrow and thymus cell culture were performed.

**Treatment with BIM/ThyFl after immunization**

**Group 4:**
(a) After 24 hr of *Salmonella typhi* 'H' Ag immunization, BIM was injected (0.5 ml ip; 0.8 μg/g of body wt.) in BDF mice.
(b) Similarly malnourished mice were treated with BIM (0.5 ml ip; 0.8 μg/g of body wt.) 24 hr after immunization.

**Group 5:**
(a) In a separate set of experiment, 24 hr after *Salmonella typhi* 'H' Ag immunization in BDF mice, ThyFl was injected (0.5 ml ip; 0.35 μg/g of body wt.).
(b) Similarly, 24 hr after immunization malnourished mice were also treated with ThyFl (0.5 ml ip; 0.35 μg/g of body wt.).

After 24 hr of BIM/ThyFl treatment the animals of both (BDF & MAL) the groups were sacrificed, bone marrow and thymus cell culture were performed.

**Treatment with BIM/ThyFl in unimmunized controls**

**Group 6:**
(a) In another set of experimental animals unimmunized BDF mice were treated with only BIM (0.5 ml ip; 0.8 μg/g of body wt.).
(b) Similarly, unimmunized malnourished mice were also treated only with BIM at a dose of (0.5 ml ip; 0.8 μg/g of body wt.).

**Group 7:**
(a) In a separate set of experiment, ThyFl was injected at a dose of ip (0.5 ml ip; 0.35 μg/g of body wt.) in unimmunized BDF control mice.
(b) Similarly unimmunized malnourished mice were treated with ThyFl (0.5 ml ip; 0.35 μg/g of body wt.).

After 24 hr of BIM/ThyFl treatment the animal of both groups were sacrificed, bone marrow and thymus cell culture were performed.

### Bone marrow cell culture

Unfractioned mouse bone marrow cells, flushed from the femurs with RPMI 1640, pH-7.3 preincubated at 37°C, were repeatedly aspirated and injected from a syringe to obtain a single cell suspension. The bone marrow cells (at a conc. of 3x10^6 cells/ml) were incubated at 37°C in serum free medium for 24, 48, 72 and 96 hr, supplemented with CPSR-2 (Sigma, USA). At the end of the incubation period the cells were separately pelleted by centrifugation (500 rpm, 10 min.) at 4°C and the supernatant fluid was collected.

The cell free soup was then subjected to 0.22 membrane filtration under N2 pressure with continuous stirring. The proteins of the crude filtrate were precipitated by 60% ammonium sulphate saturation at 4°C. The protein concentration was estimated by Bradford's method using BSA as standard. Sephadex G-10 (Sigma, USA) column chromatography of the crude protein showed three main peaks. The present study deals with the immunomodulatory effect of the pooled fractions (8-12) under the first peak, henceforth designated as BIM, mol. wt. 12.7 kD. The BIM which is used for the treatment is derived from BDF control mice cultured till 24 hr.

### Thymus cell culture

As in bone marrow cell culture, thymus cell culture was also performed in all the set of experimental animals. The thymus glands were isolated and teased in RPMI-1640 pH-7.3, preincubated at 37°C, and repeatedly syringed to obtain a single cell suspension. The thymus cells (at a conc. of 1x10^6 cells/ml) were incubated at 37°C in serum free medium; supplemented with CPSR2 (Sigma, USA) for 24, 48, 72 and 96 hr, respectively. At the end of the incubation period, the cells were separately pelleted by centrifugation (500 rpm, 10 min) and the supernatant fluid was collected.

The cell free soup was then subjected to 0.22 membrane filtration under N2 pressure with continuous stirring, as mentioned in bone marrow cell culture. The proteins of the crude filtrate were precipitated by 60% ammonium sulphate saturation at 4°C. Sephadex G-10 (Sigma USA) column chromatography of the crude protein showed five main peaks of which the first peak, Thy Fl (mol.
wt. ≥ 10 kD) showing immunomodulatory activity has been used in this study. Similarly, the ThyFl which is used for the treatment in the experimental animals is obtained from BDF control mice cultured till 24 hr.

Trypan blue exclusion test
Cell suspension of bone marrow (at a conc. of 3×10^7 cells/ml) and thymus (at a conc. of 1×10^6 cells/ml) was taken separately in a test tube. 0.9 ml of cell suspension was mixed with 0.1 ml trypan blue solution (0.4%). After 5 min, the unstained viable cells were counted under microscope using Neubauer hemocytometer and the percentage calculated accordingly.

Differential count of WBC and estimation of neutrophil phagocytic index
Blood films of sacrificed mice were fixed in methanol and stained by May Grunwald Giemsa stain. One hundred WBC were counted and percentage was calculated.

Percentage of large and small lymphocytes were counted on the basis of nucleus size. Large sized
lymphocytes showed clear blue cytoplasm on the margin of the nucleus, and in the small lymphocytes dark violet coloured nucleus almost filled the entire cell and had a rim of clear cytoplasm\textsuperscript{12}. Neutrophil phagocytic index was estimated by myeloperoxidase staining method\textsuperscript{13}. The cytoplasm of neutrophils and some monocytes, staining for myeloperoxidase showed black dots. One hundred WBC were counted and percentage of neutrophil (cells staining for +ve myeloperoxidase versus -ve cells) was determined.

Statistical evaluation was done using Student’s t test, standard deviation and standard error of mean\textsuperscript{14}.

Results

Figure 1 shows a comparative study of the effect of BIM and ThyFl on the in vitro bone marrow and thymus secretory profile after immunization with Salmonella typhi ‘H’ antigen. It is clearly evident from the figure that both the bone marrow and thymus secretory profile of BDF control mice significantly differs from the vitamin B-complex deficient malnourished mice (P<0.01). The pre immunized secretory level of bone marrow and thymus of BDF mice is higher than that of malnourished ones. In case of 24 hr post immunization (Pol), the secretion of bone marrow proteins of BDF mice is suppressed which is followed by stimulation at 96 hr Pol. In contrast the bone marrow secretory profile of malnourished mice gradually increases after immunization.

Table 1—Effect of BIM/ThyFl in immunized BDF control and malnourished mice. Values are mean ± SE (n = 5)

<table>
<thead>
<tr>
<th></th>
<th>Bone marrow</th>
<th>Thymus</th>
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</thead>
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<tr>
<td></td>
<td>secretory protein</td>
<td>secretory protein</td>
</tr>
<tr>
<td>BIM Treatment</td>
<td>µg/ml</td>
<td>µg/ml</td>
</tr>
<tr>
<td>BDF control</td>
<td>27.10 ± 3.23</td>
<td>26.48 ± 1.54</td>
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<td>malnourished</td>
<td>22.54 ± 5.26</td>
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<tr>
<td>ThyFl Treatment</td>
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<tr>
<td>BDF control</td>
<td>24.62 ± 2.34</td>
<td>29.45 ± 3.12</td>
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<tr>
<td>malnourished</td>
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<td></td>
</tr>
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</table>

PMN Neutrophil percentage % Myeloperoxidase +ve neutrophil

Fig 2—Comparison of PMN neutrophil and myeloperoxidase +ve neutrophil percentage in Control (BDF) and Malnourished (MAL) mice, immunized with Salmonella typhi ‘H’ antigen (Ag) and effect of BIM / ThyFl.
Similarly, the thymus secretory profile of BDF mice is suppressed after 24 hr Pol, like that of bone marrow, whereas in malnourished mice thymus secretory protein level gradually increases reaching its peak at 96 hr Pol.

Treatment with BIM/ThyFl separately in immunized BDF control mice resulted in no appreciable change in indigenous bone marrow and thymus secretory profile when treated with BIM / ThyFl ($P < 0.001$).

It is to be noted that, BDF control mice when treated with only BIM/ThyFl showed suppression compared to immunized control in both bone marrow and thymus secretory profile; whereas a marked stimulation is observed in malnourished mice (Table 1).

Figure 2 shows a comparison of PMN neutrophil and myeloperoxidase +ve neutrophil percentage in control (BDF) and malnourished mice, immunized with Salmonella typhi 'H' antigen and the effect of BIM/ThyFl. A lower circulating neutrophil percentage in malnourished mice was observed compared to BDF control ($P < 0.01$). On immunization, the malnourished mice showed increased myeloperoxidase +ve PMN neutrophil percentage). A significant change in percentage of neutrophil and its staining characteristics were observed in malnourished mice when treated with BIM, compared to BDF control ($P < 0.01$). Immunized BDF control mice when treated with only BIM/ThyFl showed no significant change in PMN neutrophil percentage and myeloperoxidase staining reaction.

Treatment with ThyFl in immunized and nonimmunized malnourished mice increased the lymphocyte count, but had no significant effect on the neutrophil percentage and its myeloperoxidase staining reaction.

It is evident from Fig 3 that ThyFl, irrespective of the nutritional status of the host, increased the large lymphocyte population compared to BIM ($P < 0.01$).

**Discussion**

It has been reported that enrichment and characterization of thymus repopulating cells occur in bone marrow microenvironment. It may be noted that bone marrow plays an important role in thymocyte development from progenitor cells. The T-cell precursor of bone marrow are capable of
emigrating and colonizing the thymus. It is believed that for optimum immune homeostasis, the bone marrow and thymus, function in close interdependence. The mechanism of regulation of migration of immature T-cell from bone marrow is however unknown. It seems that cytokines, secreted from thymus and bone marrow play an important role in preserving homeostasis and steady state, haemopoietic microenvironment.

Injection of Salmonella typhii 'H' antigen was observed to produce a differential effect on bone marrow and thymus secretory profile depending upon the nutritional status of the host (Fig. 1). The BDF mouse showed an initial suppression followed by stimulation. Malnourished animals on the other hand showed only stimulation. The observation is similar to that observed with brain enzymes. It is apparent from Fig. 1 that the paracrine effect on thymus secretory protein on bone marrow secretory profile is more significant (P < 0.01) than the autocrine effect of BIM in malnourished mice. BDF mouse, however, also has a significant paracrine effect on thymus irrespective of the nutritional status of the host.

Thus it seems that a bidirectional communication through cytokines exist between bone marrow and thymus. Malnourished animals are immunocompromised and suffer from a variety of infections. The immunomodulating effect of BIM has been reported earlier. Both T and B cell functions of malnourished animals were found to be improved but the BDF animals showed no appreciable change. indicating that malnourished animals gain more from cytokine treatment whereas, a negative feedback system is in operation in BDF control. In this study we further show that BIM not only improve neutrophil population or its maturation (Fig. 2). However, ThyFl, irrespective of the nutritional status of the host improves the large lymphocyte population in circulation (Fig. 3). We may perhaps be concerned not to be too indulgent in believing a subtle influence of bone marrow microenvironment on certain activities of thymus like lymphopoiesis. In conclusion, our findings further strengthen the therapeutic value of both BIM and ThyFl and their role in modulation of lymphoid tissue micro environment. There is substantial data indicating that the role of MHC class I and class II molecules in the process of colonization of the thymic microenvironment by lymphohaemopoietic cells of bone marrow. However, on the basis of the data so far discussed it may be concluded that the bone marrow and thymus probably function in finely tuned manner and play an important role in neutrophil and lymphopenia in malnourished mice.

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References

Salmonella antigen induces differential bone marrow cytokine secretion in control and malnourished rats

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Balanced diet fed (BDF) rats injected with Salmonella type B' antigen showed an initial suppression of an immunomodulatory bone marrow cytokine Fr-1 (BM-Fr-1) followed by stimulation, whereas the cytokine secretory pattern showed only stimulation in vitamin B complex malnourished rats. This nutritional-dependent differential response of a bone marrow cytokine to antigen is similar to that observed previously in brain adenosine triphosphatase (ATPase) function following immunization. An improvement in neutrophil response to S. typhimurium (P < 0.01) and neutrophil function (P < 0.05) was observed at cytokine-induced malnourished immunosuppressed rats further strengthening our previous observations that BM-Fr-1 modulates both specific and nonspecific immune systems. No significant change was observed in BDF animals indicating that malnourished rats gain more from cytokine therapy, whereas a negative feedback system might be present in BDF animals.

Key words: Salmonella typhi, bone marrow, cytokine, immunosuppression, immunotherapy, rat.

Introduction
Salmonella pathogenesis involves general immunosuppression and neutropenia. The bacteria invades both the peripheral and central immune systems. Currently under intense scrutiny, although less well understood, is how salmonella produces immunosuppression. One probable mechanism would be to disrupt the function of the bone marrow microenvironment that is controlled by cytokines regulating the production of lineage-restricted progenitor cells. There is no definite proof that this mechanism is available, except for some isolated reports on lymph nodes and thymus.

In recent years, an increasingly important role is being attributed to hematopoietic cytokines in the organism's response to infection. Because enteric infections are very much predominant in malnourished hosts, we will discuss (i) the effect of Salmonella typhi 'H' antigen on the secretion of an immunomodulatory bone marrow cytokine (BM Fr-1) of balanced diet fed (BDF) and vitamin B complex malnourished rats, and (ii) the immunomodulatory effect of BM Fr-1 in modulating the function of neutrophil and bone marrow microenvironment in BDF and bone-malnourished Salmonella antigen-injected animals.

Methods

Experimental animals
Young inbred Charles-Foster male rats (bodyweight 80-100 g) were made malnourished of vitamin B complex. Suitable controls were also kept. The animals were kept on suspended wire cages with 12 h light-dark cycle. Food and water were provided ad libitum.

Immunization of animals
The rats (BDF control and malnourished) were immunized with Salmonella typhi 'H' antigen (0.5 mL, i.p., Span, India), keeping unimmunized controls.

Bone marrow cell culture
After 24, 48, 72, 96 and 168 h post-salmonella antigen injection, the rats were killed by ether anaesthesia and cervical dislocation. The femurs were flushed with RPMI 1640 pH 7.3 preincubated to 37°C and repeatedly syringed so as to obtain a single cell suspension. Bone marrow cells (at a concentration of 106 cells/mL) were incubated at 37°C for 24 h.

Differential white blood cell count and estimation of neutrophil phagocytic index
A drop of heart blood was spread on a glass slide, fixed in cold formol-ethanol. The cells were incubated with Nadi reagent at room temperature until colour developed before being washed in distilled water, stained with safranin, washed in distilled water again and counter-stained with Giemsa stain. The cytoplasm of neutrophils and some monocytes, when stained for myeloperoxidase showed black dots. One hundred white blood cells were counted and the percentage of different cells was calculated.

Bone marrow secretory profile
The cell-free culture soup was subjected to G-10 column chromatography as described previously and the secretory proteins were estimated by Lowry's method. Three peaks were observed.
were obtained of which the first peak showing maximum immunomodulatory activity (maximum weight 12.7 kd) is discussed in this paper.

Immunomodulation by a bone marrow secretory protein

In a separate set of experiments the BDF and malnourished rats were immunized with Salmonella typhi 'H' antigen as described before. Twenty-four hour post-immunization bone marrow secretory protein Fr-I was injected with 0.5 ml (p concentration 1.2 µg/ml). Seventy-two hours after the last post-injection bone marrow cell culture was performed as before. The cell-free soup was harvested 24 h after and subjected to Sephadex G-10 column chromatography (Sigma Chemical Company, St. Louis, MO, USA). Another group of rats (control and malnourished) were treated only with the bone marrow secretory protein and 72 h after the bone marrow cells were cultured. The cell-free soup was harvested after 24 h and subjected to Sephadex G-10 column chromatography. Peripheral blood smear was stained for myeloperoxidase reaction.

**Evaluation of antibody**

Serum was isolated from the congested heart blood obtained from sacrificed rats. Antibody titre against Salmonella typhi 'H' antigen was determined after serial dilution of the serum in saline against Salmonella typhi 'H' antigen (Table 1) keeping suitable controls.

**Statistical analyses**

Statistical significance was calculated using Wilcoxon signed rank sum test and Student's t-test.

**Results**

From Fig. 1 it is evident that the secretion of bone marrow fraction 1 (BM-Fr 1) significantly differs between BDF control and vitamin B complex malnourished rats (P < 0.01). The post-immunized secretory level of BM-Fr 1 of BDF rats is higher than that of the malnourished rats. After 24 h post-immunization (POI) the secretion of BM-Fr 1 of BDF rats is suppressed, which had recovered at 48 h POI. Thereafter, a slight suppression is followed by stimulation at 168 h POI. In contrast, the BM-Fr 1 level of malnourished rats gradually increased with a slight suppression at 96 h.

It is also evident from Fig. 1 that at 168 h POI the polymorphonuclear (PMN) neutrophil percentage of both BDF and malnourished rats showed an inverse relationship with BM-Fr 1 level. However, the neutrophil myeloperoxidase staining indicated (Fig. 2) that a direct relationship seems to be present with BM-Fr 1 level.

**Figures**

**Fig. 1.** Comparison of bone marrow Fr-I cytokine secretion in response to immunization and cytokine treatment in balanced diet fed (■) and vitamin B complex deficient (□) rats. Rats were (A) non-immunized, (B) immunized, (C) immunized and BM-Fr 1 treated and (D) only BM-Fr 1 treated. We found, as is stated in Fig. 1, that the BDF-BM-Fr 1 level is much higher than that of malnourished ones (P < 0.01). After immunization the BM-Fr 1 level showed suppression.

**Fig. 2.** Relationship between bone marrow cytokine and neutrophil myeloperoxidase reaction in balanced diet fed and vitamin B complex deficient rats with or without Salmonella typhi 'H' antigen injection. (- -), BDF BM-Fr 1; (- - ), MAL BM-Fr 1; (- - -), BDF Mye; (- - -), MAL Mye; (- - -), BDF BM-Fr 1+MAL+Ag; (- - -), MAL+Ag+BM-Fr 1; (- - -), BDF+MAL+Ag+BM-Fr 1.***

**Table 1.** Effect of bone marrow cytokine Fr-I in Salmonella typhi 'H' antigen and bone marrow cytokine treatment in balanced diet fed and vitamin B complex deficient rats (Def).

<table>
<thead>
<tr>
<th>Group</th>
<th>Reciprocal Antibody titre**</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDF control+Ag</td>
<td>10-64</td>
<td>64</td>
</tr>
<tr>
<td>Def+Ag</td>
<td>2-16</td>
<td>8</td>
</tr>
<tr>
<td>BDF+Ag+BM-Fr 1</td>
<td>32-64</td>
<td>64</td>
</tr>
<tr>
<td>Def+Ag+BM-Fr 1</td>
<td>8-128</td>
<td>128</td>
</tr>
<tr>
<td>Def+Ag+TCF</td>
<td>2-16</td>
<td>8</td>
</tr>
</tbody>
</table>

Calculated on the basis of Wilcoxon composite rank sum test and in each group. Repeated twice. BM-Fr 1 injected 24 h after immunization.

**Fig. 3.** Comparison of bone marrow Fr-I cytokine secretion in response to immunization and cytokine treatment in balanced diet fed (■) and vitamin B complex deficient (□) rats. Rats were (A) non-immunized, (B) immunized, (C) immunized and BM-Fr 1 treated, and (D) only BM-Fr 1 treated.
Salmonella antigens induce differential bone marrow cytokine secretion

BM-Fr 1 cytokine is observed after immunization and the animals were immunocompetent. The immunosuppressed animals regained their immunocompetence after BM-Fr 1 treatment. The similarity of response pattern further strengthens our previous hypothesis that BM-Fr 1 and brain ATPase are closely related in physiological control of immune homeostasis. From Fig. 1 it is apparent that an increase in secretion of indigenous BM-Fr 1 seems to suppress the neutrophil percentage in peripheral circulation.

From Fig. 2 it seems that apart from its role in neutrophilic activity (Fig. 1) BM-Fr 1 regulates the biosynthesis of myeloperoxidase in neutrophils thereby controlling its bactericidal activity. In malnourished animals a suppressed neutrophil myeloperoxidase function and antibody response to S. typhi "H" antigen (Table 1) probably explains their recurrent infection status.

In order to improve this condition, the BM-Fr 1 that is under investigation in this paper and which was previously reported to have immunomodulatory functions as immunosuppressed animals. It seems from Figs 1 and 2 that the committed or immature mononuclear cells have two different mechanisms by virtue of which it responds to the salmonella antigen and BM-Fr 1. The injected bone marrow cytokine in malnourished immunized rats seem to proliferate the committed cell lineage of neutrophils (44 vs 10% in immunized malnourished control, Fig. 1) but fails to induce myeloperoxidase synthesis (44 vs 49% in immunized malnourished control, Fig. 2). On the other hand, malnourished rats treated only with the cytokine seem to induce myeloperoxidase synthesis in 46% PMN cells (Fig. 2), though its proliferative action on committed cells of neutrophil lineage is less (26%) than that of malnourished immunized and BM-Fr 1 treated rats (44%, Fig. 1).

Antibody response is also improved (Table 1) after bone marrow cytokine injection in malnourished immunized animals compared with immunized controls (P < 0.05). It seems from Figs 1 and 2 that apart from its role in neutrophilic activity, BM-Fr 1 regulates the biosynthesis of myeloperoxidase in neutrophils thereby controlling its bactericidal activity. In malnourished animals a suppressed neutrophil myeloperoxidase function and antibody response to S. typhi "H" antigen (Table 1) probably explains their recurrent infection status.

In conclusion, this report, for the first time, shows that Salmonella typhi "H" antigen produces a differential effect on bone marrow cytokine secretion depending upon the nutritional status of the host (Fig. 1). The BM-Fr 1 cytokine is observed in peripheral circulation of the cytokine under investigation in proper doses. This opens up a new area of investigation where insight into the role of cytokines as the mediators of the lymphoid tissue microenvironment suggest new therapeutic approaches.
Salmonella antigen induces differential bone marrow cytokine secretion in control and malnourished rats

Debasis Ghosal, Sarmistha Dutta and Prantosh Bhattacharyya


Abstract

The authors studied the effect of Salmonella antigen on bone marrow cytokine secretion in control and malnourished rats. Two groups of rats were injected with Salmonella typhimurium (H) and control saline solution, respectively. The bone marrow cytokine secretion was measured by assaying the supernatants of bone marrow cell cultures. The results showed that the malnourished rats had a significantly higher level of cytokine secretion compared to the control group.

Key words: Salmonella typhimurium, malnourished rats, bone marrow cytokine secretion.

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Salmonella antigen induces differential bone marrow cytokine secretion in control and malnourished rats
Debashis Ghosal, Sarmistha Dutta and Prantosh Bhattacharyya

सार

सल्मोनेल्ला अगेंट का रिक्ट्रक्सन निर्देशन करता था, इम्युनोलॉजिकल चौधरी उत्तरित मजबूत चालुक्याचिकान एक आर था। अवस्था एक आर से 1 के उत्तर्दशिक यथाच, और उसके बाद उद्देश्यों को पुनःतॊग्नित किया कबति साइटिन घाटी पदति ने इम्युनोफाइक यहाँ में इम्युनोफाइत पुनर्दृष्टिक शिफ्ट किया। एंडिकल में अवश्य प्रक्रिया चालुक्याचिकान का हीब्रिक अध्ययन ऐमनिंग प्रक्रिया वैदिक ही था। वैदिक इम्युनोफाइक एक वर्तमान स्टायल में पढ़े देखा गया था। हल्द टाइम (P < 0.05) और इम्युनोफाइक (P < 0.05) ने ऐमनिंग प्रकार में खुदर चालुक्याचिकान से अधिक फिक्स स्वतंत्र इम्युनो-यथार्थता घटनाओं में देखा गया। हालाँकि पूर्व-रायतों घिरारों और भी हुई है कि P10-18-विविक्त और अन्य सिद्धिक्त प्रोटीन इम्युनो-यथार्थता को अभिन्न नयाधिक सारांश है। B D F पत्रिका में होश वर्तमान प्रकिया निविदाय कहीं पुरा दिखाया यह वर्तमान है कि स्वतंत्र हूँ चालुक्याचिकान हो। हल्द टाइम सिद्धिक्त साइटिन नहीं हैं कक्ष प्रक्रिया सरकार अभिन्न प्रक्रिया वैदिक के पदति B D F पत्रिका में उपलब्धित हो सकती है।

पुनःतॊग्नि: जैमीक्स टाइम, उत्तरित मजबूत, चालुक्याचिकान इम्युनोफाइक इम्युनो-यथार्थता।