Human V82+ γδ T-cell tolerance to foreign antigens of Toxoplasma gondii

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Contributed by Robert A. Good, January 11, 1996

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 V82+ γδ T-cell tolerance. Analysis of γδ T cells in patients with congenital toxoplasmosis revealed evidence of anergy of these cells with or without clonal V82+ 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 repertoire of the γδ T-cell receptors were found to be within normal range. 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. V82+ γδ T cells have been considered to undergo extrathympic maturation and thus to be subject to development of peripheral tolerance. Our findings indicate that V82+ 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 intrathympic maturation (3, 4). Functional anergy after activation has been demonstrated in murine self-reactive transgenic γδ T cells during extrathympic 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, V82, 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 V82+ 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 IgM 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 TCR(61 (pan anti-γδ), 8V1 (anti-V61), 8V2 (anti-V82), and 8TC51 (anti-V81-J51/J82) mAbs were purchased from T-Cell Sciences, Cambridge, MA. FITC-conjugated TiyA (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-1a), 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.

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.

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**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 × 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 [3H]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 was determined using an enzyme-linked immunosorbent assay as described previously (17). Supernatants were also analyzed by an enzyme-linked immunosorbent assay for IL-2 (Genzyme, Cambridge, Mass.) as described above.

**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).

**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 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 antimouse 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 (TCRδ) mAb or control mAbs. Remaining γδ T cells were <1%.

**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).
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 Vδ2+ 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 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% Vδ1+, 98.8% ± 1.3% Vδ2+, 95.1% ± 8.7% Vγ9+, 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.

**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 Vγ9+Vδ2+. 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 nonresponsive 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%;

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

| Patient | CD3+ | HLA-DR+ | CD45RO+ | Total γδT | HLA-DR+ γδT | CD45RO+ γδT | Vδ1+ | Vδ2+ | Vγ9+ | Vδ1-3/δ2+ |
|---------|------|---------|---------|----------|------------|------------|------|------|------|----------|
| no.     |      |         |         |          |            |            |      |      |      |          |
| 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.
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 seropositives, P > 0.1; patients versus seronegatives, 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

| Table 2. γ-Interferon production in the presence of Toxoplasma antigens |
|-----------------------------|-----------------------------|-----------------------------|
| **Experiment**             | **Stimulator**               | **γ-Interferon, units/ml**   |
| 1. Seronegatives (n = 4)    | None                        | Live T. gondii              |
|                            | TLA                         | 0                           |
|                            | Live T. gondii              | 18.4 ± 3.8                  |
| Seropositives (n = 4)       | None                        | Live T. gondii              |
|                            | TLA                         | 21.7 ± 6.9                  |
|                            | Live T. gondii              | 77.7 ± 22.5                 |
| Patients (n = 4)            | None                        | Live T. gondii              |
|                            | TLA                         | 0                           |
|                            | Live T. gondii              | 14.0 ± 3.5                  |
| 2. Seronegatives (n = 3)    | Control mAb-depletion       | Live T. gondii              |
|                            | None                        | 24.6 ± 8.2                  |
|                            | γδTCR mAb-depletion         | None                        |
|                            | Live T. gondii              | 5.2 ± 2.9                   |
| Patients (n = 2)            | Control mAb-depletion       | Live T. gondii              |
|                            | None                        | 15.6 ± 9.8                  |
|                            | γδTCR mAb-depletion         | None                        |
|                            | 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 seropositives, P > 0.1; patients versus seronegatives, 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 Vδ2+ 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 Vδ2+ 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 TLAs, Vδ2+ 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 Vδ2+ Vδ2+ γδ 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 γδ T cells produced γ-interferon, a major mediator of host resistance against T. gondii (20), it was suggested that human γδ T cells might play a role in protection against T. gondii. In the mouse, γδ T cells have been reported to play an important role in protection against Toxoplasma infection (21).

In vivo and in vitro studies of γδ T cells in patients with congenital toxoplasmosis revealed by the analyses reported herein have shown that Vβ2+ γδ T cells became anergic with or without clonal expansion during the acute phase of congenital infection. Vβ2 clonal expansion was not associated with γδ TCR down-regulation (Fig. 3), as was observed in an animal study (22). No deletion of Vβ2+ γδ 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 Vβ2 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 γδ 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 Vβ2 subset of γδ T cells has contributed to 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 αβ T-cell and γδ 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 TLAs. Despite persistent αβ T-cell unresponsiveness, γδ T cells became reactive to live Toxoplasma-infected cells and product γ-interferon from peripheral blood mononuclear cells at an early stage of congenital toxoplasmosis reached 1 year of age. In severe patients, αβ T cells may be subject to development of a central tolerant state, whereas Vβ2+ γδ 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 γδ T cells. Further, infectious agents that induce Vβ2 expansion may contribute to loss of Vβ2 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 γδ T cells may contribute to protection against spread of Toxoplasma after 1 year of age in severe patients in which αβ T-cell unresponsiveness to TLAs persists.

To our knowledge, the present study has demonstrated for the first time that human Vβ2+ γδ 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, Vβ2+ γδ T-cell tolerance that occurs during extrathymic maturation is lost earlier than αβ 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.

We are grateful to Drs. Rumi Yamakawa, Kazue Inuma, and Munehiro Yamakoshi for providing us with samples from patients. We thank Ms. Tatsi Verjee for manuscript preparation. This work was supported in part by grants from the Ministry of Health and Welfare of Japan.

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