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Orally administered lactoferrin restores humoral immune response in immunocompromised mice

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Abstract

Cyclophosphamide (CP) is an anti-tumor drug commonly used in the chemotherapy of human cancer and autoimmune diseases. In our previous studies, we have demonstrated that lactoferrin (LF), given orally to CP-immunosuppressed mice, could reconstitute a T cell mediated immune response by the renewal of the T cell population. The aim of this present study was to evaluate the effects of LF on humoral responses in mice treated with cyclophosphamide. We demonstrate that a single, sublethal dose of cyclophosphamide (400 mg/kg body weight) profoundly inhibited the humoral immune response of CBA mice to sheep red blood cells (SRBC), as measured by the number of antibody forming cells (AFC) in the spleen after 5 weeks following CP treatment. Administration of 0.5% bovine LF in drinking water for 5 weeks partially reconstituted the AFC number (30–40% of the control values, but 7–10 × more than in CP-treated controls). Determination of T and B cell levels in the spleens by flow cytometry revealed that the content of $CD3^+$ and $CD4^+$ as well as Ig^+ splenocytes was elevated in the immunocompromised mice treated with LF. In addition, the number of peritoneal macrophages was partially restored following LF treatment. Evaluation of the proliferative response to concanavalin A (ConA) and pokeweed mitogen (PWM) demonstrated that the diminished reactivity of splenocytes from CP-treated mice was significantly enhanced by LF. In summary, we conclude that the prolonged, oral treatment of immunocompromised mice with LF led to partial reconstitution of the humoral response, associated with elevation of T and B cell and macrophage content and the proliferative response of splenocytes to mitogens.

Keywords: Lactoferrin; Cyclophosphamide; Immunocompromise

1. Introduction

Discovery of many immunosuppressive and anti-inflammatory compounds has led to a turning point in the treatment of neoplastic and autoimmune diseases, and has enabled the advent of successful organ transplantations [1] over the last half century. Cyclophosphamide (CP) is an example of such a well known and versatile compound; new applications for CP are still being discovered. CP belongs to the class of alkylating chemotherapeutics [2] that were found beneficial in treatment of leukemias [3] and such autoimmune disorders as multiple sclerosis (MS) [4] and lupus erythematosus (SLE) [5]. CP metabolites alkylate DNA and preferentially suppress the immune responses mediated by B-lymphocytes. Therefore, the main therapeutic effect of CP is to diminish abnormal B cell function [6]. The action of CP on B and T cells is differential, as recently demonstrated in a chicken embryo model [7]. CP causes strong, toxic changes in bone marrow cells and in the liver [8]. Stromal cells are also damaged by CP, and their renewal is delayed as compared with hematopoietic cells [8]. Also, it is suggested that by the selective depletion of $CD4^+$ and $CD19^+$ cells and the enlargement of $CD8^+$ cell pool, the drug can restore the immunological balance in SLE patients [5]. Analysis of CP action in thymocytes revealed that the drug primarily affects immature (PNA^+, $CD3^-$) and double positive ($CD4^+$, $CD8^+$)

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cells [9]. CP is also toxic with regard to one subpopulation of peritoneal macrophages [10].

CP has a profound effect on the humoral immune response in experimental animal models, particularly in the early events following its administration [11]. The compound may also lower the humoral response affecting IgA production in Peyer's patches, accompanied by a significant decrease in IL-2 and IL-4 production by stimulated T cells [12]. Production of antibodies, strongly reduced by CP administration, was reconstituted after 3–6 weeks, but the proliferative response of lymphocytes to mitogens was still markedly suppressed after 5 weeks [13]. CP was also shown to inhibit the cellular immune response in guinea pigs [14] and mice [15].

Toxic effects of CP on leukopoiesis and myelopoiesis may be, in part, prevented by the application of a variety of factors including: thymic hormones [16]; cytokines such as IL-1 [17], G-CSF [18], GM-CSF [19], IL-2 [20] and IL-7 [21]; bacterial cell fractions like levan [22], protein A [23] or lipid A analog [24]; and other chemical compounds [25,26].

Recently, we initiated investigations aimed at the demonstration that lactoferrin (LF) may also accelerate the reconstitution of immune cell content and the function of the immune system following the administration of a sublethal dose of CP. LF is a protein contained in secretory fluids of mammals and secondary granules of neutrophils, expressing a variety of immunoregulatory properties [27,28]. Opinions on the potential role of LF in myelopoiesis are varied [29,30], and reports on the possibility of using LF to stimulate lymphopoiesis in immunocompromised animals are lacking.

In our previous studies, we demonstrated that LF promotes differentiation of T [31] and B [32] cells from their immature precursors and stimulates recruitment of neutrophils [33]. Recently, we reported that LF is effective in the reconstruction of a T-cell mediated immune response in CP-immunocompromised mice [34]. The aim of this report is to reveal the effectiveness of oral LF administration to CP-treated mice to restore the humoral immune response to sheep erythrocytes and to restore the proliferative response of splenocytes to mitogens.

2. Materials and methods

2.1. Animals

CBA mice (males and females, 10–12 week old) were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. Mice were fed a commercial, pelleted food and water ad libitum. The animal ethics committee approved the study.

2.2. Antigens and reagents

Sheep red blood cells (SRBC) were delivered by Wroclaw Agriculture Academy. SRBC were stored in Alsever's medium at 4 °C until use. Highly purified and essentially endotoxin-free bovine LF (<4.4 E.U./mg, $<25\%$ iron saturated) was obtained from Morinaga, Japan. CP was purchased from ASTA Medica, Frankfurt, Germany. Concanavalin A (ConA) and pokeweed mitogen (PWM) were purchased from Sigma Chemical Company, MO, USA. Anti-mouse CD3 and CD4 monoclonal antibodies were purchased from BIO-TREND Chemicalien GmbH, Köln, Germany. Immunoglobulin isotype controls (FITC-conjugated rat IgG and PE-conjugated rat IgG) were purchased from BD Bioscences. All reagents were of analytical grade.

2.3. Treatment of mice with cyclophosphamide and lactoferrin

Mice were given a single intraperitoneal (i.p.) dose of CP (400 mg/kg body weight). Lactoferrin was applied as a drinking solution (0.5%) in filtered tap water for the entire duration of the experiment (30 days). The applied dose of LF was established in a separate experiment using various LF concentrations (0.2–1.0%). It is the dose that was the most effective in restoring the normal blood cell picture following CP treatment. The LF dose in drinking water corresponds to about 20 mg/day, and is consistent with other studies on rats and mice [35,36].

2.4. Immunization of mice and determination of antibody-forming cell number (AFC)

Mice were immunized with a single i.p. injection of 0.2 ml 5% SRBC suspension in PBS. After 4 days the spleens were isolated and pressed through plastic screens to obtain a single cell suspension in Hanks' medium. The determination of AFC was performed using the Mishell and Dutton method [37]. The results were presented as the mean AFC number per 10^6 viable splenocytes from six mice $+/-$ standard error (S.E).

2.5. Mitogen induced splenocyte proliferation

Spleens were pressed through a plastic screen into 0.83% NH_4Cl solution to lyse erythrocytes (5 min incubation at room temperature). The cells were then washed two times with Hanks' medium, passed through a glass wool column to remove cell debris and resuspended in a culture medium consisting of RPMI 1640, supplemented with glutamine, sodium pyruvate, 2 mercaptoethanol and 10% fetal calf serum. The cells

were distributed into 96-flat bottom tissue culture plates (Nunc) at a density of 2×10^5/100 μl/well. ConA (2.5 μg/ml) or PWM (1 μg/ml) were added to induce cell proliferation. After 3-day incubation in a cell culture incubator, the degree of cell proliferation was determined using a colorimetric MTT method [38]. The results are presented as a mean optical density (OD) measured at 550 nm wave-length ± S.E. from six mice (determinations).

2.6. Determination of the peritoneal macrophage numbers

Peritoneal cells were collected from mice 23 days following CP treatment or saline control. Cells were washed twice with Hanks' medium, resuspended in 1 ml of PBS and stained with 0.1% neutral red dye (Sigma-Aldrich). The number of stained cells was determined in a hemocytometer (Burker camera). The results are presented as a mean number of macrophages from six mice ± S.E.

2.7. Flow cytometry cell analysis

Splenocyte suspensions were prepared as described for the proliferation assays and pooled from six mice per group. Cells were resuspended in PBS containing 0.1% BSA at the concentration of 5×10^5/ml, centrifuged, and incubated with PE-labeled anti-mouse CD4 antibodies, FITC-labeled anti-mouse CD3 antibody (each Ab was added at 1 μg/10^6 cells) or FITC-labeled anti-mouse Ig antibody (Ab was added at 2.5 μg/10^6 cells) for 30 min in an ice bath. An irrelevant rabbit IgG (immunoglobulin isotype control) was negative.

Analysis of the cells was performed on a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA, USA) with a 15 mW argon ion laser at 488 nm excitation. Live gating of the forward and side scatter channels was used to exclude debris and to acquire selectively events for lymphocytes. Data were recorded on a logarithmic scale and 10 000 particles of each gated population were analyzed. The results are presented as the percentage of values from the control mice.

2.8. Statistics

For statistical evaluation of the data, the *t*-student's test was applied. Each experimental group of mice consisted of six mice. The results are presented as mean values from six determinations ± S.E. and were regarded as significant when $P < 0.05$.

3. Results

3.1. Lactoferrin partially restores the humoral immune response to SRBC in CP-treated mice

In our recent studies on CP immunocompromised mice, we demonstrated that LF given orally resulted in a renewal of the cellular immune response which was correlated with the restoration of T cell content in the spleen. Since CP preferentially causes deletion of B cells, it was also of interest whether LF would reconstitute antibody production to a T-dependent antigen-SRBC. Initial attempts to restore an almost totally abrogated immune response in mice treated with CP (400 mg/kg body weight) were unsuccessful (12-day LF treatment). Taking into account the delayed renewal of the B cell lineage following the CP injection, we extended the administration of LF to 28 days. Mice were also treated with LF after immunization with SRBC. The results of two representative experiments are shown in Fig. 1 and indicate that LF treatment can partially restore the

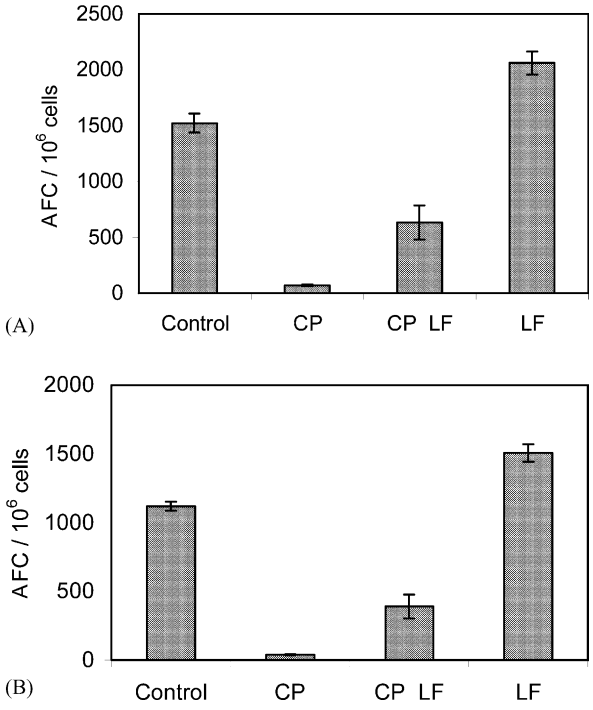

Fig. 1. Partial reconstitution of the humoral immune response to SRBC by LF in CP-treated mice. Mice were given a single i.p. dose of CP (400 mg/kg body weight) and LF as 0,5% solution in drinking water for 30 days. Then mice were immunized with a single i.p. injection of 0.2 ml 5% sheep red blood cells (SRBC) suspension in PBS. After 4 days the spleens were isolated, and determination of AFC was performed. The results were presented as the mean AFC number per 10^6 viable splenocytes from six mice ± S.E.: (A) Control/CP ($P < 0.001$), Control/CP LF ($P < 0.001$), Control/LF (< 0.01), CP/LF ($P < 0.001$), CP/CP LF ($P < 0.01$), CP LF/ LF ($P < 0.001$). (B) Control/CP ($P < 0.001$), Control/CP LF ($P < 0.001$), Control/LF (< 0.001), CP/LF ($P < 0.001$), CP/CP LF ($P < 0.01$), CP LF/ LF ($P < 0.001$).

humoral immune response expressed as AFC numbers in the spleens. Considering the fact that the immune response of mice even after 5 weeks following CP administration was virtually non-existing, the effects of LF treatment were very significant (7–10-fold elevation over CP-treated control).

3.2. Reconstitution of T, B cells and peritoneal macrophage populations by LF in CP-treated mice

The humoral immune response is dependent on the cooperation of antigen presenting cells (APC), T and B cells. Therefore, we analyzed the content of the respective cell types in the spleen and in the peritoneal cavities of the studied groups of mice. The effects of CP treatment on the splenic content of $CD3^+$ and $CD4^+$ cells, presented in Fig. 2AB, reveal that both the total T cell number and the content of T cells bearing the helper cell phenotype ($CD4^+$) are elevated following LF treatment. The analysis of the cell content positive for surface Ig demonstrated (Fig. 2C) that these cells are fully reconstituted.

Peritoneal macrophages are most probable candidates as APCs upon peritoneal SRBC immunization. Determination of the macrophage content using neutral red staining showed statistically significant restoration of that population following LF treatment (Fig. 3).

3.3. Restoration of the proliferative response of splenocytes to mitogens

We also evaluated the effects of LF on the in vitro proliferative responses of splenocytes to mitogens in mice subjected to a high dose of CP. ConA and PWM were applied, which, respectively, stimulate T and B cells. The results, shown in Figs. 4 and 5, indicate that both types of proliferative responses were significantly elevated following LF treatment.

4. Discussion

A possibility that LF, an immunoregulatory protein, may accelerate the restoration of the immune system function in immunocompromised mice has recently been explored in our laboratories. Here, we report that the humoral immune response of CBA mice to SRBC, severely depressed following administration of a single, sublethal dose of CP, can be partially restored by LF orally administered in drinking water. In addition, the proliferative responses of T and B lymphocytes to mitogens were elevated following LF treatment. The functional changes exhibited by these cells were accompanied by reconstitution of the T and B cell content in the spleens, as determined by flow cytometry, and the elevation of the number of peritoneal macrophages.

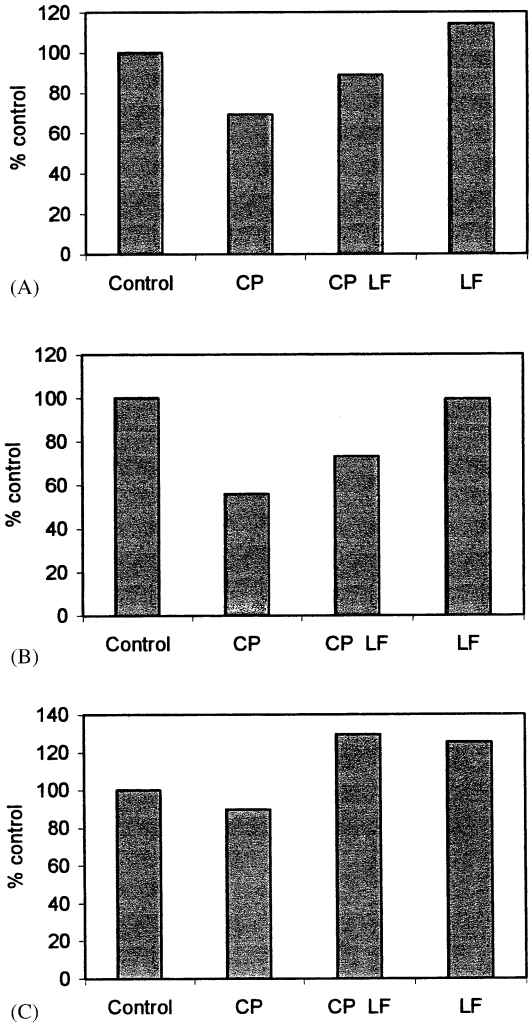

Fig. 2. Effects of LF on the splenic levels of $CD3^+$ (A), $CD4^+$ (B) and Ig^+ (C) cells in mice treated with cyclophosphamide. Mice were treated with CP and LF as described above. Splenocytes (5×10^5 cells, pooled splenocyte suspensions from six mice) were washed in PBS containing 0.1% BSA before analysis. To assess the expression of CD3, CD4 and Ig, cells were incubated with PE-labeled anti-mouse CD4 antibodies, FITC-labeled anti-mouse CD3 antibody (each Ab was added at 1 μg/10^6 cells) or FITC-labeled anti-mouse Ig antibody (Ab was added at 2.5 μg/10^6 cells). Flow cytometric analysis was performed on FACScan cytofluorometer. The results are presented as the percentage of values from the control mice.

The few reports published regarding the effects of CP on the humoral immune response in mice indicate that high (250–300 mg/kg body weight) CP doses strongly inhibit antibody responses for a prolonged period of time [11–13]. Although in one study [13] antibody production began renewal after 3–5 weeks, in our model (application of 400 mg CP/kg body weight) antibody production was still strongly inhibited after 5 weeks (over 95% inhibition). Consequently, the treatment with LF for the first 2 weeks was ineffective (data not shown), whereas the 5-week application of LF was sufficient to significantly restore the AFC number. That

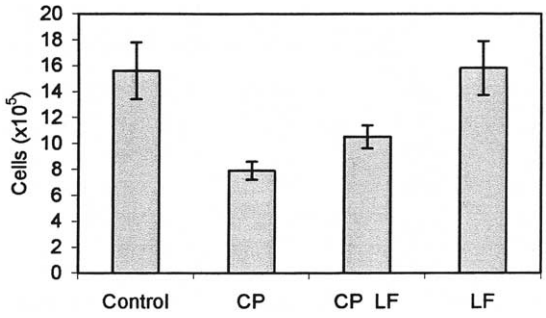

Fig. 3. Effect of LF on the content of peritoneal macrophages in CP-treated mice. Mice were treated with CP and LF as described above. Peritoneal cavities were lavaged with 10 ml of Hanks' medium. Macrophages were identified by staining with neutral red dye and the number of stained cells was counted in a hemocytometer. The data are presented as mean values from five mice/group as macrophage number per mouse ± S.E.: Control/CP ($P < 0.01$), Control/CP LF ($P < 0.05$), Control/LF (NS), CP/LF ($P < 0.01$), CP/CP LF ($P < 0.05$), CP LF/ LF ($P < 0.05$).

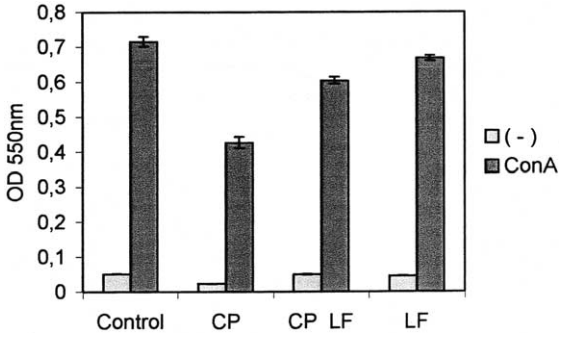

Fig. 4. Enhancement of the proliferative response of splenocytes to ConA by LF in CP-treated mice. Mice were treated with CP and LF as described above. The splenocytes was distributed in culture medium into 96-flat-bottom plates at a density of 2×10^5 cells/0.1 ml/well. ConA was added at 2.5 μg/ml and cell proliferation was determined using the colorimetric MTT method after 72 h incubation. The data are presented as mean values from five mice/group as OD at 550 nm ± S.E.: Control (−)/CP(−) ($P < 0.001$), Control (+)/CP(+) ($P < 0.001$), Control (−)/CP LF(−) (NS), Control (+)/CP LF(+) ($P < 0.001$), Control (−)/LF(−) ($P < 0.05$), Control (+)/LF(+) ($P < 0.02$), CP(−)/LF(−) ($P < 0.001$), CP(+)/LF(+) ($P < 0.001$), CP(−)/CP LF(−) ($P < 0.001$), CP(+)/CP LF(+) ($P < 0.001$), CP LF(−)/ LF (−) (NS), CP LF(+)/ LF(+) ($P < 0.001$).

result is consistent with the preferential cytotoxic effects of CP on the B cell compartment [7] and on the slower B cell renewal, compared with T cells [39,40]. Our very recent study on the effect of LF in reconstitution of the cellular immune response to ovalbumin demonstrated that 3-week treatment with LF was long enough to fully reconstitute the delayed type hypersensitivity response of mice subjected to 400 mg/kg CP dose [34]. That phenomenon was correlated with a renewal of $CD4^+$ cells as determined by a panning technique.

The analysis of cell type content in the spleens of mice subjected to the sublethal dose of CP and treated orally with LF for 5 weeks revealed a partial reconstitution of the total T cell number ($CD3^+$ cells) and T cells

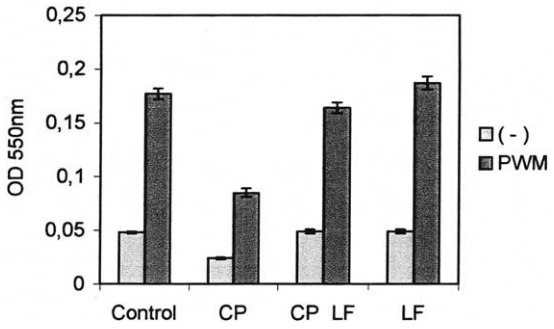

Fig. 5. Enhancement of the proliferative response of splenocytes to PWM by LF in CP-treated mice. Mice were treated with CP and LF as described above. The splenocytes were distributed in culture medium into 96-flat-bottom plates at a density of 2×10^5 cells/0.1 ml/well. PWM was added at 1 μg/ml and cell proliferation was determined using the colorimetric MTT method after 72 h incubation. The data are presented as mean values from five mice/group as OD at 550 nm ± S.E.: Control (−)/CP(−) ($P < 0.001$), Control (+)/CP(+) ($P < 0.001$), Control (−)/CP LF(−) (NS), Control (+)/CP LF(+) (NS), Control (−)/LF(−) (NS), Control (+)/LF(+) (NS), CP(−)/LF(−) ($P < 0.001$), CP(+)/LF(+) ($P < 0.001$),CP(−)/CP LF(−) ($P < 0.001$), CP(+)/CP LF(+) ($P < 0.001$), CP LF(−)/ LF (−) (NS), CP LF(+)/ LF(+) ($P < 0.01$).

expressing the helper phenotype ($CD4^+$). The Ig positive cells (B cell count) were also restored. In addition, we showed that the content of peritoneal macrophages, as the antigen presenting cells, was also partially restored. Interestingly, a subpopulation of macrophages resistant to CP [10] is characterized by a high IL-6 production, a B cell tropic cytokine [41]. Therefore, the loss in macrophage content probably did not account for much of the drastic inhibition of the humoral immune response in our model. Summarizing the analysis of cell types crucial for generation of the humoral immune response, we can conclude that a partial reconstitution of T, B cell and macrophage content contributed to a significant restoration of the virtually non-existing immune response in mice subjected to a high dose of CP.

Furthermore, the functions of T and B cells, evaluated by their ability to respond by proliferation to mitogens, were also enhanced following LF treatment. Our observation that the proliferative response of lymphocytes to mitogens was significantly inhibited after 5 weeks post CP administration is consistent with other studies [13]. The authors found a discrepancy between the impaired proliferative response of lymphocytes and minor changes in the level of lymphocyte subpopulations in the spleen determined by flow cytometry. In our model, however, the correlation between the lymphocyte content and function was rather evident. Although the level of Ig^+ cells in the immunocompromised mice did not differ much from that of control mice, the net increase of the percentage of Ig^+ cells between CP-treated and CP/LF mice was 44%. That result, together with the finding that the B cell response to PWM was

significantly increased, may explain the reconstituting effect of LF on the humoral immune response and, further, suggests that not the absolute number of Ig^+, but rather their immunocompetence, was the relevant factor in the restoration of the immune response.

The restoring effects of LF on immune functions, demonstrated in this study, can be explained by both the direct and indirect actions of LF on cells. LF given per os, can significantly stimulate both local and systemic antibody production [42]. LF is taken up by specific receptors identified on epithelial brush border cells [43]. The protein can pass through the intestinal wall partially intact [44]. Even when digested, its peptides still exhibit immunotropic properties [45]. LF is a potent inducer of cytokines as shown in vitro [46,47] and in vivo [48], among others IL-6 and IL-10, of potential significance in B cell differentiation and activation [41,49]. Recently (unpublished), we showed that LF given intravenously, induced considerable levels of IL-1 in the circulation, the cytokine belonging to B cell tropic factors as demonstrated by others [50]. A possibility also exists that LF could induce cytokine production in stromal cells able to support differentiation of T and B cells [51]. We are currently investigating such a hypothesis. However, stromal cells are particularly affected by the action of CP [8], so that the delayed effect of LF to reconstitute B cell function in our model may be, in part, attributed to a slow regeneration of stromal cells. The accelerated renewal of the T and B cell compartment may be also explained by the ability of LF to directly promote the differentiation of immature T [31] and B lymphocytes [32] and the acquisition of their immunocompetence. Our recent data (to be published) indicate that LF may also stimulate myelopoiesis in immunocompromised mice. That was an independent verification of studies conducted on healthy volunteers [33] revealing acceleration of the neutrophil turnover.

In summary, we have demonstrated that the prolonged oral treatment of mice with LF can partially reconstitute an abrogated humoral immune response and the proliferative response of splenocytes to mitogens in mice given a sublethal dose of CP that also was correlated with renewal of T and B cells as well as macrophages. Although it is difficult to distinguish the humoral events from the cellular responses during CP-induced immune suppression, here we demonstrate the ability of LF to restore the humoral arm of immunity. Per our recent reports [28,34], we propose that LF may find clinical application for immunocompromised patients as an adjuvant therapy aimed at the reconstitution of lympho- and myelopoiesis.

References

[1] A.C. Allison, Immunopharmacology 47 (2000) 63–83.
[2] B.A. Chabner, C.J. Allerga, G.A. Curt, P. Calabresi, The Pharmacological Basis of Therapeutics, ninth ed, Mc Graw-Hill, New York, 1996, pp. 1233–1287.
[3] N. Asou, H. Suzushima, S. Hishimura, T. Okubo, H. Yamasaki, M. Osato, K. Hoshino, K. Takatsuki, H. Mitsuya, Am. J. Haematol. 63 (2000) 35–37.
[4] H.L. Weiner, J.A. Cohen, Mult. Scler. 8 (2002) 142–154.
[5] J.K. Lacki, S.H. Mackiewicz, P. Leszczynski, W. Muller, Rheumatol. Int. 17 (1997) 55–60.
[6] H.A. Austin, 3rd, A.D. Patel, C.A. Cadena, D.T. Boumpas, J.E. Balow, J. Rheumatol. 24 (1997) 61–68.
[7] R.A. Hemendinger, S.E. Bloom, Immunopharmacology 35 (1996) 71–82.
[8] E. Anton, Tissue Cell 29 (1997) 1–9.
[9] A. Miyauchi, C. Hiramine, S. Tanaka, K. Hojo, Tohoku J. Exp. Med. 162 (1990) 147–167.
[10] J. Marcinkiewicz, K. Bryniarski, W. Ptak, Cytokine 6 (1994) 472–477.
[11] N.V. Masnaya, G.M. Ratner, Bull. Exp. Biol. Med. 129 (2000) 370–373.
[12] E.F. Morato, M. Gerbase-DeLima, R.M. Gorczynski, Brain Behav. Immunol. 10 (1996) 44–54.
[13] J. Moynihan, N. Cohen, Int. J. Immunopharmacol. 11 (1989) 517–527.
[14] J.E. Balow, D.L. Hurley, A.S. Fauci, J. Clin. Invest 56 (1975) 65–70.
[15] S.N. Rondinone, O.A. Giovanniello, H.A. Barrios, N.R. Nota, J. Immunol. 130 (1983) 1600–1603.
[16] H. Ohmori, M. Kamo, K. Yamakoshi, M.H. Nitta, M. Hikida, N. Kanayama, Immunopharmacol. Immunotoxicol. 23 (2001) 75–82.
[17] W.E. Fibbe, J.W. van der Meer, J.H. Falkenburg, M.S. Hamilton, P.M. Kluin, C.A. Dinarello, Exp. Hematol. 17 (1989) 805–808.
[18] J.S. Fine, X.Y. Cai, L. Justice, C.P. Jr Gommoll, L.D. Hamilton, T.A. Waters, S.K. Narula, L.A. Bober, M.J. Grace, Blood 90 (1997) 795–802.
[19] M. Santosuosso, M. Divangahi, A. Zganiacz, Z. Xing, Blood 99 (2002) 1246–1252.
[20] C.M. Walker, V. Paetkau, W.E. Rawls, K.L. Rosenthal, J. Immunol. 135 (1985) 1401–1407.
[21] G. Damia, K.L. Komschlies, C.R. Faltynek, F.W. Ruscetti, R.H. Wiltrout, Blood 79 (1992) 1121–1129.
[22] A. Siegal, S. Kopel, J. Leibovici, Cell Tissue Res. 245 (1986) 183–188.
[23] S.I. Zaidi, K.P. Singh, Raisuddin, A.K. Saxena, P.K. Ray, Immunopharmacol. Immunotoxicol. 12 (1990) 479–512.
[24] S. Ikeda, M. Kaneko, S. Nishiya, C. Nishimura, J. Clin. Lab. Immunol. 32 (1990) 189–193.
[25] I. Shalit, Y. Kletter, D. Halperin, D. Waldman, E. Vasserman, A. Nagler, I. Fabian, Eur. J. Haematol. 66 (2000) 287–296.
[26] M.T. Rejas, J.M. Rojo, G. Ojeda, I. Barasoain, Immunopharmacology 16 (1988) 191–197.
[27] B. Lonnerdal, S. Iyer, Annu. Rev. Nutr. 15 (1995) 93–110.
[28] M.L. Kruzel, M. Zimecki, Arch. Immunol. Ther. Exp. 50 (2002) 325–333.
[29] G.C. Bagby, E. Mc Call, D.L. Layman, J. Clin. Invest. 71 (1983) 340.
[30] I.N. Rich, G. Sawatzki, Blood Cells 15 (1989) 400–406.
[31] M. Zimecki, J. Mazurier, M. Machnicki, Z. Wieczorek, J. Montreuil, G. Spik, Immunol. Lett. 30 (1991) 119–123.
[32] M. Zimecki, J. Mazurier, G. Spik, J.A. Kapp, Immunology 86 (1995) 122–127.
[33] M. Zimecki, K. Spiegel, A. Wlaszczyk, A. Kubler, M.L. Kruzel, Arch. Immunol. Ther. Exp. 47 (1999) 113–118.
[34] J. Artym, M. Zimecki, M.L. Kruzel, Immunobiology 207 (2003) 1–9.

[35] H. Tsuda, K. Sekine, N. Takasuka, H. Toriyama-Baba, M. Iigo, Biofactors 12 (2000) 83–88.
[36] R.S. Bhimani, Y. Vendrov, P. Furmanski, J. Appl. Microbiol. 86 (1999) 135–144.
[37] R.I. Mishell, R.W. Dutton, J. Exp. Med. 126 (1967) 423–442.
[38] M.B. Hansen, S.E. Nielsen, K. Berg, J. Immunol. Methods 119 (1989) 203–210.
[39] D.A. Fulcher, A. Basten, Immunol. Cell Biol. 75 (1997) 446–455.
[40] B. Rocha, A.A. Freitas, A.A. Coutinho, J. Immunol. 131 (1983) 2158–2164.
[41] D.M. Hilbert, M.P. Cancro, P.A. Scherle, R.P. Nordan, J. Van Snick, W. Gerhard, S. Rudikoff, J. Immunol. 143 (1989) 4019–4024.
[42] H. Debbabi, M. Dubarry, M. Rautureau, D. Tome, J. Dairy Res. 65 (1998) 283–293.
[43] W.L. Hu, J. Mazurier, J. Montreuil, G. Spik, Biochemistry 29 (1990) 535–541.
[44] P.H. van Berkel, M.E. Geerts, H.A. van Veen, P.M. Kooiman, F.R. Pieper, H.A. de Boer, J.H. Nuijens, Biochem. J. 312 (1995) 107–114.
[45] H. Miyauchi, A. Kaino, I. Shinoda, Y. Fukuwatari, H. Hayasawa, J. Dairy Sci. 80 (1997) 2330–2339.
[46] A. Wlaszczyk, M. Zimecki, B. Adamik, G. Durek, A. Kubler, Arch. Immunol. Ther. Exp. 45 (1997) 201–212.
[47] J.K. Actor, S.A. Hwang, M. Olsen, M. Zimecki, R.L. Jr. Hunter, M.L. Kruzel, Int. Immunopharmacol. 2 (2002) 475–486.
[48] M. Zimecki, M.L. Kruzel, in: Monduzzi (Ed.), Proceedings of Fifth World Congress on Trauma, Shock, Inflammation and Sepsis, 2000, pp. 221–225.
[49] N.F. Go, B.E. Castle, R. Barrett, R. Kastelein, W. Dang, T.R. Mosmann, K.W. Moore, M. Howard, J. Exp. Med. 172 (1990) 1625–1631.
[50] J.G. Giri, P.W. Kincade, S.B. Mizel, J. Immunol. 132 (1984) 223–228.
[51] J. Tong, H. Kishi, T. Matsuda, A. Muraguchi, Immunology 97 (1999) 672–678.