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Toxoplasma gondii Inhibits the *in vitro* Induced Apoptosis of HL-60 Cells

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The integrity of the host cell may represent an important prerequisite for the intracellular survival and development of obligate intracellular pathogens. In the present study, we investigated the influence of infections with the protozoan parasite *Toxoplasma gondii* on the rate of apoptosis in the human leukemia cell line HL-60. After infection with *T. gondii* tachyzoites of the strain NTE and in uninfected controls, less than 2% of the host cells showed typical signs of apoptosis, i.e. condensation of chromatin after staining with Hoechst 33258 or internucleosomal DNA fragmentation after agarose gel electrophoresis of genomic DNA. After treatment with 0.1 to 0.5μg/ml actinomycin D for up to 16 hours, HL-60 cells considerably underwent apoptosis. However, this actinomycin D-induced apoptosis was clearly reduced after concomitant infection with *T. gondii* as shown by staining with Hoechst 33258 and by DNA fragmentation assay. Inhibition of apoptosis by the intracellular pathogen *T. gondii* might be recognized as an evasion mechanism that enables intracellular survival and establishes long-lasting persistence.

Keywords : *Toxoplasma gondii*, Apoptosis, HL-60, Actinomycin D

INTRODUCTION

Toxoplasma gondii, an obligate intracellular protozoan parasite, infects a broad spectrum of warm-blooded hosts, including up to 25% of the world's human population [21]. Infection of immunocompetent hosts with *T. gondii* is mostly asymptomatic, but leads to lifelong persistence of the parasite predominantly within the brain and muscle tissue but also within other organs [4]. However, acquisition of the parasite *in utero* may lead to abortion or overt clinical disease in newborns and children. Furthermore, *T. gondii* is a major opportunistic pathogen in immunocompromised patients, i.e. those with AIDS or those under immunosuppressive therapy, in which persisting parasites may reactivate and may lead to life-threatening cerebral toxoplasmosis [20].

T. gondii is able to actively invade essentially any cell type and may replicate within a broad range of nucleated host cells [14]. However, the integrity of the host cell is thought to be a major prerequisite for the intracellular survival and development of the parasite. It has been shown, that on one hand several intracellular pathogens, including viruses [10, 12, 22], bacteria [6] and protozoan parasites [17] down-regulate programmed cell death, i.e. apoptosis of their host cells. On the other hand, several bacteria and some viruses induce apoptosis of their host cells [18, 23, 25]. Likewise, Khan et al. [16] reported that acute infection of mice with *T. gondii* induces anergy of $CD4^+$ T cells followed by increased apoptosis of these cells *in vitro*. By contrast, it has been shown recently that *T. gondii* is also able to inhibit apoptosis in *in vitro* cultures of different murine cell types [19]. In the present study we investigated the effect of *T. gondii* infection on apoptosis of the human-derived cell line HL-60.

MATERIALS AND METHODS

Parasites and host cells

Tachyzoites of the mouse-avirulent *T. gondii* strain NTE [11] were co-cultured with L929 fibroblasts as host cells in RPMI 1640,

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containing 2 mM L-glutamine, 2 mg/ml $NaHCO_3$ and supplemented with 1% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin (all reagents from Biochrom, Berlin, Germany). Tachyzoites were harvested after initiation of host cell lysis. For infection assays, tachyzoites were isolated by centrifugation at 35 × g for 5 min to pellet contaminating host cells. The supernatant was centrifuged at 1,350 × g for 10 min and tachyzoites were resuspended in RPMI 1640 medium (as above) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Human promyelocytic leukemia (HL-60) cells were maintained in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Prior to infection with *T. gondii*, HL-60 cells were grown in the same medium supplemented with 5 nM phorbol 12-myristate 13-acetate (PMA; Sigma, Deisenhofen, Germany) for 24h to render adherent cells. For staining with Hoechst 33258, 2×10^5 HL-60 cells were cultivated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) containing 13 mm round glass coverslips. For DNA-fragmentation assays, cells were grown in 6-well tissue culture plates at a density of 2×10^6 per well.

Induction of apoptosis and *T. gondii* infection

Prior to infection, adherent HL-60 cells were washed three times with RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin to remove PMA. HL-60 cells were then infected with *T. gondii* at a parasite to host ratio of 4:1. One to two hours post infection, extracellular parasites were removed by washing three times with medium. Apoptosis was induced by adding 0.1 to 0.5 μg/ml actinomycin D (act D, Calbiochem, La Jolla, CA) to the cells, which were then incubated for additional 12 or 16 hours at 37℃ and 5% CO_2 in saturated humidity.

DNA fragmentation assay

The DNA fragmentation assay was performed as described previously [7]. Briefly, 2×10^6 HL-60 cells from each sample were lysed in 2 ml of 7 M guanidine hydrochloride. Genomic DNA was then extracted with a Wizard® Plus Minipreps DNA Purification System (Promega, Madison, WI). Finally, DNA was electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

Morphological detection of apoptosis

After 8 h incubation, cells were fixed with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS, pH 7.4, Biochrom, Berlin) for 30 min at room temperature. After washing with PBS cells were stained with 50 ng/ml Hoechst 33258 (Sigma) in PBS for 1 h. Finally, coverslips

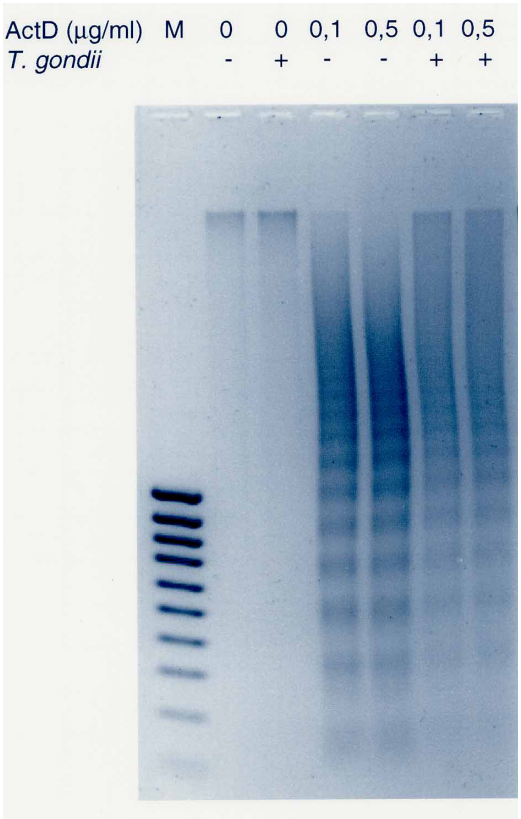
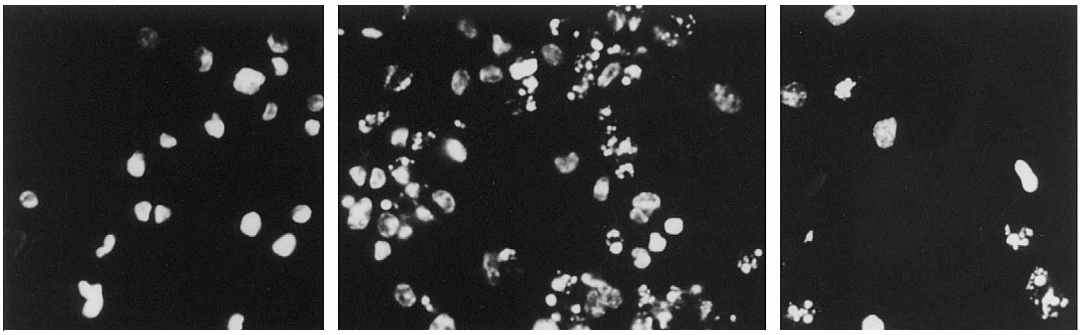


Fig. 1 DNA fragmentation in actinomycin D-treated HL-60 cells after infection with *Toxoplasma gondii* and in uninfected controls. HL-60 cells were infected at a parasite to host ratio of 4:1 and incubated with 0.1 or 0.5 μg/ml actinomycin D as indicated. Sixteen hours later genomic DNA was electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. As a marker (M), a 100 bp DNA ladder was separated in parallel.



a b c

Fig. 2 Influence of *Toxoplasma gondii* infection on actinomycin D-induced apoptosis in HL-60 cells. Condensation of chromatin after 12 h incubation was visualized by staining with Hoechst 33258. In (a) non-infected HL-60 cells were cultivated in the absence of actinomycin D (act D), the nuclei show no condensation of chromatin. (b) Treatment of HL-60 cells with 0.5 μg/ml act D induced condensation of chromatin. Apoptotic nuclei show a granular pattern. (c) Act D-treated HL-60 cells concomitantly infected with *T. gondii* at a parasite to host ratio of 4:1, leading to reduction of cells with chromatin condensation.

were mounted with Mowiol (Calbiochem, San Diego, CA) and were examined by conventional fluorescence microscopy.

Viability assay

To distinguish apoptosis and necrosis, the viability of the cells was tested by trypane blue exclusion for each apoptosis assay. In each experiment, an additional set of coverslips was subjected to staining with 0.1% (w/v) trypane blue (Biochrom) in PBS.

RESULTS

The DNA fragmentation assay was used to determine the effect of *T. gondii* on the rate of apoptosis in a host cell population. Sixteen hours after infection of HL-60 cells with *T. gondii* tachyzoites of the strain NTE at a parasite to host ratio of 4:1, fragmentation of genomic DNA was not increased as compared to uninfected control cells (Fig. 1). After incubation of HL-60 cells with 0.1 or 0.5 μg/ml act D for 16 hours, genomic DNA from these cultures was highly fragmented into multimers of 180 bp as judged by agarose gel electrophoresis, indicating that these cells underwent apoptosis. However, if act D-treated cultures were concomitantly infected with *T. gondii* at a parasite to host ratio of 4:1, fragmentation of genomic DNA clearly diminished which indicates a reduction of apoptosis in these cultures as compared to non-infected cultures treated with act D.

To quantify the reduction of act D-induced apoptosis by *T. gondii*, cell cultures were stained with Hoechst 33258 to visualize nuclear DNA condensation as a typical feature of apoptosis (Fig. 2). In the absence of act D, only single cells underwent apoptosis and this was similarly observed in non-infected cultures (Fig. 2A) as well as *T. gondii*-infected cell cultures (data not shown). After treatment with 0.1 to 0.5 μg/ml act D for 12 h, the number of apoptotic cells increased dramatically (Fig. 2B). In act D-treated cultures concomitantly infected with *T. gondii* at a parasite to host ration of 4:1 however, the total number of apoptotic cells considerably decreased (Fig. 2C).

Fig. 3 summarizes the results of two independent experiments after infection with *T. gondii*. In untreated HL-60 cultures, the percentage of apoptotic cells was smaller than 2% and this did not increase after infection with *T. gondii*. However, apoptosis was dose dependently induced after treatment with 0.1 to 0.5 μg/ml act D. In contrast, the rate of act D-induced apoptosis was clearly reduced in HL-60 cultures that were infected with *T. gondii* at a host to parasite ratio of 4:1, irrespective the concentration of act D. The rate of apoptosis decreased by 62%, 58% and 45% after treatment with 0.1 μg/ml, 0.25 μg/ml and 0.5 μg/ml act D, respectively.

In parallel to all experiments, the viability of HL-60 cells was tested by trypane blue exclusion to distinguish apoptotic from

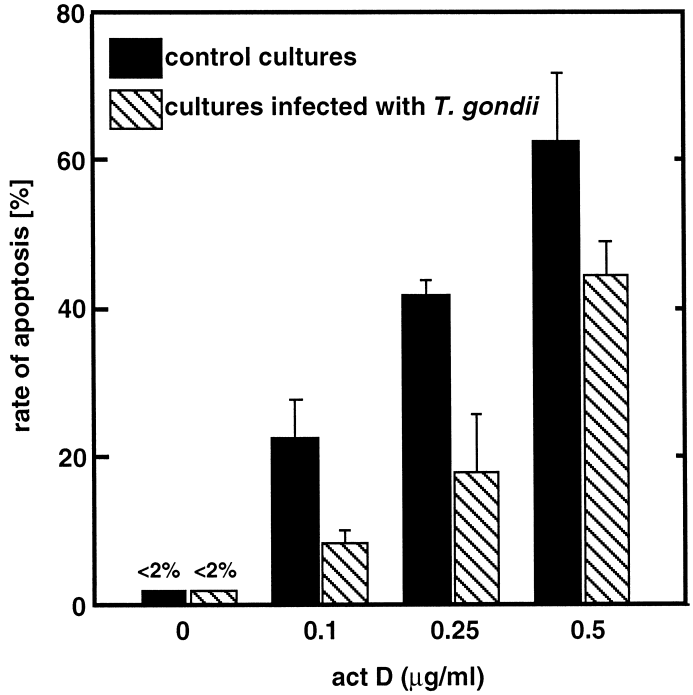


Fig. 3 Percentages of apoptotic HL-60 cells after infection with *Toxoplasma gondii* and treatment with 0.1, 0.25 and 0.5 μg/ml actinomycin D (act D) for 12 hours. HL-60 cultures were concomitantly infected with *T. gondii* at a parasite to host ratio of 4:1. Cell cultures were stained with Hoechst 33258 to visualize nuclear DNA condensation as a typical feature of apoptosis. At least 500 cells of each sample were examined using fluorescence microscopy. Data represent means ± standard error from two independent experiments.

necrotic cells. In all cases, less than 6% of the host cells underwent necrosis (data not shown).

DISCUSSION

Several intracellular pathogens, including bacteria and some viruses have been shown to increase the rate of apoptosis of their host cells [1, 15, 18, 25], while other viral infections inhibit apoptosis [1]. However, little is known about the effect of more complex eukaryotic parasites on programmed cell death of their host cells. *T. gondii* stimulated apoptosis of lymphocytes from infected mice [16], and the closely related protozoan parasite *Plasmodium falciparum* stimulated apoptosis of human mononuclear cells [24]. In contrast, a decrease in the rate of apoptosis was described after infection of murine host cells with *Leishmania donovani* [17] or *T. gondii* [19].

The results presented herein clearly show that *T. gondii* is able to prevent act D-induced apoptosis of human-derived HL-60 cells. This confirms the finding of Nash et al. [19] who showed that cultures of several murine cell types infected with *T. gondii* resist programmed cell death that was initiated by different apoptosis-inducing treatments. The molecular mechanism of this parasite-host interaction remains to be elucidated. For viruses, several apoptosis-regulator genes are known which can inhibit host cell apoptosis, e.g. genes for members of the Bcl-2 family or caspases [1]. The bacterial pathogen *Chlamydia trachomatis* suppresses host cell apoptosis by blockade of mitochondrial cytochrome c release and caspase activation [8], whereas *Mycobacterium tuberculosis* is thought to down regulate the apoptosis of its host cells by stimulation of TNF-α production [6]. The protozoan parasite *Leishmania donovani* also stimulates the production of TNF-α together with GM-CSF by its host cells to prevent their apoptosis [17].

A proapoptotic effect of *T. gondii* on

murine $CD4^+$ T cells has been described by Khan et al. [16]. In that study, infection of mice with *T. gondii* rendered $CD4^+$ T lymphocytes unresponsive to mitogenic or antigenic stimulation and consequently apoptotic when cultured *ex vivo* for several days. Induction of programmed cell death of $CD4^+$ T cells [16] as well as macrophages [13] may represent a mechanism to evade the host's immune response in the acute phase of infection and this is part of a strategy that establishes persistence of *T. gondii* in its host. The induction of apoptosis in T cells has been similarly described for *Trypanosoma cruzi* [3] and *P. falciparum* [24] and this possibly represents a more general feature of protozoan parasites. However, at least those parasites that persist intracellularly have to rely on the integrity of the host cell. Prevention of host cell apoptosis, as reported herein and by others [1, 17] may facilitate long-term survival of the parasite. *T. gondii* is thought to persist predominantly within neurons in the brain of infected hosts [9]. In this cell type, apoptosis is unlikely to interfere with bradyzoite persistence since functionally active mature neurons do not undergo apoptosis in healthy hosts. More importantly, however, cases of acute toxoplasmosis were observed in patients that were seronegative prior to organ transplantation indicating that *T. gondii* is able to persist in organs besides the CNS [2]. This has additionally clearly been shown in *Toxoplasma*-infected animals [5]. Persistence in cells of such organs may be established by inhibition of apoptosis by the parasite. Our report and that of Nash et al. [19] demonstrate that *T. gondii* indeed is able to inhibit host-cell apoptosis in different cell types. This may represent a mechanism that facilitates long-term survival of *T. gondii*.

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REFERENCES

1) Barry M, McFadden G: Apoptosis regulators from DNA viruses. Curr Opin Immunol 10: 422-430, 1998.
2) Derouin F, Devergie A, Auber P, Gluckman E, Beauvais B, Garin YJF, Lariviere M: Toxoplasmosis in bone marrow-transplant recipients: report of seven cases and review. Clin Infect Dis 15: 267-270, 1992.
3) DosReis GA, Fonseca MEF, Lopes MF: Programmed T-cell death in experimental chagas disease. Parasitol Today 11: 390-394, 1995.
4) Dubey JP, Peattie CP: Toxoplasmosis of animals and man. CRCPress, Inc., Boca Raton, Florida, USA, 1988.
5) Dubey JP: Tissue cyst tropism in *Toxoplasma gondii*: a comparison of tissue cyst formation in organs of cats, and rodents fed oocysts. Parasitology 115: 15-20, 1997.
6) Dürrbaum-Landmann I, Gercken J, Flad HD, Ernst M: Effect of *in vitro* infection of human monocytes with low numbers of *Mycobacterium tuberculosis* bacteria on monocyte apoptosis. Infect Immun 64:5384-5389, 1996.
7) Eldadah BA, Yakovlev AG, Faden AI: A new approach for the electrophoretic detection of apoptosis. Nucl Ac Res 24: 4092-4093, 1996.
8) Fan T, Lu H, Hu H, Shi L, McClarty GA, Nance DM, Greenberg AH, Zhong G: Inhibition of apoptosis in *Chlamydia*-infected cells: blockade of mitochondrial cytochrome c release and caspase activation. J Exp Med 187: 487-496, 1998.
9) Ferguson DJP, Hutchison WM: An ultrastructural study of the early development and tissue cyst formation of *Toxoplasma gondii* in the brains of mice. Parasitol Res 73: 483-461, 1987.
10) Gregory CD, Dive C, Henderson S, Smith CA, Williams GT, Gordon J, Rickinson AB: Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature 349: 612-614, 1991.
11) Gross U, Müller WA, Knapp S, Heesemann J: Identification of a virulence-associated antigen of *Toxoplasma gondii* by a mouse monoclonal antibody. Infect Immun 59: 4511-4516, 1991.
12) Henderson S, Rowe M, Gregory C, Croom-Carter D, Wang F, Longnecker R, Kieff E, Rickinson A: Induction of bcl-2 expression by Epstein-Barr Virus Latent Membrane Protein 1 protects infected B cells from programmed cell death. Cell 65: 1107-1115, 1991.
13) Hisaeda H, Sakai T, Ishikawa H, Maekawa Y, Yasutomo K, Good RA, Himeno K: Heat shock protein 65 induced by $\gamma\delta$ T cells prevents apoptosis of macrophages and contributes to host defense in mice infected with *Toxoplasma gondii*. J Immun 159: 2375-2381, 1997.
14) Joiner KA, Dubremez JA: *Toxoplasma gondii*: a protozoan for the nineties. Infect Immun 61: 1169-1172, 1993.
15) Keane J, Katarzyna B-S, Remold HG, Chupp GL, Meek BB, Fenton MJ, Kornfeld H: Infection by *Mycobacterium tuberculosis* promotes human alveolar marcrophage apoptosis. Infect Immun 65: 298-304, 1997.
16) Khan IA, Matsuura T, Kasper LH: Activation-mediated $CD4^+$ T cell unresponsiveness during acute *Toxoplasma gondii* infection in mice. Int Immunol 8: 887-896, 1996.
17) Moore KJ, Matlashewski G: Intracellular infection by *Leishmania donovani* inhibits macrophage apoptosis. J Immunol 152: 2930-2937, 1994.

18) Müller A, Hacker J, Brand BC: Evidence for apoptosis of human macrophage-like HL-60 cells by *Legionella pneumophila* infection. Infect Immun 64: 4900-4906, 1996.
19) Nash PB, Purner MB, Leon RP, Clarke P, Duke RC, Curiel TJ: *Toxoplasma gondii* -infected cells are resistant to multiple inducers of apoptosis. J Immunol 160: 1824-1830, 1998.
20) Porter S, Sande MA: Toxoplasmosis of the central nervous system in the acquired immunodeficiency syndrome. N Engl J Med 327: 1643-1648, 1992.
21) Remington JS, Desmonts G: Toxoplasmosis. In: J. S. Remington, J. O. Klein (eds): Infectious diseases of the fetus and newborn infant. W. B. Saunders, Philadelphia: 89-195, 1990.
22) Revilla Y, Cebrian A, Baixerás E, Martínez A, Vinuela E, Salas ML: Inhibition of apoptosis by the african swine fever virus Bcl-2 homologue: Role of the BH1 domain. Virology 228: 400-404, 1997.
23) Takizawa T, Matzukawa S, Higguchi Y, Nakamura S, Nakamishi Y, Fukuda R: Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. J Gen Virol 74: 2347-2355, 1993.
24) Toure-Balde A, Sarthou JL, Aribot G, Michel P, Trape JF, Rogier C, Roussilion C: *Plasmodium falciparum* induces apoptosis in human mononuclear cells. Infect Immun 64: 744-750, 1996.
25) Zychlinsky A, Prevost MC, Sansonetti JS: *Shigella flexneri* induces apoptosis in infected macrophages. Nature 358: 167-169, 1992.