Immunomodulation of malnourished mice bearing Dalton's lymphoma

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The immunomodulatory effect of a mouse bone-barrow-derived cytokine (BIM), (mol wt<10 kd), was studied in mice bearing Dalton's lymphoma. It was observed that this factor increased the life-span of mice malnourished with respect to vitamin B-complex and ascorbic acid and infected with Daltons' lymphoma, by 40 ± 4 days when compared to malnourished lymphoma controls while in animals maintained on balanced diet (BDF) the increase in life-span 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[1]. 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[2]. If a specific defect lies within the host then reatinal 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[3]. 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[4–6] 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 proves fatal in untreated malnourished control[3], 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[4,5].

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 2×10^4 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 secretory 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 3×10^6 cells/l. At the end of the incubation period, the cells were pelleted by centrifugation (500 rpm) at 4°C and the supernatant fluid was collected[6]. The cell-free crude extract was then subjected to membrane filtration under N_2 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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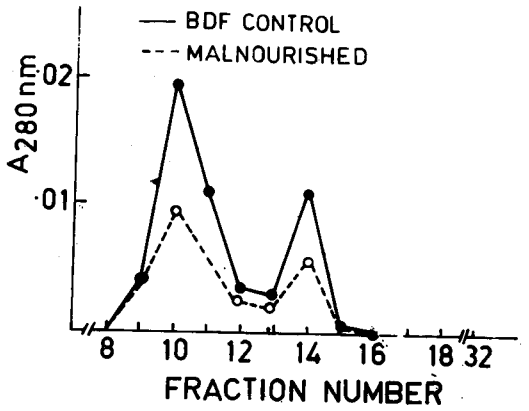

Figure 1. Sephadex G-10 column chromatography of mouse bone marrow cell secreted factor (mol wt < 10 kd). Pooled fraction 8–12 under the first peak is BIM_1.

and Fr B (mol wt < 10 kd). To locate the presence of active fractions both the fractions were screened for antilymphoma activity on BDF mice bearing 6-day-old lymphoma. Crude Fr A/B 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 increment of 8 ± 4 days over saline-treated controls.

Studies on Fr B (mol wt < 10 kd)

The proteins of the crude filtrate (mol wt < 10 kd) were precipatated by 60% ammonium sulphate cut at 4°C, reconstituted in 0.9% NaCl, dialized against dd H_2O at 4°C overnight in benzoylated tubing (Sigma, USA), lyophilized and again reconstituted in saline. The protein concentration was estimated by Lowry's method[7] using BSA as standard. The protein sample (conc.435 mg/ml) was applied to a Sephadex G-10 (Sigma USA) column (1.8 cm × 27 cm) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.2. The flow rate of the column was maintained at 8.0ml/h and 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-1 (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 conc 0.3 µg/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–Meir probability curve and Students 't'-test.

Results

Figure 2 shows that the deficient mice bearing Dalton's lymphoma (D-DL) died within 20 days after the onset of experimental diet, ie within 14 days post-lymphoma introduction (PLI). Respiratory distress was observed within 9 days PLI and the deaths, as observed from postmortem (PM) and histopathological findings, were primarily due to pneumonia

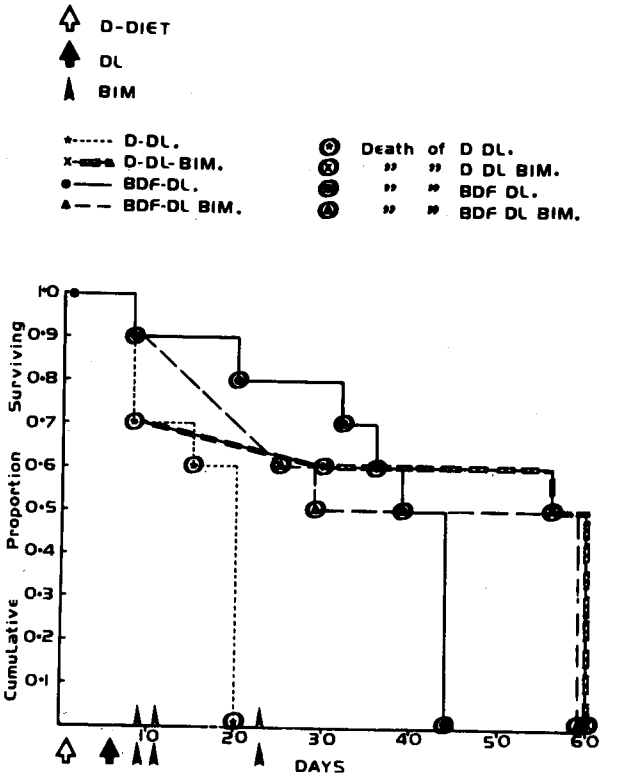

Figure 2. Immunomodulatory effect of mouse bone marrow cell secreted BIM_1 on mice infected with Dalton's Lymphoma. BDF = Balanced Diet Fed; D = Deficient (8-complex vitamins and ascorbic acid) diet fed; DL = Dalton's Lymphoma; BIM = Bio-immunomodulator.

of bacterial origin (Figure 3). The spleen (49 ± 8 mg) and the thymus (2 ± 1 mg) were minimal in size. There were peticheal haemorrhages on the spleen and histology showed oedematous fluid, 'stary' 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 peticheal haemorrhage and pus-filled whitish growth on the surface and microscopically showed infiltration with MN cells (Figure 3). Fatty changes, perivascular cupping by MN cells, collection of oedematous fluid and some nodules of the lymphoma cells 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 53 ± 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).

After 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 giant eosinophilic and PMN cells in the aveolor cavity which contained eosinophilic exudation. The liver showed profuse haemorrhagic spots, oedematous changes, and infiltration by PMN and MN cells. Giant anaphase stage basophilic cells were found in clusters that formed nodules (Figure 3). The peritoneum was covered with the lymphoma

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

Organ	BDF control	D control	BDF-DL	BDF-DL-BIM	D-DL	D-DL-BIM
Thymus	23 ± 2	4 ± 2*	4 ± 1	16 ± 5*	2 ± 1	2 ± 1
Spleen	52 ± 6	30 ± 8*	105 ± 10*	105 ± 16	49 ± 8*	120 ± 20*

BDF: Basal diet fed, D: deficient *: $P < 0.001$.

cells like a white sheet that was viscous in nature.

Fifty per cent of BIM-1 treated mice were alive after 60 ± 2 days, ie 55 ± 2 days PLI; 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.

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, ie there was only 15 ± 3 increment 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 thymus 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 were also present. 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 basophilic secretion 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 lumen of arteries 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-1-treated mice lived approximately 45 days longer (P 0.001) compared to DL-controls.

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 ($P<0.001$) while the spleen weighed 105 ± 16 mg (no significant difference from BDF-DL controls). The spleen was pale and peticheal 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 follicles. The liver showed fatty degeneration and evident necrosis. There was infiltration by MN cells and lymphoma cells were present in nodular formation (Figure 3). The lungs showed exudatins containing RBC in the alveolar space and rupture of alveolar wall (Figure 3).

Table 1 shows that in BDF-DL mice with atrophy of the thymus there was gain in weight and in cellularity (evidenced by histological studies) after BIM treatment. The spleen showed initial hypertrophy after lymphoma injection, but there was no change in gross organ weight following BIM treatment. The malnourished lymphoma-bearing mice showed changes in microscopic thymus structure (Figure 3) following BIM treatment. Little change in macroscopic structure was observed. This is in sharp contrast to that observed in malnourished animals without lymphoma[6]. The spleen, on the other hand, showed hypertrophy after lymphoma injection and again after BIM treatment.

The bone marrow secretory profile of BDF-DL mice showed an initial overproduction of BIM-1, but as the lymphoma grew a suppressed production of cytokine was observed (Figure 4).

Discussion

Reduced socio-economic status, presumably implying impaired health status, has been reported in association with increased incidence of lymphoma[1]. Thus malnourished mice, with low levels of immune competence have unsurprisingly also exhibited a rapid spread of lymphoma[6,8] – see also Figure 2. However BIM-1 treated malnourished mice survived longer than untreated mice on a balanced diet while untreated manourished controls died very early (10 ± 4 days PLI).

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[4–6]. This may also explain the immune suppression observed during parasitism[9, 10] and in the case of Burkitt's lymphoma[11]. 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 mice 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 lymphoma stimulates indigenous BIM production (Figure 4) so as to counter the threat of lymphoma by increasing the production of WBC in circulation[6]. We injected BIM in the early

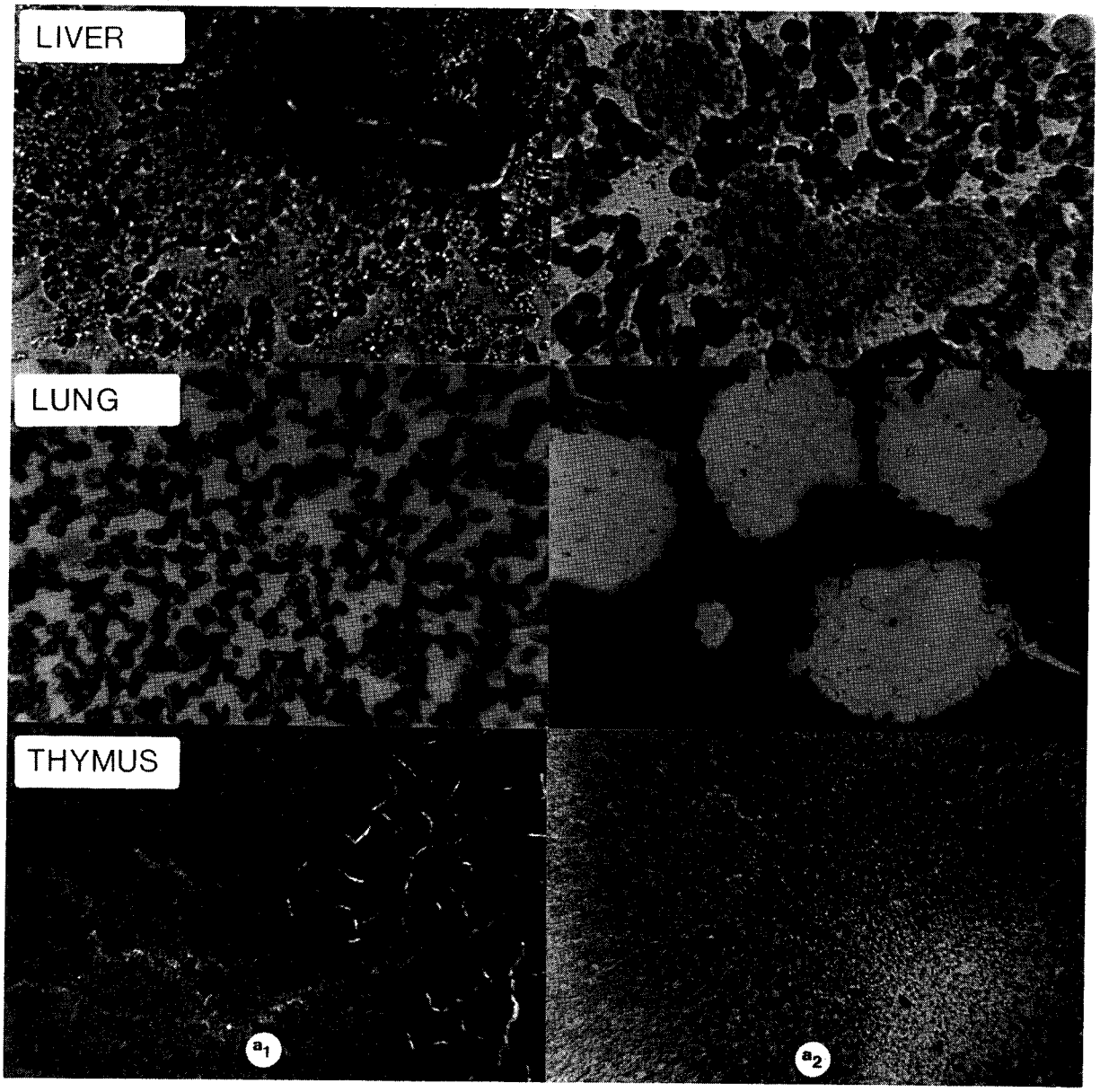

Figure 3. Histological changes observed in swiss mice, (basal diet fed (BDF) and malnourished infected with Daltons' lymphoma (DL) and treated with BIM_1. Giemsa's stain. a_1= BDF-DL; a_2 = BDF-DL-BIM_1; a_3 = D-DL; a_4 = D-DL-BIM_1.

Liver. a_1– a_3: Infiltration of neutrophil and mononuclear cells in hepatic tissue, oedema, necrosis and formation of cancer nodules by lymphoma cells. a_4: Lesser amount of infiltration of WBC in hepatic tissue. Cancer nodules very small in size and few in number. × 400.

Lung. Bronchopneumonia. a_2 and a_4 show reduction in microbial load compared to a_1 and a_3. × 400.

Thymus. a_1 and a_3: atrophy of thymus. a_2 and a_4: regeneration of thymus cortex more than medulla × 100.

stage of lymphoma establishment and this might trigger a negative feed-back system as suggested earlier[6] and as has also recently been observed with LIF [12]. 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[6], 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.[13] 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[13].

Our studies on brain lysosmal Na^+-K^+-ATPase and CA^{2+}-Mg^{2+}-ATPase[14] 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, prob-

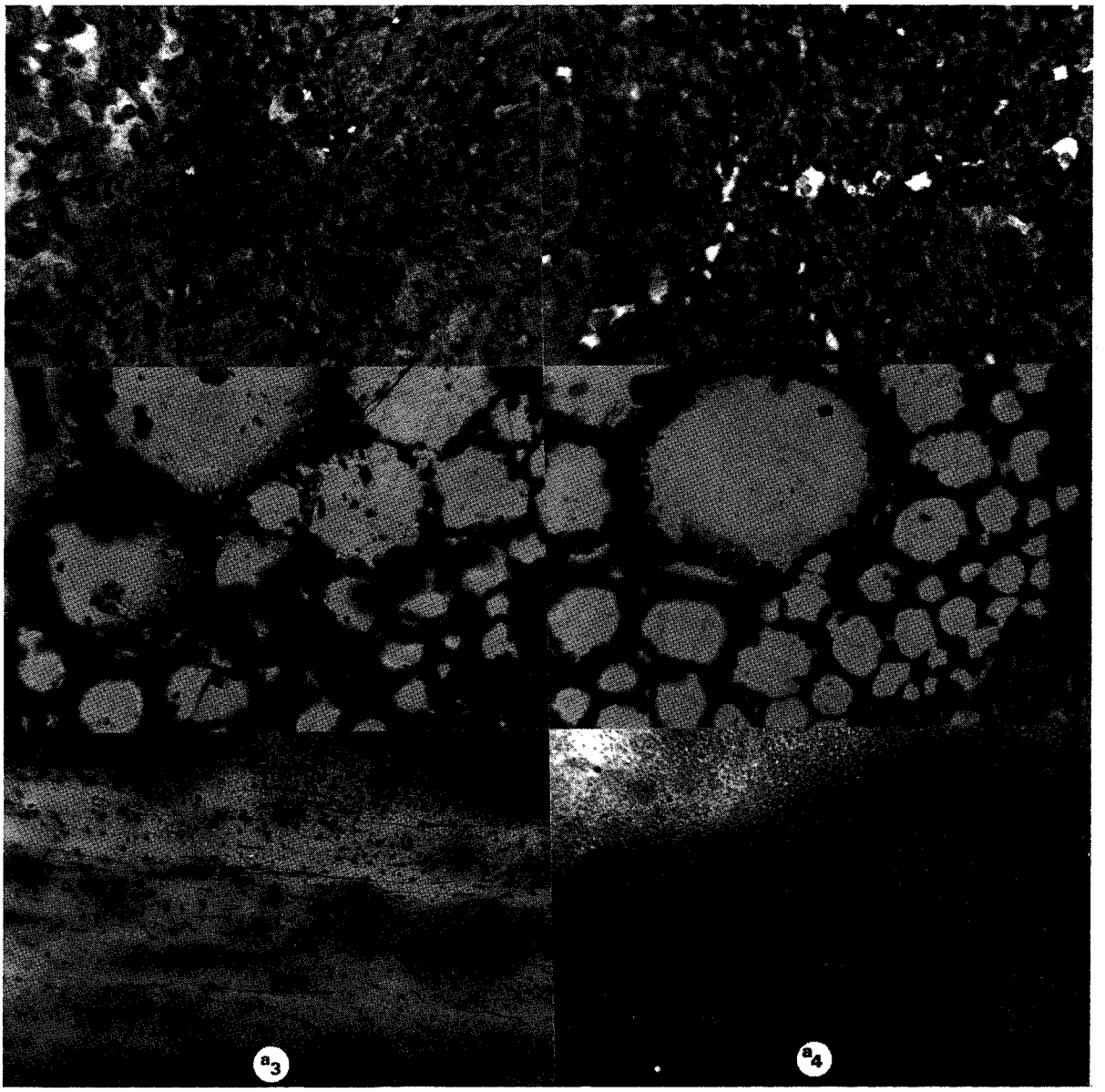

Figure 3 continued. For caption see opposite page.

ably alters the EC/IC ionic balance of the malignant cells tilting 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[6] that D-mice gain more from BIM treatment.

Conclusion

In conclusion, from our previous communication[4, 6] 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.

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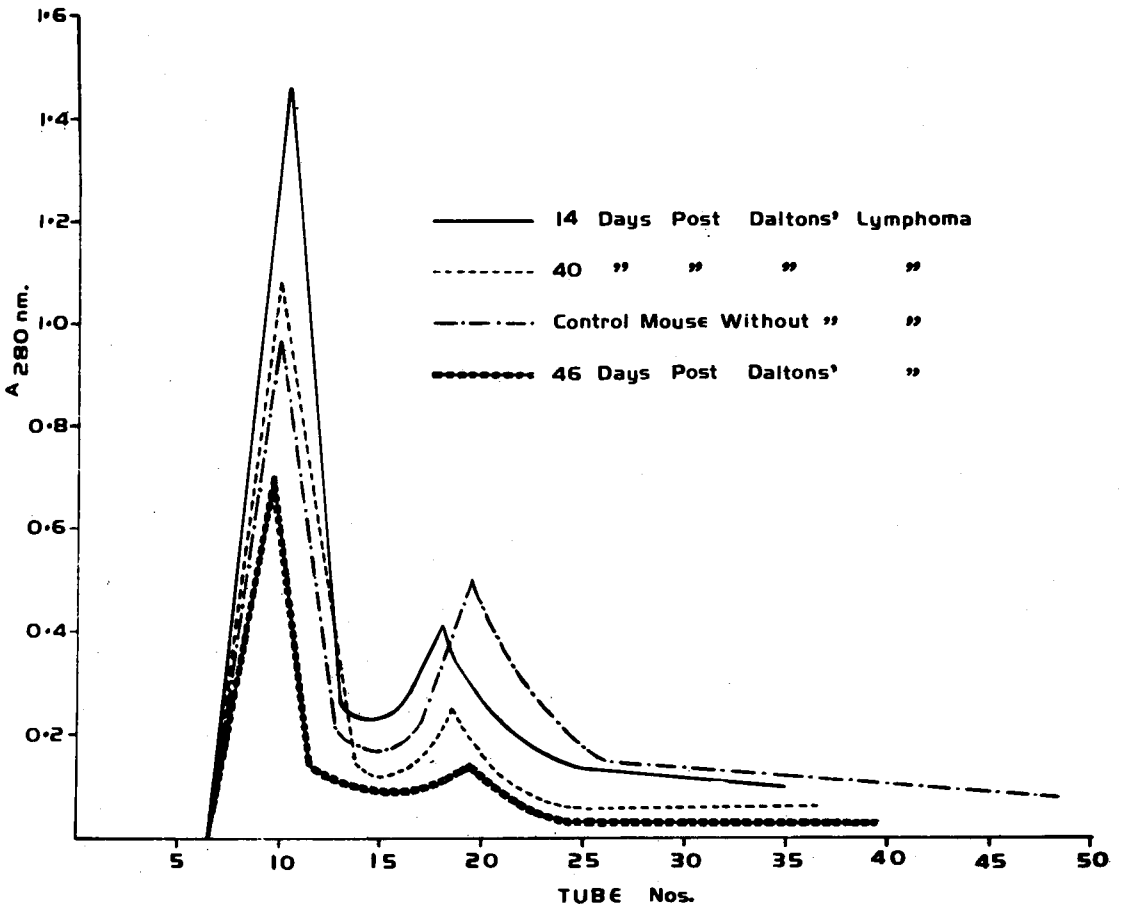

Figure 4. BIM_1 secretion from bone marrow at different periods after introduction of Daltons' lymphoma (DL) in Swiss mice.

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सारांश

वर्णान्धो की लसिका (डाल्टन लिम्फोमा) पर चूहे की हड्डी की मज्जा से उत्पन्न साइटोकाइन (बी आइ एम), (एम. वजन < 10 केडी) की असंक्रमणीय (इम्युनोमोडुलेटरी) भूमिका का अध्ययन किया गया। यह देखा कि यह कारक बिटामिन बी- काम्पलेक्स एवं एस्कार्विक अम्ल से कुपोषित चूहों के जीवन काल को बढ़ा देता है जो वर्णान्धों की लसिका से 40±4 दिनों से संक्रमित होते है जब उनकी तुलना कुपोषित लसिका नियत्रण से की जाती है। जबकि संतुलित आहार वाले पशुओं के जीवन काल में केवल 11±2 दिनों की वृद्धि होती है। लासिका कोशिकाओं को सन्निविष्ट करने के बाद विभिन्न समय अंतराल पर हड्डी की मज्जा के कल्चर से पता चला कि लसिका कोशिकाओं को सन्निविष्ट करने पर बी आइ एम$_1$ का स्राव बढ़ गया। जैसे ही लसिका विकसित हुई बी आइ एम। का स्राव संतुलित आहार वाले चूहों की अपेक्षा कुपोषित चूहों में बहुत पहले ही धर गया। यह प्रेक्षण हमारे पूर्ववर्ती निष्कर्षों को और भी सुदृढ करता है कि बी आइ एम एफ असंक्राम्यनियंत्रक (इम्युजोमोड्यूलेटर) के रूप में संतुलित आहार वाले पशुओं की अपेक्षा कुपोषित पशुओं पर अधिक कारगर रूप से कार्य करता है जहाँ पुननिविशन निरोधी प्रभाव पड़ सकता है।

營養不良並患有 Daltons' 淋巴瘤的小鼠的免疫調節

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 Daltons' 淋巴瘤 (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瘤細胞到骨髓培養基中，可見引進的淋巴瘤細胞會增加 BIM_1 的分泌。由於淋巴瘤的生長， BIM_1 分泌減弱，這種現象遠遠較營養不良的 BDF 小鼠爲早。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，較進食平衡膳食 (BDF) 小鼠要好得多，因爲後者可能出現反饋抑制。