STEM CELLS AND DEVELOPMENT 14:548–555 (2005)

Original Research Paper

Lactoferrin Accelerates Reconstitution of the Humoral and Cellular Immune Response During Chemotherapy-induced Immunosuppression and Bone Marrow Transplant in Mice

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ABSTRACT

Experimental evidence from previous studies supports the conclusion that orally administered lactoferrin (LF) restores the immune response in mice treated with a sublethal dose of cyclophosphamide (CP). The aim of this study was to elucidate potential benefit of LF in mice undergoing chemotherapy with busulfan (BU) and CP, followed by intravenous (i.v.) injection of bone marrow cells. CBA mice were treated orally with busulfan (4 mg/kg) for 4 consecutive days, followed by two daily doses of CP delivered intraperitoneally (i.p.) at a dose of 100 mg/kg and reconstituted next day with i.v. injection of 10^7 syngeneic bone marrow cells. One group of these mice was given LF in drinking water (0.5% solution). After treatment, mice were immunized with ovalbumin (OVA) to subsequently measure delayed type hypersensitivity responsiveness and with sheep red blood cells to determine humoral immunity by evaluation of splenic antibody-forming cells. As expected, both humoral and cellular immune responses of mice that were treated with these chemotherapeutic agents was markedly impaired. Here we report that this impairment was remarkably attenuated by oral administration of LF. Humoral immunity fell to levels that were 66–88% lower than that of untreated animals. Humoral immunity of LF-treated animals was equivalent to that of untreated mice within 1 month. Cellular immune responses were inhibited by chemotherapy treatment to a lesser degree, reaching levels that were approximately 50% lower than those of untreated animals. Again, LF mitigated this decrease, resulting in responses that were only slightly lower than those observed in untreated animals. Furthermore, when mice were given a lethal dose of BU (4 × 25 mg daily doses, i.p.) followed by a bone marrow transplant, LF caused enhanced lympho-, erythro-, and myelopoiesis in the bone marrow and appearance of transforming splenic lymphoblasts, similar to effects caused by administration of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF). In summary, our study suggests that LF may be a useful agent to accelerate restoration of immune responsiveness induced by chemotherapy in bone marrow transplant recipients.

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INTRODUCTION

IMMUNOSUPPRESSIVE AGENTS are predominantly used in chemotherapy of autoimmune and neoplastic diseases, and in prevention of allograft rejection and graft-versus-host disease. Cyclophosphamide (CP), an alkylating, immunosuppressive drug (1), is fairly effective in treatment of systemic lupus erythematosus (2), nephritis (3), multiple sclerosis (4), rheumatoid arthritis (5), and lymphomas (6). CP treatment, however, leads to profound leukopenia (7) and neutropenia (8). The compound preferentially affects B lymphocytes (9), probably because of their longer life span compared with T cells (10). Consequently, the humoral immune response is more profoundly inhibited than T cell-mediated cellular responses (11). CP is also used with other chemotherapeutics, like busulfan (BU), in the conditioning regimen of patients scheduled for bone marrow transplantation (12,13). Busulfan, a myeloablative, but not an immunosuppressory agent, is cytotoxic for early hematopoietic cells providing similar benefit to patients as whole-body irradiation (14). BU is more often applied for the conditioning regimen of patient before bone marrow transplant than irradiation, in part, due to faster renewal of the immune function (15). A combinatory chemotherapy in mice with application of cyclophosphamide, cisplatin and 1,3-bis(2-chloroethyl)-1-nitrosourea (16) leads to a differential renewal of neutrophils, T and B cells with a restoration time of 14, 10–18, and 18–32 days, respectively.

In the early period following bone marrow transplantation (BMT), patients are particularly susceptible to infection due to severe immune cell depletion. To accelerate immune reconstitution after chemotherapy and BMT, cytokines such as interleukin-2 (IL-2) (17), granulocyte-macrophage colony-stimulating factor (GM-CSF) (18), G-CSF (19), and IL-7 (20), thymic hormones (21), bacterial (22), and other substances (23,24) have been tested in various animal models. In particular, recombinant G-CSF showed significant reduction in the duration of severe neutropenia, leading to common use in the clinic (25). The potential utility of lactoferrin (LF), an iron-binding protein and constituent of neutrophils (26), in treatment of various immunosuppressive conditions was recently demonstrated. Oral treatment of CP-immunocompromised mice with LF facilitated much faster reconstitution of both cellular (27) and humoral (28) immune response, which was associated with renewal of T cell, B cell, and macrophage levels (27,28) and normalization of the main cell types in the peripheral blood (29). To assess the clinical benefit of LF in reconstitution of immune functions, a typical chemotherapy regimen used for patients (12) was adopted to animal models by applying BU and CP treatment followed by BMT. In this study, we further examine oral administration of LF to develop faster and more pronounced immune reconstitution in mice undergoing BU/CP/BMT treatment. LF was also assessed to facilitate engraftment of a suboptimal bone marrow cell (BMC) dose by histological evaluation of the bone marrow and spleenic tissue.

MATERIALS AND METHODS

Animals

Female CBA mice, 10–12 weeks old, were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy (Wroclaw). Mice were fed a granulated, commercial food and filtered tap water ad libitum. The local animal ethics committee approved the study.

Antigens and reagents

Sheep red blood cells (SRBC) were supplied by Wroclaw Agriculture Academy (Wroclaw, Poland). SRBC were stored in Alserver's solution at 4ºC until use. Ovalbumin (OVA) was purchased from Sigma Chemical Co. (St. Louis, Missouri). Highly purified and essentially endotoxin-free bovine lactoferrin (<4.4 E.U./mg, <25% iron saturated) was obtained from Morrinaga Milk Industry Co., Ltd, (Zama Japan). Complete (cFa) and incomplete (iFa) Freund's adjuvant were from Sigma Chemical Co. (St. Louis, MO). CP was purchased from ASTA Medica, (Frankfurt, Germany), BU from Glaxo-Wellcome, (London, UK), and filgrastim (Neupogen®) from Roche (Basel, Switzerland).

Experimental design

For the evaluation of LF efficacy in the restoration of the immune fuction in immunocompromised mice, we applied the following protocol. Mice were given BU [4 mg/kg body weight (b.w.)] daily doses for four consecutive days (−6, −5, −4, and −3), followed by 2-day administration of CP (100 mg/kg b.w. per day), intraperitoneally (i.p.) (days −2, −1). On the next day, mice received 10^7 syngeneic bone marrow cells by intravenous injection into a lateral tail vein. LF was given in drinking water (0.5% filtered solution), following the bone marrow transfer and for the continuation of experiments (7–31 days). Control mice were untreated, fed LF orally or treated with BU/CP and given BMT. At various time intervals, the ability of mice to develop cellular or humoral immune response was determined. A different experimental protocol was used to assess LF effect on the bone marrow cell engraftment in mice devoid of stem cells. For that purpose mice were treated with four daily doses of BU (25 mg/kg b.w.), followed by a BMT [10^5 cells, intravenously (i.v.)], 48 h later.

Mice were given free access to 0.5% LF in drinking water. The following control groups were used: (1) mice treated with BU and not reconstituted with BMC, (2) mice treated with BU, reconstituted with BMC, and (3) mice treated with BU, reconstituted with BMC and given four, daily subcutaneous (s.c.) injections of filgrastim (25 ug/kg b.w., corresponding to 2.5×10^6 units/kg b.w. each). The filgrastim group was included to confirm its known effects on myelopoiesis after BMT. The spleens and femurs were isolated 8 days later for histological examination.

Isolation of bone marrow cells

Femurs of mice were aseptically isolated, and bone marrow cells were obtained by flushing the bones with Hanks' medium (0.45-mm needles using 5 ml syringes). Then, a single cell suspension was prepared by dispersion of cells in a syringe. Cells were washed two times with Hanks' medium, passed through a sterile cotton wool to remove cell debris, and counted in a hemocytometer. The cells were finally resuspended in 0.9% NaCl and given i.v. into a lateral vain at a number of 10^7 or 10^5 in a 0.2 ml volume depending on experimental protocol.

Immunization of mice and determination of antibody-forming cell number

Mice were immunized i.p. with a single dose (0.2 ml) of 5% SRBC suspension, and after 4 days they were sacrificed and the spleens were isolated. The determination of antibody-forming cells (AFC) was perfomed using the Mishell and Dutton method (30). The results were presented as a mean AFC number per 10^6 viable splenocytes from 6 mice ± standard error (SE).

Preparation of histological sections

Femur and spleen sections were fixed in 4.0% formalin solution for 24 h. Fragments of spleen were dehydrated and embedded in paraffin. Sections of femur were subjected to demineralization using a mixture of sodium citrate and formic acid at a 1:1 ratio, followed by dehydration and paraffin embedding. Fragments of the preparations were subsequently serially cut (6- to 7-μm sections) and stained with Hematoxylin & Eosin (H&E). The sections were evaluated using a light microscope at 400× magnification by a pathologist who was blinded with regard to the experimental groups.

Statistics

For statistical evaluation of the data, the analysis of variance (ANOVA) test was applied with Tukey test post hoc. Each experimental group of mice in the determination of the humoral and cellular immune response consisted of 6 mice, and the BMC engraftment experiment of 12 mice. The results are presented as mean values from at least six determinations ± SE and were regarded as significant when $p < 0.05$.

RESULTS

Effect of LF on restoration of delayed type hypersensitivity in BU/CP-treated and bone marrow cells-reconstituted mice

Mice were treated with the drugs and reconstituted with BMC as described in the Materials and Methods. Figure 1 shows the effects of LF administration on the magnitude of OVA-specific DTH response in mice on days 7 and 14 following BMT. The delayed-type hypersensitivity (DTH) response was significantly suppressed in BU/CP-treated mice (21% and 53.5% of the control value on days 7 and 14). However, LF reduced the suppression to 67.8% and 86.2% of the response in control mice.

Effect of LF on restoration of the humoral immune response in BU/CP-treated and bone marrow cell-reconstituted mice

The results shown in Fig. 2 indicate that the humoral immune response, determined as the number of AFC against SRBC and expressed as the percentage of the control value, was profoundly depressed on days 14 and 21

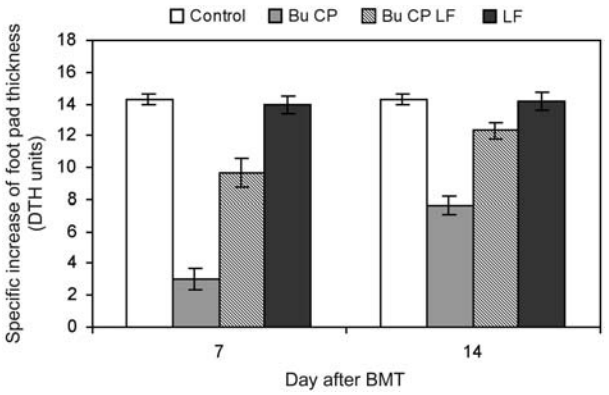

FIG. 1. Effect of LF on reconstitution of DTH in BU/CP-treated and BMC-reconstituted mice. There were significant differences in DTH by ANOVA analysis ($p < 0.05$). A Tukey test showed significant differences in DTH between day 7 (control/BU CP $p = 0.0001$; control/BU CP LF $p = 0.0002$; BU CP/BU CP LF $p = 0.0001$; BU CP/LF $p = 0.0001$; BU CP LF/LF $p = 0.0002$) and day 14 (control/BU CP $p = 0.0002$; BU CP/BU CP LF $p = 0.0002$; BU CP/LF $p = 0.0002$).

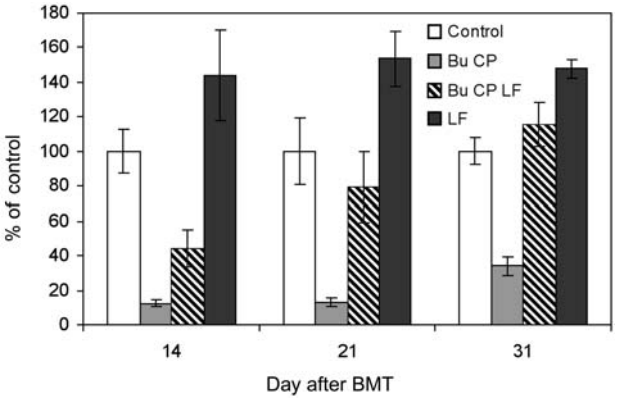

FIG. 2. Effect of LF on reconstitution of humoral immune response in BU/CP-treated and BMC-reconstituted mice. There were significant differences in AFC number by ANOVA analysis ($p < 0.05$). A Tukey test showed significant differences in AFC number between day 14 (control/BU CP $p = 0.00119$; control/BU CP LF $p = 0.049$; LF/BU CP LF $p = 0.0005$; LF/BU CP $p = 0.0001$), day 21 (control/BU CP $p = 0.0059$; BU CP/Bu CP LF $p = 0023$; LF/BU CP LF $p = 0.019$; LF/BU CP $p = 0.0001$), and day 31 (control/BU CP $p = 0.0004$; BU CP/BU CP LF $p = 0.0002$; control/LF $p = 0.006$; LF/BU CP $p = 0.0001$).

after BMT with a sign of recovery on day 31. The treatment of mice with LF led to reduced suppression on days 14 and 21 and complete reconstitution of the immune response by day 31.

Effects of LF on hematopoietic renewal in mice treated with BU and reconstituted with syngeneic BMC

Histological examination of spleens and femurs isolated from mice treated with 100 mg of BU/kg b.w. and reconstituted with 10^5 syngeneic BMC on day 8 following BMT was performed by the pathologist, who was blinded to type of experiment and treatment. Analysis of organ sections in control mice (without BMT) revealed typically deserted areas in the spleen and bone marrow. Mainly fibroblasts and fibrocytes were found in the bone marrow (Fig. 3B). In mice reconstituted with BMC, diminution of cell number of the hematopoietic lineage, particularly erythro- and lymphoblasts, as compared to the normal organ picture (Fig. 3A), was seen (Fig. 3C). Pluripotential hematopoietic cells showed a "resting" state. Some reticular cells exhibited metaplasis to adipocytes. Numerous macrophages were present, particularly among lympho- and erythropoietic colonies. A distinct dilation of marrow blood vessels was also noted. In the spleen of these mice (Fig. 4C), deserted areas in the periphery of lymphatic follicles were observed. The central zone of lymphatic follicles, formed by reticular cells and lymphoblasts, was distinctly thinned, with appearance of a higher number of macrophages. That phenomenon was also observed in periellipsoidal lymphatic tissue, where deserted areas were sometimes found. In the reticular tissue, constituting red pulp, areas of increased erythrocyte degradation were registered. Mice treated with chemotherapy and LF showed in the bone marrow, among trabeculi of spongy bone tissue, numerous cells of hematopoietic lineage with domination of erythro- and lymphoblastic area (Fig. 3D). Pluripotential, hematogenic cells were also abundant. Fibroblasts and fibrocytes were present in the vicinity of endothelium, in the zone of the tunica adventitia. Single macrophages were also found in the colonies of hematopoietic cells. Distinctly formed lymphatic follicles of white pulp were observed in the spleen of LF-treated mice (Fig. 4D). The central zone of these follicles, as well as the periellipsoidal lymphatic tissue, contained numerous reticular cells, transforming lymphocytes and macrophages. In the group of mice administered filgrastim, a significant colonization of pluripotential cells within bone marrow, accompanied by myelo- and lymphopoiesis around the colonies, was noted (Fig. 3E). In the spleen (Fig. 4E), the deserted areas were rare and gradually repopulated with cells of the lymphoblastic lineage.

DISCUSSION

In this investigation, it was demonstrated that LF given to mice orally during BU- and CP-induced immunosuppression and bone marrow cells reconstitution accelerates renewal of the humoral and cellular immune response. At least two mechanisms may account for these observations: (1) direct and indirect effects of LF on maturation and differentiation of immunocompetent cells from the grafted bone marrow cells and (2) facilitation of bone marrow cell engraftment as shown by the histological analysis of the bone marrow and spleen of mice given a suboptimal dose of BMC and treated with LF.

The data presented here are clearly in agreement with recent findings that LF reconstituted both cellular (27) and humoral (28) immune responses in mice given a sublethal dose of CP. Although the results obtained in the former (27,28) and present models show similar reconstitution effects of LF on immune function, they apparently differ in kinetics of reconstitution. Whereas the control, humoral immune response, measured as AFC number after a high CP (350 mg/kg b.w.) dose was virtually absent on day 30 following CP injection and LF caused only 40% reconstitution (28). The control response in BU/CP/BMT mice (this study), constituting 34% of the response of normal mice, was completely restored by LF. Similar, but less marked, differences were observed between the cellular (DTH) response in the for-

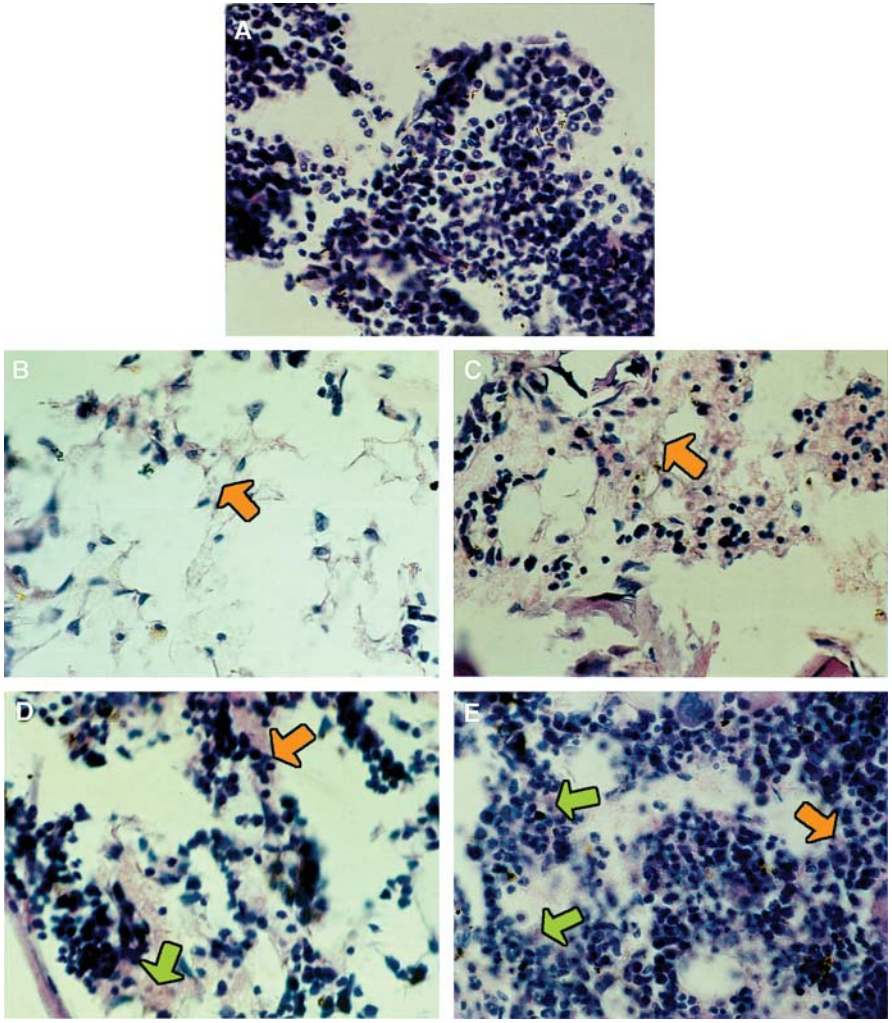

FIG. 3. Histological evaluation of the bone marrow; H&E staining; magnification, 400×. (**A**) Normal mice. (**B**) Mice treated with BU, no reconstitution with BMC: deserted areas and the presence of fibroblasts and fibrocytes. (**C**) Mice treated with BU and reconstituted with BMC: diminution of erythroblasts and lymphoblasts. (**D**) Mice treated with LF: the areas of lymphopoiesis (orange arrow) and erythropoiesis (green arrow). (**E**) Mice treated with filgrastim: induction of myelopoiesis (green arrows) and lymphopoiesis (orange arrow).

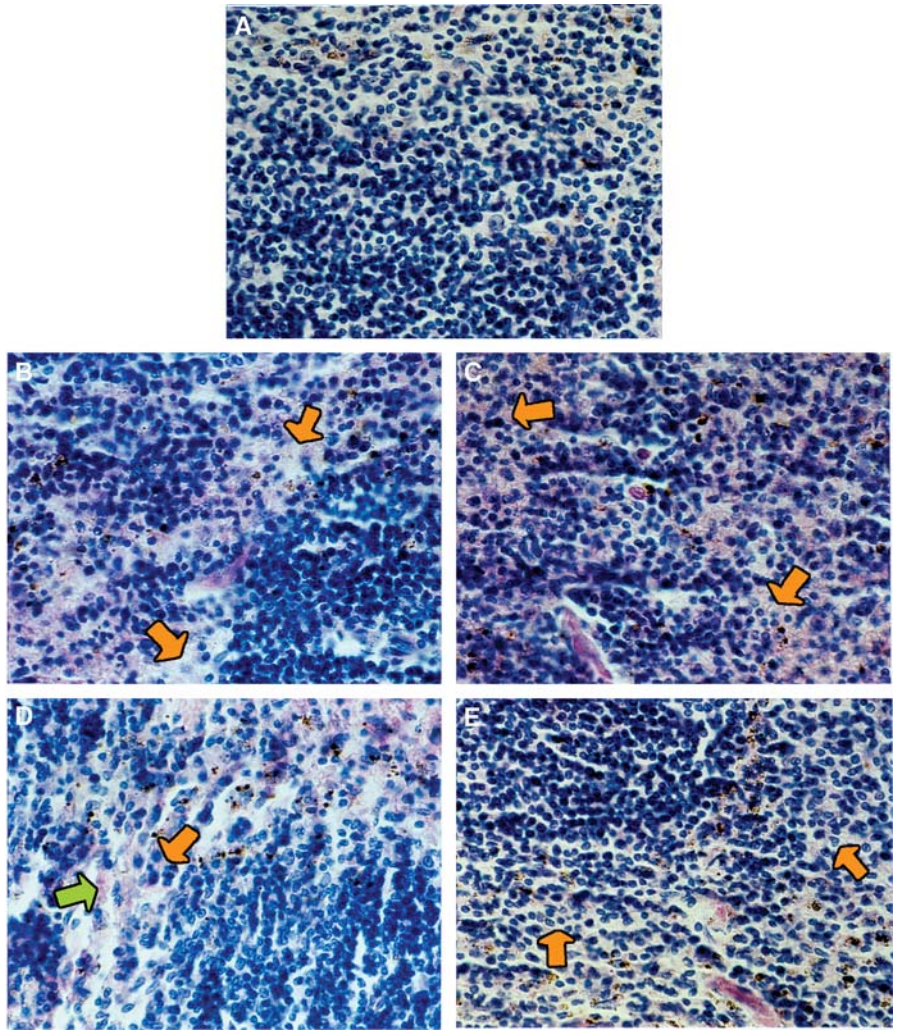

FIG. 4. Histological evaluation of the spleen. (**A**) Normal mice. (**B**) Mice treated with BU, no reconstitution with BMC: deserted areas. (**C**) Mice treated with BU and reconstituted with BMC: deserted areas in the periphery of lymphatic follicles, the central zone of lymphatic follicles distinctly thinned, high number of macrophages; increased erythrocyte degradation in the reticular tissue areas. (**D**) Mice treated with LF: areas of lymphopoiesis (orange arrows) and erythropoiesis (green arrow). (**E**) Mice treated with filgrastim: decrease of deserted areas, repopulation with cells of lymphoblastic lineage.

merly described experimental system (27) and this investigation.

In comparing the results obtained here with previous studies, it should be noted that the initial immune status of mice before LF administration was different. Administration of high CP dose led to severe depletion of lymphocytes (7), neutrophils (8), and macrophages (31), leaving early hematopoietic progenitor cells intact (1). Mice treated with BU and CP are essentially devoid of early hematopoietic cells (14); reconstitution with BMC provides a pool of T and B precursors and cells of myelocytic lineage. Hence, the delayed reconstitution of the humoral immune response by LF in CP-immunocompromised mice could be explained by a lack of or a very limited number of early B cell progenitors. On the other hand, in BMC-reconstituted mice, LF could act on immature B cells contained in bone marrow. It was previously shown that LF can promote differentiation of immature B cells from neonatal spleens (32). Although anti-SRBC humoral immune response is T cell dependent, immunocompetent T cells are not a limiting factor because the DTH response is reconstituted more quickly in both CP (27) and CP/BU/BMT mice (Fig. 1). That could result from a short life span of T cells and a direct maturation of double-negative T cell precursors in thymus under the influence of LF (33). T cell precursors are also contained in bone marrow (34,35) and could be a target for LF action in BMC-reconstituted mice. Renewal of the immune system function by LF may also be associated with its action on stromal bone marrow cells by facilitating BMC engraftment and by inducing colony-stimulating factors and IL-6. It has been demonstrated that LF can induce IL-6 in other mouse models (36), and, interestingly, IL-6 up-regulation facilitates BMC engraftment (37). LF was also shown to increase the number of 8-day CFU in the spleens (not shown). Such colonies, except of cells of megakaryocyte/erythroid lineage, contain common myeloid precursors and even committed precursors of T and B cells (34), and so they may contribute to faster renewal of T cell and B cell dependent immune response upon LF treatment.

Histological examination of the bone marrow and spleens in BU-injected, BMC-reconstituted, and LF-treated mice, were consistent with results demonstrating accelerated renewal of the immune system function in BU/CP-treated and BMC-reconstituted mice (Figs. 1 and 2) and prolongation of the survival time in mice treated with BU and reconstituted with suboptimal 10^5 BMC (not shown). Generally, the cytotoxic effect of BU treatment was much more pronounced in the bone marrow because the drug acts on early hematopoietic cells (14).The application of LF to mice treated with BU and given a low dose of BMC was associated with initiation of lymphopoiesis and erythropoiesis in the bone marrow and transforming lymphocytes evident in the spleen. That finding was in accord with previous data in mice treated with a sublethal dose of CP and given LF in drinking water, where the percentage of circulating lymphocytes was preferentially elevated (29). On the other hand, the treatment of mice with filgrastim (Figs. 3 and 4) led to anticipated changes (myelopoiesis with a less marked lymphopoiesis), which could result from synergistic effects of G-CSF with other cytokines (38,39).

The phenotypic characteristics of the cell target for LF within the hematopoietic compartment is currently under investigation. Data on the effects of LF in CP-immunocompromised mice showed that LF accelerated renewal of neutrophils and macrophages (28,29), the main phagocytic cell types responsible for host defense against infection. There are several reasons why LF, a pleiotropic, immunoregulatory protein, should be more advantageous in reconstruction of the immune system function than other immunoregulatory agents. There is ample evidence that oral administration of LF is very effective (40) and carries no toxic effect to the patient. In addition to being protective for the intestinal mucosa (41,42), LF appears to promote maturation of the progenitor cells of the lymphoid and myeloid lineage by inducing endogenous production of cytokines (36,43–45) and acting directly on immunologically committed cells (32,33). In addition LF has antibacterial (46) and antitumor (47) properties. In summary, this study demonstrated the potential benefit of LF as an adjuvant therapy for immune system recovery in patients subjected to chemotherapy and reconstituted with pluripotential stem cells.

ACKNOWLEDGMENT

We thank Dr. Jeffrey K. Actor for reviewing this manuscript and for helpful discussions.

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Received April 9, 2005; accepted June 7, 2005.