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

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Introduction
Salmonella pathogenesis involves general immunosuppression1–3 and neutropenia.4 The bacteria invades both the peripheral and central immune systems.2 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.5 There is no definite proof that this mechanism is available, except for some isolated reports on lymph nodes and thymus.6,7

In recent years an increasingly important role is being attributed to haematopoietic cytokines in the organism’s response to infection.8–12 Because enteric infections are very much predominant in malnourished hosts,1 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 in-bred Charles–Foster male rats (bodyweight 80–100 g) were made malnourished of vitamin B complex. Suitable controls were also kept.9 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 105 cells/mL) were incubated at 37°C for 24 h.9 The cells were removed by centrifugation (500 r.p.m., 5 min) and the supernatant fluid was collected. The protein concentration of the fluid was measured by Lowry’s method13 using bovine serum albumin as standard.

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 reagent14 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. A similar calculation was also done for neutrophils (cells staining for positive myeloperoxidase vs negative cells).

Bone marrow secretory profile
The cell-free culture soup was subjected to G-10 column chromatography as described previously10 and the secretory proteins were estimated by Lowry’s method.13 Three peaks

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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 F 1 was injected with 0.5 mL i.p (concentration 1.2 mg/mL). Seventy-two hours after the last protein 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).10

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.

**Estimation of antibody**

Serum was isolated from the coagulated 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.9

**Statistical analysis**

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 pre-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.

Figure 3 shows a comparison between (A) non-immunized, (B) immunized, (C) immunized and BM-Fr 1 treated and (D) only BM-Fr 1 treated, BDF and malnourished rats. 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 shows sup-

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**Table I. Effect of bone marrow cytokine Fr 1 in *Salmonella typhi ‘H’* antigen (Ag) immunized balanced diet food (BDF) control and vitamin B complex deficient rats (Def)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Reciprocal range</th>
<th>Antibody titre**</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDF control+Ag</td>
<td>16–64</td>
<td>64</td>
<td><em>P</em> = 0.001</td>
</tr>
<tr>
<td>Def+Ag</td>
<td>2–16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>BDF+Ag+BM-Fr 1*</td>
<td>32–64</td>
<td>64</td>
<td>NS</td>
</tr>
<tr>
<td>BDF+Ag+TCF</td>
<td>16–64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Def+Ag+BM-Fr 1*</td>
<td>8–128</td>
<td>128</td>
<td><em>P</em> = 0.001</td>
</tr>
<tr>
<td>Def+Ag+TCF</td>
<td>2–16</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*Calculated on the basis of Wilcoxon composite rank sum test. *n* = 6 in each group. Repeated thrice. * BM-Fr 1 injected 24 h after immunization. ** Antibody titre at 168 h post-immunization.
pression in BDF rats while a stimulation is seen in malnourished ones. BM-Fr 1 treatment of immunized BDF rats showed further suppression in indigenous BM-Fr 1 secretion. Immunized malnourished rats treated with BM-Fr 1 showed a suppression in indigenous BM-Fr 1 level than its immunized counterpart, whereas a stimulation is seen compared with the malnourished control. Balanced diet fed rats injected only with BM-Fr 1 showed suppression unlike the unimmunized control, whereas a slight stimulation is observed in malnourished rats.

From Fig. 1 we find that injection of BM-Fr 1 in immunized and non-immunized malnourished rats produced different effects on neutrophils. The non-immunized malnourished rats showed a lower neutrophil value compared with immunized rats ($P < 0.05$). Figure 2 shows the neutrophil myeloperoxidase staining reaction in the above-mentioned animals. The non-immunized malnourished BM-Fr 1 treated groups have more myeloperoxidase positive PMN neutrophils than do immunized and BM-Fr 1 treated rats ($P < 0.05$). No significant changes in percentage of neutrophils and its staining characteristics is observed in BDF rats treated with BM-Fr 1.

From Table 1 we observe that the malnourished rats have a significantly lower antibody titre to *S. typhi* 'H' antigen than do the BDF rats ($P < 0.001$). After BM-Fr 1 injection the malnourished immunized animals showed a higher antibody titre compared with immunized control ($P < 0.001$). No significant change is observed in BDF animals.

**Discussion**

The marrow environment is regulated in a finely tuned manner by cytokines. During inflammation or immune activation the marrow responds by producing appropriate cells in large numbers.$^{5,8}$ On the other hand, certain disease conditions suppress production of certain cells.$^2$ The underlying molecular mechanism is yet to be fully clarified. It is reasonable to believe that by modulation of secretion of certain haematopoietic cytokines the production or suppression of lineage-restricted progenitor cells could be achieved.$^6,7$ Although there is no definite evidence to date regarding the modulation of the bone marrow microenvironment by invading micro-organism$^{15}$ or its product, this paper indicates such a possibility as was observed in the case of Dalton’s lymphoma$^{10}$ and *Escherichia coli*.$^{16}$

Salmonella is known to invade the bone marrow$^2$ where antigen presenting cells (macrophage or dendritic) might present MHC class I restricted antigens to CD$^8$ T cells.$^{17}$

In order to study the effect of the Salmonella antigen on bone marrow we injected *Salmonella typhi* 'H' antigen which showed a differential effect on the BM-Fr 1 production depending upon the nutritional status of the host (Fig. 1). The BDF rats showed an initial suppression in cytokine secretion followed by stimulation. Malnourished animals showed only stimulation in BM-Fr 1 secretion (Fig. 1). Is a suppressive mechanism not in operation in malnourished animals?

Comparison of the brain microsomal membrane adenosine triphosphatase (ATPase) activity$^{18,19}$ with the present BM-Fr 1 secretion profile revealed that in BDF rats suppression is observed in both cases after immunization, followed by stimulation at the peak of immune response.$^{18,19}$

In malnourished rats, stimulation in brain ATPase$^{18,19}$ and BM-Fr 1 cytokine is observed after immunization and the animals were immunoincompetent. 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.$^{19}$ 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 neutropoiesis (Fig. 1) BM-Fr 1 regulates the biosynthesis of myeloperoxidase in neutrophils thereby controlling its bacteriocidal activity.$^{20}$ In malnourished animals a suppressed neutrophil myeloperoxidase function and antibody response to *S. typhi* 'H' antigen (Table 1) probably explain 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 in malnourished animals,$^9$ was injected *in vivo*.

It seems from Figs 1 and 2 that the committed or immature neutrophils 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 (14 vs 40% 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 to malnourished immunized animals compared with immunized controls ($P < 0.01$).$^{9,11}$ Balanced diet fed immunized animals did not show any improvement in antibody titre,$^{9,11}$ neutrophil percentage or myeloperoxidase reaction after bone marrow cytokine treatment. This further proves the presence of a negative feedback system (Fig. 3).$^9$ It seems that the injected BM-Fr 1 has a differential autocrine effect on bone marrow cells depending upon the nutritional status of the host, a negative feedback system in BDF animals and a stimulatory effect in malnourished animals. Moreover, it is also apparent that the malnourished immunosuppressed animals gain more from the cytokine treatment than do the BDF rats.$^{10}$

**Conclusion**

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; the cytokine secretion pattern is similar to that reported in the case of brain ATPase of BDF and malnourished immunized rats, and importantly the immunosuppression observed in malnourished animals, following salmonella antigen injection, could be improved by introducing the cytokine under investigation in proper doses. This opens up a new area of investigation where insight into the role of cytokines in the modulation of the lymphoid tissue microenvironment suggest new therapeutic approaches.
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