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

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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 RH 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).

CDS* T cells play a crucial role in the control of infection with intracellular microbes. The mechanisms underlying the CDS* T-cell-mediated clearance of the intracellular pathogen *Toxoplasma gondii* are, however, not completely understood. The effect of CDS* 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 CDS* 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.

**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-l cells were generated by stable transfection of M12.4.1 cells (a gift from L. Glimcher, Harvard Medical School, Boston, Mass.) 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-2b-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 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 CDS* CDS* phenotype (data not shown). The cytolytic activity of the T-cell lines was determined by a standard 3Cr release assay as described previously (1). The percentages of specific 3Cr release by M12.4.1 (H-2b), P81S mastocytoma (H-2b), and EL4 thymoma (H-2b) 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-2b.

To induce CTL specific for *T. gondii*, BALB/c mice were primed twice by intraperitoneal inoculations with *T. gondii* RH tachyzoites (10⁶/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 × 10⁶/ml) were cultured for 5 days in the presence of mitomycin C-treated *T. gondii* tachyzoites (10⁴/ml) to induce CTL specific for *T. gondii*.

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DNase treatment of killed *T. gondii* and DNA preparation. Complement-mediated killing of free tachyzoites (10^5) was performed at 37°C for 30 min with rat anti-*T. gondii* serum prepared from a Lewis rat after priming with X-ray-irradiated (120 Gy) RH tachyzoites and with rabbit complement. The trypsin blue dye exclusion test indicated that 100% of the parasites treated with antibody plus complement were dead after the treatment (data not shown). Cells were incubated in phosphate-buffered saline (PBS) containing DNase (50 μg/ml) for 30 min at 37°C. After the cells were washed once with PBS, genomic DNA was prepared with DNAzol (Gibco BRL) in accordance with the manufacturer’s instructions. Glycogen (10 μg) (Boehringer GmbH, Mannheim, Germany) was included in the DNAsol solution as a carrier.

Preparation of DNA from *T. gondii*-infected target cells after CDR \( ^{*} \) T-cell-mediated cytolysis. M12-neo-1 cells were infected with *T. gondii* at a multiplicity of infection of 3:1 for 2 h at 37°C. Free tachyzoites were removed from infected cells by two rounds of low-speed centrifugation (70 × g for 10 min) followed by use of a magnetic cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). The infected cells were incubated with rat anti-*T. gondii* serum at 4°C for 10 min. After being washed with PBS, the cells were incubated with microbeads coated with mouse anti-rat immunoglobulins (20 μl of magnetic probes per 10^6 cells) in 1 ml of PBS containing 5% bovine serum albumin and 0.1 mM EDTA at 8°C for 20 min. Free tachyzoites were subsequently removed in a magnet field of 0.6 T with an MS column (Miltenyi Biotec) in which the flowthrough fraction was collected. The number of surviving free tachyzoites was less than 2% of M12-neo-1 cells, as determined by light microscopy. The proportion of the cells infected with *T. gondii* was 40 to 50%. After coculturing of infected M12-neo-1 cells (10^6) with CTL (10^5), samples were treated with DNase (50 μg/ml) for 30 min at 37°C and DNA was extracted. In some experiments (see Fig. 3), *T. gondii*-infected M12-neo-1 cells were passed through a 30-gauge needle several times to force the intracellular parasites out of the parasitophorous vacuoles as described previously (4). The materials were treated or not treated with anti-*T. gondii* serum and complement before DNase treatment.

QC-PCR of SAG-1 and Neo genes. The number of parasites was determined by QC-PCR of the SAG-1 gene as previously described (12). Briefly, genomic DNA (>1 μg) extracted from *T. gondii* or infected cells was coamplified with a constant amount of competitor DNA (i.e., approximately 90 copies of the truncated SAG-1 gene) by use of a set of SAG-1-specific primers for 36 cycles in a final volume of 50 μl in a TCR-300 thermal sequencer (IWAKI Glass Co. Ltd., Chiba, Japan). The amplified products were separated by agarose gel electrophoresis and stained with ethidium bromide. The ratios of the staining intensities of the target and competitor sequences were determined by densitometry (IPLab Gel densitometer; Signal Analytical Corp., Vienna, Va.). By comparing the ratio thus obtained to a standard curve, the copy number of SAG-1 DNA was calculated. The detection limit was seven copies of SAG-1 DNA per sample. Because SAG-1 is a single-copy gene, the copy number of SAG-1 DNA is equal to the number of *T. gondii* tachyzoites. The tachyzoites were expressed as the mean parasite number per template DNA, corresponding to 100 input tachyzoites or 300 input M12-neo-1 cells ± 1 standard deviation (SD). For statistical comparisons of two groups, an unpaired two-tailed Student’s t test was used. A P value <0.05 was considered significant.

The number of live M12-neo-1 cells was determined by QC-PCR of the Neo gene. The SAG-1 gene was amplified by PCR as described above. The PCR product from live parasites (Fig. 1, lane 2; Table 1) was treated with DNase to remove the DNA template within killed *T. gondii*, assuming that DNase can gain access to the DNA within dead but not viable cells. This pretreatment reduced the amount of the PCR product from dead parasites more than 100-fold (Fig. 1, lanes 3 and 4, Table 1). This reduction was selective for dead parasites, because the same treatment did not significantly reduce the amount of the PCR product from live parasites (Fig. 1, lane 2; Table 1, P = 0.268).

To determine that this method can be applied to a mixture of live and dead *T. gondii*, a sample containing a 1:1 mixture of live and dead parasites was treated with DNase, and the number of viable *T. gondii* parasites was quantitated by QC-PCR. As expected, the number of SAG-1 gene copies obtained from the DNase-treated samples (88 ± 2) was approximately half that in the untreated samples (163 ± 16) (Fig. 1, lanes 5 and 6; Table 1). In a separate experiment, we confirmed that the gene copy numbers estimated by this method had a linear relationship with the ratio of viable parasites within the mixture of dead and live *T. gondii* (data not shown). These results indi-

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### RESULTS

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

The number of intracellular *T. gondii* organisms can be comparatively estimated by plotting the ratios of the peak areas of the QC-PCR product of *T. gondii* SAG-1 and its competitor sequences against a standard curve (12). Initially, we examined whether this method can selectively amplify DNA of viable parasites. DNA extracted from free viable tachyzoites and those killed by anti-*T. gondii* serum plus complement were used to estimate the number of *T. gondii* organisms by QC-PCR (Fig. 1 and Table 1). The ratio of the amplified target and competitor sequences did not differ significantly (Fig. 1, lanes 1 and 3; Table 1, P = 0.207), indicating that the DNA extracted from complement-killed *T. gondii* can become a template for PCR, like that extracted from viable *T. gondii*. Therefore, we modified this method by introducing DNase treatment to exclude DNA from dead parasites and determined whether the number of live parasites can be estimated by QC-PCR. Free *T. gondii* was treated with DNase to remove the DNA template within killed *T. gondii*, assuming that DNase can gain access to the DNA within dead but not viable cells. This pretreatment reduced the amount of the PCR product from dead parasites more than 100-fold (Fig. 1, lanes 3 and 4, Table 1). This reduction was selective for dead parasites, because the same treatment did not significantly reduce the amount of the PCR product from live parasites (Fig. 1, lane 2; Table 1, P = 0.268).

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### 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>Anti-<em>T. gondii</em> antibody + complement</th>
<th>Mixture of not treated + anti-<em>T. gondii</em> antibody + complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not treated</td>
<td>(P = 0.268)</td>
<td>(P &lt; 0.001)</td>
</tr>
<tr>
<td>135 ± 8</td>
<td></td>
<td>149 ± 17</td>
</tr>
<tr>
<td>128 ± 5</td>
<td></td>
<td>&lt;7</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), as determined by the trypsin blue dye exclusion test. These tachyzoites or their 1:1 mixture was treated (+) or not treated (−) with DNase. DNA was extracted and subjected to QC-PCR of the SAG-1 gene in triplicate. The results are expressed as the mean SAG-1 copy number per template DNA, corresponding to approximately 100 input tachyzoites ± 1 SD. The detection limit was seven copies.

b) Data are for Student’s t test between DNase-treated and untreated groups.
determined whether DNase is able to reach the DNA within infected host cells. A cell line allowed quantitation of the number of viable line M12-neo-1 was used as a target. The use of a neo transfectant, the infected target cells were incubated with H-2d, and CTL activity was assessed by the 51Cr release assay. The percentages of specific 51Cr release by the target cells during 4 and 6 h of culturing were 76 and 84%, respectively. Similar results were obtained in two independent experiments.

Speculated 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 and host tissue.

Viability of T. gondii in infected CTL target cells. We applied this method to determine the fate of intracellular tachyzoites after the lysis of host cells by CD8+ CTL. An alloreactive CTL line specific for H-2d 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-2d-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, perhaps 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 the lysis of host cells by CD8+ CTL and DNase treatment indicated that DNase treatment effectively eliminates the SAG-1 gene within dead T. gondii.

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 51Cr 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 51Cr release suggested that permeability to DNase may be a more sensitive method than 51Cr release in determining CTL activity. Thus, DNase is able to access cellular DNA when target cells are lysed by CTL.

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 51Cr release</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND</td>
<td>48</td>
</tr>
<tr>
<td>1</td>
<td>ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND</td>
<td>67</td>
</tr>
<tr>
<td>2</td>
<td>ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND</td>
<td>78</td>
</tr>
<tr>
<td>3</td>
<td>ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND/ND</td>
<td>82</td>
</tr>
</tbody>
</table>

Note: *51Cr-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 51Cr 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 quantitate 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 3H-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+ cells 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 cells 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
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 parasiticidal (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 apoptosis by CD8+ T lymphocytes may result in the phagocytosis of apoptotic cells by macrophages (17), resulting in enzymatic digestion of the parasites in phagolysosomes. Thus, one of the roles of CTL may be to prepare infected cells for engulfment by macrophages. In our CTL assay, only target cells and CTL were mixed in vitro, and intracellular T. gondii survived the CTL-mediated lysis of the host cells. We believe that it is likely that macrophages are involved in the clearance of T. gondii in vivo. Our preliminary study indeed suggests that this mechanism may be operative.

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