Polish Journal of Microbiology
2004, Vol. 53, Suppl., 75–80

The Modulation of Transferrin Receptors Level on Mouse Macrophages and Fibroblasts by *Toxoplasma gondii*

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A b s t r a c t

Macrophage-mediated early nonspecific immunological response is an important part of the immunity against intracellular parasite *Toxoplasma gondii.* The immunological functions of macrophages are closely connected with iron metabolism and acquiring of iron mainly from transferrin by the receptor-mediated endocytosis. The level of specific transferrin receptors can be modulated by different soluble exogenous and endogenous factors and also by microbial pathogens. The goal of our study was to determine the influence of *T. gondii* infection and toxoplasma lysate antigen (TLA) on the expression level of transferrin receptors (TfRs) on mouse macrophages and fibroblasts which can serve as host cells for the parasite replication. The level of TfRs was measured using CELISA assay. Strong down-regulation of the receptors level, started about 18 hours after infection of macrophages with a high number of freshly harvested tachyzoites *T. gondii.* Stimulation of the mouse cells with TLA antigen did not cause any changes in TfRs expression. In our studies we did not observe any differences in the TfRs level on mouse fibroblasts even after incubation with high concentrations of TLA antigen or inoculation with a high number of tachyzoites.

K e y w o r d s: *Toxoplasma gondii*, transferrin receptors, macrophages functions.

Introduction

An essential part of the immunity against intracellular parasite *Toxoplasma gondii* is early nonspecific T-cell independent immunological response mediated by macrophages, NK cells, dendritic cells and granulocytes. Macrophages are immune cells also responsible for the controlling of tachyzoite replication and the development of the later specific T-cell mediated response (Denkers and Gazzinelli, 1998; Denkers *et al*., 2004; Gazzinelli *et al.*, 1996; Hauser and Tsai, 1986; Sher *et al*., 1993). The physiological and immunological functions of macrophages, like other mammalian cells, are strictly connected with iron metabolism. Changes in iron content affect macrophage microbicidal function and also macrophage-mediated cytotoxicity, for instance by involving iron in catalyzing the formation of highly toxic hydroxyl radicals *via* the Fenton reaction (Aisen *et al*., 2001; Weinberg, 2000).

Most cells of vertebrate hosts acquire iron from the main serum iron-carrier, transferrin, by the receptor-mediated endocytosis. Transferrin is the physiological source of most of the iron required by different types of cells (Aisen *et al*., 1999; Qian and Tang, 1995). The uptake of iron on the transferrin-dependent pathway is initiated with the binding of iron-saturated protein (holo-transferrin) to the specific receptors on the cell membrane followed by the endocytosis of the receptor-holo-transferrin complex (internalization) and release of iron from the carrier-protein by a decrease in endosomal pH (Ponka and Lok, 1999; Qian and Tang, 1995).

The transferrin receptors are membrane homodimeric glycoproteins with two subunits linked by two disulfide bonds and they are mainly responsible for the controlled cellular uptake of iron from transferrin (Ponka and Lok, 1999). With the exception of some cells, for example mature erythrocytes, resting T and B lymphocytes and circulating monocytes, transferrin receptors are expressed at various levels probably on all erythroid and non-erythroid cells including immune cells such as differentiated macrophages and activated

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lymphocytes (Galbraith and Galbraith, 1983; Ponka and Lok, 1999). The expression of the transferrin receptors on the macrophage surface could be modulated by various endogenous and exogenous stimuli. It was showed that J774 cells strongly suppressed the transferrin receptors expression after stimulation with IFN-γ and LPS, and the observed down-regulation appeared to be NO-independent (Mulero and Brock, 1999). The decrease in the macrophage transferrin receptors synthesis was also found to be involved in the inhibition of multiplication of different microbial pathogens, like *Legionella pneumophila* and *Mycobacterium avium* (Byrd and Horwitz, 2000; Zhong *et al*., 2001). Additionally, it was noted that the intestinal epithelial cell line IEC-6 and rat primary enterocytes pretreated with IFN-γ inhibited the intracellular replication of *T. gondii* and the observed inhibition was connected with the limitation of the availability of intracellular iron (Dimier and Bout, 1993; Dimier and Bout, 1998).

The presented study documents that infection with parasite *T. gondii* which utilizes fibroblasts as well as macrophages as host cells for its replication could modulate the expression of transferrin receptors on the immune cells but not on fibroblasts. The observed down-regulation of the macrophage receptors expression might be an important mechanism of the macrophage-mediated host defense against protozoan parasite involved in the decreasing of intracellular iron and could result in the inhibition of *T. gondii* replication and even elimination of the pathogen.

Experimental

Materials and Methods

Tissue culture media and reagents. Iscove's medium (Sigma) supplemented with 5% FCS (Cytogen), 2 mM L-glutamine (Sigma), 50 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin and 100 μg/ml streptomycin (Polfa) for culturing of mouse fibroblast line L929; RPMI 1640 medium (The Institute of Immunology and Experimental Therapy PAN, Wrocław) with 10% FCS (Cytogen) or with 10% NCS (Sigma) with L-glutamine and antibiotics at concentration as above for culturing of mouse macrophages lines Ana-1 and J774, respectively; PBS without Ca^{2+} and Mg^{2+} with 0.5% formaline for cell fixation.

Cell lines. Murine fibroblasts line L929 obtained from the Institute of Medical Microbiology and Virology, University of Düsseldorf, Germany; two murine macrophages lines derived from two strains of inbred mice differing significantly in their natural resistance to toxoplasmosis: J774 (BALB/c origin) and Ana-1 (C57Bl/6 origin) received from the Institute of Medical Microbiology and Virology, University of Düsseldorf, Germany.

Culture of *Toxoplasma gondii* BK (intraspecies subgroup I). Parasites were maintained by serial passage in confluent monolayers of mouse L929 fibroblasts grown in Iscove's culture medium. Tachyzoites of *T. gondii* were harvested 3 or 4 days after infection, resuspended in culture medium appropriate for each cell line and after counting used for infection of murine macrophages or fibroblasts.

Preparing of *Toxoplasma* Lysate Antigen (TLA). Washed twice and resuspended in PBS, tachyzoites of *T. gondii* were 10 times freeze-thawed in liquid nitrogen and at 37°C in warm water bath, respectively. Obtained extract was centrifuged at 10 000 × g (20 min, 4°C) to remove cellular debris and then the concentration of the protein was determined spectrophotometrically using Bradford standards. The average yield of the used procedure was 1–2 mg protein/10^9 parasites. The samples of TLA antigen were stored at –70°C and thawed directly prior to stimulation.

CELISA assay. To detect expression of transferrin receptor (TfR) on mice fibroblasts (L-929) and macrophages (Ana-1 and J744) the cellular enzyme-linked immunosorbent assay (CELISA) was used. A total number of 2×10^6 cells was plated into tissue culture dishes ∅ 3 cm (Nunc) for 3 hours followed by incubation with four different concentrations (1, 3, 10 and 30 μg/ml) of TLA antigen or infection with freshly harvested tachyzoites of *Toxoplasma gondii* at the ratios 1:3, 1:10 and 1:30 parasites per mouse cell at 37°C in humidified atmosphere of 5% (RPMI 1640 medium) or 10% (Iscove's medium) CO_2 in the air. Three, 6 or 18 hours postincubation the cells were collected using culture media, centrifuged at 65 × g for 10 min, washed and resuspended with PBS. After counting, the cells were fixed using 0,5% formaline (POCH) in PBS, aliquoted (1×10^4/well) into 96-well ELISA plate and dried at 37°C. Then the wells were blocked with 1% milk (Oxoid) at 4°C overnight and the rat IgG2a anti-mouse transferrin receptor monoclonal antibodies (obtained from postculture supernatants of hybridoma cell line R17.217.1.3., received from the Institute of Immunology, University of Mainz, Germany) were added. Incubation with primary antibodies was performed at 37°C for 2 h and followed by washing with PBS + 0.05% Tween-20 (Sigma). The reaction was developed using secondary peroxidase-conjugated goat anti-rat IgG + IgM polyclonal antibodies at dilution 1:2000 (Jackson ImmunoResearch Laboratories, Inc.) and ABTS [diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate), Sigma] as a chromogen. The absorbance values for all samples were determined at $\lambda = 405$ nm using the automatic microtiter plate reader (Labsystems). The results are expressed as a mean of eight values at least from three independent experiments. Statisitcal analysis was performed by U Mann-Whitney test ($p < 0.05$).

Results

Transferrin receptors expression on mouse fibroblasts and macrophages during *T. gondii* infection. In the study the CELISA assay was used to examine the changes in the expression of TfRs on murine fibroblasts and macrophages after *T. gondii* infection. To determine the ability of the parasite to modulate

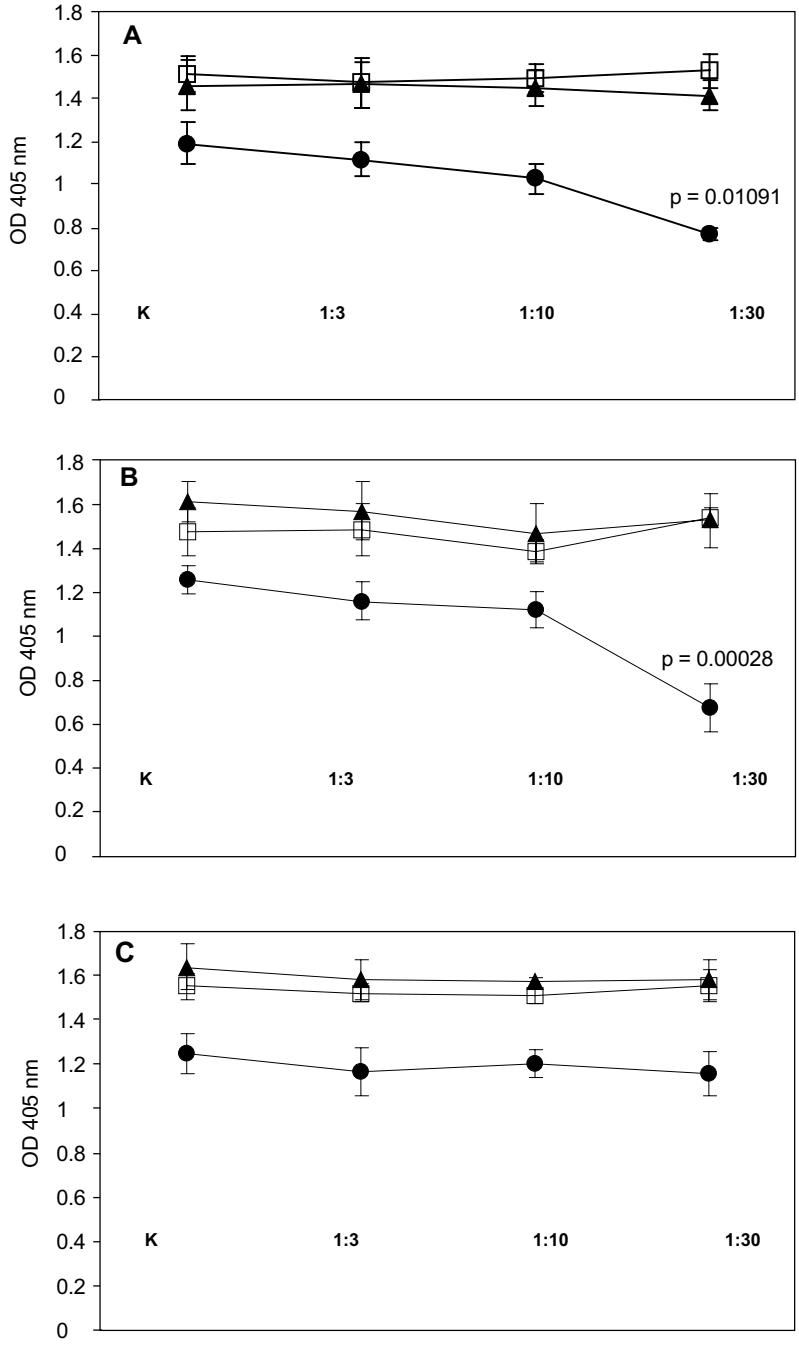

Fig.1. Transferrin receptors level on mouse J774 (A) and Ana-1 (B) macrophages and L929 fibroblasts (C) after infection with *Toxoplasma gondii* BK.
K – control (no toxoplasms), 1:3, 1:10, 1:30 – the ratio of cells: *Toxoplasma gondii*; ▲ – 3 h; □ – 6 h; ● – 18 h postinfection.

the level of expression, fibroblasts and Ana-1 macrophages were inoculated with viable *T. gondii* BK. Mouse J774 (Fig.1A) and Ana-1 (Fig. 1B) macrophages infected with a high number of the parasites at a ratio 1:30 displayed an essential decrease in TfRs level. A slight inhibition of TfRs expression on these cells occurred also after inoculation of cells with 10 tachyzoites per macrophage. A down-regulation of TfRs expression on mouse macrophages started about 18 hours postinfection; we did not find any changes in TfRs level 3 or 6 hours after inoculation of *T. gondii.* In the performed experiments we did not observe any alternation in the expression of TfRs on mouse fibroblasts (Fig. 1C) and there was no effect of low number of parasites (ratio 1:3) on the TfRs expression on mouse macrophages.

Effect of TLA antigen on the expression of transferrin receptors. To determine the ability of *T. gondii* antigens to modulate the level of transferrin receptors, mouse macrophage lines J774 and Ana-1 were

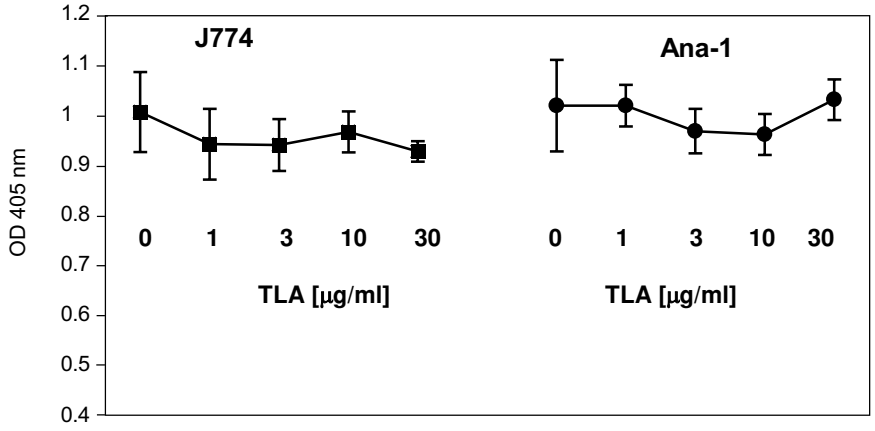

Fig.2. The expression of transferrin receptors on mouse J774 (■) and Ana-1 (●) macrophages after 18 h incubation with TLA antigen.

incubated with four different final concentrations (1, 3, 10 or 30 μg/ml) of the TLA for 18 h. The antigen stimulation of macrophages did not result in the changes of the expression of studied receptors compared to control unstimulated cells (Fig. 2). Even high concentrations of TLA antigen (10 and 30 μg/ml) used for experiments were unable to alter the transferrin receptors expression level on mouse macrophages.

Discussion

Toxoplasma gondii is an obligate intracellular pathogen that causes toxoplasmosis in many endothermic vertebrate hosts including humans. The essential part of the immunity against this parasitic infection is a nonspecific macrophage-mediated immunological response involved in the controlling of tachyzoite replication (Denkers and Gazzinelli, 1998). Iron is a crucial element that modulates the immunological functions of macrophages (Weinberg, 2000). On the other hand, iron is essential for multiplication of many bacterial and parasitic pathogens (Wilson and Britigan, 1998). Similarly to macrophages, pathogenic protozoa, such as *Trypanosoma brucei*, *Trypanosoma cruzi, Leishmania* spp. utilize mammalian iron-transporting protein, transferrin, as a source of iron during parasite replication in vertebrate hosts (Britigan *et al*., 1994; Lima and Villalta, 1990; Steverding *et al*., 1995). Recent research revealed that the possible way in which mammalian cells could inhibit the intracellular growth of *T. gondii* is limitation of the availability of intracellular iron, however, the mechanism of this limitation is not fully clear (Dimier and Bout, 1998). We examined the transferrin receptors (TfRs) level on mouse fibroblasts line L929 and macrophages line J774 and Ana-1 during infection with *T. gondii* BK or after stimulation with TLA antigen. We found that mouse macrophages but not fibroblasts inoculated with a high number of 30 tachyzoites per cell essentially decreased the expression of TfRs at 18 hours postinfection. The similar down-regulation of TfRs on mouse macrophages was noted during infection with *M. avium*. The treatment of the peritoneal cells with live mycobacteria resulted in a decreased TfRs level. Simultanously the expression of natural resistance-associated macrophage proteins Nramp1 mRNA and Nramp2 mRNA involved in the transporting of iron essential in the production of highly toxic hydroxyl radicals increased and correlated with the progress of infection (Zhong *et al*., 2001). These and our results could suggest that observed suppression in TfRs level on the immune cells during intracellular pathogens infections might be an important mechanism by which macrophages limit the availability of transferrin-associated iron. It would effect the growth of surviving parasite and result in the elimination of the pathogen. Also, experiments with human monocytes infected *in vitro* with *L. pneumophila* show that limiting of iron-saturated transferrin availability is crucial in the host antimicrobial immunological response (Byrd and Horwitz, 2000). Both macrophage lines used in our study responded very similarly to *T. gondii* infection what could suggest that the differences in natural susceptibility to toxoplasmosis between BALB/c (relatively resistant) and C57Bl/6 (relatively susceptible) mice are independent from the ability of macrophages to limit the TfRs expression.

Contrary to the earlier study by Gail *et al.* (2001) we did not find any changes in the TfRs level on mouse fibroblasts. The dissimilarity in the obtained results could be a consequence of the different cell lines used

for experiments and studying the TfRs expression by estimation of TfRs mRNA or very TfRs. Lack of any modification in fibroblasts TfRs expression might be related to the role of these cells in the organism. It is likely that opposite macrophages, the inhibition of transferrin-bound iron acquisition in fibroblasts did not result in the limitation of *T. gondii* replication. It could be connected with the presence of other proteins that serve as a source of intracellular iron as fibroblasts are very efficient permissive cells usually used in many laboratories to multiply toxoplasms *in vitro*.

We did not find any influence of TLA antigen on the TfRs level on mouse macrophages. Inability of TLA to modify the TfRs expression might be related to the freeze-thawing procedure used for extracting the antigen. This technique is not sufficient for isolation of all, particularly membrane tachyzoite antigens families with a high yield. It is known that any surface *T. gondii* components (SAG and SRS-antigens, lectins, laminin) mediate adhesion of the parasite on the host cells (Boothroyd *et al*., 1998; Furtado *et al*., 1992; Grimwood and Smith, 1996; Jacquet *et al*., 2001; Manger *et al*., 1998; Ortega-Barria and Boothroyd, 1999) which could be correlated with the modification of the cellular mechanisms participating in the controlling of parasite replication. Moreover, stimulation with soluble toxoplasma antigens might be not adequate for TfRs modification that could require direct parasite-cell interaction.

In conclusion, we postulate that down-regulation of TfRs by *T. gondii* infected-macrophages is one of the possible mechanisms responsible for inhibition of the parasite replication, and the observed decrease in TfRs level is time-dependent and correlated with progress of the infection.

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