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Immunology

Human Vδ2⁺ γδ T-cell tolerance to foreign antigens of *Toxoplasma gondii*

TOSHIRO HARA*†, SHIZUKO OHASHI*, YUSHIRO YAMASHITA‡, TOSHIAKI ABE§, HAJIME HISAEDA¶, KUNISUKE HIMENO¶, ROBERT A. GOOD‖, AND KENZO TAKESHITA*

*Department of Child Neurology, Institute of Neurological Sciences, Tottori University School of Medicine, Tottori, Japan; ‡Department of Pediatrics, Faculty of Medicine, Kurume University, Kurume, Japan; §Department of Pediatrics, Faculty of Medicine, Teikyo University, Tokyo, Japan; ¶Department of Parasitology and Immunology, University of Tokushima, School of Medicine, Tokushima, Japan; and ‖Department of Pediatrics, All Children's Hospital, University of South Florida, St. Petersburg, FL 33701

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ABSTRACT Little is known about the mechanisms involved in human γδ T-cell tolerance to self or to foreign antigens. Patients with congenital toxoplasmosis offer a unique opportunity to examine Vδ2⁺ γδ T-cell tolerance. Analysis of γδ T cells in patients with congenital toxoplasmosis revealed evidence for anergy of these cells with or without clonal Vδ2⁺ γδ T-cell expansion in the acute phase of the *Toxoplasma* infection. T cells in general were unresponsive and did not proliferate upon exposure to mitogens or to *Toxoplasma* lysate antigens or in response to live *Toxoplasma*-infected cells when the congenitally infected infants were 1 month of age, and they exhibited selective anergy to *Toxoplasma* lysate antigens and live *Toxoplasma*-infected cells when the infants were aged 5 months. During the chronic phase of congenital toxoplasmosis in the patients who were more than 1 year of age, the repertoires of the γδ T-cell receptors were found to be within normal ranges. In addition, in the chronic phase, the γδ T cells proliferated and secreted γ-interferon in response to exposure to live *Toxoplasma*-infected cells. By contrast, αβ T cells remained anergic. Vδ2⁺ γδ T cells have been considered to undergo extrathymic maturation and thus to be subject to development of peripheral tolerance. Our findings indicate that Vδ2⁺ γδ T-cell tolerance was lost in these infected infants earlier than αβ T-cell tolerance. These findings suggest that γδ T cells play a role in protection against *Toxoplasma gondii* in the chronic phase when congenitally infected children are more than 1 year of age, especially in those in whom αβ T cells continue to exhibit deficits in specific immune responses to *Toxoplasma* antigens.

αβ T-cell tolerance to self and foreign antigens has been extensively studied in mice (1). Clonal deletion or anergy of reactive murine αβ T cells has been reported when self or foreign antigens are introduced into the thymus at an early stage of T-cell development (2). Only a few reports have appeared concerning γδ T-cell tolerance toward self antigens in mice. Elimination and immunologic inactivation have recently been reported for murine self-reactive transgenic γδ T cells during intrathymic maturation (3, 4). Functional anergy after activation has been demonstrated in murine self-reactive transgenic γδ T cells during extrathymic maturation (5, 6).

In humans, few data are available on γδ T-cell tolerance to self or foreign antigens. Congenital infections in humans often result in long-term T-cell unresponsiveness to the pathogens (7). However, the precise mechanisms of both αβ and γδ T-cell tolerance in the course of congenital infections remain to be clarified. Among the pathogens capable of causing congenital infections in humans, *Toxoplasma gondii* is of special interest. In patients with acquired *T. gondii* infection, expansion of cells bearing a particular V region, Vδ2, of γδ T-cell receptor (TCR) has been observed (8, 9).

Therefore, the repertoire and function of γδ T cells were analyzed, and the mechanism of Vδ2⁺ γδ T-cell tolerance to foreign antigens of *T. gondii* was examined in patients with congenital toxoplasmosis.

MATERIAL AND METHODS

Patients. The diagnosis of congenital toxoplasmosis was made in infants who expressed characteristic clinical features including chorioretinitis and central nervous system abnormalities (cerebral calcification and/or hydrocephalus) as well as characteristic serological evidence of congenital *Toxoplasma* infection and who did not have any evidence of cytomegalovirus infection. By serological studies, patients 1–6 had *Toxoplasma*-specific IgG and IgM antibodies, even in early infancy. Patient 7 had persistent *Toxoplasma*-specific IgG antibody and exhibited serologic evidence of intrauterine *Toxoplasma* infection (at 12 weeks of gestation with a titer of <1/128 and at 38 weeks a titer of 1/2048). In patients 1 and 2, *T. gondii* DNA was found in the cerebrospinal fluid by polymerase chain reaction (A. Yano, Nagasaki University, Nagasaki, Japan; personal communication). Patients 1–3 with acute phase of congenital toxoplasmosis were studied at the ages of 2 weeks, 1 month, and 3 weeks, respectively. Patients with chronic phase of congenital toxoplasmosis were examined at the age of 1 year (patients 4 and 5) and 6 years (patients 6 and 7). Ten seronegative, age-matched children aged 1 month to 7 years, six seronegative adults, two seropositive children with no evidence of congenital infection, and four seropositive mothers of the patients served as controls. Blood was obtained from the patients and controls after obtaining informed consent for our study.

Monoclonal Antibodies (mAbs). Fluorescein isothiocyanate (FITC)-conjugated TCRδ1 (pan anti-γδ), δV1 (anti-Vδ1), δV2 (anti-Vδ2), and δTCS1 (anti-Vδ1-Jδ1/Jδ2) mAbs were purchased from T-Cell Sciences, Cambridge, MA. FITC-conjugated TiγA (anti-Vγ9), phycoerythrin (PE)-conjugated anti-HLA-DR (I2), and PE-conjugated anti-CD56 (NKH1) mAbs were from Coulter. FITC- and PE-conjugated anti-CD3 (NU-T3), FITC-conjugated anti-HLA-DR (NU-Ia), and FITC-conjugated anti-CD45RO (UCHL1) were from Nichirei Company, Tokyo. FITC-conjugated anti-αβ TCR (WT-31), PE-conjugated anti-CD4 (Leu3), anti-CD8 (Leu2), and anti-CD45RO (Leu45RO) mAbs were from Becton Dickinson.

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Abbreviations: TLA, *Toxoplasma* lysate antigen; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; FBS, fetal bovine serum; TCR, T-cell receptor.

†To whom reprint requests should be addressed at: Department of Child Neurology, Institute of Neurological Sciences, Tottori University School of Medicine, 36-1 Nishimachi, Yonago City 683, Japan

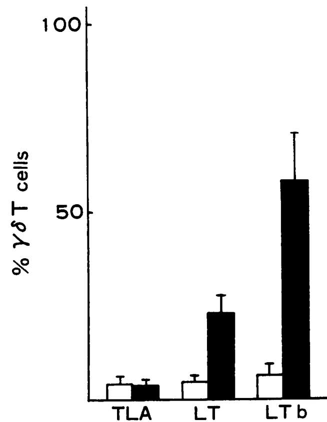

FIG. 1. γδ T-cell expansion *in vitro* in the presence of various forms of *Toxoplasma* antigens. PBMC from six seronegative individuals were cultured for 8 days in the presence (solid bars) of TLAs, irradiated, live *Toxoplasma*-infected PBMC (LT), or irradiated, live *Toxoplasma*-infected PHA blasts (LTb) and analyzed with a flow cytometer. PBS, irradiated PBMC, and irradiated PHA blasts were used as controls (open bars) for TLA, LT, and LTb, respectively. Statistically significant differences were observed in percent γδ T cells expanded in the presence of TLAs versus LT or LTb ($P < 0.01$) and LT versus LTb ($P < 0.01$).

Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Depletion of γδ T Cells. PBMC from the patients or control subjects were isolated using Ficoll/Hypaque density gradient centrifugation (10). For depletion of γδ T cells, PBMC were labeled for 30 min at 4°C with anti-Vδ2 and Vγ9 or control irrelevant mAbs and washed and resuspended in RPMI 1640 medium with 10% fetal bovine serum (FBS; CLS, Victoria, Australia). To this was added ice-washed goat anti-mouse Ig-coated M-450 immunomagnetic beads (Dynabeads; Dynal, Oslo), in RPMI 1640 medium with 10% FBS at a beads-to-target cell ratio of 40:1 (11). Rosetting cells were removed with a magnet (Dynal). Depletion was repeated with anti-pan γδ T cells (TCRδ1) mAb or control mAbs. Remaining γδ T cells were <1.0%.

Flow Cytometric Analysis. Cells were stained with an appropriate mAb at 4°C for 30 min. After washing, single-color or two-color flow cytometric analysis was done using a FACScan (Becton Dickinson Immunocytometry Systems) as described (12).

Preparation of Live *T. gondii* and *Toxoplasma* Lysate Antigens (TLAs). The RH strain of *T. gondii* was passaged in 8- to 10-week-old female ddY mice (Shimizu Laboratory Supplies, Kyoto, Japan). Free tachyzoites were recovered from the peritoneal cavity after 5 ml of PBS was instilled. Isolated tachyzoites were passed through a 27-gauge needle and then a 3-μm filter (Costar) to remove murine macrophages, and they were then centrifuged at $200 \times g$ for 10 min according to the method described (13). The pellet containing free tachyzoites was washed and resuspended in RPMI 1640 medium with 10% FBS and was used as a live *T. gondii* preparation.

TLA was prepared as described (7). Its protein concentration was determined by the Bradford method (Bio-Rad).

In Vitro Infection with *T. gondii*. Phytohemagglutinin (PHA)-activated blasts (PHA blasts) were obtained by incubating PBMC and 5 μg of PHA-P (Difco) per ml of RPMI 1640 medium with 10% FBS for 4 days. Autologous PBMC or PHA blasts were infected with RH strain tachyzoites at a multiplicity of 2:1 to 5:1 for 2 h in RPMI 1640 medium with 10% FBS. Extracellular tachyzoites were removed by Ficoll/Hypaque density gradient centrifugation in experiments with PBMC. Extracellular tachyzoites and a few contaminating murine macrophages were removed by centrifugation with a continuous gradient of Percoll (Pharmacia) in experiments with PHA blasts (14). After irradiation with 12,000 rad (1 rad = 0.01 Gy; ref. 15), *T. gondii*-infected PBMC or PHA blasts were used as stimulators.

***In Vitro* Stimulation of T Cells.** PBMC or separated T cells were cultured at 2×10^5 cells per ml in RPMI 1640 medium with 10% FBS/1 mM glutamine in the presence or absence of 1 μg of mitogenic CD3 mAb per ml that was prepared in our laboratory (10), 5 μg of TLA per ml, and 4×10^4 cells per ml of irradiated *Toxoplasma*-infected PBMC or PHA blasts for the indicated days. In some experiments, recombinant human interleukin-2 from Shionogi Pharmaceutical Company, Osaka, Japan, was added at a final concentration of 100 units/ml. For proliferation assays, the cells were pulsed with [^{3}H]thymidine during the last 12 h and harvested onto glass fiber filters. Incorporated radioactivity was determined by liquid scintillation counting as described (16).

γ-Interferon Assay. Supernatants of PBMC or separated T cells cultured in the presence or absence of a stimulator were obtained 2 days following initiation of culture. γ-Interferon

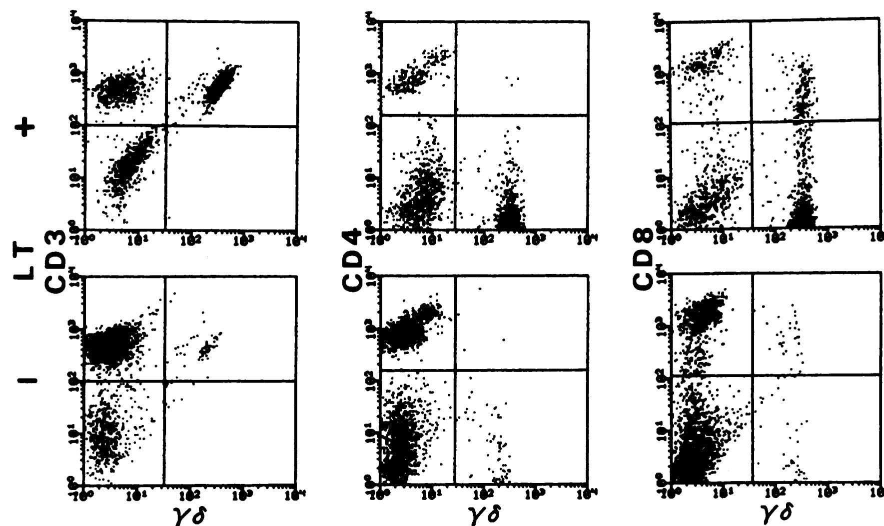

FIG. 2. Flow cytometric analysis of γδ T cells expanded *in vitro* with live *Toxoplasma*. In the presence (+) or absence (−) of irradiated, live *Toxoplasma*-infected PHA blasts (LT), γδ T cells were 55.8% and 2.5% of lymphocytes, respectively. The expanded γδ T cells are $CD4^-$ and some are $CD8^+$.

Table 1. Surface marker analysis of the T cells in patients with congenital toxoplasmosis

Patient no.	$CD3^+$	HLA-DR^+ CD3	$CD45RO^+$ CD3	Total $\gamma\delta$T	HLA-DR^+ $\gamma\delta$T	$CD45RO^+$ $\gamma\delta$T	$V\delta1^+$	$V\delta2^+$	$V\gamma9^+$	$V\delta1$–$J\delta1$/ $J\delta2^\dagger$
1	39.1	1.1	3.3	2.3	0.1	0.8	1.1	1.3	1.0	0.0
2	79.2	17.4	20.6	17.6	12.8	14.7	0.0	17.7	11.7	0.1
3	67.3	10.3	19.8	16.2	8.2	13.3	0.3	16.0	12.4	0.2
4	70.5	0.5	15.9	3.2	0.3	1.8	2.1	1.2	1.3	1.9
5	62.7	0.2	6.2	2.4	0.6	1.4	0.3	2.1	1.0	0.4
6	65.1	0.8	21.1	5.6	0.8	4.1	0.5	5.2	4.9	0.3
7	60.4	2.5	18.2	8.3	1.1	7.2	0.8	7.4	7.3	0.9

Data are expressed as percent positive cells.

was measured with a human interferon-γ enzyme-amplified sensitivity immunoassay kit (Medgenix Diagnostics, Fleurus, Belgium).

Statistics. Comparisons were made with Mann–Whitney test or Student's *t* test.

RESULTS

***In Vitro* Expansion of γδ T Cells with Live *Toxoplasma*-Infected Cells.** To investigate $V\delta2^+$ γδ T-cell function *in vitro*, we first established the system to selectively stimulate γδ T cells with *T. gondii*. With soluble TLAs, no γδ T-cell expansion (3.5% ± 2.1%: mean ± SD of total lymphocytes) was observed as shown in Fig. 1. However, a marked γδ T-cell expansion was reproducibly demonstrated with irradiated, live *Toxoplasma*-infected PBMC (23.5% ± 4.8%) and irradiated, live *Toxoplasma*-infected PHA blasts (58.2% ± 12.4%). γδ T-cell proliferation was not induced by PBMC or PHA blasts incubated with *T. gondii*-free peritoneal lavage fluids (data not shown). The expanded γδ T cells with irradiated, live *Toxoplasma*-infected PHA blasts were 1.8% ± 0.2% $V\delta1^+$, 98.8% ± 1.3% $V\delta2^+$, 95.1% ± 8.7% $V\gamma9^+$, $CD4^-$, and 7.8% ± 3.1% $CD8^+$ (Fig. 2). $CD56^+$ γδ T cells were detected with a marked individual variation from 5.3% to 37.6% (14.8% ± 13.1%) of total γδ T cells. No significant expansion (17.6% ± 4.8% versus 16.1% ± 5.1%: $P > 0.1$) of γδ TCR^-CD56^+ cells (natural killer cells) in the presence or absence of irradiated, live *Toxoplasma*-infected PHA blasts was observed in this system. Thus, *T. gondii*-infected cells were used to investigate *Toxoplasma*-specific immune functions of γδ T cells.

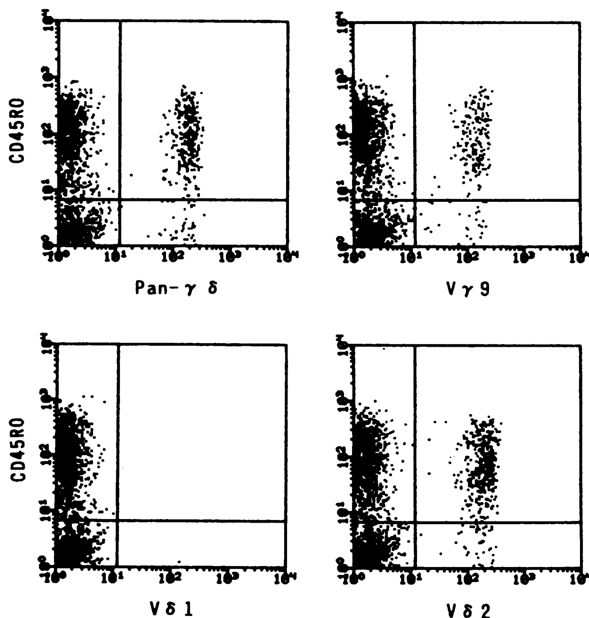

FIG. 3. γδ TCR repertoire in the acute phase of congenital toxoplasmosis. The expanded γδ T cells in acute phase *in vivo* consist of $V\gamma9^+V\delta2^+$ γδ T cells with CD45RO phenotype (patient 2). No down-regulation of γδ TCRs is observed compared with those of controls.

Anergy with or without Vδ2 Clonal Expansion in Acute Phase of Congenital Toxoplasmosis. In three patients with acute-phase congenital toxoplasmosis, patient 1 had lymphopenia and was considered to have T-cell anergy *in vivo* because there was neither activation (HLA-DR^+CD3^+ cells, 1.1%; $CD45RO^+CD3^+$ cells, 3.3%) nor expansion of αβ T cells or γδ T cells (Table 1). Patient 1 was too ill to permit functional studies of T cells and died of disseminated toxoplasmosis on the 23rd day of life. Patients 2 and 3 had significantly (>2 SD) increased percentages of HLA-DR^+ and $CD45RO^+CD3^+$ cells, most of which were HLA-DR^+ and $CD45RO^+$ γδ T cells, respectively. Age-matched controls (n = 10) showed 1.3% ± 1.0% (mean ± 2 SD) HLA-DR^+CD3^+ cells, 8.4% ± 5.4% $CD45RO^+CD3^+$ cells, and 1.9% ± 1.8% γδ T cells. The expanded γδ TCRs were mostly $V\gamma9^+V\delta2^+$. No down-regulation of γδ TCRs was observed as shown in Fig. 3. Functional studies *in vitro* showed that the patients' T cells were not responsive to stimulation with a T-cell mitogen, CD3 mAb, TLA, or irradiated, live *Toxoplasma*-infected cells when obtained from the infected infants at 1 month of age, and they became weakly reactive to CD3 mAb (patients versus controls; $P < 0.1$) and remained nonreactive to irradiated, live *Toxoplasma*-infected cells or TLAs at 5 months of age (Fig. 4).

Functional Recovery of γδ T cells in Chronic Phase of Congenital Toxoplasmosis. In four patients (patients 4–7) with congenital toxoplasmosis studied at ages 1–6 years, no increases in HLA-DR^+CD3^+ cells or $CD45RO^+CD3^+$ cells were observed compared with those of age-matched controls (mean ± 2 SD; percent HLA-DR^+CD3^+ cells, 2.1% ± 1.7%;

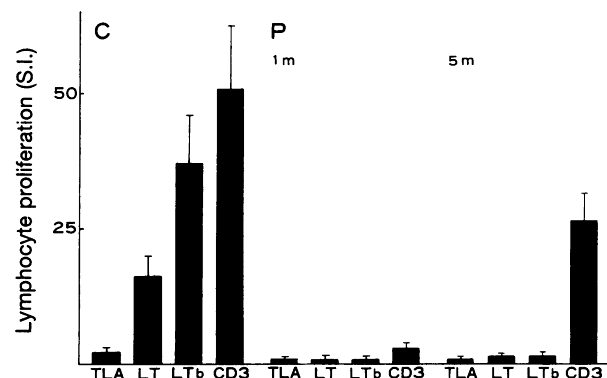

FIG. 4. Lymphocyte proliferation by T-cell mitogen and *Toxoplasma* antigens in the acute phase of congenital toxoplasmosis. PBMC from four seropositive individuals (C) and from the patients (P) were cultured for 4 days in the presence or absence of CD3 mAb and for 8 days in the presence or absence of TLA, irradiated live *Toxoplasma*-infected PBMC (LT), or irradiated live *Toxoplasma*-infected PHA blasts (LTb). DNA synthesis was measured with [^{3}H]thymidine incorporation. S.I., stimulation index. Patients' lymphocyte proliferations (patients 2 and 3) were measured at the age of 1 month (1 m) and 5 months (5 m).

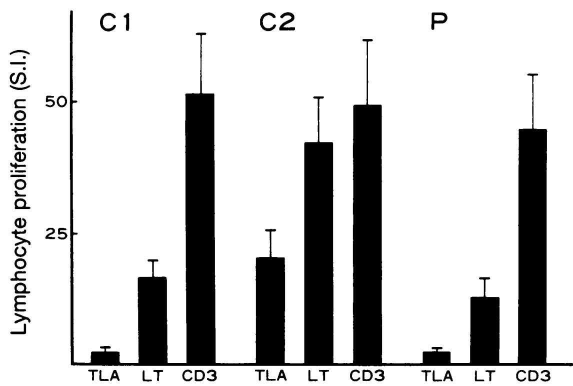

FIG. 5. Lymphocyte proliferation by T-cell mitogen and *Toxoplasma* antigens in chronic phase of congenital toxoplasmosis. PBMC from six seronegative controls (C1), four seropositive controls (C2), and four patients (P) were cultured and assayed in the same way as described in Fig. 4.

percent $CD45RO^+CD3^+$ cells, 14.8% ± 7.8%; n = 10). γδ T cells showed neither deletion nor expansion, but they showed age-dependent increases as compared to those of age-matched controls (4.3% ± 4.1%) as shown in Table 1.

Functional studies *in vitro* showed that the four patients with congenital toxoplasmosis exhibited normal T-cell responses to CD3 mAb (patients versus seropositives or seronegatives, $P > 0.1$), a slightly weakened response to irradiated, live *Toxoplasma*-infected cells (patients versus seronegatives, $P > 0.1$; patients versus seropositives $P < 0.01$), and no response to TLA (Fig. 5). Seronegative controls showed proliferative responses to irradiated, live *Toxoplasma*-infected cells but not to TLAs, whereas seropositive controls had responses to TLAs as well as to irradiated, live *Toxoplasma*-infected cells. To analyze which cells responded to respective antigens, flow cytometric analysis was performed (Fig. 6). In patients with congenital toxoplasmosis and seronegative controls, γδ T cells were selectively activated and expanded in response to live *Toxoplasma*-infected PHA blasts, and no significant responses of αβ T cells to TLAs or live *Toxoplasma*-infected cells were observed. In seropositive controls, αβ T cells were responsive to TLAs and live *Toxoplasma*-infected cells, and γδ T cells were responsive to live *Toxoplasma*-infected cells.

To assess protective T-cell functions against *T. gondii*, the supernatants stimulated by TLAs or live *Toxoplasma*-infected

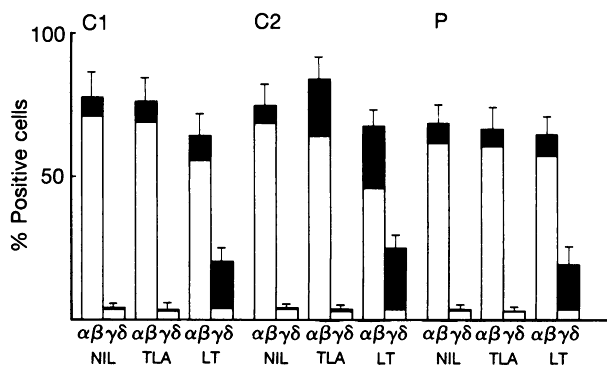

FIG. 6. Flow cytometric analysis of αβ and γδ T cells with the activation antigen, HLA-DR. PBMC from six seronegative individuals (C1), four seropositive individuals (C2), and four patients with congenital toxoplasmosis (P) were cultured in the absence (NIL) or presence of TLA or irradiated, live *Toxoplasma*-infected PHA blasts (LT) for 8 days. Two-color flow cytometric analysis of the cultured PBMC was performed with FITC-conjugated anti-pan αβ or γδ TCR mAb and PE-conjugated HLA-DR mAb. Solid areas show percentages of $HLA-DR^+$ αβ or γδ T cells.

Table 2. γ-Interferon production in the presence of *Toxoplasma* antigens

Experiment	Stimulator	γ-Interferon, units/ml
1. Seronegatives (n = 4)	None	0
	TLA	0
	Live *T. gondii*	18.4 ± 3.8
Seropositives (n = 4)	None	0
	TLA	21.7 ± 6.9
	Live *T. gondii*	77.7 ± 22.5
Patients (n = 4)	None	0
	TLA	0
	Live *T. gondii*	14.0 ± 3.5
2. Seronegatives (n = 3)		
Control mAb	None	0
depletion	Live *T. gondii*	24.6 ± 8.2
γδTCR mAb	None	0
depletion	Live *T. gondii*	5.2 ± 2.9
Patients (n = 2)		
Control mAb	None	0
depletion	Live *T. gondii*	15.6 ± 9.8
γδTCR mAb	None	0
depletion	Live *T. gondii*	4.1 ± 3.1

For experiment 1, PBMC were cultured for 2 days in the presence or absence of a stimulator. Supernatants were collected and assayed for γ-interferon.

For experiment 2. After 6-day culture in the presence or absence of a stimulator, PBMC were treated with control or γδ TCR mAb for γδ T-cell depletion. Then, control mAb-depleted or γδ TCR mAb-depleted PBMC were cultured for an additional day in the presence of recombinant human interleukin-2. Supernatants were assayed for γ-interferon.

γ-Interferon data is expressed as mean ± SD.

cells were assayed for γ-interferon production. As shown in Table 2, the patients' T cells produced significant amounts of γ-interferon in the presence of irradiated, live *Toxoplasma*-infected cells (patients versus seronegatives, $P > 0.1$; patients versus seropositives, $P < 0.01$) but not in the presence of TLAs. The fact that depletion of γδ T cells markedly decreased γ-interferon production indicated that γδ T cells were responsive for most γ-interferon production in the presence of live *Toxoplasma*-infected cells.

DISCUSSION

Human γδ T cells comprise two major subsets, Vδ1 and Vδ2. The Vδ1 subset predominates in the thymus, whereas the Vδ2 subset becomes predominant in the periphery and increases with age (17). The Vδ2 subset appears to undergo extrathymic maturation and to be implicated in defenses against various microorganisms (17, 18).

Patients with congenital *Toxoplasma* infection offer the unique possibility to examine $V\delta2^+$ γδ T-cell tolerance because *T. gondii* induces expansion of a particular V region, Vδ2, of γδ TCR in acquired infection (8, 9). To study the mechanisms of $V\delta2^+$ γδ T-cell tolerance in congenital *Toxoplasma* infection, an *in vitro* system to evaluate specific γδ T-cell function was established. In response to irradiated (12,000 rad), live *Toxoplasma*-infected cells, but not in response to TLA, $V\delta2^+$ γδ T cells predominantly proliferated and secreted γ-interferon. Irradiation with 3000 rad was not sufficient to kill nonattenuated *T. gondii*. Subauste *et al.* (19) have recently reported that human $V\gamma9^+V\delta2^+\gamma\delta$ T cells were preferentially activated and expanded in response to irradiated (3000 rad) PBMC infected with ultraviolet light-attenuated *T. gondii* or temperature-sensitive ts-4 strain of *T. gondii*. Absolute numbers of γδ T cells expanded with live *T. gondii*-infected PBMC showed a 24-fold increase, whereas those of αβ T cells showed a 1.2-fold increase. For γδ T-cell expansion, intact tachyzoites were required in both studies. Because in our studies as well as

in those of Subauste *et al.* human $\gamma\delta$ T cells produced γ-interferon, a major mediator of host resistance against *T. gondii* (20), it was suggested that human $\gamma\delta$ T cells might play a role in protection against *T. gondii*. In the mouse, $\gamma\delta$ T cells have been reported to play an important role in protection against *Toxoplasma* infection (21).

In vivo and *in vitro* studies of $\gamma\delta$ T cells in patients with congenital toxoplasmosis revealed by the analyses reported herein have shown that $V\delta2^+$ $\gamma\delta$ T cells became anergic with or without clonal expansion during the acute phase of congenital infection. $V\delta2$ clonal expansion was not associated with $\gamma\delta$ TCR down-regulation (Fig. 3), as was observed in an animal study (22). No deletion of $V\delta2^+$ $\gamma\delta$ T cells was observed. Patients 2 and 3 showed total T-cell anergy at 1 month of age and *Toxoplasma*-specific anergy at 5 months of age. T-cell unresponsiveness to *Toxoplasma* antigens was observed during the acute phase of acquired *Toxoplasma* infection as well (23, 24). It is likely that $V\delta2$ clonal expansion followed by unresponsiveness might be a more frequent phenomenon in acute phase than no clonal expansion. This concept is attractive because all four patients with congenital toxoplasmosis in a recent report showed increased $CD45RO^+$ (memory) T cells in the cord blood (25), and most of the $CD45RO^+$ T cells were $\gamma\delta$ T cells in our series of patients. The present study also revealed that, in addition to the findings by Michie and Harvey (25), $CD45RO^+$ T cells were not always elevated in congenital toxoplasmosis, especially when the disease was very severe as in our patient 1. Clonal expansion of reactive T cells preceding anergy has been documented only in extrathymic peripheral tolerance in mice (22, 26, 27). Indeed, the $V\delta2$ subset of $\gamma\delta$ T cells is considered to undergo extrathymic maturation and to be subject to development of peripheral tolerance rather than central tolerance in the thymus (17).

Long-term (5–19 years) reduction of T-cell response *in vitro* has been demonstrated in congenital infections as well as in prenatal exposure to foreign antigens (7, 28). McLeod *et al.* (7) reported that infants with severe congenital toxoplasmosis tended to show low T-cell responsiveness to TLAs. Because it is difficult to investigate differences between $\alpha\beta$ T-cell and $\gamma\delta$ T-cell tolerance in mild patients with early recovery of T-cell responsiveness to *Toxoplasma* antigens, we elected to investigate only severe patients with persistent T-cell unresponsiveness to TLA. Despite persistent $\alpha\beta$ T-cell unresponsiveness, $\gamma\delta$ T cells became reactive to live *Toxoplasma*-infected cells and produced γ-interferon after the infants with congenital toxoplasmosis reached 1 year of age. In severe patients, $\alpha\beta$ T cells may be subject to development of a central tolerant state, whereas $V\delta2^+$ $\gamma\delta$ T cells are subject to development of peripheral tolerance. Peripheral tolerance can be reversed when antigen is absent or upon exposure to certain other infectious agents (27, 29, 30). After 1 year, it is likely that *Toxoplasma* antigens have been removed from direct contact with $\gamma\delta$ T cells. Further, infectious agents that induce $V\delta2$ expansion may contribute to loss of $V\delta2$ tolerance in congenital toxoplasmosis. Because the treatment of congenital toxoplasmosis is usually discontinued after the children reach 1 year of age without evidence of relapse, the reversal of peripheral tolerance of $\gamma\delta$ T cells may contribute to protection against spread of *Toxoplasma* after 1 year of age in severe patients in which $\alpha\beta$ T-cell unresponsiveness to TLA persists.

To our knowledge, the present study has demonstrated for the first time that human $V\delta2^+$ $\gamma\delta$ T cells may become anergic with or without clonal expansion during the acute phase of congenital toxoplasmosis. In the chronic phase in infants more than 1 year old, $V\delta2^+$ $\gamma\delta$ T-cell tolerance that occurs during extrathymic maturation is lost earlier than $\alpha\beta$ T-cell tolerance in severe patients. Additional analyses of the precise mechanism or mechanisms involved in T-cell tolerance in congenital infections should contribute to better understanding of the pathophysiology of persistent infections as well as better understanding of late complications that may be mediated by immune mechanisms.

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1. Geenan, V. & Kroemer, G. (1993) *Immunol. Today* **14,** 573–575.
2. Pircher, H., Bürki, K., Lang, R., Hengartner, H. & Zinkernagel, R. M. (1989) *Nature (London)* **342,** 559–561.
3. Dent, A. L., Matis, L. A., Hooshmand, F., Widacki, S. M., Bluestone, J. A. & Hedick, S. M. (1990) *Nature (London)* **343,** 714–719.
4. Bonneville, M., Ishida, I., Itohara, S., Verbeek, S., Berns, A., Kanagawa, O., Haas, W. & Tonegawa, S. (1990) *Nature (London)* **344,** 163–165.
5. Barret, T. A., Delvy, M. L., Kennedy, D. M., Lefrancois, L., Matis, L. A., Dent, A. L., Hedick, S. M. & Bluestone, J. A. (1992) *J. Exp. Med.* **175,** 65–70.
6. Barret, T. A., Tatsumi, Y. & Bluestone, J. A. (1993) *J. Exp. Med.* **177,** 1755–1762.
7. McLeod, R., Mack, D. G., Boyer, K., Mets, M., Roizen, N., Swisher, C., Patel, D., Beckmann, E., Vitullo, D., Johnson, D. & Meier, P. (1990) *J. Lab. Clin. Med.* **116,** 623–635.
8. De Paoli, P., Basaglia, G., Gennari, D., Crovatto, M., Modolo, M. L. & Santini, G. (1992) *J. Clin. Microbiol.* **30,** 729–731.
9. Scalise, F., Gerli, R., Castellucci, G., Spinozzi, F., Fabietti, G. M., Crupi, S., Sensi, L., Britta, R., Vaccaro, R. & Bertotto A. (1992) *Immunology* **76,** 668–670.
10. Hara, T. & Fu, S. M. (1985) *J. Exp. Med.* **161,** 641–656.
11. Vartdal, F., Kvalheim, G., Lea, T. E., Bosnes, V., Gaudernack, G., Ugelstad, J. & Albrechtsen, D. (1987) *Transplantation* **43,** 366–371.
12. Hara, T., Fu, S. M. & Hansen, J. A. (1985) *J. Exp. Med.* **161,** 1513–1524.
13. Chao, C. C., Gekker, G., Hu, S. & Peterson, P. K. (1994) *J. Immunol.* **152,** 1246–1252.
14. Gmelig-Meyling, F. & Waldmann, T. A. (1980) *J. Immunol. Methods* **33,** 1–9.
15. Curiel, T. J., Krug, E. C., Purner, M. B., Poignard, P. & Berens, R. L. (1993) *J. Immunol.* **151,** 2024–2031.
16. Hara, T., Mizuno, Y., Nagata, M., Okabe, Y., Taniguchi, S., Harada, M., Niho, Y., Oshimi, K., Ohga, S., Yoshikai, Y., Nomoto, K., Yumura, K., Kawa-Ha, K. & Ueda, K. (1990) *Blood* **75,** 941–950.
17. Haas, W., Pereira, P. & Tonegawa, S. (1993) *Annu. Rev. Immunol.* **11,** 637–685.
18. Hara, T., Mizuno, Y., Takaki, K., Takada, H., Akeda, H., Aoki, T., Nagata, M., Ueda, K., Matsuzaki, G., Yoshikai, Y. & Nomoto, K. (1992) *J. Clin. Invest.* **90,** 204–210.
19. Subauste, C. S., Chung, J. Y., Do, D., Koniaris, A. H., Hunter, C. A., Montoya, J. G., Porcelli, S. & Remington, J. S. (1995) *J. Clin. Invest.* **96,** 610–619.
20. Beaman, M. H., Wong, S.-Y. & Remington, J. S. (1992) *Immunol. Rev.* **127,** 97–117.
21. Hisaeda, H., Nagasawa, H., Maeda, K., Maekawa, Y., Ishikawa, H., Ito, Y., Good, R. A. & Himeno, K. (1995) *J. Immunol.* **83,** 347–352.
22. Rocha, R. & von Boehmer, H. (1991) *Science* **251,** 1225–1228.
23. Anderson, S. E. M., Jr., Krahenbuhl, J. L. & Remington, J. S. (1979) *J. Clin. Lab. Immunol.* **2,** 293–297.
24. Chan, J., Siegel, J. P. & Luft, B. (1986) *Cell. Immunol.* **98,** 422–433.
25. Michie, C. & Harvey, D. (1994) *Lancet* **343,** 1259–1260.
26. Webb, S., Morris, C. & Sprent, J. (1990) *Cell* **63,** 1249–1256.
27. Rocha, B., Tanchot, C. & von Boehmer, H. (1993) *J. Exp. Med.* **177,** 1517–1521.
28. Steel, C., Guinea, A., McCarthy, J. S. & Ottesen, E. A. (1994) *Lancet* **343,** 890–893.
29. Ohashi, P. S., Oehen, S., Bürki, K., Pircher, H., Ohashi, C. T., Odermatt, B., Malissen, B., Zinkernagel, R. M. & Hengartner, H. (1991) *Cell* **65,** 305–317.
30. Rocken, M., Urban, J. F. & Shevach, E. M. (1992) *Nature (London)* **359,** 79–83.