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Cytotoxic T-Lymphocyte-Mediated Lysis of Toxoplasma gondii-Infected Target Cells Does Not Lead to Death of Intracellular Parasites

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CD8+ T cells play a crucial role in the control of infection with intracellular microbes. The mechanisms underlying the CD8+ T-cell-mediated clearance of the intracellular pathogen Toxoplasma gondii are, however, not completely understood. The effect of CD8+ cytotoxic T-lymphocyte (CTL)-mediated lysis of host cells on the viability of intracellular T. gondii was investigated. Quantitative competitive PCR of the gene encoding T. gondii major surface antigen (SAG-1) was combined with treatment of the parasites with DNase, which removed the DNA template of nonviable parasites. The induction by CD8+ CTLs of apoptosis in cells infected with T. gondii did not result in the reduction of live parasites, indicating that intracellular T. gondii remains alive after lysis of host cells by CTLs.

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects all warm-blooded animals, including humans. After the primary infection in an immunocompetent individual, the immune response of the host limits the replication of tachyzoites, resulting in the formation of the bradyzoite form, a dormant stage of the parasite. Because of this effective immune reaction against parasites, chronic infection of an immunocompetent individual with T. gondii is usually asymptomatic (3). However, in patients with AIDS as well as other immunocompromised states, reactivation of chronic toxoplasmosis results in excessive cellular destruction, often leading to severe morbidity and mortality (10).

Protective immune responses against infection with T. gondii have been studied with experimental murine systems. Vaccination with an attenuated mutant (22, 24) or irradiated tachyzoites (18, 25) induces protective responses against subsequent infection with the virulent RH strain. CD8+ T-cell-mediated immunity is one of the major protective mechanisms. Mice primed with T. gondii succumb to lethal infection when depleted of CD8+ T cells prior to challenge infection, and host resistance can be adoptively transferred to naive animals by primed CD8+ T cells (22, 25) or by CD8+ T-cell clones specific for T. gondii major surface antigen (SAG-1) (8). However, the underlying mechanisms of CD8+ cell-mediated protective immunity are not completely understood. The protection depends on the presence of gamma interferon, which can be secreted by CD8+ T cells as well as CD4+ cells (8, 23). T. gondii-specific CD8+ T cells also lyse parasite-infected target cells in a class I major histocompatibility complex-restricted manner (7, 11, 14, 21, 26). Lysis of host cells may directly damage intracellular microbes or simply release viable parasites into the extracellular space. Apoptosis of infected host cells induced either chemically (13) or by CD8+ cytotoxic T lymphocytes (CTL) (20) appears to be coupled to damage to intracellular mycobacteria. Little is known, however, about the fate of T. gondii parasites in host cells lysed by CD8+ CTL.

The present study was therefore designed to determine whether CD8+ CTL-mediated lysis of host cells is associated with the killing of intracellular T. gondii tachyzoites. We developed a novel method to determine the number of live T. gondii parasites within host cells undergoing apoptosis. We used a combination of DNase treatment of the samples prior to DNA extraction, which removed DNA within dead parasites, and quantitative competitive PCR (QC-PCR) of the T. gondii SAG-1 gene (12). The results indicated that CD8+ CTL-mediated apoptosis of target cells does not lead to the death of intracellular T. gondii tachyzoites.

MATERIALS AND METHODS

Animals, parasites, and cell lines. BALB/cAnNCrj and C57BL/6NCrj mice and Lewis/Crj rats were purchased from Charles River Japan (Kanagawa, Japan). Animals were housed in the Laboratory Animal Center for Biomedical Research at the Nagasaki University School of Medicine (Nagasaki, Japan) and were used at 8 to 10 weeks of age. T. gondii RH was maintained as previously described (16, 26). M12-neo-1 cells were generated by stable transfection of M12.4.1 cells (a gift from L. Glimcher, Harvard Medical School, Boston, Mass.) (9) with linearized Rc/CMV (Invitrogen, Carlsbad, Calif.) by use of a Gene Pulser (Bio-Rad, Hercules, Calif.). Transfectants were selected in culture medium containing G418 (0.5 mg/ml) (Gibco BRL, Grand Island, N.Y.) and cloned by a limiting-dilution method.

H-2d-specific CTL lines were established from nonadherent splenocytes of C57BL/6 mice by repeated stimulation with X-ray-irradiated (20 Gy) BALB/c splenocytes in RPMI 1640 (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 U of penicillin per ml, 100 μg of streptomycin per ml, 50 μM of mercaptoethanol, and 20 μM of human recombinant interleukin 2 (Shionogi, Osaka, Japan) per ml. These T cells expressed the T-cell-receptor β chain and were of the CD3+ CD4- CD8+ phenotype (data not shown). The cytolytic activity of the T-cell lines was determined by a standard 51Cr release assay as described previously (1). The percentages of specific 51Cr release by M12.4.1 (H-2d), P815 mastocytoma (H-2b), and EL4 thymoma (H-2d) cells at an effector/target cell ratio of 2.5:1 were 74, 69, and 1%, respectively, indicating that these cell lines were specific for H-2d.

To induce CTL specific for T. gondii, BALB/c mice were primed twice by intraperitoneal inoculations with T. gondii RH tachyzoites (107/mouse) which had been inactivated by treatment with mitomycin C (200 μg/ml) for 2 h at 37°C. Two weeks after the final priming, mice were sacrificed and spleens were removed. After lysis of erythrocytes, spleen cells (4 × 108/ml) were cultured for 5 days in the presence of mitomycin C-treated T. gondii tachyzoites (107/ml) to induce CTL specific for T. gondii.

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The DNA within the Neo gene sequences was amplified with a pair of primers which contained a constant amount of competitor DNA. The amplified competitor DNA was coamplified with the DNA from infected cells. The results were expressed as the mean SAG-1 copy number per template DNA, corresponding to approximately 100 input tachyzoites.

The number of live M12-neo-1 cells was determined by QC-PCR of the Neo gene. Because SAG-1 is a single-copy gene, the copy number of SAG-1 DNA is equal to the number of tachyzoites. The results were expressed as the mean SAG-1 copy number per template DNA, corresponding to 100 input tachyzoites or infected cells per template DNA, corresponding to 300 input target cells.

**RESULTS**

**Estimation of the number of live T. gondii organisms by QC-PCR.**

The number of intracellular T. gondii organisms can be estimated by calculating the ratio of the amplified target and competitor sequences to the amplified competitor DNA. The results were expressed as the mean SAG-1 copy number per template DNA, corresponding to 100 input tachyzoites or infected cells per template DNA, corresponding to 300 input target cells.

**Table 1. Number of SAG-1 gene copies in live and dead T. gondii after DNase treatment**

<table>
<thead>
<tr>
<th>DNase treatment</th>
<th>Not treated (P = 0.268)</th>
<th>Anti-T. gondii antibody + complement (P &lt; 0.001)</th>
<th>Mixture of not treated plus anti-T. gondii antibody + complement (P = 0.01)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>135 ± 8</td>
<td>149 ± 14</td>
<td>163 ± 16</td>
</tr>
<tr>
<td></td>
<td>128 ± 5</td>
<td>&lt;7</td>
<td>88 ± 2</td>
</tr>
</tbody>
</table>

a Tachyzoites were not treated or were treated with anti-T. gondii serum and complement. Their viability was nearly 100% (not treated) or 0% (treated). b Data are for Student’s t-test between DNase-treated and untreated groups.
cated that DNase treatment effectively eliminates the SAG-1 gene within dead *T. gondii* and that this method can be applied to the quantitation of live parasites in the presence of dead *T. gondii* host tissue.

Viability of *T. gondii* in infected CTL target cells. We applied this method to determine the fate of intracellular *T. gondii* after the lysis of host cells by CD8^+^ CTL. An alloreactive CTL line specific for H-2^k^ was generated by repeated stimulation of C57BL/6 lymphocytes with BALB/c spleen cells. The stable cell line M12-neo-1 was used as a target. The use of a neo transfectant cell line allowed quantitation of the number of viable target cells in the presence of CTL. M12-neo-1 cells were infected with *T. gondii* in vitro. After the removal of free *T. gondii*, the infected target cells were incubated with H-2^k^ specific CTL for 0 to 12 h. Cells were treated with DNase, and DNA was extracted and subjected to QC-PCR to determine the number of live *T. gondii* parasites within the target cells (Fig. 2). During the initial 8 h of coculturing, the number of tachyzoites within the target cells did not change significantly. After 8 h, the number increased, possibly due to the DNA synthesis of *T. gondii* within the infected cells. Interestingly, this increase was observed even when the target cells were lysed by CTL. We speculate that some of the parasites within the apoptotic target cells infected and multiplied within effector CTL. Indeed, light microscopic inspection of the cells after 12 h of culturing revealed that approximately 8% of the effector T cells were infected by tachyzoites (data not shown). In the same experiment, ^51^Cr release by the target cells reached 84% during 6 h of culturing (Fig. 2). These results indicate that CTL-mediated lysis of cells infected with tachyzoites does not lead to the death of the intracellular parasites.

One caveat of this interpretation is that there remained a possibility that DNase might not be able to access DNA within cells lysed by CTL and therefore might be unable to digest DNA of intracellular *T. gondii*. To rule out this possibility, we determined whether DNase is able to reach the DNA within lysed target cells (Table 2). The M12-neo-1 cell line was used as a target for this purpose. Thus, the Neo gene is present in target and not in effector cells, enabling us to determine the number of viable target cells in the target cell-effector cell mixture. M12-neo-1 cells were incubated with alloreactive CTL, and QC-PCR of the Neo gene was performed with DNA extracted from these cells. Coculturing of M12-neo-1 cells with CTL resulted in the reduction of the Neo gene copy number in parallel with an increase in ^51^Cr release by the target cells. In contrast, M12-neo-1 cells cultured without CTL maintained the same copy number of the Neo gene after DNase treatment. The comparison of QC-PCR with ^51^Cr release suggested that permeability to DNase may be a more sensitive method than ^51^Cr release in determining CTL activity. Thus, DNase is able to access cellular DNA when target cells are lysed by CTL.

Intracellular *T. gondii* resides within parasitophorous vacuoles. It was thus still not clear whether DNase could reach the DNA of intracellular *T. gondii* after CTL-mediated lysis of host cells. Thus, we examined the viability of intracellular *T. gondii* after CTL-mediated lysis of host cells by an alternative method. We examined whether intracellular *T. gondii* within host cells lysed by CTL maintains an intact cell membrane. After coculturing with CTL, parasites were released from target cells by mechanical disruption. DNA was extracted from these samples after DNase treatment and subjected to QC-PCR (Fig. 3). To confirm that dead parasites were DNase accessible in this experiment, one of the disrupted samples was treated with anti-*T. gondii* antibody and complement prior to DNase treatment. The results indicated that *T. gondii* within CTL target cells was resistant to DNase treatment, supporting the conclusion that the parasites were alive.

Finally, to determine that intracellular *T. gondii* was indeed viable after the lysis of host cells by *T. gondii*-specific CTL, we determined the numbers of Neo and SAG-1 gene copies in *T. gondii*-infected M12-neo-1 cells after incubation with *T. gondii*-specific CTL and DNase treatment (Table 3). CTL were induced by coculturing of primed BALB/c spleen cells with mitomycin C-treated *T. gondii* tachyzoites. These CTL were specific for *T. gondii* because treatment of the *T. gondii*-infected target cells with DNase after coculturing with the CTL resulted in a 64% reduction in the Neo gene copy number (137 versus 60), while no significant reduction was observed when the target cells were not infected (124 versus 122). The same DNA samples were used to assess the viability of *T. gondii* within the target cells. The SAG-1 gene copy number did not change significantly after DNase treatment (102 versus 110),

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**TABLE 2. Number of Neo gene copies of M12-neo-1 cells after lysis by CTL and DNase treatment**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Neo gene copy no. with the following CTL/DNase treatment</th>
<th>% Specific ^51^Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>195 ± 30, 180 ± 17, ND, ND, ND</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>ND, ND, 199 ± 24, 64 ± 3, 48</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>ND, ND, 210 ± 36, 26 ± 12, 67</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>ND, ND, 188 ± 35, 4 ± 2, 78</td>
<td>4</td>
</tr>
</tbody>
</table>

*a* ^51^Cr-labeled M12-neo-1 cells (10^6^) were incubated or not incubated with alloreactive CTL cells (10^5^) for 1 to 4 h, and treated or not treated with DNase. The number of Neo gene copies was determined by QC-PCR and is expressed as the mean copy number per template DNA, corresponding to 300 input M12-neo-1 cells ± 1 SD. The percent specific ^51^Cr release was determined by a standard method in the same experiment. Similar results were obtained in three independent experiments. ND, not done.
indicating that intracellular *T. gondii* was alive after lysis of the target cells by the *T. gondii*-specific CTL.

**DISCUSSION**

A novel method to selectively quantify the number of live *T. gondii* parasites in a mixture of dead *T. gondii* and host tissue was developed. The quantitation of live *T. gondii* parasites was previously performed by determining their ability to grow intracellularly or lyse host cells by a $^3$H-uracil incorporation assay or a plaque assay, respectively (15). However, only 20 to 30% of the free tachyzoites lysed from hosts are infective for other cells, and these methods are difficult to apply in quantifying viable tachyzoites within apoptotic host cells, because individual tachyzoites cannot be segregated from the apoptotic cell, which tends to clump. Therefore, we used membrane permeability as a measure of cell viability and overcame these difficulties by a combination of DNase treatment and QC-PCR, which allowed simple and rapid quantitation of viable tachyzoites in the cell mixture. This method was applied to determine whether CD8$^+$ CTL can kill intracellular tachyzoites when they lyse target cells. The results indicated that CTL specific for *T. gondii* are unable to kill intracellular *T. gondii* tachyzoites.

The induction of apoptosis in target cells by CD8$^+$ CTL is mediated by perforin and granzyme B or the engagement of Fas on the target cells (7, 11). In our assay system, it is likely that the lysis of *T. gondii*-infected target cells was mediated by perforin and granzyme B, because the CTL used in our assay system are conventional CD8$^+$ CTL and because M12-neo-1 target cells do not express Fas (reference 5 and unpublished data). Thus, we believe that the lysis of *T. gondii*-infected cells through the release of cytotoxic granules by CTL does not lead to the death of intracellular parasites. Indeed, perforin does not appear obligatory for protection against *T. gondii* infection, because *T. gondii*-primed perforin knockout mice retain resistance to challenge infection with the parasite (2). It is formally possible that Fas-mediated lysis of host cells has distinct effects on intracellular *T. gondii*. We think it is unlikely, however, because the induction of apoptosis in Fas$^+$ A20 infected with *T. gondii* by anti-Fas monoclonal antibodies did not kill intracellular parasites (data not shown). Perforin- or granzyme-mediated lysis of infected macrophages by CTL has been shown to result in the death of intracellular *Mycobacterium tuberculosis*, whereas Fas-mediated lysis has not (20). The discrepancy between our study and theirs may be due to the differences in the pathogens used (*T. gondii* versus *M. tuberculosis*) and in the host species (mouse versus human). Alternatively, differences in host cells may explain this difference. We used a B-cell tumor which does not have the phagocytic ability of apoptotic cells, while they used macrophages as infected targets. Therefore, the possibility that the death of the intracellular bacteria was due to the phagocytosis of apoptotic macrophages by neighboring macrophages was not completely ruled out in their study. It is also possible that a subset of CD8$^+$ CTL can kill intracellular *T. gondii*.

We previously demonstrated that protective immunity against a virulent strain of *T. gondii* can be transferred to naive animals by adoptive transfer of primed CD8$^+$ cells (25). If

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**TABLE 3. Effect of *T. gondii*-specific CTL on the viability of intracellular *T. gondii* $^a$**

<table>
<thead>
<tr>
<th>CTL</th>
<th>Infection of target cells with <em>T. gondii</em></th>
<th>Neo copies</th>
<th>SAG-1 copies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ M12-neo-1 cells (10$^5$) which were infected (+) or not infected (−) with *T. gondii* were incubated or not incubated with CTL specific for *T. gondii* for 4 h at an effector/target cell ratio of 50:1 prior to DNase treatment. The numbers of Neo and SAG-1 gene copies were determined by QC-PCR as described in Materials and Methods and are expressed as the mean copy number for triplicate experiments ± 1 SD. Fifty-one percent of the target cells were infected with *T. gondii*, as determined by light microscopy. ND, not done.
CD8+ CTL are not themselves cytotoxic for intracellular *T. gondii*, how are tachyzoites cleared in vivo? The lysis of host cells by CTL may release tachyzoites from their sequestered environment into the extracellular space, where other effector molecules and cells are accessible. These include antibody, complement, NK cells, a population of CD8+ T cells which are directly parasitocidal (8), and macrophages (6). It is unclear which molecules and cells are the most critical for clearing tachyzoites after host cell lysis. Alternatively, tachyzoites may be cleared even prior to their release into the extracellular fluid after the induction of host cell apoptosis. induction of tachyzoites after host cell lysis. Alternatively, tachyzoites may directly parasitocidal (8), and macrophages (6). It is unclear from the Ministry of Education, Science, Culture and Sports.

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