Acta virologica **64**: 271 – 275, 2020 doi: 10.4149/av_2020_303

MINIREVIEW

Obstacles and limitations of transfer factor biological activity assay design

V. KEMPOVÁ, M. ZAŤOVIČOVÁ, I. KAJANOVÁ, L. JELENSKÁ, L. KLIMKO, J. KOPÁČEK, V. ZELNÍK*

Institute of Virology, Biomedical Research Center Slovak Academy of Sciences, Dúbravská cesta 9, 845 05 Bratislava, Slovak Republic

Received May 28, 2020; accepted July 15, 2020

Summary. – Transfer factor (TF) is a heterogeneous mix of low-molecular weight molecules obtained from dialyzed leukocyte extract that is capable of transferring cell-mediated immunity. As an immunostimulatory drug TF is used to improve treatment of infectious diseases, allergies, cancer and immune deficiencies. The main benefit of TF preparations as immunotherapeutic agents is the induction of a rapid immune response and the potential of TF as an adjuvant in combination with other drugs might lead to development of novel approaches to combat various diseases in the future. The process of TF preparation is rather simple. However, with respect to fact that TF is composed by several multifunction molecules, it is likely that during the activity measurement based only on one single parameter, other TF biological activities might be overlooked. In addition, separated TF components might display synergetic activity effect. According to recent European Pharmacopoeia there is no general protocol for immuno-stimulatory drugs (including TF) activity measurement available. Nevertheless, for the process of TF preparation, control of input material and for final pharmaceutical product batches it is inevitable to guaranty proper quality control including appropriate *in vivo* or *in vitro* test(s) for TF biological activity assay. The animal-origin materials and *in vivo* assays convey a considerable logistic, ethic and economic problem, meanwhile the available *in vitro* assays have been reported with limited reproducibility and sometimes contradictory results. The currently used method for testing biological activity of TF is the *in vitro* MTT cells proliferation assay that is recognized by control authorities in Slovak Republic.

Keywords: immune system; transfer factor; dialysable leukocyte extract; diseases; MTT cells proliferation assay

Introduction

The immune system plays a pivotal role in the life of each individual and its interaction with surrounding ecosystem. Activation of our immune system occurs only when the organism encounters a pathogen and our body is exposed to infection. Therefore, in general, the greater exposure of the organism to the pathogen means the better immunity. Neonates acquire passive immunity from breast milk, which has been shown to contain also immunostimulants like lactoferrin (Sánchez *et al*., 1992) and transfer factor (TF), which are non-antigen specific, making them universally effective for a wide range of pathogens (Krishnaveni, 2013). TF known also as the dialysable leukocyte extract (DLE) consists of small peptides and oligoribonucleotides with a molecular weight of 3.5–6.0 kDa (Sanchez-Gonzalez *et al*., 2011). Lawrence first described TF in 1955 based on the finding that the

*E-mail: viruzelo@savba.sk; phone: +421-2-5930-2463.

Abbreviations: Aza = azathioprine; BrdU = bromodeoxyuridine; DLE = dialysable leukocyte extract; DTH = delayed-type hypersensitivity; IFN-γ = interferon gamma; IL = inteleukin; TB = tuberculosis; TF = transfer factor

dialysable extracts of human leukocytes can passively transfer delayed-type hypersensitivity (DTH) to allergens from an immune donor to a naïve recipient. He prepared an intracellular extract from circulating leucocytes of patients who had been exposed to tuberculosis (TB) and then injected the leukocyte extract into volunteer patients non-exposed to TB demonstrating that the immune system of non-exposed to TB patients treated with leukocyte extract can recognize TB and respond to it, as if it has already fought it (Lawrence, 1955).

Since commercial TF preparations are formed as a collection of leukocyte extracts from several hundred donors, considerable heterogeneity and complexity of the molecules contained in TF is thus ensured. Therefore, determining the exact composition of TF or at least its major components is complicated.

Mechanism of transfer factor action

From the adaptive immunity point of view, TF contains peptide molecules capable transfer part of cell-mediated immunity (CMI) of (sensitized) donors to (unimmunized) individuals.

The TF is produced by CD4+Th1 cells during the immune response to an antigen. Increased Th1 in turn repress the production of Th2 and its cytokines like interleukin (IL)-4, IL-5, IL-6, and IL-13. A remarkable feature of transfer factor is eliciting multiple, contradictory functions (Borkowsky and Lawrence, 1983; Alvarez-Thull and Kirkpatrick, 1996). One activity is the presence of the inducer and helper functions, so-called inducer factor and antigen-specific factor. An additional activity is the presence of the suppressive (regulatory) function, so-called suppressor factor. The inducing factor sends a specific signal to the cells of the immune system and, by culturing a population of non-immune leukocytes with the inducing factor, is able to react to a specific antigen (Lawrence and Borkowsky, 1996). In addition, inducer factor enhances the antigenic stimulus, which causes the production of interferon gamma (IFN-γ), IL-2 and tumour necrosis factor alpha (TNF-α) by CD4+Th1 cells. As a consequence, cell-mediated immune response develops against the target antigen. Here, antigen specific factors or fractions aid the function of recognizing and memorizing pathogenic organisms in a faster manner. Secondly, inducer fraction increases the antigenic stimulus whereas, suppressor fraction acts by releasing IL-10, an inhibitory cytokine from Th2 cells, playing a vital role in controlling immune over reactions, mistargeted reactions in the development of autoimmune disorders (Krishnaveni, 2013). While stimulating cell mediated immunity, it does not increase antibody secretion nor its responses against the same specific antigen. So, transfer factors develop cell mediated responses in patients who are suffering from immunodeficiency, infectious diseases, as well as in disorder with certain allergies. Maturation of naïve T cells as well as increased cell mediated immunity are regulated by thymus factors. It is agreed that TF is more efficient in educating naïve cells about the approaching danger (Khan *et al.*, 1975).

Kirkpatrick (2000) analyzed the peptide partial conserved sequences of TF and found a novel amino acid consensus sequence LLYAQDL/VEDN, which was found in each of the analyzed TF preparations. This sequence binds with high affinity to specific receptors (TF receptors) of target cells. However, tyrosine and glycine are always more concentrated in TF (Berrón-Pérez *et al.*, 2007).

Obtaining transfer factor preparations

The first TF preparations were obtained in the Lawrence laboratory by dialysis of human leukocyte cryolysates (Lawrence, 1955). An adapted method according to Lawrence (1974) is still used to prepare DLE preparations. DLEs are extracted from peripheral blood leukocytes (buffy coats) which have been isolated from more than 1000 healthy human blood donors. After several cycles of freeze and thaw, cell lysis occurs, after which the DLE are dialyzed, concentrated by lyophilisation and ultra-filtered with tangential flow filtration (TFF) system, using a 10 kDa membranes and heated at 60°C. Finally, the DLE preparations are filter sterilized, aliquoted in ampules and lyophilized (Lawrence, 1974). Later, dialysis was replaced by ultrafiltration using membrane filters of size <12 kDa (Fudenberg and Pizza, 1994; Perepechkina and Perepechkin, 1999; Vacek *et al.*, 2002). The processes of TF preparation are still in use nowadays, but are not very favourable and alternative ways of TF preparation are explored. During the DLE concentration there is concentrated not only the TF but also salts content causing the pain during and after injection application of the drug under the skin of the patient. The dose volume administered is also in a rather large volume. Therefore, the researchers focused mainly to find a method that can accurately determine the biological activity of TF, to find a better way to prepare TF, in which the preparation does not have as much salts, and to reduce the volume of drug administered.

The specific TF preparations are obtained from both, animal and human sources by injecting them with certain pathogen to produce specific TF (Krishnaveni, 2013).

No adverse contraindications and side effects have been reported so far with transfer factor treatment (Khan *et al.*, 1978), and it is valuable when administered orally as

well as by injection (Kirkpatrick *et al*., 1995; Kirkpatrick, 1996). Long-term oral administration is conveniently safe (Pizza *et al*., 1996a) and easily accepted by infants or elderly people who are at the risk for numerous infections.

Transfer factor in diseases treatment

Transfer factors influence the activities of various immune components and also regulate cytokines profiles (Kirkpatrick, 1996). Imbalances in the production of transfer factor lead to the development of rheumatoid arthritis, cancer, Alzheimer's disease, heart disease, hepatitis and other disorders. The time taken for complete development of immature immune response or delayed hypersensitivity is 10–14 days, but the TF induces an immune response in within 24 h (Lawrence and Borkowsky, 1996). During the treatment with TF there has to be considered antigenic specificity, strength of the extract and recipients' immune status and also the proper dose (Fudenberg and Fudenberg, 1989).

Transfer factor and cancer

In study by Pineda *et al.* (2005), the authors demonstrated the beneficial effect of TF use in immunotherapy in experimental therapy of C6 malignant glioma in rats. Carmustine was used together with TF. This synergistic effect of TF and chemotherapy resulted in a decrease in tumour size in rats as well as an increase in the number of CD2+, CD4+, CD8+, as well as the number of natural killer (NK) cells. Based on this study, the authors concluded that due to the synergistic effect of both substances, it is possible to reduce the doses of chemotherapy itself in the future (Pineda *et al*., 2005). In additional *in vitro* studies it was shown that the presence of TF caused the ability of lymphocytes to destroy cancer cells (Franco-Molina *et al*., 2006).

Franco-Molina *et al.* (2008) tested the use of adjuvant immunotherapy with bovine DLE (in the form of preparation IMMUNEPOTENT CRP) against lung cancer. Patients receiving conventional therapy with IMMUNEPOTENT CRP showed an increase in total leukocyte and T-lymphocyte subpopulations CD4+, CD8+, CD16+ and CD56+ as well as an increased quality of patients' lives. Combination of classical radiation and chemotherapy together with IMMUNEPOTENT CRP has suggested immunologic protection against chemotherapeutic side effects in non-small cell lung cancer (NSCLC) patients (Franco-Molina *et al*., 2008).

Other human diseases

For more than 30 years, an effective anti-HIV vaccine has been sought through immunotherapy. Initial results of studies of the specific TF effect in the treatment of AIDS have shown overall clinical improvement and restoration of the skin test reactivity of patients and a slight increase in their CD4+ cell counts (Viza *et al*., 1987), DTH was restored to recall antigen and CD4+ and CD8+ cell counts of the patients were increased (Pizza *et al*., 1996b). Such an increase in CD8+, as well as an increase in total leukocyte number and IL-2 level, in AIDS patients after treatment with HIV-specific TF has been also reported by other authors (Raise *et al*., 1996). Bovine DLE can reduce the transcription of HIV-1 and inactivate the nuclear factor-kappa B (NF-κB) signalling pathway (Lara *et al*., 2011).

The effect of TF preparations has also been studied in the treatment of infections caused by herpes simplex virus, both HSV-1 (cold sores) and HSV-2 (genital) (Khan *et al*., 1981), as well as in the treatment of varicella-zoster infections. Patients treated with TF had elevated CD4+ cells, CD4/CD8 ratio as well as γ-interferon level, which is very important in the treatment of varicella zooster infections (Steele *et al*., 1980; Bowden *et al*., 1985; Estrada-Parra *et al*., 1998). These findings bring TF to the forefront over other conventional antiviral agents. The greatest effect of TF preparations has been reported in the treatment of cytomegalovirus (CMV) infections (Viza *et al*., 2013).

TF preparations have also been tested in the treatment of other viral diseases such as hepatitis B and human papillomavirus (Pizza *et al*., 1979; Roda *et al*., 1985), as well as alternatives to vaccines against emerging deadly influenza viruses (Viza *et al*., 2013).

In addition to viral diseases, the spectrum of use of TF preparations is relatively broad. As early as 40 years ago, TF preparations were used in the treatment of mycobacterial infections (*Mycobacterium tuberculosis*) (Whitcomb and Rocklin, 1973) and later found that the treatment of these infections was dose-dependent (Viza *et al*., 2013).

Biological activity of DLE

For the DLE production and quality control it is necessary to test DLE biological activity, because there is a possibility that the DLE biological activity can have a complex cell regulation mechanism due to the DLE bipartite molecular structure, putative DLE cellular receptors and the DLE-immune specific binding with corresponding antigen (Lawrence and Borkowsky, 1983), which has not yet been confirmed. Despite strong clinical evidence of the potential use of DLE in the treatment of various diseases, the molecular mechanisms responsible for the

DLE biological activity are still unclear. Part of the general disinterest in the DLE basic research is the absence of an adequate and universally accepted DLE *in vitro* and/or *in vivo* model (Cardoso *et al.*, 2017). There are several models used for DLE biological activity quantification, including: E-rosette test (Valdimarsson and McGuire, 1977), induction of delayed type hypersensitivity in mice (Kirkpatrick *et al.*, 1995), leukocyte migration (Pizza *et al.*, 1996a) and IFN-γ secretion (Medina-Rivero *et al.*, 2014). However, these assays, *in vivo* or *in vitro*, also have their limitations and insufficiencies.

MTT assay is a widely accepted colorimetric method for cell proliferation assay which is used to measure the TF biological activity (Mosmann, 1983; Gerlier and Thomasset, 1986). The MTT assay is a method by which cell proliferation can be monitored and also the viability of metabolically active non-dividing cells can be quantified. The amount of generated formazan is proportional to the number of cells and their metabolic activity (Berridge and Tan, 1993; Bernas and Dobrucki, 2002).

Cardoso *et al.* (2017) tested a new way to determine the biological activity of the commercial preparation TF (IMMODIN®) cell proliferation, by using two methods – MTT and/or BrdU (bromodeoxyuridine) incorporation assays. When the cells, A20 or Jurkat were treated with +Aza/+DLE (azathioprine) they observed a significantly higher proliferation, when compared with +Aza/-DLE. In the absence of Aza, cells did not present any proliferation difference between –DLE or +DLE treatments. Both assays, MTT and BrdU showed similar results. Although using MTT assay, to test the biological activity of DLE on Jurkat cells, they found consistent results with acceptable reproducibility and linearity of the assay, this treatment of the cells did not result into significant IL-2 or IFN-γ secretion, and known lymphocyte proliferative drugs failed to rescue Jurkat cells viability in the presence of +Aza, as +DLE treatment. In conclusion, they were able to prove that their novel cell proliferation-based MTT assay for TF biological activity displays considerable consistency, robustness and cost effectiveness, presenting important advantages over previous DLE activity *in vitro* and *in vivo* assays (Cardoso *et al.*, 2017).

Conclusion

The role of the immune system is to protect the organism against foreign or self-modified endogenous substances. Various pathogens have developed a number of strategies to trick our immune system and thus to avoid immune reactions. Therefore, the immune system must constantly evolve, adapt to new threats and eliminate antigens by identifying each cell. In the search for new approaches to immunotherapy for infectious, oncological and autoimmune diseases, DLE has been discovered, along with TF as a part of it. For further extended potential use of TF in immunotherapies, standardized and validated method(s) have to be developed for TF biological activity assay and quality control of its production. Such methods have to be fully accepted and supported by state drug control authorities.

Acknowledgments. This work was supported by the contribution of the Slovak Research and Development Agency under the project APVV-15-0720.

References

Alvarez-Thull L, Kirkpatrick CH., Biotherapy 9, 55–59, 1996. https://doi.org/10.1007/BF02628657

Bernas T, Dobrucki J., Cytometry 47, 236–242, 2002. https://doi.org/10.1002/cyto.10080

Berridge MV, Tan AS., Arch. Biochem. Biophys. 303, 474–482, 1993. https://doi.org/10.1006/abbi.1993.1311

Berrón-Pérez R, Chávez-Sánchez R, Estrada-García I, Espinosa-Padilla S, Cortez-Gómez R, Serrano-Miranda E, Ondarza-Aguilera R, Pérez-Tapia M, Pineda Olvera B, Jiménez-Martínez Mdel C, Portugués A, Rodriguez A, Cano L, Pacheco PU, Barrientos J, Chacón R, Serafín J, Mendez P, Monges A, Cervantes E, Estrada-Parra S., Rev. Alerg. Mex. 54, 134–139, 2007.

Borkowsky W, Lawrence HS, In Kirkpatrick CH, Burger DR, Lawrence HS (Eds): Immunobiology of Transfer Factor. Academic Press, New York, pp. 75–89, 1983. https://doi.org/10.1016/B978-0-12-409850-3.50012-7

Bowden RA, Siegel MS, Steele RW, Day LM, Meyers JD., J. Infect. Dis. 152, 1324–1327, 1985. https://doi.org/10.1093/infdis/152.6.1324

Cardoso FM, Tomkova M, Petrovajova D, Bubanova M, Ragac O, Hornakova T., J. Pharmaceut. Biomed. 138, 100–108, 2017. https://doi.org/10.1016/j.jpba.2017.01.052

Estrada-Parra S, Nagaya A, Serrano E, Rodriguez O, Santamaria V, Ondarza R, Chavez R, Correa B, Monges A, Cabezas R, Calva C, Estrada-Garcia I., Int. J. Immunopharmacol 20, 521–535, 1998. https://doi.org/10.1016/S0192-0561(98)00031-9

Franco-Molina MA, Mendoza-Gamboa E, Miranda-Hernández D, Zapata-Benavides P, Castillo-León L, Isaza-Brando C, Tamez-Guerra RS, Rodríguez-Padilla C., Cytotherapy 8, 408–414, 2006. https://doi.org/10.1080/14653240600847266

Franco-Molina MA, Mendoza-Gamboa E, Zapata-Benavides P, Vera-García ME, Castillo-Tello P, García de la Fuente A, Mendoza RD, Garza RG, Témez-Guerra RS, Rodríguez-Padilla C., Cytotherapy 10, 490–496, 2008. https://doi.org/10.1080/14653240802165681

Fudenberg HH, Fudenberg HH., Annu. Rev. Pharmacol. 29, 475–516, 1989. https://doi.org/10.1146/annurev.pa.29.040189.002355

Fudenberg HH, Pizza G., Prog. Drug Res. 42, 309–400, 1994. https://doi.org/10.1007/978-3-0348-7153-2_7

Gerlier D, Thomasset N., J. Immunol. Methods 94, 57–63, 1986. https://doi.org/10.1016/0022-1759(86)90215-2

Khan A, Hansen B, Hill NO, Loeb E, Pardue AS, Hill JM., Dermatologica 163, 177–185, 1981. https://doi.org/10.1159/000250157

Khan A, Sellars W, Grater W, Graham MF, Pflanzer J, Antonetti A, Bailey J, Hill NO., Ann. Allergy. 40, 229–232, 1978.

Khan A, Sellars WA, Gabela P, Thometz D., New. Engl. J. Med. 292, 868–869, 1975. https://doi.org/10.1056/NEJM197504172921616

Kirkpatrick CH., Biotherapy 9, 13–16, 1996. https://doi.org/10.1007/BF02628651

Kirkpatrick CH., Mol. Med. 6, 332–341, 2000. https://doi.org/10.1007/BF03401941

Kirkpatrick CH, Hamad AR, Morton LC., Cell. Immunol. 164, 203–206, 1995. https://doi.org/10.1006/cimm.1995.1162

Krishnaveni M., Drug Invention Today 5, 153–156, 2013. https://doi.org/10.1016/j.dit.2013.04.002

Lara HH, Ixtepan-Turrent L, Garza-Trevino EN, Badillo-Almaraz JI, Rodriguez-Padilla C., BMC Res. Notes 4, 474–482, 2011. https://doi.org/10.1186/1756-0500-4-474

Lawrence HS., J. Clin. Invest. 34, 219–230, 1955. https://doi.org/10.1172/JCI103075

Lawrence HS., Harvey Lect. 68, 239–350, 1974. https://doi.org/10.1177/0145482X7406800513

Lawrence HS, Borkowsky W., Cell. Immunol. 82, 102–116, 1983. https://doi.org/10.1016/0008-8749(83)90145-4

Lawrence HS, Borkowsky W., Biotherapy 9, 1–5, 1996. https://doi.org/10.1007/BF02628649

Medina-Rivero E, Merchand-Reyes G, Pavon L, Vazquez-Leyva S, Perez-Sanchez G, Salinas-Jazmin N, Estrada-Parra S, Velasco-Velazquez M, Perez-Tapia SM., J. Pharmaceut. Biomed. 88, 289–294, 2014. https://doi.org/10.1016/j.jpba.2013.09.004

Mosmann T., J. Immunol. Methods 65, 55–63, 1983. https://doi.org/10.1016/0022-1759(83)90303-4

Perepechkina NP, Perepechkin LP., J. Membrane Sci. 160, 1–6, 1999. https://doi.org/10.1016/S0376-7388(99)00045-9

Pineda B, Estrada-Parra S, Pedraza-Medina B, Rodriguez-Ropon A. Pérez R, Arrieta O., J. Exp. Clin. Canc. Res. 24, 575–583, 2005.

Pizza G, De Vinci C, Fornarola V, Palareti A, Baricordi O, Viza D., Biotherapy 9, 175–185, 1996a. https://doi.org/10.1007/BF02628677

Pizza G, Chiodo F, Colangeli V, Gritti F, Raise E, Fudenberg HH, De Vinci C, Viza D., Biotherapy 9, 41–47, 1996b. https://doi.org/10.1007/BF02628655

Pizza G, Viza D, Roda A, Aldini R, Roda E, Barbara L., New Engl. J. Med. 300, 1332, 1979. https://doi.org/10.1056/NEJM197906073002311

Raise E, Guerra L, Viza D, Pizza G, De Vinci C, Schiattone ML, Rocaccio L, Cicognani M, Gritti F., Biotherapy 9, 49–54, 1996. https://doi.org/10.1007/BF02628656

Roda E, Viza D, Pizza G, Mastroroberto L, Phillips J, De Vinci C, Barbara L., P. Soc. Exp. Biol. Med. 178, 468–475, 1985. https://doi.org/10.3181/00379727-178-42033

Sánchez L, Calvo M, Brock JH., Arch. Dis. Childhood. 67, 657–661, 1992. https://doi.org/10.1136/adc.67.5.657

Sanchez-Gonzalez DJ, Sosa-Luna CA, Vasquez-Moctezuma I., Med. Clin. (Barc.) 137, 273–277, 2011. https://doi.org/10.1016/j.medcli.2010.05.002

Steele RW, Myers MG, Vincent MM., New Engl. J. Med. 303, 355–359, 1980. https://doi.org/10.1056/NEJM198008143030702

Vacek A, Hofer M, Hromas J, Luksíková E, Svoboda J, Schneiderová H., Immunopharm. Immunot. 24, 651–664, 2002. https://doi.org/10.1081/IPH-120016049

Valdimarsson H, McGuire RL., Clin. Exp. Immunol. 29, 261–265, 1977.

Viza D, Fudenberg HH, Palareti A, Ablashi D, De Vinci C, Pizza G., Folia Biol. (Prague) 59, 53–67, 2013.

Viza D, Lefesvre A, Patrasco M, Phillips J, Hebbrecht N, Laumond G, Vich JM., J. Exp. Pathol. 3, 653–659, 1987.

Whitcomb ME, Rocklin RE., Ann. Intern. Med. 79, 161–166, 1973. https://doi.org/10.7326/0003-4819-79-2-161