Research Article

Non-specific immunostimulatory effects of transfer factor

Abstract

The focus of this study was to confirm the hypothesis that food supplementation with colostrum- and egg-derived transfer factor can positively influence immune reaction. Our results found that phagocytosis, proliferation of spleen cells, NK cell cytotoxicity, IL-2 and IFN-γ production were significantly stimulated by feeding with transfer factor. Production of antibodies and IL-4, IL-5, IL-10 and TNF-α and the cellular composition of spleen was not affected. From our data we conclude that this type of transfer factor affects both cellular and humoral branch of immune reactions.

Keywords: NK cell cytotoxicity, spleen cells, IL-2 and IFN-γ

Vetvicka V, Fernandez-Botran R
University of Louisville, Department of Pathology, Louisville, KY, USA

Correspondence: Vaclav Vetvicka, University of Louisville, Department of Pathology, 511 S. Floyd, Louisville, KY 40202, USA, Email vaclav.vetvicka@louisville.edu

Introduction

Transfer factor essentially represents small immune messenger molecules that are produced by all higher organisms and are involved in inter-cellular communication. Transfer factor was originally described as undefined immune molecules that are derived from blood or spleen cells and can cause antigen-specific cell-mediated immunity, primarily delayed hypersensitivity and the production of lymphokines.[1] In general, transfer factors represent a combination of proteins and peptides containing antigen-specific information which educates and improves the immune system and helps maintain immune system balance.

The original transfer factor was isolated from human blood cells, but the advent of HIV strongly lowered the interest in blood-related products. However, the subsequent research confirmed that transfer factors are able to transmit some parts of the cell-mediated immunity from sensitized donors to naïve recipients, which further stimulated interest among scientist. Among others, transfer factor has been found to improve diseases such as herpes infection, tuberculosis or parasitic infections.[2-5]

A second use of the term transfer factor applies to a potentially different entity derived from cow colostrum or chicken egg yolk.[6] At present, neither the precise chemical nature, nor the exact molecular mechanisms of action of either version of transfer factor have been defined. However, these materials have been shown to provide both therapeutic and prophylactic benefits.[7,8]

Colostrum is a form of milk produced by the mammary glands of mammals in late pregnancy. Colostrum also contains multiple immune modulating molecules, including antibodies. Egg extracts have a variety of physiological effects, including antibacterial, immunomodulatory and antidepressive effects.[8]

In our study we evaluated the biological effects of Transfer Factor Plus Tri-Factor Formula which combines transfer factors obtained by proprietary filtration methods from bovine colostrum (UltraFactor XF® and NanoFactor®) and chicken egg yolk (OvoFactor®) sources as well as other ingredients.

Material and methods

Animals

Female, 6 week old BALB/c mice were purchased from the BioLASCO (Taiwan). Ten mice/group were used in this study. Each animal was weighted once a week during the study period.

Materials

Concanavalin A (Con A), lipopolysaccharide (LPS), propidium iodine were purchased from Sigma (St. Louis, USA).

Cell lines

Cancer cell line YAC-1 (ATCC, Manassas, VA, USA) was maintained in culture at 37 °C in a humidified atmosphere supplemented with 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS.

Transfer factor

Transfer Factor Plus Tri-Factor sample was kindly donated by 4Life Research, Sandy, UT, USA. Three different doses, 273 mg/kg/day (low), 546 mg/kg/day (medium) and 1,365 mg/kg/day (high) were used. Individual samples and negative control (sterile water) were administered daily by oral gavage for 6 weeks.

Sample collection

Blood samples were collected at the end of experiments by heart puncture. Serum was obtained and stored at -30°C before experiments. Peritoneal macrophages were obtained after injection of cold HBSS buffer into the peritoneal cavity.

Cell proliferation

Splenocytes at $2x10^5$ cells/well concentration were treated with Con A or LPS for 72 hrs at 37°C. Cell proliferation was measured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) at 490 nm.

Phagocytosis

The Phagocytosis Assay Kit using FITC-labeled *E. coli* was used according to manufacturer's instruction (Cayman Chemicals, Ann Arbor, MI, USA).

NK cell activity

YAC-1 cells were pre-labeled with PKH67 dye by PKH67 Fluorescent Cell Linker Kit (Sigma). Splenocytes were incubated with labeled YAC-1 cells at the 25:1 ratio for 4 hrs at 37°C. After incubation with 50μl of propidium iodine, the NK cell-mediated cytotoxicity was measured by flow cytometry.

Surface markers

Splenocytes were labeled with fluorescence-conjugated monoclonal antibodies at the concentration of $2x10^5$ cells/well. Antibodies recognizing T lymphocytes (CD4$^+$/CD3$^+$), cytotoxic T lymphocytes (CD8$^+$/CD3$^+$), B lymphocytes (CD19$^+$/CD45$^+$) and NK cells (PanNK$^+$/CD45$^+$) were used and the individual subpopulations were evaluated by flow cytometry.

Cytokine analysis

Splenocytes at 1-$2x10^6$ cells/well concentration were treated with Con A or LPS for 72 hrs at 37° C. After centrifugation, supernatants were collected and levels of IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN−γ were evaluated by ELISA using appropriate ELISA kits (Biolegend, San Diego, CA, USA).

Serum immunoglobulins

Whole blood was centrifuged at 2,200 g for 15 minutes and serum samples were analysed for levels of individual classes of immunoglobulins by ELISA using an ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA).
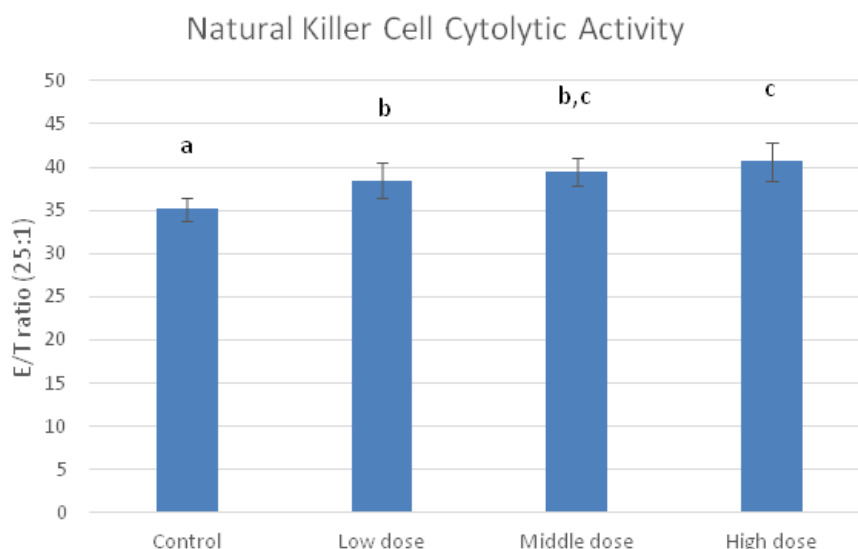
Statistics

One way ANOVA followed by Duncan's test were used to statistically analyze the data.

Results

No clinical signs of illness were observed during our study. Mean body weight at the beginning of the study was 18.4 -18.6 g, the mean weight at the end of the study was 21.4 – 21.6 g. Table 1 shows that the body weight or spleen-to-body weight ratio did not statistically differ among tested groups (Table 1).

Proliferative response to LPS or Con A stimulation showed significant increase by product supplementation. Increase in dose of tested samples caused a significant increase in proliferation, but this increase was dose-dependent (Table 2). Additional results showed significant induction of cytotoxic activity of splenic NK cells (Figure 1) and phagocytic activity of peritoneal macrophages (Figure 2) with a dose-response effect.

Table 1 Body Weight Changes and Spleen-to-body Weight Ratios

Group†	NC	TA-L	TA-M	TA-H
Body weight (g)				
Week 0	18.6 ± 0.5 [a]	18.4 ± 0.6 [a]	18.6 ± 0.8 [a]	18.4 ± 0.8 [a]
Week 1	19.0 ± 0.5 [a]	18.8 ± 0.9 [a]	18.8 ± 0.9 [a]	18.4 ± 0.5 [a]
Week 2	19.2 ± 0.7 [a]	19.1 ± 0.8 [a]	19.3 ± 0.9 [a]	18.9 ± 0.8 [a]
Week 3	19.5 ± 0.8 [a]	19.5 ± 0.7 [a]	19.5 ± 1.0 [a]	19.6 ± 0.8 [a]
Week 4	20.7 ± 1.0 [a]	20.4 ± 0.8 [a]	20.5 ± 0.9 [a]	20.2 ± 0.9 [a]
Week 5	20.9 ± 1.1 [a]	20.8 ± 0.7 [a]	20.8 ± 0.7 [a]	20.6 ± 1.1 [a]
Week 6	21.5 ± 0.8 [a]	21.6 ± 0.5 [a]	21.6 ± 1.0 [a]	21.4 ± 1.4 [a]
Spleen-to-body weight ratio (%)#				
	0.433 ± 0.033 [a]	0.472 ± 0.110 [a]	0.448 ± 0.038 [a]	0.451 ± 0.023 [a]

Data are expressed as mean ± standard deviation (S.D.) of 10 mice

Spleen-to-body weight ratios= [Spleen weight (g)/ body weight (g)] x 100

†NC, negative control; TA-L: test article low dose, TA-M: test article middle dose; TA-H: test article high dose

Table 2 Proliferative Response of Mouse Splenocytes

Group†	Dose (mg/kg/day)	Stimulation index (S.I.)# Con A (5.0 μg/mL)	LPS (10.0 μg/mL)
NC	—	4.81 ± 0.46 [a]	2.18 ± 0.36 [a]
TA-L	273	5.55 ± 0.75 [b]	2.54 ± 0.30 [b]
TA-M	546	5.79 ± 0.73 [b]	2.58 ± 0.28 [b]
TA-H	1365	6.09 ± 0.48 [b]	2.72 ± 0.28 [b]

Data are expressed as mean ± standard deviation (S.D.) of 10 mice, and were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters in same column are significantly different ($p < 0.05$)

Stimulation index (S.I.) = OD490 nm of Con-A or LPS-stimulated cells / OD490 nm of unstimulated cells. Con A: Concanavalin A; LPS: Lipopolysaccharide

†NC, negative control; TA-L: test article low dose, TA-M: test article middle dose; TA-H: test article high dose

Figure 1 Data are expressed as mean ± standard deviation (S.D.) of 10 mice. Data were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters are significantly different ($p < 0.05$). E/T ratio: Effector cell (NK cell)/ target cell (YAC-1 cell) ratio.
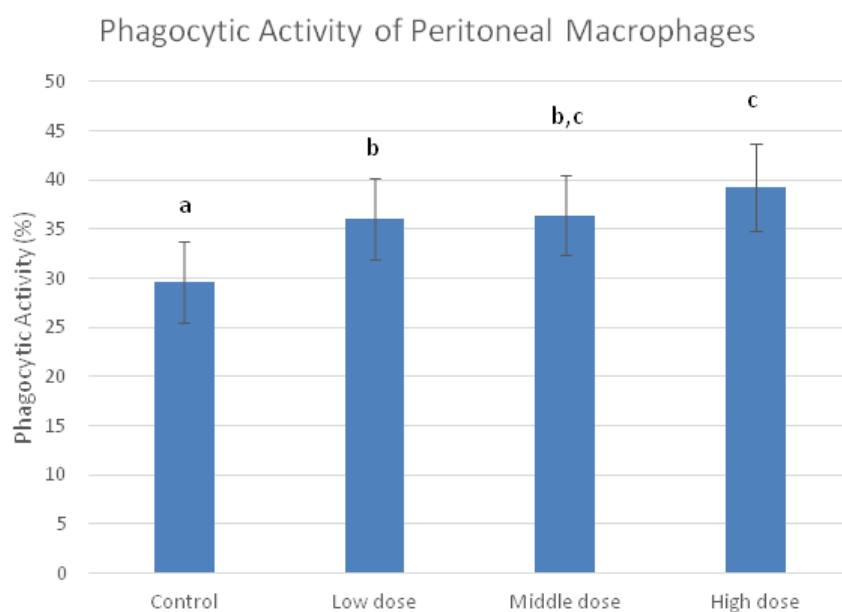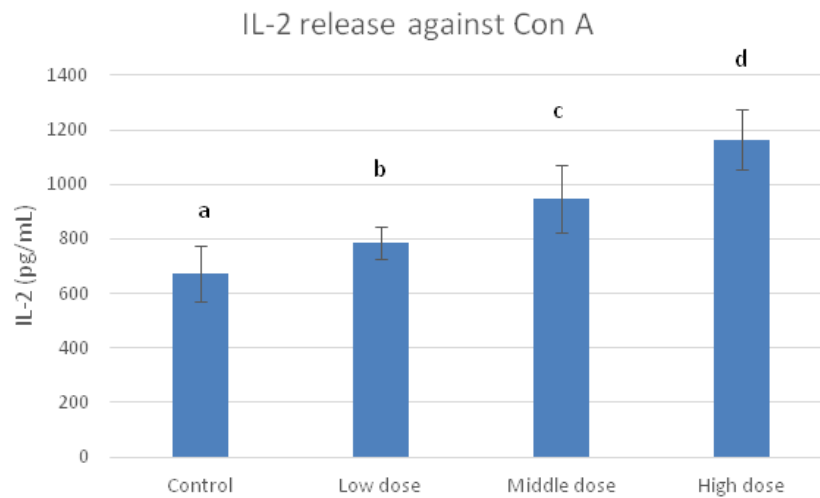

Figure 2 Data are expressed as mean ± standard deviation (S.D.) of 10 mice. Data were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters are significantly different ($p < 0.05$). Peritoneal macrophages isolated from experimental animal of each group were incubated with fluorescein-labeled *E. coli*. Peritoneal macrophages positive for *E. coli* uptake were determined by flow cytometry. Phagocytic activity was indicated as the percentage of macrophages with phagocytized fluorescein-labeled *E. coli*.

Next, we focused on effects of tested transfer factor product on production of cytokines. There were no significant differences in basal levels, i.e. without any mitogen stimulation) of IL-2 secretion among experimental and control groups. However, after Con A treatment, the levels of IL-2 were significantly increased and these increases were found to be dose-dependent (Figure 3). In addition, elevated IL-2 levels were found in the medium and high doses after LPS stimulation (Figure 4). IFN-γ secretion was again the same under unstimulated conditions, but significantly and dose-dependently increased upon Con A stimulation (Figure 5). Stimulation with LPS resulted in some increase of production, but the stimulation was not significant (Figure

6). Secretion of IL-4, IL-5 and IL-10 were not changed by transfer factor feeding regardless the presence or absence of Con A or LPS stimulation (data not shown). With respect of TNF-α, there were no changes at basal levels and the increase upon stimulation was dose-dependent, but not statistically significant (data not shown).

Studies measuring the effect of transfer factor stimulation on production of immunoglobulins found no significant changes for IgG, IgM, IgA and IgE levels (Table 3). Similarly, no changes were found during phenotyping of splenocytes with no changes in numbers of T and B lymphocytes, NK cells nor CD4+ or CD8+ lymphocytes (Table 4).
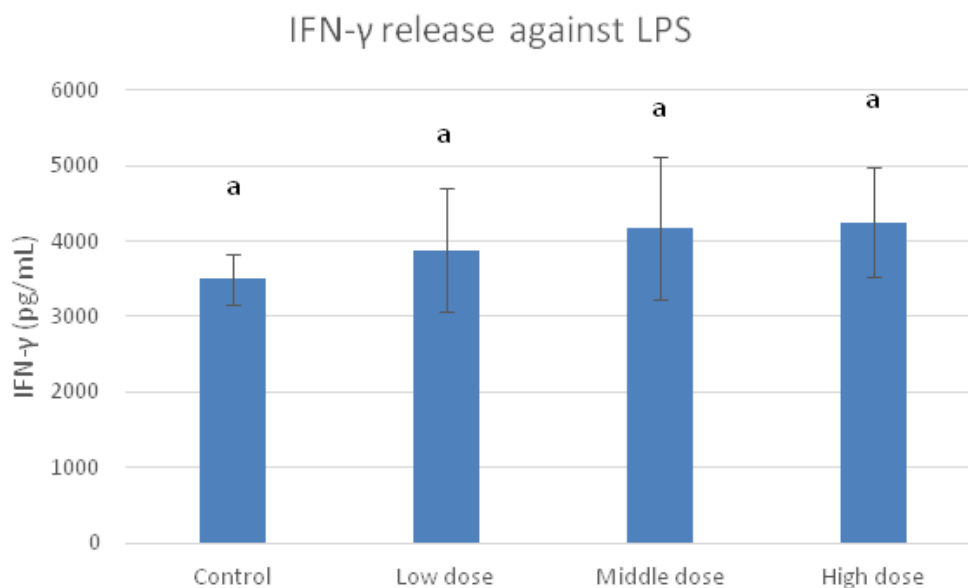
Figure 3 Interleukin-2 (IL-2) stimulation from Concanavalin A (Con A) are expressed as mean ± standard deviation (S.D.) of 10 mice. Data were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters are significantly different (p < 0.05).
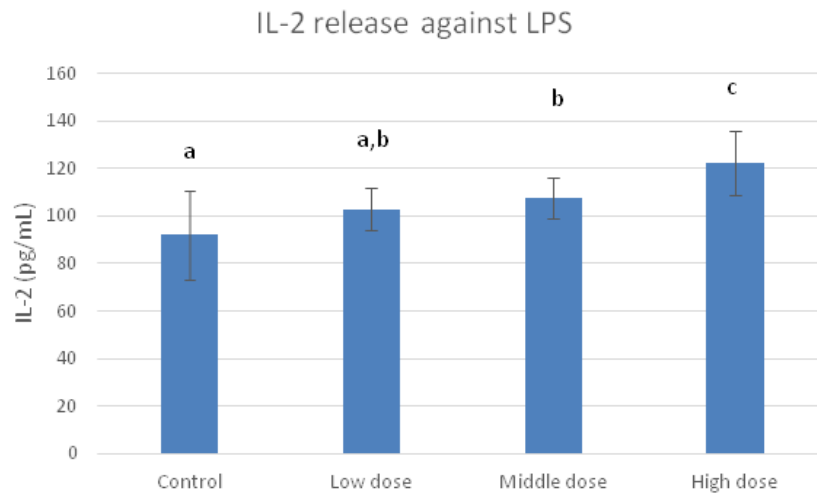
Figure 4 Interleukin-2 (IL-2) stimulation from lipopolysaccharide (LPS) are expressed as mean ± standard deviation (S.D.) of 10 mice. Data were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters are significantly different (p < 0.05).
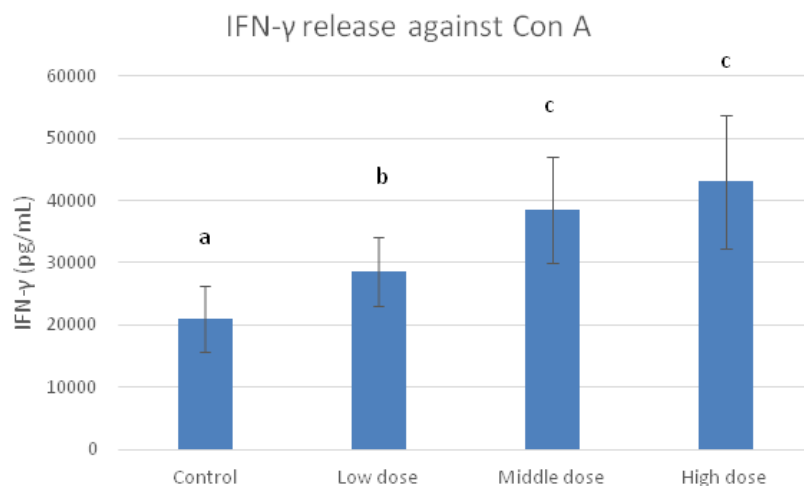
Figure 5 Interferon-gamma (IFN-γ) stimulation from Concanavalin A (Con A) are expressed as mean ± standard deviation (S.D.) of 10 mice. Data were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters are significantly different (p < 0.05).

Figure 6 Interferon-gamma (IFN-γ) stimulation from lipopolysaccharide (LPS) are expressed as mean ± standard deviation (S.D.) of 10 mice. Data were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters are significantly different ($p < 0.05$).

Table 3 Serum Immunoglobulins Levels

Group[†]	Serum antibody (µg/mL)			
	IgG	IgM	IgA	IgE
NC	3003.4 ± 285.0 [a]	356.6 ± 64.3 [a]	220.8 ± 50.0[a]	0.24 ± 0.07 [a]
TA-L	3225.4 ± 410.8 [a]	355.9 ± 59.2 [a]	222.8 ± 42.0[a]	0.26 ± 0.07 [a]
TA-M	3260.5 ± 713.0 [a]	358.5 ± 90.1 [a]	226.7 ± 39.1[a]	0.21 ± 0.2 [a]
TA-H	3323.5 ± 796.7 [a]	356.2 ± 40.0 [a]	227.0 ± 24.6[a]	0.23 ± 0.05 [a]

Data are expressed as mean ± standard deviation (S.D.) of 10 mice, and were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters in same column are significantly different ($p < 0.05$)

[†]NC, negative control; TA-L: test article low dose, TA-M: test article middle dose; TA-H: test article high dose

Table 4 Immunophenotyping of Murine Splenocytes

Group[†]	Immune cell type (%)				
	T4 cell (CD4^{+},CD3^{+})	T8 cell (CD8^{+},CD3^{+})	B cell (CD19^{+},CD45^{+})	T cell (CD3^{+},CD45^{+})	NK cell (PanNK^{+},CD45^{+})
NC	33.60 ± 2.46 [a]	12.22 ± 2.91 [a]	43.32 ± 7.18[a]	50.29 ± 6.70 [a]	5.41 ± 0.91 [a]
TA-L	32.73 ± 2.98 [a]	12.85 ± 2.34 [a]	45.02 ± 7.62 [a]	48.72 ± 5.99 [a]	6.05 ± 0.52 [a]
TA-M	32.78 ± 2.84 [a]	11.93 ± 2.42 [a]	45.04 ± 6.66 [a]	48.05 ± 5.61 [a]	5.85 ± 1.14 [a]
TA-H	32.41 ± 2.89 [a]	11.00 ± 2.53 [a]	48.43 ± 3.12 [a]	48.55 ± 3.62 [a]	6.20 ± 01.19 [a]

Data are expressed as mean ± standard deviation (S.D.) of 10 mice, and were analyzed using one-way ANOVA followed by Duncan's multiple range test. Values with different alphabet letters in same column are significantly different ($p < 0.05$).

[†]NC, negative control; TA-L: test article low dose, TA-M: test article middle dose; TA-H: test article high dose

Discussion

The use of natural products as a possible remedy is weaved into the history of mankind. Dietary supplements are preparations that conceptually fall into an in-between category that lies somewhere between food and drugs. Hundreds and hundreds of botanicals and their mixtures are used in complementary and alternative medicine or as a part of healthy nutrition. The use of dietary supplements is steadily growing each year with about 50% or the American adult population consuming dietary supplements.

One of these natural supplements is transfer factor, which is used for several decades. In fact, the first observations and establishing of the concept date back to the early 1950s.[1] Transfer factors are small proteins that can transfer cell-mediated immune reactions from immune donors to immunologically naïve recipients.[10] The complete structure of these molecules is still unknown, but most of the studies indicate that the responsible molecules are oligoribonucleopeptides with a MW around 5 kDa. In addition, some conserved sequences have already been established.[11] Both specific and nonspecific transfer factor were prepared traditionally from human and animal leukocytes,[12] but newer research suggested that similar molecules can be found in the immunized egg yolk.[13] Subsequent studies confirmed that egg extracts contain molecules with transfer factor activities.[14] Readers seeking a summary of the current knowledge on transfer factor should see Viza et al.[15]

This study is a follow up of the previously published study showing effects of short-term supplementation of mice with different versions of transfer factor.[15] Throughout the entire experimental period, the animals showed normal growth and weight gains without any signs of clinical problems.

First, we focused on cell-mediated immunity. Professional phagocytes (cells of the monocyte-macrophage lineage and neutrophils) often form the first line of defense. At the same time, activation of their activities represents an important step in a complex process of immune activation. Not surprisingly, phagocytosis is usually one of the first reactions evaluated in studies of immunological effects of natural immunomodulators and supplements. We used a flow cytometry technique[17] and found significant effects on phagocytic activity of peritoneal macrophages. Similar findings were found when we measured the effects of transfer factor supplementation on cytotoxic effects of NK cells. In addition, we found a significant increase in Con A- or LPS-stimulated proliferation of splenocytes, allowing us to conclude that transfer factor can be considered to be a natural stimulator of cell-mediated branch of immune reactions.

The second part of the study focused on humoral immunity. Our data showed some effects, particularly on IL-2 and IFN-γ secretion. Some of these stimulations were dose-dependent. However, several other tested cytokines showed an increase in secretion, similar to the effects on antibody formation, allowing us to conclude that the effects of supplementation with transfer factor has only limited effects on this second branch of immune reactions. We can conclude that in addition to the classical, more established cell-derived transfer factor, the less controversial colostrum- and/or egg-derived transfer factors also offer interesting and substantial immunostimulating capacity and can contribute to the battle for health.

Acknowledgments

Conflicts of interest

The authors have no conflict to declare.

References

1. Lawrence HS. The transfer of generalized cutanous hypersensitivity of the delayed tuberculin type in man by means of the constituents of disrupted leucocytes. *J Clin Invest.* 1954;33:951–952.

2. Estrada Parra S, Chavez Sanchez R, Ondarza Aguilera R, et al. Immunotherapy with transfer factor of recurrent herpes simplex type 1. *Arch Med Res.* 1995;26:S87–S92.

3. Wilson GB, Metcalf JF, Fudenberg HH. Treatment of *Mycobacterium fortuitum* infection with 'transfer factor'. New methodology for evaluation TF potency and predicting clinical response. *Clin Immunol Immunopath.* 1982;23:478–491.

4. Delgado O, Romano EL, Belfort E, et al. Dialyzable leukocyte extract therapy in immunodepressed patients with cutaneous leishmaniasis. *Clin Immunol Immunopathol.* 1981;19:351–259.

5. Fabre RA, Perez TM, Aguilar LD, et al. Transfer factors as immunotherapy and supplement of chemotherapy in experimental pulmonary tuberculosis. *Clin Exp Immunol.* 2004;136:215–223.

6. Wilson GB, Paddock GV. Process for obtaining transfer factor from colostrum, transfer factor so obtained and use thereof. US Patent US4816563A, 1984.

7. Wilson GB, Poindexter C, Fort JF, et al. *De novo* initiation of specific cell–mediated immune responsiveness in chicken by transfer factor (specific immunity inducer) obtained from bovine colostrum and milk. *Acta Virol.* 1988;32(1):6–18.

8. Radosevich JK, Scott GH, Olson GB. Delayed–type hypersensitivity responses induced by bovine colostral components. *Am J Vet Res.* 1985;46(4):875–878.

9. Solberg E. The effects of powdered fertilized eggs on depression. *J Med Food* 2011;14(7-8):870–875.

10. Lawrence HS. Transfer factor in cellular immunity. *The Harvey Lectures, Series.* 1974;68:239–350.

11. Kirkpatrick CH. Transfer factors: Identification of conserved sequences in transfer factor molecules. *Mol Med.* 2000;6(4):332–341.

12. Kirkpatrick CH. Structural nature and function of transfer factors. *Ann N Y Acad Sci.* 1993;685:362–368.

13. Chen DY, Xie MR, Xu G. The study of hyperimmune egg yolk dialysates on transferring delayed type hypersensitivity. Zhongguo Sheng Hua Yao Wa Za Zhi 1999;20:281–282.

14. Xu YP, Zou W, Zhan J, et al. Preparation and determination of immunological activities of anti–HBV egg yolk extraction. *Cell Mol Immunol.* 2006;3(1):67–71.

15. Viza D, Fudenberg HH, Palareti A, et al. Transfer factor: an overlooked potential for the prevention and treatment of infectious diseases. *Fol Biol.* 2013;59(2):53–67.

16. Vetvicka V, Vetvickova J. Effects of transfer factor supplementation on immune reactions in mice. *J Nutr Health Sci.* 2019;6: e301.

17. Bassoe CF. Flow cytometric studies on phagocyte function in bacterial infections. *Acta Pathol Microbiol Immunol Scand.* 1984;92:167–171.