

Effects of Transfer Factor Supplementation on Immune Reactions in Mice

Vetvicka V* and Vetvickova J

University of Louisville, Department of Pathology, Louisville, KY, USA

*Corresponding author: Vetvicka V, University of Louisville, Department of Pathology, 511 S. Floyd, Louisville, KY 40202, USA, Tel: 5028521612, E-mail: vaclav.vetvicka@louisville.edu

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Abstract

Colostrum-derived transfer factors are among the highest potential natural immunostimulatory food supplements. In our study, we evaluated the possible effects of supplementation with various compositions that included transfer factors in phagocytosis, TNF- α and IL-2 secretion, antibody formation and NK cells activity. We found significant improvements of all these immune reactions after 7 days of supplementation.

Keywords: Transfer Factor; Phagocytosis; IL-2; Cytokines; Antibodies, NK Cells

Introduction

The term "transfer factor/s" has various unrelated meanings in science. One definition was developed by H. Sherwood Lawrence, originated from human cells and consisted entirely of amino acids. A second use of the term transfer factor applies to a potentially different entity derived from cow colostrum or chicken egg yolk. Recently, transfer factor has been harvested from sources other than blood, and administered orally, as opposed to intravenously. This use of transfer factors from sources other than blood has not been accompanied by the same concerns associated with blood-borne diseases, since no blood is involved. These are marketed as an oral dietary supplement under the same name citing claims of benefit to the immune system. Generally, transfer factors are a chemical material taken from either humans or animals with already established immunity against certain diseases [1,2].

The idea originated in Chase's observation that cells taken from the peritoneum of guinea pigs that had been immunized could transfer immunity when injected into guinea pigs that had never been exposed to the antigen [3]. Subsequent research attempted to uncover how the cells imparted their effects. Later, Henry Sherwood Lawrence discovered that partial immunity could be transferred even when the immune cells had undergone lysis. This indicates that cells did not need to be fully intact in order to produce immune effects [4]. The original definition described this material as dialyzable leukocyte extract that can transfer antigen-specific immunity from a person whom tests positive for the antigen in a delayed hypersensitivity skin test manner to a person negative for the same antigen. Transfer factor is a small molecule, and it has been the center of a scientific mystery, in part because Dr. Lawrence and other scientists were unable to identify it precisely. Some scientists suspect that transfer factor represents bits of many molecules. At present, neither the precise chemical nature, nor the exact molecular mechanisms of action have been defined. However, transfer factors have been shown to provide both therapeutic and prophylactic benefits [5,6].

However, a setback occurred in the 1980s with the first blood screening tests for HIV infection and the fear of contracting HIV through blood borne products. Additional problems were caused by the scandal from two researchers at Harvard working with transfer factors, who noted that "original positive results may not have been obtained by the procedures described". Culmination of these events set back the interests of research community for decades. Subsequently it took decades before serious research in transfer factor started again [7].

Most recently, transfer factors have been harvested from sources other than blood, and administered orally, as opposed to intravenously. This use of transfer factors from sources other than blood has not been accompanied by the same concerns associated with blood-borne diseases, since no blood is involved. Transfer factor-based nutritional supplements have become extremely popular throughout the world. However, the way transfer factors work is still not clear.

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. Based on studies noting an overlap in the observed *in vitro* effects between molecules contained in colostrum called colostrinin and the dialyzable leukocyte extract, a hypothesis formed that the two were

the same. There has been no recent research investigations comparing the two entities and thus there is no verifiable evidence that either colostrum or egg whites do or do not contain the cellular product that shares the name transfer factors. The orally available transfer factors are not obtained from humans or from blood products of any mammal or animal and does not carry the presumed risks of contracting blood borne or animal tissue derived diseases. It is true that few studies found positive effects of colostrum-derived transfer factor, but it was obtained from previously immunized cows against specific antigens. This type of material was able to transfer the immunity from cows to chickens [5]. Similarly, it transferred the immunity to young calves [6]. In all cases, the transfer factors and the subsequent transfer of immunity was specific [8].

In our study, we focused on various combinations of transfer factor composition and evaluated not only the direct immunological effects of transfer factor alone, but also possible synergistic effects of additional bioactive molecules.

Materials and Methods

Animals

Female, 8 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO₂ asphyxiation.

Cell line

Cancer cell line YAC-1 (ATCC, Manassas, VA) was maintained in culture at 37 °C in a humidified atmosphere supplemented with 5% CO₂ in RPMI 1640 medium supplemented with 10% FCS.

Material

Wright stain and RPMI 1640 medium were purchased from Sigma (St. Louis, USA), fetal calf serum (FCS) from Hyclone Laboratories (Logan, UT, USA).

Transfer Factor Samples

Sample Name	Ingredient Composition	Dose (mg/kg)
Transfer Factor Plus (TFP)	Colostrum ultrafiltrate Colostrum nanofiltrate Egg yolk Soy phytosterols IP-6 Maitake mushroom Shiitake mushroom <i>Cordyceps sinensis</i> <i>Agaricus blazeii</i> Baker's yeast Lemon peel Zinc methionine Oat seed Olive leaf Aloe vera	506
Transfer Factor Tri-Factor Formula, (TF)	Colostrum ultrafiltrate Colostrum nanofiltrate Egg yolk	123
Transfer Factor Plus without Tri-Factor Formula, (TFP – TF)	Soy phytosterols IP-6 Maitake mushroom Shiitake mushroom <i>Cordyceps sinensis</i> <i>Agaricus blazeii</i> Baker's yeast Lemon peel Zinc methionine Oat seed Olive leaf Aloe vera	383
Wheymune, (WM)	Enzyme-cleaved sweet dairy whey proteins	123
Transfer Factor Tri-Factor Formula + Wheymune, (TF + WM)	Colostrum ultrafiltrate Colostrum nanofiltrate Egg yolk Enzyme-cleaved sweet dairy whey proteins	246

Table 1: Composition of samples

All samples were kindly donated by 4Life Research, Sandy, UT, USA and by Glanbia Nutritionals, Carlsbad, California, USA (Whey-mune). The animals were supplemented with individual samples orally for 7 days (for 21 days in case of antibody response) at doses shown in Table 1.

Phagocytosis

The technique employing phagocytosis of synthetic polymeric microspheres was described earlier [9]. Briefly: peripheral blood cells were incubated with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; 5×10^8 /ml). The test tubes were incubated at 37 °C for 60 min., with intermittent shaking. Smears were stained with Wright stain which allowed us to see the microparticles inside the cells the cells with three or more HEMA particles were considered positive. The same smears were also used for evaluation of cell types.

IL-2 Secretion

Purified spleen cells (2×10^6 /ml in RPMI 1640 medium with 5% FCS) obtained from mice fed with samples were added into wells of a 24-well tissue culture plate. Cells were incubated for 48 hrs in a humidified incubator (37 °C, 5% CO₂/95% air). Addition of 1 µg of Concanavalin A (Sigma) was used as a positive control. At the endpoint of incubation, supernatants were collected, filtered through 0.45 µm filters and tested for the presence of IL-2 using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

TNF-α Secretion

Mice were sacrificed, blood collected, serum prepared and filtered through 0.45 µm filters. The level of TNF-α was determined using Quantikine mouse TNF-α kit (R & D Systems, Minneapolis, MN, USA) as described earlier [10].

Antibody Formation

The technique was described earlier [10]. Briefly: formation of antibodies was evaluated using ovalbumin (Sigma) as an antigen. Mice were injected twice (two weeks apart) with 100 µg of albumin and the serum was collected 7 days after last injection. Experimental groups were getting daily ip. injection of glucan. Level of specific antibodies against ovalbumin was detected by ELISA. As a positive control, a combination of ovalbumin and Freund's adjuvant (Sigma) was used.

NK Cell Assay

Splenic cells were isolated from the spleen of mice by standard methods. A cell suspension was generated by pressing minced spleen against the bottom of a petri dish containing PBS. After elimination of erythrocytes by 10-second incubation in distilled water, and five washes in cold PBS, the cells were resuspended in PBS and counted. The viability was determined by trypan blue exclusion. Only cells with viability better than 95% were used in subsequent experiments. Splenocytes (10^6 /ml; 0.1 ml/well) in V-shaped 96-well microplates were incubated with 50 µl of target cell line YAC-1. After spinning the plates at $250 \times g$ for 5 min, the plates were incubated for 4 h at 37 °C. The cytotoxic activity of cells was determined by the use of CytoTox 96 Non-Radioactive Cytotoxicity Assay from Promega (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 10 µl of lysis solution was added into appropriate control wells 45 min before the end of incubation. The plates were spun at $250 \times g$ for 5 min, followed by transferring 50 µl of the supernatant into flat-bottomed, 96-well microplates. After 50 µl of reconstituted substrate was added into each well, plates were covered and incubated for 30 minutes at room temperature in the dark. The optical density was determined by using a STL ELISA reader (Tecan U.S., Research Triangle Park, NC, USA) at 492 nm. Specific cell-mediated cytotoxicity was calculated using the formula:

Percent-specific killing (% cytotoxicity) = $100 \times [(OD_{492} \text{ experimental} - OD_{492} \text{ spontaneous}) / (OD_{492} \text{ maximum} - OD_{492} \text{ spontaneous})]$ as described in the manufacturer's instructions, where spontaneous release was that from target cells incubated with medium alone and maximum release was that obtained from target cells lysed with the solution provided in the kit.

Statistical Analysis

Paired t-test statistical significance was evaluated (GraphPad Prism 5.04; GraphPad Software, USA). An average and standard deviation was evaluated after determining standard value composition (D'Agostino, Pearson).

Results

To test the effects of our samples on cellular immunity, we used phagocytosis. We employed synthetic hydroxyethyl methacrylate particles known for minimal nonspecific adhesion to the membrane of phagocytosing cells [9], and evaluated the effects on phagocytic ability of peripheral blood monocytes and neutrophils. Data shown in Figure 1 demonstrate the effects of our samples on phagocytosis of peripheral blood cells. Several trends can be observed – Transfer Factor Plus had no effects compared to the control, but Tri-Factor significantly stimulated phagocytic activity of both cell types. Surprisingly, when we used Transfer Factor Plus without the Tri-Factor, we found the stimulation be the same as Tri-Factor alone and significantly higher than Transfer Factor Plus alone. Wheymune alone had no activity compared to the control, and in combination with Tri-Factor we found significant synergistic effects.

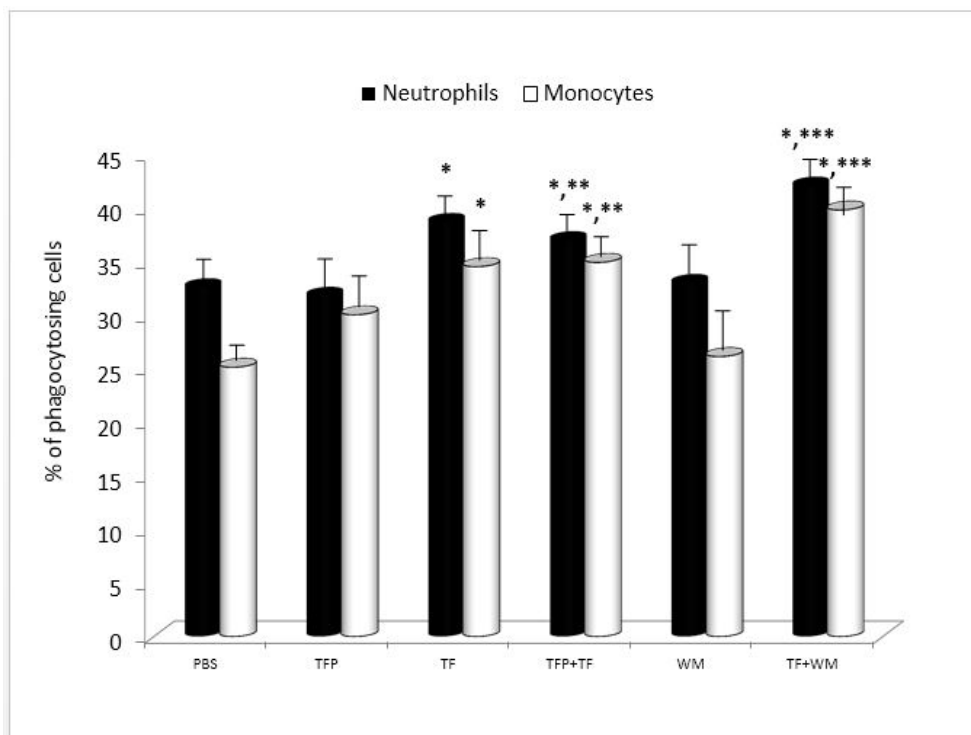


Figure 1: Effects of supplementation with individual samples on the phagocytosis of microparticles by peripheral blood neutrophils and monocytes. Experiments were repeated three times, results are given as mean ± SD. *Represents significant differences between control and samples at P ≤ 0.05 level, ** significant differences between TFP alone and TFP-TF combination, and *** significant differences between Welmune alone and TP-WM combination.

For evaluation of the effects on cytokine production, we measured the production of TNF-α in the blood (*in vivo* experiment) and IL-2 by splenocytes (*in vitro* experiments). In the case of TNF-α, all samples significantly increased the production of this cytokine, with the strongest effects of Transfer Factor Plus without the Tri-Factor (Figure 2). The TFP-TF combination was significantly more active than the individual parts alone. Addition of Tri-Factor to Wheymune strongly potentiated the effects of Wheymune alone. In the case of IL-2 production, all samples significantly increased the IL-2 secretion, with no significant differences between individual samples (Figure 3).

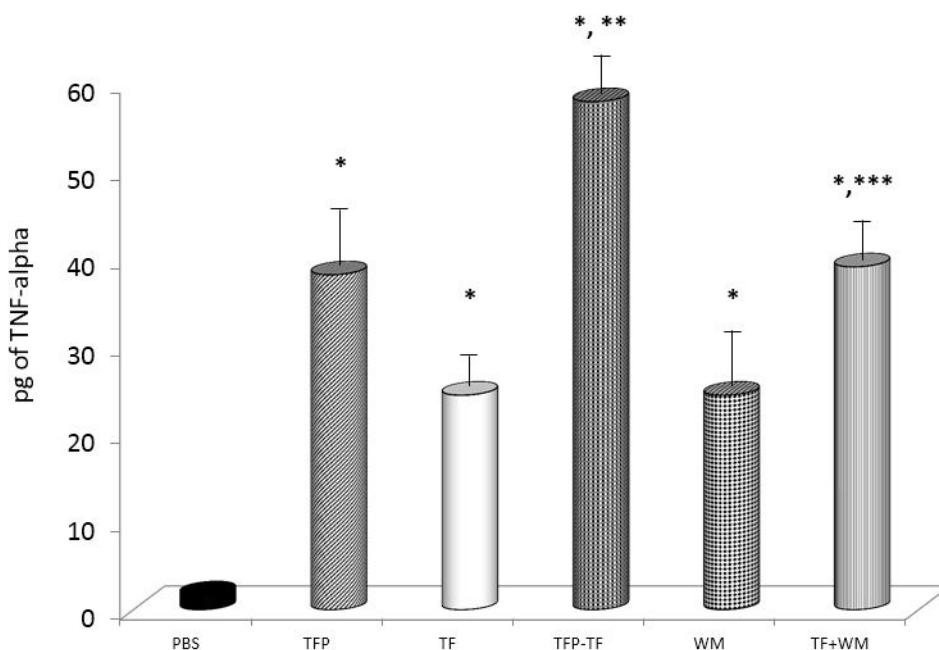


Figure 2: Effects of supplementation with individual samples on secretion of TNF-α. Experiments were repeated three times, results are given as mean ± SD. *Represents significant differences between control and samples at P ≤ 0.05 level ** significant differences between TFP alone and TFP-TF combination, and *** significant differences between Welmune alone and TP-WM combination

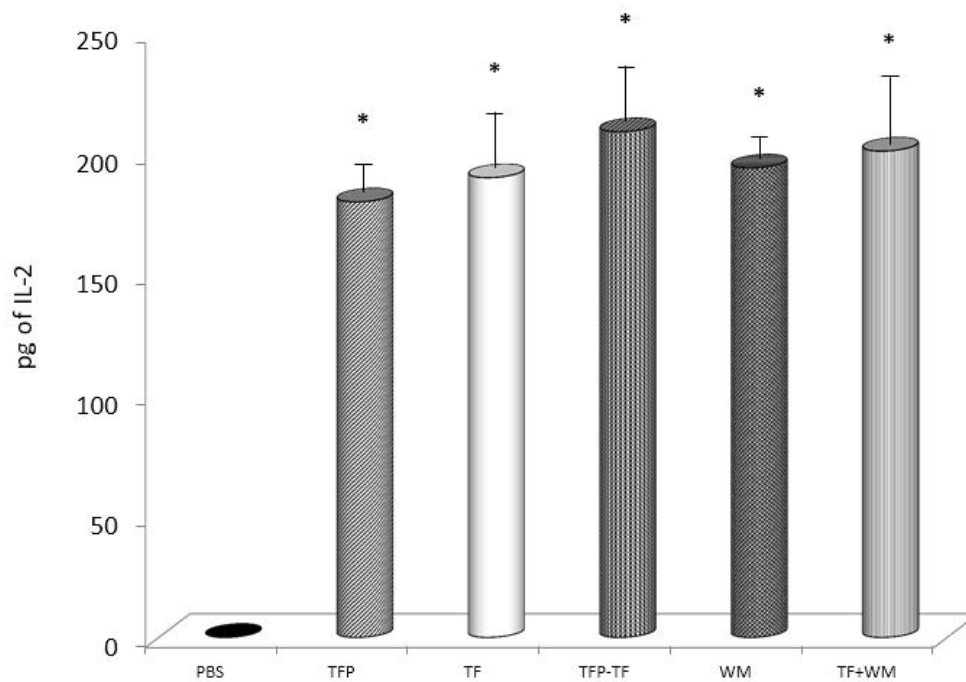


Figure 3: Effects of supplementation with individual samples on secretion of IL-2 by splenocytes. Experiments were repeated three times, results are given as mean ± SD. *Represents significant differences between control and samples at P ≤ 0.05 level

In the next step, we evaluated a lesser known area of transfer factor effects - antibody response. We immunized mice with ovalbumin, where samples were applied together with two separate intraperitoneal injections of antigen. As a positive control, ovalbumin was used with Freund’s adjuvant. The results summarized in Figure 4 showed that all samples significantly improved antibody response, with Transfer Factor Plus without the Tri-Factor and Tri-Factor with Wheymune being the most active ones.

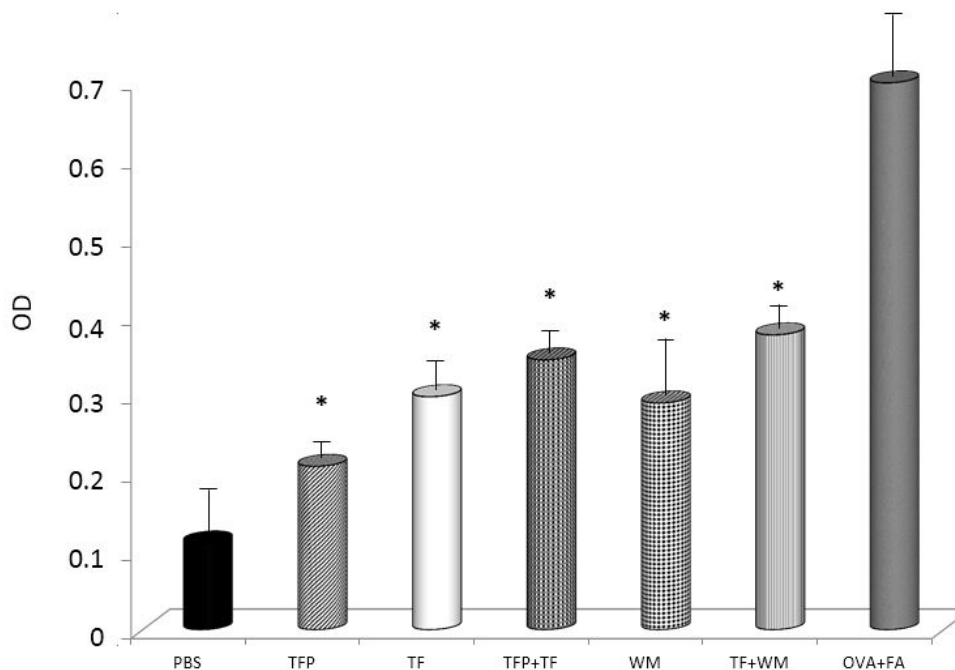


Figure 4: Effects of three week oral delivery of tested samples on formation of antibodies against ovalbumin. Animals were injected twice (two weeks apart) with antigen and the serum was collected 7 days after last injection. Level of specific antibodies against ovalbumin was detected by ELISA. As positive control, Freund’s adjuvant was used. *Represents significant differences between control (ovalbumin alone) and samples at P ≤ 0.05 level.

In the last part of our study, we tested the effects on NK cell activity. We found that all samples increased the killing of YAC-1 cells, with the highest effects from Transfer Factor Plus without the Tri-Factor. In the case of Wheymune, addition of Tri- Factor had significant effects (Figure 5) on NK cell stimulation.

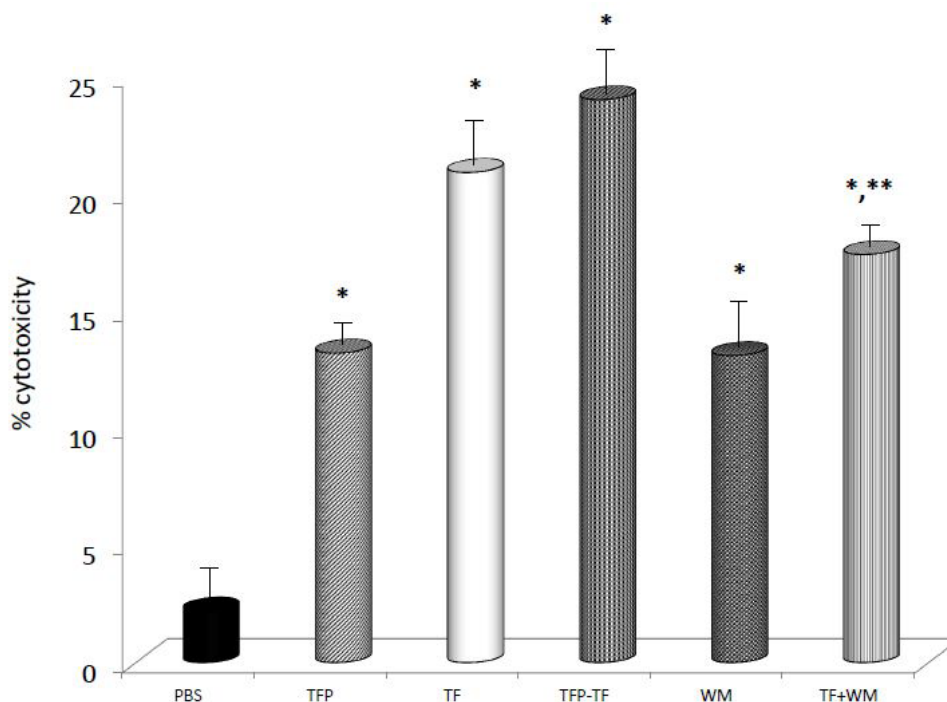


Figure 5: Effect of tested samples on NK cell activity. Ratio of 50:1 was used. Each value represents the mean \pm SD. *Represents significant differences between control and experimental samples at $P \leq 0.05$ level, ** significant differences between Welmune alone and TP-WM combination

Discussion

People have been appealing to nature for curing various diseases since ancient times. One natural supplement is transfer factor, used for several decades. Most of scientific and/or clinical studies of transfer factors have been based on the specificity of each transfer factor, e.g., transfer factor that is specific for *Herpes* infection will prevent recurrent infection with this virus, but not with anything else. Transfer factors were found to selectively enhance development of specific T lymphocytes [11], express anti-HBV activities [12], be specific to human sperm antigen [13], be useful as immunotherapeutic and supplemental agents in therapy of pulmonary tuberculosis [14], to have some synergy with zidovudine in HIV patients [15], to extend some effects on malignant melanomas [16], and to help to improve experimental glioma [17].

It is important to note, however, the companies marketing most current transfer factors use a different approach - they often ignore the specificity and make nonspecific claims of boosting the immune system. The aim of our study was to find if regular colostrum- based transfer factors had any immunological activity and, in case of positive effects, this activity can be positively changed by changing the formula. Despite significant progress in elucidation of the effects and mechanisms of action of numerous natural immunomodulators, the search for improved biological properties is still on. One of the possible ways how to improve the stimulation of the immune responses is the combination of various immunomodulators. Polysaccharides are one of the most promising immunomodulators, therefore they were added to the formula to see any possible synergistic effects.

For a long time transfer factors were used only by injection, most of all by subcutaneous injection. However, some studies using mouse models showed that there are no substantial differences in response to either oral or injected transfer factor [18]. Despite some skepticism for its use in clinical practice, the use of transfer factors as tailor-made immunotherapy in the treatment of some diseases is quite valuable. But before its administration, it is important to evaluate the specificity, potency, and the best dose. In our study, we focused on evaluation of the effects of colostrum-derived transfer factor on immune reactions.

The best known effects of various natural immunomodulators consist of the augmentation of phagocytosis of professional phagocytes – granulocytes, monocytes, macrophages and dendritic cells. Activation of professional phagocytes represents an important element of more complicated processes, when mediator molecules secreted by them (such as interleukin-1, interleukin-9, or tumor necrosis factor) initiate inflammation reactions. Not surprisingly, we started our evaluation of glucan activities by phagocytosis. We used the 2-hydroxyethyl methacrylate particles that have only a slight negative charge and thus do not nonspecifically adhere to the cell surface. This guarantees that only phagocytosing cells will engulf these particles and it significantly lowers the chance of false negativity [19]. Our results showed solid effects on cellular branch of immune reactions (phagocytosis and NK cell activity) suggesting that transfer factor can be considered to be a natural immunomodulator.

Several cytokines are known to be affected by supplementation with natural immunomodulators. In our study, we focused on production of IL-2 by splenocytes *in vitro* and on production of TNF- α *in vivo*. Under normal steady-state conditions, splenocytes produce no IL-2, which is the reason why all samples showed significant effects. However, none of them had effects comparable to positive effects of Concanavalin A (1,229 pg of IL-2, data not shown). Similar data were found in case of TNF- α secretion. These results confirmed the hypothesis that transfer factor is not only a stimulator of cellular immunity, but extends its effects on humoral branch, too.

Conclusion

With respect of the two aims of this study, the first one was answered quite clearly – there was significant immune-stimulating activity in a wide spectrum of immune reactions. To answer the second question is more difficult, as the results of experiments trying to find the optimal composition are not clear. On the one hand, a combination of transfer factors with Wheymune clearly improved Wheymune's immunostimulating activity. On the other hand, however, sample of Transfer Factor Plus without the transfer factor reagents consistently showed better or similar results than Transfer Factor Plus or Transfer Factor alone. Experiments aiming on answering this question are currently in progress.

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