Immunomodulation of malnourished mice bearing Dalton’s lymphoma

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The immunomodulatory effect of a mouse bone marrow-derived cytokine (BIM), (mol wt 10 kD), was studied in mice bearing Dalton’s lymphoma. It was observed that this factor increased the lifespan of mice malnourished with respect to vitamin B-complex and ascorbic acid and infected with Dalton’s lymphoma, by 40 ± 4 days when compared to malnourished lymphoma controls while in animals maintained on balanced diet (BDF) the increase in lifespan was just over 11 ± 2 days. In cultured bone marrow cells at different time intervals after introduction of lymphoma cells it was shown that introduction of lymphoma cells increased the secretion of BIM. While the lymphoma developed the secretion of BIM diminished much earlier in malnourished than in BDF mice. This observation further strengthens our previous findings that the BIM acted as an immunomodulator much more effectively in malnourished animals than in animals fed a balanced diet, where a feed-back inhibitory effect might be present.

Introduction

Immunodeficiency due to malnutrition paves the way for the development of many types of lymphoma. It is currently posited that functional defect of the lymphoid tissue compartment, as a consequence of defective interaction between host and cell, may be one of the possible reasons for lymphoma development. If a specific defect lies within the host then restimul therapy correcting the microenvironment of the host would be more effective than eliminating the neoplastic cell. Thus conventional radiotherapy and chemotherapy are now widely in use. Recently, as part of monoclonal antibody technology, immunological techniques including antibody therapy and biological response modifiers, eg interferons, have begun to be explored. Bone marrow transplantation in patients who are otherwise resistant to conventional treatment is now being explored and this technique may be more effective in the early stage of the disease. But bone marrow transplantation is a very expensive and difficult approach in an already immunocompromised lymphoma-affected host. In this paper we discuss a possible immunomodulatory approach to treating lymphoma by bone marrow cytokine.

Earlier reports showed that a rodent bone marrow cell-secreted factor immunomodulated malnourished immunosuppressed mice by not only improving the T and B cell population and functions in immunocompetent organs, but also the bone marrow cellular compartment and peripheral blood profile. An increased resistance towards lung and gastrointestinal infections, which otherwise prove fatal in untreated malnourished control, was also observed. To further study the immunomodulatory activity of this factor it was subsequently tested in mice infected with Dalton’s lymphoma.

Materials and method

Male Swiss mice (age 30 days; body wt 16 ± 2 g) were maintained on ad libitum balanced diet for 7 days under 12-h light–dark cycle in suspended wire cages. The mice were then divided into two batches of 20 each and one batch rendered malnourished in regard to B-complex vitamins and ascorbic acid. Development of lymphoma

A Dalton’s lymphoma (DL) cell line is maintained in male Swiss mice at Bose Institute. The lymphoma cells were collected from the peritoneum in normal saline in an aseptic condition and pelleted in cold centrifugation (500 rpm for 10 min). The viability of cells was studied by the trypan blue exclusion method (>95% viable) and each experimental mouse received 2 x 10⁶ cells, intraperitoneally. The lymphoma was allowed to grow in vivo for 3 days, in both the balanced-diet-fed (BDF) and B-complex and ascorbic-acid-deficient (D) groups.

At different time periods bone marrow cell cultures were performed in the BDF-DL group by the method described below and the secretary profile of the bone marrow factors evaluated, compared and contrasted with uninfected controls.

Preparation of bio-immunomodulator (BIM)

Unfractionated mouse bone marrow cells, flushed from the femurs of healthy young animals, were repeatedly aspirated and ejected from a syringe to obtain single cell suspensions. The cells were cultured in RPMI 1640 (pH 7.3) serum-free medium at 37°C for 18 h at a concentration of 3 x 10⁵ cells/1. At the end of the incubation period, the cells were pelleted by centrifugation (500 rpm) at 4°C and the supernatant fluid was collected. The cell-free crude extract was then subjected to membrane filtration under N₂ pressure with continuous stirring and with a molecular cut-off range at 10 kD (Amicon, USA). Two fractions were obtained, Fr A (mol wt > 10 kD)

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IMMUNOMODULATION OF MALNOURISHED MICE BEARING DALTON’S LYMPHOMA

Table 1. Effect of a bone marrow-derived biocommodulator (BIM) on mean organ wt (in mg) of Swiss mice with Dalton’s lymphoma (DL)

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<tr>
<th>Organ</th>
<th>BDF control</th>
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</tr>
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<tbody>
<tr>
<td>Thymus</td>
<td>23 ± 2</td>
<td>4 ± 2*</td>
<td>4 ± 1</td>
<td>16 ± 5*</td>
<td>2 ± 1</td>
<td>2 ± 1*</td>
</tr>
<tr>
<td>Spleen</td>
<td>52 ± 6</td>
<td>30 ± 8*</td>
<td>105 ± 10*</td>
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BDF: Basal diet fed; D: Deficient; *P < 0.001.

Discussion
Reduced socio-economic status, presumably implying impaired health status, has been reported in association with increased incidence of lymphoma. Thus malnourished mice, with low levels of immune competence have unsurprisingly also exhibited a rapid spread of lymphoma. It seems also that the establishment of lymphoma some factor(s) involved which directly or indirectly suppress bone marrow cytokine secretion that is essential for optimum immune response. This may also explain the immune suppression observed during parasitism and in the case of Burkitt’s lymphoma. Whether this initial upsurge followed by suppression of BMDP-1 production has got anything to do with establishing a significant difference from BDF-DL controls. The spleen was pale and petechial haemorrhage was observed. Under the microscope the spleen showed degeneration of B-cell centres more than T-cell centres. There was infiltration by lymphoma cells. The thymus showed degeneration of cortex and oedematous fluid. In the absence of T cells, there was degeneration of corticomedullary junction and evidenced by absence of T cells. There was infiltration by lymphoma cells and lymphoma cells were present in nodular formation (Figure 3). The lungs showed exudations containing RBC in the alveolar space and rupture of alveolar wall (Figure 3).

Table 2 shows that the BIM-treated mice showed a significant increase in the number of lymphocytes and a decrease in the number of plasma cells. The spleen was pale and petechial haemorrhage was observed. Under the microscope the spleen showed degeneration of B-cell centres more than T-cell centres. There was infiltration by lymphoma cells. The thymus showed degeneration of cortex and oedematous fluid. In the absence of T cells, there was degeneration of corticomedullary junction and evidenced by absence of T cells. There was infiltration by lymphoma cells and lymphoma cells were present in nodular formation (Figure 3). The lungs showed exudations containing RBC in the alveolar space and rupture of alveolar wall (Figure 3).

Table 1 indicates that BIM seems to act directly on thymus and spleen depending upon the nutritional status of the lymphoma-bearing host. In BDF animals introduction of BMDP stimulation of the thymus (Table 2) so as to counter the threat of lymphoma by increasing the production of WBC in circulation. We injected BIM in the early

Figure 1. Sphingosine G-10 column chromatography of bone marrow cell secretion factor (wt < 0.1 kg). Pooled fraction 8-12 under the first peak is BIM.

and Fr B (wt < 0.1 kg). To locate the presence of active fractions both the fractions were screened for antithymoma activity on BDF mice bearing 6-day-old lymphoma. Crude Fr B/A was injected ip in three divided doses at an interval of 5 days to BDF-DL-bearing mice and it was seen, as will be discussed in detail later, that Fr B contained factor(s) capable of increasing life-span of lymphoma-bearing mice, with a mean increase of 4 ± 8 days over saline-treated controls.

Studies on Fr B (wt < 0.1 kg) The proteins of the crude filtrate (wt < 0.1 kg) were precipitated by 60% ammonium sulphate at 4°C, reconstituted in 0.9% NaCl, dialyzed against dd H2O at 4°C overnight in benzoylated tubing (Sigma, USA), lyophilized and again reconstituted in saline.

The protein concentration was estimated by Lowry’s method using BSA as standard. The protein sample (con: 4.5 mg/ml) was applied to a Sephadex G-10 (Sigma USA) column (1.8 cm x 27 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.2. The flow rate of the column was maintained at 8.0 ml/h. The 2-ml fractions were collected. The protein of the collected fractions was measured at 280 nm (Figure 1). The present paper deals with the immunomodulatory effect of pooled fraction 8-12 under the first peak, henceforth known as BIM (Figure 1).

Immunomodulatory effect of BIM-1

Three days after the introduction of lymphoma in both BDF and D mice, three doses of BIM-1 (days 9, 11 and 23 after introduction of the deficient diet; protein cone 0.3 mg/kg dose) was injected ip, saline being injected into a control mouse.

The mice were weighed every alternative day, their death recorded and a postmortem (PM) examination along with histopathology was performed.

Statistical calculations

Statistical evaluation was done using the Kaplan–Meier probability curve and Students ‘t’ test.

Results

Figure 2 shows that the deficient mice bearing Dalton’s lymphoma (DL-DL) died within 20 days after the onset of experimental diet, ie within 14 days post-lymphoma introduction (PLI). Radioautographic studies were carried within 9 days PLI and the deaths, as observed from postmortem (PM) and histopathological findings, were primarily due to pneumonia of bacterial origin (Figure 3). The spleen (49 ± 8 mg) and the thymus (2 ± 1 mg) were minimal in size. There were petechial haemorrhages on the spleen and histology showed oedematous fluid, ‘starry’ appearance of the cortex and atrophy of the follicular region. The later observation was also found in the thymus (Figure 3). The liver was pale, with petechial haemorrhage and pin-pointed whitish growth on the surface and microscopically showed infiltration with MN cells (Figure 3). Fatty changes, perivascular cuffing by MN cells, collection of oedematous fluid and some nodules of the lymphoma were also evident. The kidney and intestine showed signs of haemorrhage.

In contrast, 50% malnourished BIM-1 injected lymphoma-bearing mice showed no respiratory distress till 3 ± 3 days of malnourishment, ie 48 ± 3 days PLI. The abdominal circumference did not show appreciable growth of the lymphoma in these animals till 30 ± 8 days PLI (45 ± 6 days of malnourishment) and 22 ± 5 days after the last injection of BIM-1 (Figure 2).

A 30 ± 8 days of malnourishment 40% of animals died. Their thymus was rudimentary (wt 2 ± 1 mg) though the spleen was normal in size (wt 120 ± 20 mg). Histopathological examination showed collection of oedematous fluid and fibrous changes. The thymus had giant eosinophilic cells at the corticomedullary junction. There was haemorrhage of the lungs, collapse of alveoli, infiltration of MN, and the spleen showed degeneration of B-cell centres more than T-cell centres. There was infiltration by lymphoma cells. The thymus showed degeneration of cortex and oedematous fluid. In the absence of T cells, there was degeneration of corticomedullary junction and evidenced by absence of T cells. There was infiltration by lymphoma cells and lymphoma cells were present in nodular formation.

Cells fifty per cent of BIM-1 treated mice were alive after 60 ± 2 days, in 5 ± 2 days PLI, 45 ± 5 days after the last injection of BIM-1. This was in marked contrast to the fate of untreated animals which died within 20 days of malnourishment, ie 14 days PLI. The postmortem and histological findings were similar to those described above.

The BDF-DL mice showed a steady growth of the tumour. Fifty per cent of the animals died by 35 ± 4 days PLI. All the animals were dead by 39 ± 2 days PLI, there was only 15 ± 3 increase in life-span of the BDF-DL mice over the malnourished lymphoma control (P<0.001). These mice initially showed no signs of infection; however, after 25 ± 4 days PLI lung infection was observed. In postmortem studies thymus and spleen were larger than those of malnourished lymphoma controls (thymus 4 ± 1 mg, spleen 105 ± 10 mg as against lymphoma 2 ± 1 mg, spleen 49 ± 8 mg P<0.001, Table 1). The abdomen was completely filled with yellowish-red ascites fluid and there were cancerous nodules on the intestine and peritoneal walls. Histological studies of the thymus showed collection of oedematous fluid and fibrous changes (Figure 3). The lymphocytes were found to be at different stages of development and some non-stained cells of various sizes also appeared. The lungs showed collapse of alveoli and infiltration by MN cells, and PMN cells, with vacuolated cytoplasm, were found in alveolar space (Figure 3) where there was sometimes pyknotic or degeneration and infiltration by lymphoma cells. Spleen showed infiltration by DL cells, fibrous changes and disintegration of pulp. The liver had oedematous changes, damage to the epithelial lining of the lesser omentum and veins and cancer nodules were observed (Figure 3). In some mice, 30 days PLI, the spleen was found to be enormous (wt 335 ± 50 mg). Histological studies showed degeneration of lymphoid follicle and infiltration by macrophage and lymphoma cells.

Fifty per cent of BDF-DL BIM-treated mice were dead by 25 ± 2 days PLI. All the animals died by 52 ± 2 days PLI, thus an increase in lifespan by only 11 ± 2 days over BDF-DL controls was observed. In contrast DDL-BIM-BIM-treated mice lived approximately 45 days longer (P<0.001) compared to DDL-control mice.

No significant difference in longevity was observed between the BIM-treated groups. The thymus of the BDF-DL-BIM mice weighed 16 ± 5 mg vs BDF-DL controls 4 ± 1 mg (P<0.001). The spleen weighed 105 ± 16 mg (no significant difference from BDF-DL controls). The spleen was pale and petechial haemorrhage was observed. Under the microscope the spleen showed degeneration of B-cell centres more than T-cell centres. There was infiltration by lymphoma cells. The thymus showed degeneration of cortex and oedematous fluid. In the absence of T cells, there was degeneration of corticomedullary junction and evidenced by absence of T cells. There was infiltration by MN cells and lymphoma cells were present in nodular formation. (Figure 3). The lungs showed exudations containing RBC in the alveolar space and rupture of alveolar wall (Figure 3).
cells like a white sheet that was viscous in nature. Fifty per cent of BDF-1 treated mice were alive after 60 ± 2 days, in 5 ± 2 days PLI and 45 days after the last injection of BIM-1. This was in marked contrast to the fate of untreated animals which died within 20 days of malnourishment, ie 14 days PLI. The postmortem and histological findings were similar to those described above.

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BDF: Bush diet fed, D: deficient; *: P < 0.001.

Discussion

Reduced socio-economic status, presumably implying impaired health status, has been reported in association with increased incidence of lymphoma. Thus malnourished mice, with low levels of immune competence have unsurprisingly also exhibited a rapid spread of lymphoma 16-18 see also Figure 2. However BIM-1 treated malnourished mice survived longer than untreated mice on a balanced diet while untreated malnourished controls died very early (10 ± 4 days) post-injection.

The growth of the lymphoma has a suppressive effect on bone-marrow secretory profile (Figure 4), similar to that observed during malnourishment (Figure 1). It seems that in the establishment of lymphoma some factor(s) are involved which directly or indirectly suppress bone marrow cytokine secretion that is essential for optimum immune response 16,17. This may also explain the immune suppression observed during parasitism 18-20 and in the case of Burkitt’s lymphoma 19. Whether this initial upsurge followed by suppression of BIM-1 production has got anything to do with establishment of infection/tumour remains to be confirmed. Because secretory products believed to be present in the serum of lymphoma-bearing mice, suppress BIM secretion, injection of BIM-1 was used to find out whether it has any immunomodulatory effect on mouse bearing Dalton’s lymphoma whether the mice are well-fed or malnourished.

Table 1 indicates that BIM seems to act differentially on thymus and spleen depending upon the nutritional status of the lymphoma-bearing host. In BDF animals introduction of the lymphoma stimulation is sufficient (as shown in Figure 4) so as to counter the threat of lymphoma by increasing the production of WBC in circulation. We injected BIM in the early...
The stage of lymphoma establishment and this might trigger a negative feedback system as suggested earlier and as has also recently been observed with LIP. As the lymphoma established itself, the bone marrow showed hypoplasia (observed from cyto-centrifuged smears). This may be one of the causative factors for suppression in indigenous BIM production.

In malnourished mice, in whom BIM production was suppressed much earlier, external BIM injection seemed to revive immunocompetence more effectively and to be able to prevent rapid growth of tumour cells.

Earlier studies showed that malignant cells display elevated Na⁺\textsuperscript{+}-K⁺-ATPase activity and increased intracellular (IC) Na⁺ ion concentration. Sodium ions now appear to be a favoured candidate for the role of a major 'early' mediator of cell division. Moreover, reduction in the extracellular (EC) Ca²⁺ ion has also been found to favour continued growth of malignant cells.

Our studies on brain lysosomal Na⁺\textsuperscript{+}-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase showed a different effect of BIM in BDF control and malnourished rats. In BDF animals immunization suppressed the ATPase activity while in malnourished animals an increase in activity was noticed. BIM injection in BDF animals showed no significant changes in ATPase activity, compared to immunized BDF controls, while in malnourished animals an initial suppression was noticed followed by opening of the ion channel. This opening of the ion channel, as evidenced from increased ATPase activity, probably alters the EC/IC ionic balance of the malignant cells tiling it in favour of the normal cellular microenvironment. This correction of ionic microenvironment seems to be able to prevent rapid tumour growth in D-DL-BIM mice, as stated above, thereby supporting our previous observation that D-mice gain more from BIM treatment.

Conclusion

In conclusion, from our previous communication\textsuperscript{4, 5} it was evident that the bone-marrow secreted factor showing immunomodulatory activity was active in malnourished immunosuppressed animals more effectively than BDF controls. In this communication similar observations were also noted. Here the bone-marrow secreted factor worked better in malnourished lymphoma bearing mice than in controls fed a balanced diet thus strengthening our previous hypothesis that a physiological feed-back inhibitory activity might be present in BDF controls at an early stage of lymphoma development.

Acknowledgement - The authors thankfully acknowledge the help and encouragement received from Prof. AK Barua, Department of Chemistry and Prof. SK Chakraborty, Department of Microbiology, Bose Institute. Research support was provided by the Indian Council of Medical Research.
stage of lymphoma establishment and this might trigger a negative feed-back system as suggested earlier$^9$ and as has also recently been observed with LIF$^{12}$. As the lymphoma established itself, the bone marrow showed hypoplasia (observed from cyto-centrifuged smear). This may be one of the causative factors for suppression in indigenous BIM production.

In malnourished mice, in whom BIM production was suppressed much earlier$^9$, external BIM injection seemed to revive immunocompetence more effectively and to be able to prevent rapid growth of tumour cells.

Earlier studies showed that malignant cells display elevated Na$^+$.K$^+$.ATPase activity and increased intracellular (IC) Na$^+$ ion conc.$^{11}$ Sodium ions now appear to be a favoured candidate for the role of a major ‘early’ mediator of cell division. Moreover, reduction in the extracellular (EC) Ca$^{2+}$ ion has also been found to favour continued growth of malignant cells$^{11}$.

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Figure 4. BIM secretion from bone marrow at different periods after introduction of Daltons' lymphoma (DL) in Swiss mice.

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Figure 4. BIM secretion from bone marrow at different periods after introduction of Dalton's lymphoma (DL) in Swiss mice.

References

營養不良並患有 Dalton's 淋巴癌的小鼠的免疫調節

摘要

作者研究了從小鼠骨髓取得的細胞活素（BIM）、（m. wt. < 10 Kd）對 Dalton's 淋巴癌（DL）的免疫調節作用。結果發現，細胞活素（BIM）會增加複合維生素B和維生素C營養不良、並染有DL小鼠的壽命達40±4天；而維持平衡膳食（BDF）、營養不良的DL小鼠僅增加壽命11±2天。在不同時間引進淋巴癌細胞到骨髓培養基中，可見引進的淋巴癌細胞會增加BIM的分泌。由於淋巴癌的生長，BIM分泌減弱，此種現象遠遠較營養不良的BDF小鼠為早。這資料進一步加強了我們以前的發現，那就是BIM作爲營養不良小鼠的免疫調節者，較維持平衡膳食（BDF）小鼠好得多，因緒後者可能出現相反抑制。
Determinants of serum levels of retinol, β-carotene and α-tocopherol in men and women born in Australia, Greece and Italy

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Serum retinol, β-carotene and α-tocopherol levels were measured in a volunteer sample of 764 Australian-, Greek- and Italian-born adult residents of Melbourne, Australia. There was no difference among the ethnic groups in mean levels of serum retinol or α-tocopherol. Mean β-carotene levels were between 11 and 22% higher for Australian-born subjects. Serum β-carotene was higher in females, retinol was higher in males. The serum levels of retinol, β-carotene and α-tocopherol were significantly positively associated with serum cholesterol. Serum triglyceride was positively associated with serum retinol and α-tocopherol but negatively associated with serum β-carotene. A positive association with retinol and an inverse association with β-carotene was found for alcohol consumption. Serum α-tocopherol was positively associated with dietary vitamin E. Serum β-carotene was correlated with carotenoid intake among subjects who had never smoked. Serum retinol increased with age in women only. These data provide a degree of cross-cultural robustness to previous findings in regard to the determinants of serum retinol, β-carotene and α-tocopherol in healthy men and women.

Introduction

There is some epidemiological evidence of an inverse association between the level of retinol in blood and cancer risk. The suggested mechanism is that retinol inhibits tumour promotion through the regulation of cell growth and development. Post-hepatic conversion to retinol is one possible mechanism that might explain a reduction in cancer risk associated with β-carotene, although the more conventional explanation involves its antioxidant properties. Another dietary antioxidant that has been proposed to have a preventive role in the pathogenesis of cancer and coronary heart disease (CHD) is vitamin E. Cross-sectional surveys in Australia show that migrants from southern Europe consume large amounts of leafy green vegetables and vegetable oils; both rich sources of antioxidants. It is possible that Italian- and Greek-born Australians obtain some protection against CHD and cancer from dietary antioxidants because their mortality advantage is not explicable in terms of established risk factors such as serum cholesterol, cigarette smoking, obesity or physical inactivity.

It is well recognized that positive associations exist between the dietary intake of β-carotene and α-tocopherol and their levels in serum or plasma. No such relationship is evident for retinol, although elevated serum retinol levels have been reported among individuals taking daily vitamin A supplements. In recent years attention has focused on identifying other factors associated with the serum levels of retinol, β-carotene and α-tocopherol. The list includes: serum cholesterol and triglycerides, age, gender, smoking status, alcohol consumption, energy intake, relative body weight, use of antihypertensive medication, and season of the year in which the blood was taken. Establishing the relative importance of the various endogenous and exogenous determinants of the level of a nutrient in serum can best be done by performing multivariate analysis with all of the independent variables included in one regression model. In addition, the extent to which the determinants are cross-culturally robust is likely to be of biological relevance yet this has not been adequately addressed. Serum levels of retinol, β-carotene and α-tocopherol were therefore measured in a field study conducted in Melbourne, the Australian city with the largest Italian and Greek communities. The aims of the study were threefold: to establish whether there were differences in the serum levels of these nutrients on the basis of ethnicity; to describe a normal reference range for these nutrients in healthy Australian men and women, and to identify their determinants within a heterogeneous population using multiple linear regression.

Materials and methods

Study population and recruitment

The study population (Table 1) consisted of a volunteer sample of 764 healthy men and women who took part in the feasibility trial of the Melbourne Collaborative Cohort Study. The sampling strategy was to obtain people who were likely to volunteer to be in a long-term study of their health.

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