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Effect of Lactoferrin on the Methotrexate-induced Suppression of the Cellular and Humoral Immune Response in Mice

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Abstract. *Our previous studies revealed that lactoferrin (LF) reconstitutes the cellular and humoral immune response in cyclophosphamide-treated mice. The aim of this investigation was to establish whether the suppressory effects of methotrexate (MTX) on the cellular and humoral immune response can be modulated by LF. We found that MTX, given intraperitoneally (i.p.) at a dose of 200 mg/kg b.w., 48 h following sensitization of CBA mice with ovalbumin (OVA), reduced by 80% the delayed type hypersensitivity (DTH) response. Co-administration of LF in drinking water (0.5% solution) for the duration of the experiment (4 days) restored the DTH response almost to the control level. However, LF was not able to restore the primary humoral immune response, measured by the number of antibody-forming cells (AFC) to sheep erythrocytes (SRBC) in the spleens when MTX (1 mg/kg b.w.) was administered to mice i.p. 48h post immunization. On the other hand, mice treated with LF after second challenge with SRBC showed significant restoration of the MTX-suppressed humoral immune response following the booster immunization. In addition, LF (1 μg/ml) restored the secondary humoral immune response to SRBC in vitro when MTX (0.05-1 mM) was added to cell cultures on day 2 following cell culture initiation. These data demonstrate that LF preferentially restores the cellular immune response impaired by MTX treatment. It seems that LF also prevents the block of the activity of T memory cells in the secondary, humoral immune response. Taken together, we demonstrated that LF given orally can reduce the toxic effects of MTX.*

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Methotrexate (MTX), an immunosuppressory drug, is an antimetabolite used for treatment and prophylaxis of several disorders such as: rheumatoid arthritis (1), systemic lupus erythematosus (2), psoriasis (3) and, together with other drugs, in treatment of leukemias (4). MTX, an antagonist of folic acid synthesis (5), causes apoptosis in activated cells, primarily in the G_1- and S-phases of the cell cycle (6), block of cell division (7) and inhibition of synthesis of several proinflammatory cytokines (8) possibly by suppression of NF-Î B activation (9). MTX was found to inhibit the humoral and cellular immune response in several animal models (5, 10-13). The compound was most effective when applied 24-48 h following immunization (6) or activation with mitogens (7). Psychic stress (14) or infection (15) worsened the side-effects of MTX treatment. The toxic effects of MTX may be ameliorated by application of plant extracts (16), leukovorine (17) or TGF-α (18).

Lactoferrin (LF) is an 80 kDa protein, involved in iron metabolism of mammals (19). Receptors for LF were described on several cell types including macrophages/monocytes (20), T (21) and B (22) lymphocytes and intestinal brush border cells (23). The protein exhibits a variety of protective, immunological activities, such as: antibacterial (24), antiviral (25), antifungal (26) and antiparasitic (27). LF may also protect animals against tumors (28) and autoimmune diseases (29). Other interesting, immunotropic properties of LF include promotion of T (30) and B (31) cell maturation, adjuvanticity (32) and, in general, immunoregulation (33).

Recently, we demonstrated that LF, given to mice in drinking water, can significantly accelerate renewal of the cellular and humoral (34, 35) response after administration of a sublethal dose of cyclophosphamide (CP). That phenomenon was correlated with a more rapid recovery of the number of peripheral leukocytes and normalization of the blood cell picture (36). Since MTX is commonly used in many therapeutical protocols, also together with CP, our objective was to evaluate the effectiveness of LF in reducing the suppressory actions of MTX in models of the humoral and cellular immune response in mice.

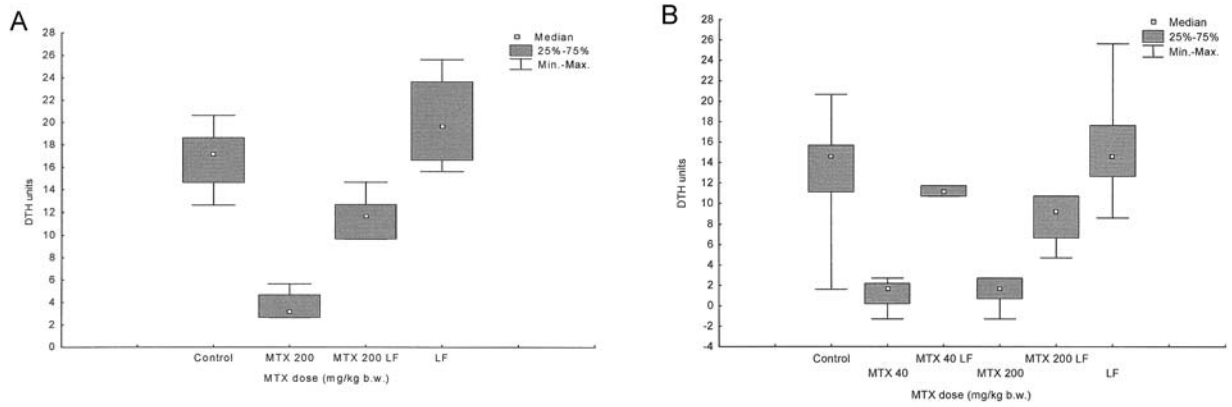

Figure 1. *Effect of LF on delayed type hypersensitivity supressed by MTX. Mice were sensitized with OVA and treated with MTX i.p. with a dose of 200 mg/kg b.w. (A) or 40 and 200 mg/kg b.w. (B) 48h following sensitization. LF (0.5% water solution) was given to mice for the whole duration of the experiments. Mann-Whitney test showed significant differences in DTH between: A. Control/MTX ($p<0.001$); Control/MTX LF ($p<0.01$); MTX /MTX LF ($p<0.001$); LF/MTX ($p<0.001$). B. Control/MTX 1 ($p<0.001$); Control/MTX 5 ($p<0.001$); MTX 1/MTX 1 LF ($p<0.001$); MTX 5/MTX 5 LF ($p<0.001$); MTX 5/MTX 5 LF ($p<0.001$); Control/MTX 5 LF ($p<0.001$).*

Materials and Methods

Mice. Twelve-week-old CBA male and female mice were delivered by the Animal Facility of the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The mice were fed a granulated, commercial food and filtered tap water *ad libitum*. The local ethics committee approved the study.

Reagents. Sheep red blood cells (SRBC) were provided by the Wroclaw Agriculture Academy. SRBC were kept in Alsever's solution until use. Ovalbumin (OVA) was purchased from Sigma, and methotrexate (MTX) was the product of LACHEMA (Czech Republic). Low endotoxin bovine milk lactoferrin (0.16 E.U./mg, <25% iron saturated) was obtained from Morinaga Milk Industry Co., Japan.

Treatment of mice and cell cultures with methotrexate and lactoferrin. In the cellular immune response, mice were given MTX intraperitoneally *(i.p.)* at a dose of 40 and 200 mg/kg b.w., 48 h after sensitization of mice with OVA. LF was administered to mice as a 0.5% solution (~20mg/day) from the time of immunization to determination of the DTH response.

In generation of the humoral immune response *in vivo,* MTX was administered *i.p.* at a dose 1 mg/kg b.w., 48 h following immunization (primary immune response) or 48h after booster antigenic dose (secondary immune response). LF was administered to mice as a 0.5% solution from the time of immunization to determination of the AFC number (in the primary response) and from the time of booster immunization to the AFC assay (the secondary response).

In the secondary humoral immune response *in vitro,* MTX was added to the cell cultures at a final concentration 0.05-1 mM, 24 h after initiation/immunization of cultures. LF was added to the cell cultures (1 μg/ml) at the beginning of 4-day incubation.

The cellular immune response. Mice were sensitized subcutaneously (*s.c.*) with 10 mg OVA emulsified in Freund's complete adjuvant into the tail base. After 4 days, the mice were challenged with 50 mg OVA in Freund's incomplete adjuvant into both hind foot pads. Following the next 24 h, the delayed type hypersensitivity reaction was measured as the foot pad edema using a caliper with 0.05 mm accuracy. The background, nonspecific response was elicited by administration of an eliciting dose of OVA in naive mice and was subtracted from the response of sensitized mice. The results are shown as median values, 25 and 75% quartile and min-max values from 5 mice/group (10 determinations), and expressed in DTH units (37) – one unit = 0.1 mm.

The humoral immune response in vivo. For development of the primary immune response, mice were given *i.p.* 0.2 ml of 2.5% SRBC suspension in 0.9% NaCl. After 4 days, the splenocytes were isolated and the number of antibody-forming cells (AFC) was determined by the local hemolysis assay (38). The secondary immune response was measured in mice primed with 2.5% SRBC suspension and challenged after 14 days with 1% SRBC. Again, the number of AFC in the spleens was determined 4 days after the booster antigen dose. The results are shown as mean AFC values from 5 mice/group, calculated per 10^6 viable splenocytes +SE.

The secondary humoral immune response in vitro. Mice were primed with 0.2 ml 1% SRBC suspension *i.p.* After 4 days, the splenocytes were isolated and a single cell suspension was prepared in a culture medium consisting of RPMI 1640, supplemented with 10% fetal calf serum, glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics. The cells were incubated in 24-well culture plates ($5x10^6$/ml/well) with addition of 50 μl 0.005% SRBC. After 4 days, the number of AFC was determined. The results are shown as mean values of AFC number from 5 wells ±SE, calculated per 10^6 viable cells.

Statistics. In humoral immune response analysis the differences across groups were determined by analysis of variance after testing homogeneity of variance by Levenéa test. Individual grades were then compared using the Tukey test for multiple comparisons. The

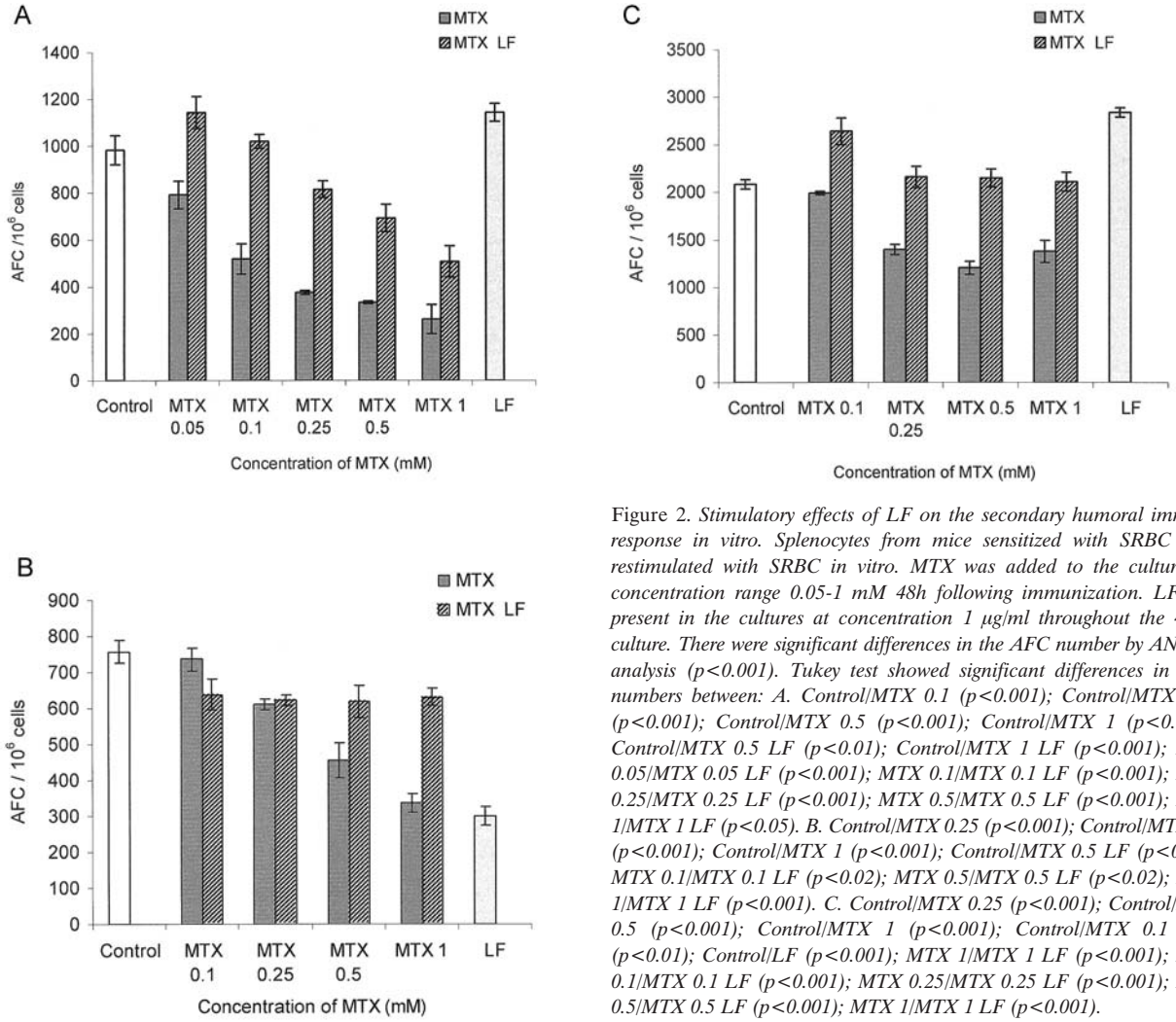

Figure 2. *Stimulatory effects of LF on the secondary humoral immune response in vitro. Splenocytes from mice sensitized with SRBC were restimulated with SRBC in vitro. MTX was added to the cultures at concentration range 0.05-1 mM 48h following immunization. LF was present in the cultures at concentration 1 μg/ml throughout the 4-day culture. There were significant differences in the AFC number by ANOVA analysis ($p<0.001$). Tukey test showed significant differences in AFC numbers between: A. Control/MTX 0.1 ($p<0.001$); Control/MTX 0.25 ($p<0.001$); Control/MTX 0.5 ($p<0.001$); Control/MTX 1 ($p<0.001$); Control/MTX 0.5 LF ($p<0.01$); Control/MTX 1 LF ($p<0.001$); MTX 0.05/MTX 0.05 LF ($p<0.001$); MTX 0.1/MTX 0.1 LF ($p<0.001$); MTX 0.25/MTX 0.25 LF ($p<0.001$); MTX 0.5/MTX 0.5 LF ($p<0.001$); MTX 1/MTX 1 LF ($p<0.05$). B. Control/MTX 0.25 ($p<0.001$); Control/MTX 0.5 ($p<0.001$); Control/MTX 1 ($p<0.001$); Control/MTX 0.5 LF ($p<0.01$); MTX 0.1/MTX 0.1 LF ($p<0.02$); MTX 0.5/MTX 0.5 LF ($p<0.02$); MTX 1/MTX 1 LF ($p<0.001$). C. Control/MTX 0.25 ($p<0.001$); Control/MTX 0.5 ($p<0.001$); Control/MTX 1 ($p<0.001$); Control/MTX 0.1 BLF ($p<0.01$); Control/LF ($p<0.001$); MTX 1/MTX 1 LF ($p<0.001$); MTX 0.1/MTX 0.1 LF ($p<0.001$); MTX 0.25/MTX 0.25 LF ($p<0.001$); MTX 0.5/MTX 0.5 LF ($p<0.001$); MTX 1/MTX 1 LF ($p<0.001$).*

data are expressed as a mean±SE (standard error). The Mann-Whitney test was used to evaluate the changes in cellular immune response (DTH). Median values, 25 and 75% quartile and min-max values are shown. For all tests, differences were considered significant when p was less than 0.05. The statistical analysis was performed using STATISTICA 6.0 for Windows.

Results

Effect of LF on the cellular immune response to OVA in MTX-treated mice. Mice were sensitized with OVA and treated with MTX and LF as described in Materials and Methods. The results (Figure 1) showed that in MTX-treated mice the magnitude of DTH response was deeply inhibited (by 80%). However, mice given access to LF in drinking water demonstrated a level of DTH close to that of the untreated counterparts. Mice treated with LF alone exhibited insignificant elevation of the cellular response.

Lactoferrin restores the secondary humoral immune response in vitro and in vivo. The experiments aimed at reconstitution of MTX-suppressed primary humoral immune response to SRBC revealed that LF treatment was insufficient to restore AFC number in mice given MTX (200-0.5 mg/kg b.w.), 48 h following immunization (data not shown). Interestingly, when we used splenocytes from SRBC-primed mice for induction of the secondary immune response *in vitro* (Figure 2A,B,C), LF (1 μg/ml) significantly increased the AFC number in cultures treated with 0.05 mM-1 mM of MTX 24 h following initiation of the 4-day incubation, however, LF alone was slightly stimulatory (Figure 2A,C) or inhibitory (Figure 2B). Similarly, LF given to mice in drinking water from the day of administration of the booster antigen dose almost completely restored the secondary immune response of mice, deeply inhibited (by 90%) upon administration of MTX (1mg/kg b.w.) 48h following antigen challenge (Figure 3A,B).

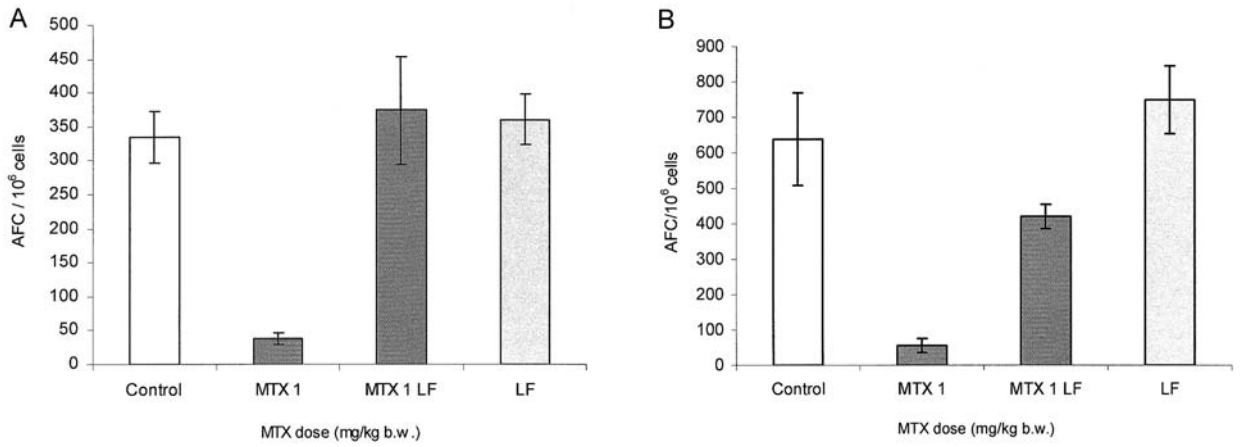

Figure 3. *Reconstituting effects of LF on the secondary humoral immune response in vivo. Mice were given a second dose of SRBC 14 days following sensitization. MTX (1 mg/kg b.w.) was given i.p. 48h after second immunization. LF (0.5% solution) was administered to mice from the day of the second antigen challenge. There were significant differences in the AFC number by ANOVA analysis ($p<0.001$). Tukey test showed significant differences in AFC numbers between: A. Control/MTX ($p>0.01$), MTX/LF ($p<0.01$), MTX/MTX LF ($p<0.001$). B. Control/MTX ($p>0.001$), MTX/MTX LF ($p<0.05$).*

Discussion

In this investigation, we showed that LF can reverse the suppressory action of MTX in generation of DTH and in the secondary immune response in mice. LF given orally at 20 mg/day was insufficient to reconstitute the primary immune response to SRBC inhibited by MTX (not shown) and the proliferative response of splenocytes to T and B cell mitogens (concanavalin A, pokeweed mitogen and lipopolysaccharide) when MTX was used in a broad range of concentrations.

The results in this study are consistent with other data demonstrating that MTX was most effective when added 24-48h following immunization (6). In the generation of DTH response to OVA (not shown), MTX was unable to suppress that response when administered 24h before sensitization of mice. Some authors showed that MTX suppressed the DTH when given to rats before sensitization (39), possibly due to transient preservation of MTX in cells in a form of polyglutamates (10) which is released when T cells become activated.

Our results suggest that LF interferes with MTX activity, which typically induces apoptosis in activated cells. Our findings are in line with other reports on LF and MTX effects on gut epithelial cells (40, 41). First, LF was found to protect the epithelial cells *in vivo* against MTX-mediated damage (40). The protective activity of LF may be explained by inhibition of cell proliferation as suggested for gut epithelial CaCO2 cells (41). In that model, LF interfered with the glucagon-like peptide 2 - induced cell proliferation. Others have found that compounds interfering with the synthesis or signaling pathways for IL-2 synthesis may also be protective against MTX action (7). That phenomenon may be analogous to the action of cyclosporine A (42) in activation-induced cell death in the immature B cell line WEHI 231. Our previous studies on the immunotropic action of LF showed that LF may also inhibit proliferation and IL-2R expression on antigen-specific T cells (43). In addition, our data revealed that the antibodies anti-IgM-induced death of the immature B cell line WEHI 231 could be inhibited by LF. That phenomenon was associated with a decrease of expression of surface IgM and IL-2R. Thus, the anti-apoptotic action of LF could be, in that case, associated with promotion of cell maturation and inhibition of cell proliferation (44). The anti-apoptotic properties of LF were also described in relation to rat osteoblasts (45). Lastly, since MTX was shown to inhibit expression of adhesion molecules (46), the protective effect of LF could be related to up-regulation of LFA-1 expression (47) and ICAM-1 (unpublished data).

It was of interest that presumably T cells, supporting the primary humoral immune response, were irreversibly blocked by MTX, in contrast to another report (12). However, the reconstituting effect of LF in the antigen-specific secondary humoral immune response indicates that LF may prevent suppression of memory T cell function. That may be reminiscent of other, long-term studies on breast cancer patients subjected to chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil) (48), which showed that memory T cells may be resistant to chemotherapy. It appeared that women vaccinated for tick-borne-encephalitis (TBE) before chemotherapy could

develop significant anti-TBE antibody titers, but not in patients vaccinated in the course or after chemotherapy. It can, therefore, be concluded that preexisting memory T cells in the humoral immune response of that particular model were not inactivated by chemotherapy. Although, in our model, memory T cells were susceptible to MTX action, LF apparently prevented loss of their function, possibly by interference with MTX-induced apoptosis and/or by ensuring delivery of costimulatory signals to antigen-specific B cells. The protective effect of LF on MTX-induced inactivation of memory T cells may also be associated with LF ability to reduce the production of inducible nitric oxide (49). It appeared that lack of inducible nitric oxide synthase (iNOS) led to higher frequencies of both $CD4^+$ and $CD8^+$ memory T cells in response to immunization, accompanied by an increase of the levelof the anti-apoptotic proteins Bcl-2 and Bcl-xL (50). More interestingly, iNOS inhibitors did not affect the primary immune response, which suggests that a prolonged survival, but not enhanced activation was responsible for the elevated numbers of memory cells. These findings could explain the differential action of LF on the primary and secondary T cell-dependent humoral immune response in mice treated with MTX. Thus, the anti-apoptotic property of LF (45) may be relevant in that phenomenon.

Taken together, we demonstrated that LF can reduce the toxic effects of MTX in the generation of antigen-specific immune response. The lack of toxicity and excellent bioavailability of LF at oral administration predisposes that protein for future consideration in human clinical protocols.

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