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Toxoplasma gondii: Inhibition of the Intracellular Growth by Human Lactoferrin

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A b s t r a c t

In the present paper we focused on the potential role of human lactoferrin for the intracellular replication rate of *T. gondii* BK tachyzoites following our earlier observation that *Toxoplasma gondii* was able to bind human lactoferrin but not serum transferrin. The study was performed *in vitro* on human CaCo-2 epithelial cells and mouse L929 fibroblasts. We found that the multiplication of the parasite was inhibited by lactoferrin in both cell lines used. However, the direct cytotoxic effect on the parasite and the host cells was not observed. The intracellular growth of *T. gondii* was not affected when tachyzoites or host cells were only pre-coated with human lactoferrin. The results suggest that lactoferrin does not influence parasites penetration into host cells but could trigger unknown antiparasitic mechanisms in the infected cells.

K e y   w o r d s: *Toxoplasma gondii*, host cells, human lactoferrin

Introduction

All parasitic protozoa have evolved specific mechanisms to scavenge iron from their hosts. Since iron is largely insoluble at physiological pH, the parasites utilize iron bound to inorganic or organic chelates, such as extracellular iron-transporting proteins (serum transferrin and lactoferrin) or intracellular iron compounds (hemin, hemoglobin, ferritin and others) (Wilson and Britigan, 1998; Aisen *et al.*, 2001; Długońska *et al.*, 2005).

Up to now, little is known about iron metabolism of *Toxoplama gondii*, cosmopolitan intracellular protozoan, which attacks all nucleated cells of many endothermic hosts, including humans. The invasion of *T. gondii* occurs usually benignly and asymptomatically, but can be very serious for immunodeficient patients (AIDS, transplant recipients *etc.*) and fetuses infected by transplacental transmission of parasites from their mothers with primary parasitaemia. Natural infection is usually acquired from tissue cysts or oocysts by the oral route. The parasites multiply initially in *lamina propria* and later in epithelium, in the environments rich in lactoferrin (Petersen and Dubey, 2001).

Lactoferrin (Lf), a cationic iron-binding glycoprotein of the transferrin family, is produced and secreted by glandular epithelial cells and neutrophils. It is found on mucosal surfaces, in many biological fluids (milk, saliva *etc.*) and within specific granules of neutrophilic granulocytes. The protein molecule comprises two homologous lobes and can reversibly bind two iron (Fe^{3+}) atoms. Lactoferrins of different mammal species share a high structural and amino acid sequence homology (bovine and human lactoferrins 69%). Due to a strongly basic and flexible region (lactoferricin) close to N-terminus, lactoferrin displays iso-electric point of approximately 9.0 and is able to bind to a large number of acidic molecules. Lactoferrin is known as a multifunctional protein involved in innate antimicrobial and antitumour defence, immunoregulation, iron adsorption, granulopoiesis *etc.* For many years the broad and extensive antimicrobial activity of lactoferrin was only attributed to its ability to sequester iron required for the growth of almost all pathogens, however, newer findings indicate also direct antimicrobial action of both lactoferrin and its fragment, lactoferricin (Brock, 1995; Farnaud and Evans, 2003). Lactoferrin is able to inhibit the

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growth of numerous parasitic protozoans, among them Apicomplexan intracellular pathogens of human and endothermic animals: *Plasmodium falciparum* (Fritsch *et al.*, 1987), *Toxoplasma gondii* (Tanaka *et al.*, 1996) and *Babesia caballi* (Ikadai *et al.*, 2005).

Recently, we found that extracellular *T. gondii* tachyzoites of BK strain (genotype I) bound specifically human lactoferrin but not serum transferrin (Dziadek *et al.*, 2005). The biological aspects of the observed phenomenon are still unknown. As one of the most prominent humoral factors of innate immunity in mammals, lactoferrin is particularly important in parasitic invasions acquired by enteric route. Up to now, there is no report on the effect of human lactoferrin on the growth of *T. gondii*, common human parasite. In the present paper we studied the influence of human lactoferrin on *in vitro* intracellular replication of *T. gondii* BK tachyzoites in homologous (human) and heterologous (mouse) host cells. Both host cells and tachyzoites were exposed to Lf transiently (30 min pre-incubation) or continuously to determine whether even short-time contact could influence the intracellular growth rate of *T. gondii*.

Experimental

Materials and Methods

Preparing *T. gondii* tachyzoites for *in vitro* experiments. Tachyzoites of *T. gondii* BK strain (Winser *et al.*, 1948) were propagated *in vitro* in L929 mouse fibroblasts cultivated in Iscove's medium (Sigma) supplemented with 10% heat-inactivated FBS (fetal bovine serum, Cytogen), 2 mM L-glutamine (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Polfa) and 5×10^{-5} M 2-mercaptoethanol (Sigma). L929 fibroblasts were infected 1:10 with tachyzoites of BK strain and after a 3-day incubation in humidified 10% CO_2 atmosphere at 37°C, egressed parasites were harvested from culture supernatants by filtration (5 µm, Sartorius) and pelleted at $800 \times g$ for 15 min. Purified parasites were washed 2 times with PBS + 0.1% (w/v) BSA (phosphate-buffered saline + bovine serum albumin, Sigma) and resuspended in the same medium to 1×10^7/ml.

Kinetics of human lactoferrin (Lf) binding by tachyzoites of *T. gondii* BK estimated by cellular ELISA (cELISA). Human iron-saturated lactoferrin (holo-lactoferrin, Sigma) was added to 1 ml samples of 1×10^7 live tachyzoites in eppendorf tubes, to the final concentration of 0.1, 1 and 10 µg/ml. At different time points of incubation (humidified 10% CO_2 atmosphere, 37°C), from 0–180 min, tachyzoites were pelleted by centrifugation ($800 \times g$, 15 min), washed twice with PBS + 0.1% BSA, next fixed with 0.5% formaline

(POCH) in PBS and plated (2×10^6) in quadruplicates into wells of polystyrene 96-well titration plate (Medlab). The plates were dried overnight at 37°C, washed 4 times with washing buffer (PBS + 0.05% v/v Tween 20), blocked with 1% w/v skim milk (Oxoid) in PBS, washed again and developed using rabbit anti-human lactoferrin polyclonal antibody (Sigma). Goat anti-rabbit IgG labeled with horseradish peroxidase (Jackson ImmunoResearch) were used as a secondary antibody and ABTS (Sigma) as a chromogen.

In separate experiments, 2×10^6 tachyzoites/well were firstly fixed with 0.5% formaline in 96-well polystyrene titration plate (Medlab). After 4-fold washing and blocking procedure, as described above, 50 µl biotinylated human holo-lactoferrin (0.1, 1, 10 and 100 µg/ml), prepared according to the protocol of Tanaka *et al.* (2003), was added; the amount of lactoferrin per 1×10^7 fixed tachyzoites – 0.5, 5, 50 and 500 µg. After the incubation time of 0–180 min at 37°C tachyzoites were washed again and the reaction was developed with streptavidin-peroxidase (Sigma) and ABTS as chromogen (Sigma).

The influence of human lactoferrin pre-coating of tachyzoites, host cells or both on the intracellular multiplication of the parasites. Samples of 1×10^7 live tachyzoites were pelleted, then suspended in Iscove's medium supplemented with 0.1% (w/v) BSA and human holo-lactoferrin at the final concentrations from 1 to 1000 µg/ml and incubated for 30 min. After 2-fold washing with PBS + 0.1% BSA, the parasites were resuspended in culture medium and in number of 2×10^5/ well added to 2×10^4/well of L929 (murine C3H/An fibroblasts, ATCC BS CL 56) or CaCo-2 (colon adenocarcinoama of epithelial origin, ATCC Colo2 HTB 37) confluent cell monolayers in 96-flat bottom microplates (Nunc); host cells: parasites ratio = 1:10. For experiments with pre-coated host cells, the human lactoferrin binding procedure was performed directly with cell monolayers incubated for 30 min with the protein at concentrations as above. Then the cells were washed twice with PBS + 0.1% BSA and the monolayers were inoculated with coated with human lactoferrin or uncoated *T. gondii* tachyzoites. After 48 h of incubation, 37 kBq of [^{3}H]uracil (Moravek Biochemicals, Inc., USA) was added to each microculture for further 24 h. The amount of the isotope incorporated into the parasite nucleic acid pool, corresponding to the parasite growth, was measured by liquid scintillation counting (Pfefferkorn and Pfefferkorn, 1977).

The impact of human lactoferrin present in culture medium on intracellular proliferation of *T. gondii* BK tachyzoites, L929 fibroblasts growth and extracellular tachyzoites viability. Samples of pelleted 1×10^7 tachyzoites were suspended in 1 ml culture medium supplemented with human holo-lactoferrin (1–1000 µg/ml) and in a number of 2×10^5/

well added to confluent monolayers of host cells (L929 fibroblasts and CaCo-2 epithelial cells) grown in 96-well culture microplates (Nunc). The proliferation intensity of intracellularly multiplying tachyzoites was evaluated with [³H]uracil uptake assay.

To check the possibility of a direct cytotoxic effect of human lactoferrin on host cells and extracellular tachyzoites, 1×10^4 L929 fibroblasts or 5×10^6 tachyzoites in 200 μl Iscove's medium + 0.1% BSA were plated into wells on microtiter plates and then human holo-lactoferrin was added to the final concentrations of 1, 10 and 100 μg/ml. After 1, 2, 3, 24, 48, 72 and 96 h of incubation in humidified 10% CO_2 atmosphere at 37°C, the cells were washed once with Iscove's medium + 0.1% BSA and 10 ml of 5% (w/v) MTT (Sigma) in PBS was added to each well followed by another 4-hour incubation in the same conditions. The formed formazane crystals were pelleted (583 × g, 10 min, at room temperature), solubilized in 150 μl DMSO and 25 μl 0.1 M glycine buffer pH 10.5 and the absorbance was measured at λ = 550 nm.

Statistics. Statistical analysis was performed with U Mann-Whitney and Wilcoxon tests using STATISTICA 5.0 PL software. Differences were considered significant with p<0.05.

Results

Human lactoferrin binding by tachyzoites of *T. gondii* BK. We found that human lactoferrin binding by live tachyzoites was concentration dependent, started immediately after the addition of the protein and at the point 0, corresponding to the time of the first centrifugation step (*ca.* 20 min), reached the maximum and remained constant for next 3 hours (Fig. 1). At the concentration of 10 μg/ml of lactoferrin the maximum binding value was noticed 30 min

later (0–30 min, p = 0.027), therefore this incubation time was chosen for the next experiments including pre-coating of parasites with the protein. The kinetics of lactoferrin binding by formaline-fixed, non-permeabilized tachyzoites revealed quite a different course (Fig. 2). The binding intensity grew gradually along with the incubation time and, for samples containing 0.1–10 μg/ml of Lf, it achieved the maximum at the end of the experiment (120–180 min). Only at the highest concentration of lactoferrin (100 μg/ml) the binding process occurred rapidly with the maximum value being reached as soon as after 5 min.

The effect of human lactoferrin pre-coating of *T. gondii* BK tachyzoites (or/and pre-coating of host cells) on the parasite replication *in vitro*. Thirty minutes pre-coating of either *T. gondii* BK tachyzoites or host cells (human CaCo-2 epithelial cells and murine L929 fibroblasts) or both tachyzoites and host cells with human lactoferrin did not influence significantly intracellular replication of the parasites (p>0.05) (Fig. 3).

The impact of culture medium supplementation with human lactoferrin on the intracellular replication of *T. gondii* tachyzoites. The cultures of human CaCo-2 epithelial cells and murine L929 fibroblasts growing in the medium supplemented with human lactoferrin (1–1000 μg/ml) were infected with *T. gondii* BK. The highest protein concentrations (100 and 1000 μg/ml) showed statistically significant (p<0.05) antitoxoplasmic action. Compared to the control without lactoferrin, the incorporation of [³H]uracil in those cultures decreased by 38,4/41,4 and 63,4/63,9%, respectively (Fig. 4). As microscopically evaluated, in control cultures all host cells were completely destroyed by the egress of parasites after 3 days post-infection whereas in those carried out with 100 and 1000 μg/ml of human lactoferrin the monolayer of host cells was only partly damaged.
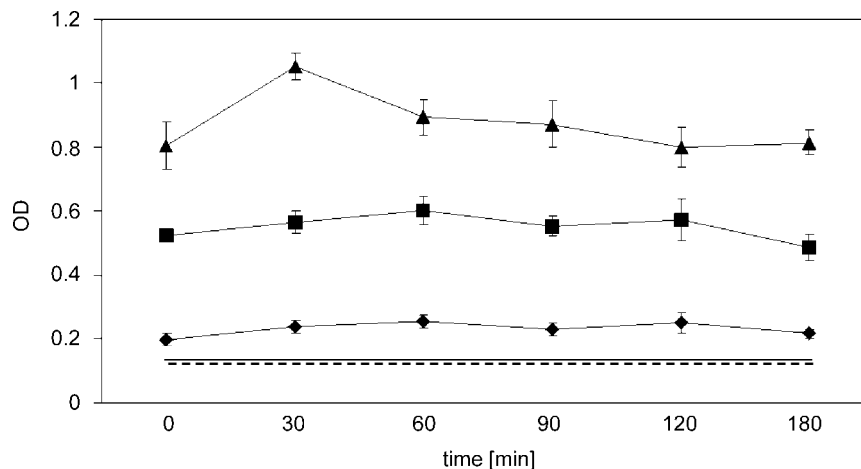

Fig. 1. Kinetics of the *in vitro* binding of human lactoferrin to live *T. gondii* BK tachyzoites (2×10^6) evaluated by cELISA. Tachyzoites incubated with Lf at the concentrations of: ▲ – 10, ■ – 1, ◆ – 0.1 μg/ml; ----- control without LF; each point represents a mean absorbancy value of 2 experiments with 3 repeats per sample ± SD.
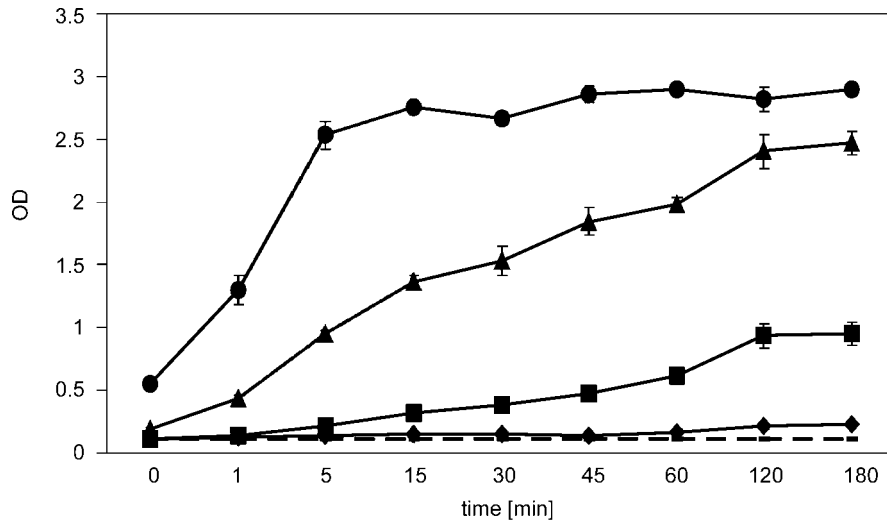
Fig. 2. Kinetics of the *in vitro* binding of human lactoferrin to formaline-fixed *T. gondii* BK tachyzoites (2×10^6) determined by cELISA.
Tachyzoites incubated with LF at the concentrations of: ● – 100, ▲ – 10, ■ – 1, ◆ – 0.1 µg/ml, ----- control without Lf; the results are expressed as mean absorbancy value of 4 experiments with 3 repeats per sample ± SD.
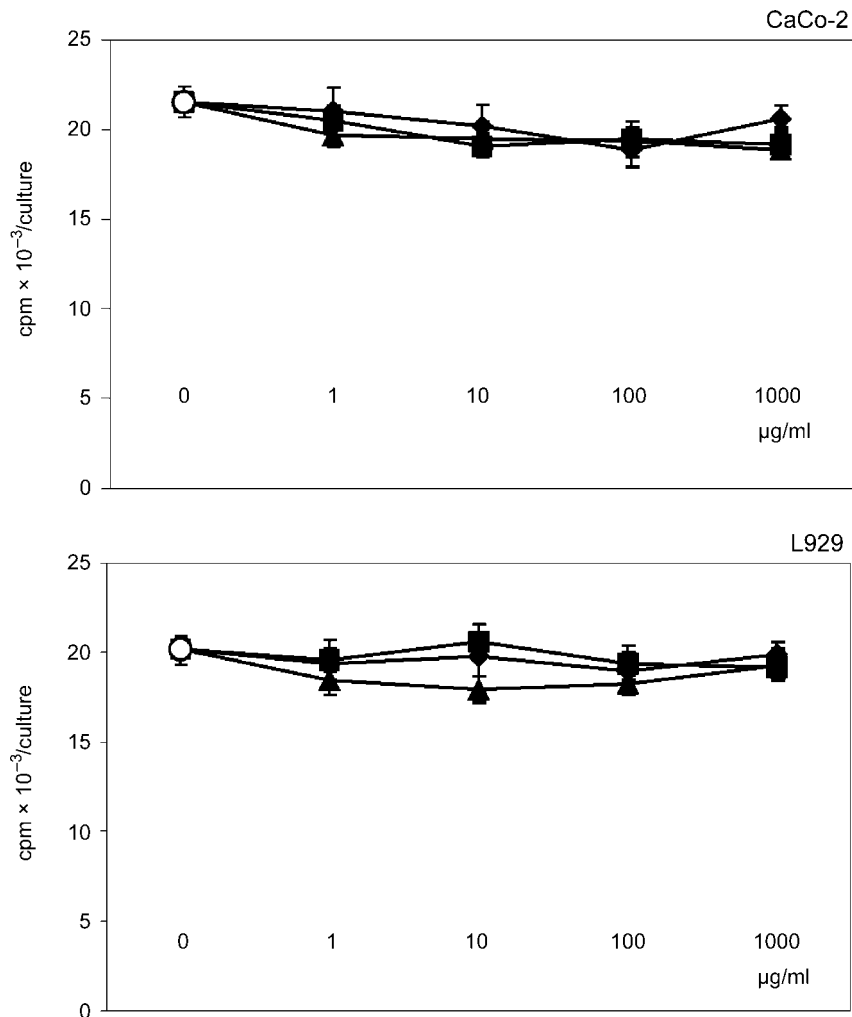

Fig. 3. Replication intensity of *T. gondii* BK tachyzoites in host cell cultures evaluated by incorporation of [^{3}H]-uracil into parasites.
■ – tachyzoites pre-coated with human lactoferrin; ◆ – host cells (human CaCo-2 and mouse L929) pre-coated with human lactoferrin;
▲ – both host cells and tachyzoites pre-coated with human Lf before co-culturing; ○ – control cultures of host cells
and tachyzoites uncoated with Lf; results are expressed as a mean cpm values of quadruplicate cultures from 3 experiments
± SD; control cultures without *T. gondii* <247 cpm, control cultures without host cells <112 cpm.
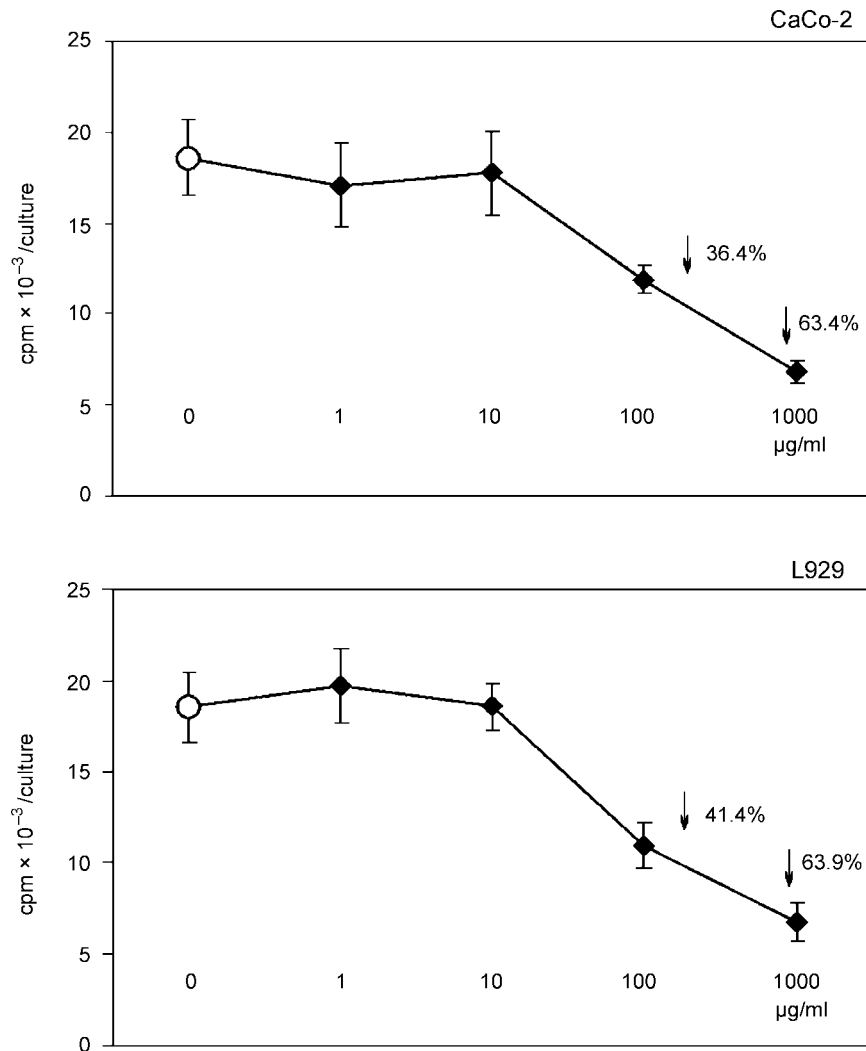
Fig. 4. Intracellular growth rate of *T. gondii* BK tachyzoites, multiplying in CaCo-2 and L929 cells cultivated in medium supplemented with human lactoferrin, evaluated by incorporation of [^{3}H]-uracil into parasites.

O – control medium without human Lf; results expressed as mean cpm values of quadruplicate cultures

from 3 experiments $\pm$ SD; control cultures without *T. gondii* <247 cpm, control cultures without host cells <112 cpm.

Additionally, we studied the effect of human lactoferrin on extracellular *T. gondii* tachyzoites survival and host cells (L929) growth in short-and long-termed cultures and found no cytotoxicity (Fig. 5). The viability of extracellular tachyzoites diminished gradually, independent of the concentration of human protein in the culture medium (Fig. 5A). L929 fibroblasts used as host cells for *Toxoplasma* were not damaged by human lactoferrin, either, and even higher (10 and 100 µg/ml) protein concentrations supported significantly (p<0.05) the cell growth rate, starting from 24 h culture (Fig. 5B).

Discussion

Following our previous observation that *T. gondii* tachyzoites bind specifically human lactoferrin but not transferrin (Dziadek *et al.*, 2005), in the present study we determined kinetics of the binding. As expected, fixed and non-permeabilized tachyzoites bound lactoferrin in a concentration dependent and saturable manner. In contrast, live tachyzoites bound lactoferrin rapidly and the amount of the bound protein was constant within a 3-hour observation. The fate of the bound ligand, including its uptake *via* non-specific pinocytosis or receptor-specific endocytosis (Botero-Kleiven *et al.*, 2001), turnover and possible recycling, is not known at all and will be an interesting subject for future study.

Our study showed that human lactoferrin at the concentrations of 100 and 1000 µg/ml significantly inhibited the *in vitro* replication of *T. gondii* in human epithelial and murine fibroblast cell lines. These concentrations, although relatively high, are physiological. For example, the mean level of lactoferrin in tears of normal humans of 3rd decade was evaluated as 1.48 mg/ml, whereas in human colostrum it can even
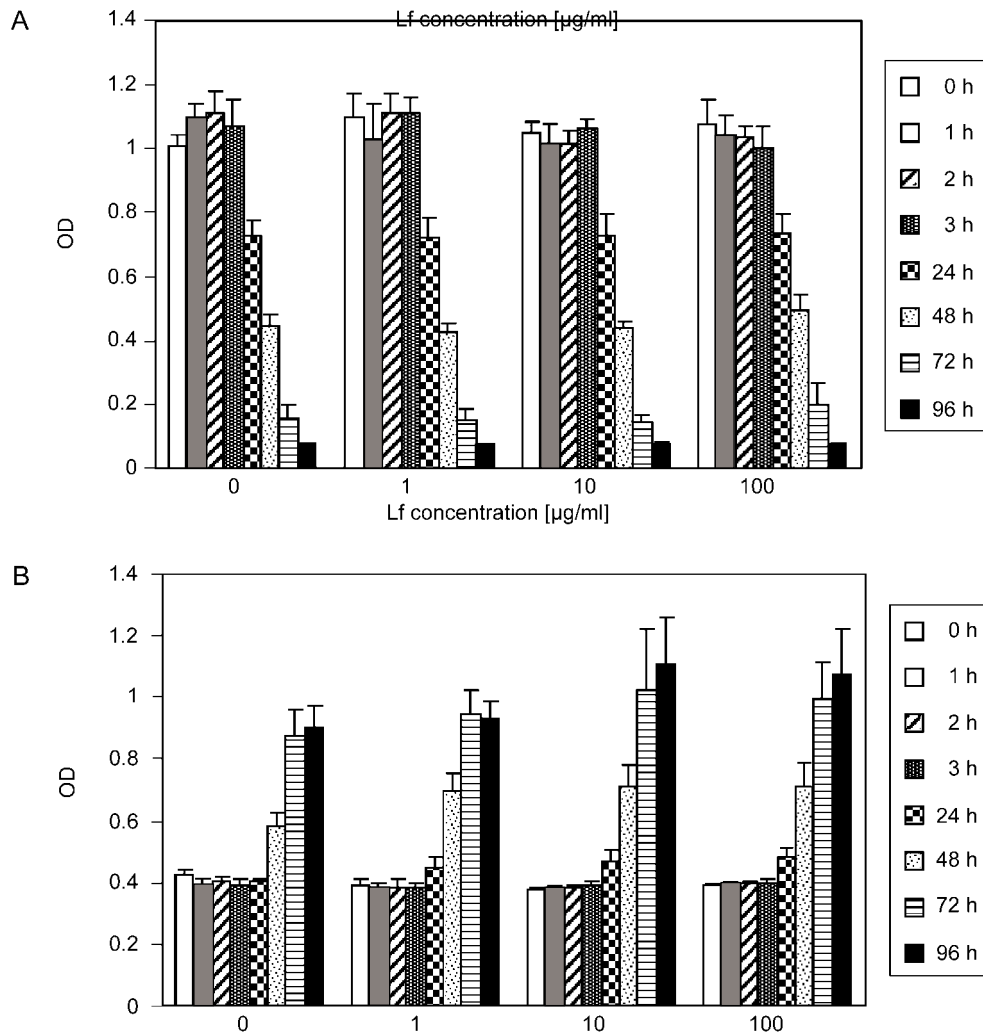
Fig. 5. The influence of human lactoferrin on extracellular *T. gondii* BK tachyzoites viability (A)
and L929 fibroblasts (B) growth rate estimated by cell metabolic activity using MTT reduction test.
Results are presented as mean absorbancy values of 2 experiments with 3 repeats per sample ± SD.

reach 7 mg/ml (Jensen *et al.*, 1986; Farnaud and Evans, 2003). Similarly to our findings, Tanaka *et al.* (1996) observed that intracellular development of *T. gondii* was inhibited linearly to the increasing concentration of bovine lactoferrin present in cell culture medium. Since the receptors for human lactoferrin are expressed on many human cells, they could provide a mechanism for the interactions between lactoferrin-binding *T. gondii* tachyzoites and host cells (Suzuki *et al.*, 2005). Additionally, our experiments revealed that short-time pre-coating of tachyzoites with lactoferrin did not affect the parasite replication in both human epithelial CaCo-2 cells and murine L929 fibroblasts. Most probably, the tachyzoite bound lactoferrin does not mediate or facilitate the penetration process and the amount of lactoferrin introduced into parasitophorous vacuole with one penetrating tachyzoite is not sufficient to cover the demand for iron of many nascent parasites and therefore we did not observe any

"promicrobial" activity of human lactoferrin to *T. gondii*. Moreover, no change in parasite replication intensity was noticed by lactoferrin pre-coating of host cells (CaCo-2 and L929) or both host and parasite cells. The lack of effect could be due to the host cells used and to the time of pre-coating. According to Tanaka *et. al.* (1996), 24 h pre-treatment of murine macrophages with bovine lactoferrin, in contrast to mouse embryonal cells, did not show any inhibitory effect. Besides, our experiments indicated that antitoxoplasmic action of human lactoferrin does not rely on its direct parasiticidal acitivity aimed at extracellular tachyzoites or/and its cytotoxic action on host cells. In the latter case, human lactoferrin was even utilized as factor promoting cell replication.

The mechanism of antimicrobial action of lactoferrin is not fully understood. The anti – *T. gondii* growth activity was found to be independent of bovine lactoferrin saturation with ferric ions (Tanaka *et al.*,

1996), since both holo-lactoferrin and apo-lactoferrin as supplements of culture medium inhibited the intracellular development of parasites but not the penetration of parasites into host cells. On the other hand, Lima and Kierszenbaum (1987) noticed that the presence of iron in lactoferrin molecule is required for human lactoferrin-stimulated intracellular killing of other intracellular pathogens: *Trypanosoma brucei* amastigotes and *Listeria monocytogenes* in mouse macrophages. Tanaka *et al.* (1997) found that the inhibitory activity of bovine lactoferrin was not mediated by O_2^- or $NO^{\bullet}$ molecules, however, they observed that the lactoferrin-induced tyrosine phosphorylation in mouse macrophages resulted in their activation to restrict intracellular *Toxoplasma* growth (Tanaka *et al.*, 1998). In contrast, earlier study of Lima and Kierszenbaum (1985) on *Trypanosoma brucei* amastigotes indicated that reactive oxygen intermediates are involved in the intracellular killing of the parasites by human lactoferrin-treated mouse macrophages. Recently the results of Sakamoto *et al.* (2006) showed that human lactoferrin interacts with molecules containing the RGD motif (Arg-Gly-Asp), present in numerous proteins on the cell surface and extracellular matrix. Such a wide spectrum of potential target molecules for human lactoferrin may explain its multifunctionality. Up to now the mechanisms, signaling, metabolic pathways and mediators involved in significant inhibition of toxoplasma growth in different host cells which live in the environments rich in this protein, are not known and need to be elucidated. The inhibitory activity of lactoferrin seems to be due to the biology of the protein. The study on differentiated CaCo-2 cells showed that human lactoferrin was specifically bound, then internalized from apical cell side and localized in the nucleus, whereas transferrin was taken up from basolateral side and localized in the cytoplasm (Ashida *et al.*, 2004). The binding of lactoferrin to DNA could initiate genes transcription and activation of antimicrobial mechanisms.

High concentrations of human lactoferrin in mucosa could prevent effectively the intensive replication of *T. gondii* at the site of infection in gut. Particularly active against toxoplasma seems lactoferricin, the N-terminal amino acid peptide derived from lactoferrin by pepsin cleavage. Omata *et al.* (2001) observed the reduction of infectivity for mice of *T. gondii* sporozoites pre-incubated with bovine lactoferricin. After crossing the intestinal wall barrier, toxoplasma is transferred to the environments almost free from lactoferrin (with the exception of polymorphonuclear leucocytes inside) which correlates with development of systemic infection (acute toxoplasmosis). A low concentration of human lactoferrin in cerebrospinal fluid (Maffei *et al.*, 1999) is not sufficient to restrict toxoplasma growth and protect the host from brain cyst formation in the chronic phase of primary infection. Lactoferrin does not either prevent reactivation of chronic infection in brain in immunocompromised individuals which results in neurotoxoplasmosis or systemic toxoplasmosis. Only strong protective cellular immunity, dominated by the production of pivotal cytokine, IFN-γ which primarily activates macrophages, inhibits the parasite replication (Hunter and Reichmann, 2001).

Summarizing, human lactoferrin at high concentrations (≥ 100 μg/ml) significantly restricts intracellular growth of *T. gondii* without any cytotoxic effect on the very parasites or/and host cells. The mechanism of its antitoxoplasmic activity is so far unknown and needs further studies at the cellular and molecular level, the results of which could be applied in the prevention and therapy of toxoplasmosis.

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