[Jpn. J. Parasitol., Vol. 44, No. 3, 210–217, June, 1995]

# Comparative Study of Interferon-γ Production by *Toxoplasma gondii*-infected B Lymphoma Cell-specific CD8$^+$ CTL and *Toxoplasma gondii*-infected Melanoma Cell-specific CD4$^+$ CTL

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(Accepted May 16, 1995)

## Abstract

Two types of *Toxoplasma gondii* (*T.gondii*)-infected cell-specific cytotoxic T lymphocyte (CTL) lines have been established from peripheral blood leukocytes (PBL) of a patient with chronic toxoplasmosis. One type is CD8$^+$ CTL line specific for *T.gondii*-infected B lymphoma cells in association with human leukocyte antigens (HLA)-A2 molecules and the other type is CD4$^+$ CTL line specific for *T.gondii*-infected melanoma cells in association with HLA-DR molecules. Both CD8$^+$ CTL and CD4$^+$ CTL lines specific for *T.gondii*-infected cells produced equally high levels of interferon-γ (IFN-γ) upon the antigen-specific stimulation. The surface expression of HLA-DR molecules of *T.gondii*-infected melanoma target cells but not that of *T.gondii*-infected B lymphoma target cells, was dose-dependently up-regulated by the exogenous human recombinant IFN-γ (rIFN-γ). Further, CD4$^+$ CTL showed a markedly enhanced cytotoxicity against *T.gondii*-infected melanoma that was co-cultured with rIFN-γ while the lytic activity of CD8$^+$ CTL against rIFN-γ treated *T.gondii*-infected B lymphoma was not enhanced. Interestingly, the expression of intercellular adhesion molecule (ICAM-1) of *T.gondii*-infected melanoma, but not that of *T.gondii*-infected B lymphoma, was augmented by the treatment with rIFN-γ, indicating that ICAM-1 might be a possible molecule participating in the enhanced cytotoxicity of CD4$^+$ CTL against rIFN-γ treated *T.gondii*-infected melanoma. The biological function of secreted IFN-γ from CD8$^+$ CTL or CD4$^+$ CTL was comparatively discussed.

**Key words:** *Toxoplasma gondii*-infection; antigen presentation; interferon-γ; CD8$^+$ CTL; CD4$^+$ CTL.

## Introduction

Human antigen-presenting cells (APC) infected by the intracellular parasitic protozoan *T.gondii* are capable of presenting *T.gondii*-infected cell-specific antigens to HLA class I- and class II-restricted T cells (Yano *et al.*, 1989). We have already reported the establishment of two types of CTL lines specific for *T.gondii*-infected cells derived from PBL of a patient with chronic toxoplasmosis. One is HLA-A2 molecule-restricted CD8$^+$ CTL line which was induced by the stimulation with *T.gondii*-infected B lymphoma. To CD8$^+$ CTL, *T.gondii*-infected cell-specific antigen was shown to be presented by *T.gondii*-infected B lymphoma through the endogenous antigen presenting pathway. Electron microscopic analysis of the infected APC revealed that the outer membrane of *T.gondii* fused with some parts of the vacuolar membranes building channellike structures in *T.gondii*-infected APC. The direct entry of *T.gondii* antigen into the cytoplasm was detected by cytofluoromeric studies (Yano *et al.*, 1992). Naturally processed peptides recognizable by *T.gondii*-infected cell-specific CD8$^+$ CTL were isolated from HLA-A2 molecules of *T.gondii*-infected B lymphoma (Aosai *et al.*, 1994). The other one is HLA-DR molecule-restricted CD4$^+$ CTL line which

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This work was supported in part by a Grant-in Aid for Scientific Research from the Ministries of Education and National Health of Japan.

was induced by the stimulation with *T.gondii*-infected melanoma. Thus, not only B lymphoma but also melanoma works as APC in antigen presentation of *T.gondii*-infected cell-specific antigens for CTL in association with HLA molecules. We have recently postulated a hypothesis that both *T.gondii*-infected melanocytes (as APC) and $CD4^+$ CTL participate in the pathogenesis of toxoplasmic retinochoroiditis (Yang *et al.*, 1995).

IFN-γ has been shown to be a major protective cytokine in immunity to *T.gondii* (Chinchilla and Frenkel, 1984; Suzuki *et al.*, 1988; Suzuki and Remington, 1990; Gazzineli *et al.*, 1992). $CD8^+$ CTL or natural killer (NK) cells have been thought as major sources of IFN-γ production (Suzuki and Remington, 1990; Gazzinelli *et al.*, 1991; Denkers *et al.*, 1994; Sher *et al.*, 1993), although the participation of $CD4^+$ T cells or $CD4^-CD8^-$ cells were also reported (Gazzinelli *et al.*, 1991; Gazzinelli *et al.*, 1992; Kasper and Khan, 1993; Johnson *et al.*, 1993). We have found that both $CD8^+$ CTL and $CD4^+$ CTL lines specific for *T.gondii*-infected cells produced equally high amounts of IFN-γ upon the antigen specific stimulation. The biological effects of IFN-γ production by $CD8^+$ CTL and $CD4^+$ CTL lines are comparatively examined.

## Materials and Methods

*T.gondii-infected cell-specific CTL lines*

$CD8^+$ CTL lines (*T.gondii* (RH strain)-infected human B lymphoma-specific, HLA-A2-restricted) established from PBL of a patient with chronic toxoplasmosis (Aosai *et al.*, 1994) were maintained by weekly *in vitro* stimulation with *T.gondii*-infected human B lymphoma ARH-77 (ARH) cells or EBV-Ya cells (Epstain Barr Virus-transformed autologous B lymphoma; a kind supply from Dr. K. Yamashita) in IMDM tissue culture medium supplemented with 5% FCS, $5\times10^{-5}$ M 2-mercaptoethanol, penicilline-streptomycin, and 10 U/ml human recombinant IL-2 (rIL-2) (Shionogi Pharm. Co., Osaka, Japan).

$CD4^+$ CTL lines (*T.gondii*-infected human melanoma-specific, HLA-DR-restricted) established from PBL of the same patient (Yang *et al.*, 1995) were maintained by weekly *in vitro* stimulation with *T.gondii*-infected human melanoma, SK-MEL28 (P36) cells which shared HLA-DR4 molecules with the patient. Preparation of *T.gondii*-infected cells was done as previously described (Yano *et al.*, 1989). Tenets of the Declaration of Helshinki were followed. Informed consent was obtained from the patient after the nature of the procedure had been explained.

*Assay of IFN-γ production*

Two $\times 10^6$ $CD8^+$ or $CD4^+$ CTL were cultured with $2\times10^4$ of either *T.gondii*-infected or uninfected stimulators cells ($CD8^+$ CTL were cultured with either *T.gondii*-infected or uninfected ARH or EBV-Ya cells. $CD4^+$ CTL were cultured with either *T.gondii*-infected or uninfected P36 cells) in a 24-well tissue culture plate in IMDM without rIL-2. Supernatants were harvested 48 hours later and IFN-γ activities in the supernatants were assayed by ELISA (Otsuka Pharm. Co., Tokyo, Japan).

*Cell treatment with IFN-γ and flow cytometric analysis of surface expression of HLA, LFA-1 or ICAM-1 molecules*

To test the effects of human recombinant IFN-γ (rIFN-γ) on the surface expression of the HLA molecules of target cells, *T.gondii*-infected or uninfected ARH (or EBV-Ya) or P36 cells were cultured with or without 60 ng/ml human rIFN-γ for 24 hours. The cells were then incubated either with anti-HLA-DR monoclonal antibody (mAb) B33.1.3 (IgG2a; a kind gift of Dr. P. Bice, Jefferson Cancer Institute, PA, USA) or anti-HLA-A,B,C mAb W6/32 (IgG2a; purchased from American Type Culture Collection (ATCC)) for 30 minutes on ice and were stained with second-layer fluorescein (FITC)-conjugated goat anti-mouse immunoglobulins (Igs) for another 30 minutes on ice. The FITC intensity of the stained cells was analysed with a FACScan (Becton Dickinson, Mountain View, CA, USA). For control staining, cells were exposed to second-layer antibodies only.

The effects of rIFN-γ on the surface expression of LFA-1 and ICAM-1 molecules of target cells were also examined by incubating the rIFN-γ treated or untreated target cells (*T.gondii*-infected or uninfected ARH (or EBV-Ya) or P36 cells) either with anti-human LFA-1α mAb TS1/22.1.1.13 (CD11a) (IgG1; purchased from ATCC), anti-hu-

man LFA-1$\beta$ mAb TS1/18.1.2.11.4 (CD18) (IgG1; purchased from ATCC), or anti-human ICAM-1 mAb 84-H-10 (CD54) (IgG1; Medical Biology Laboratories, Nagoya, Japan) for 30 minutes on ice followed by the staining with second-layer FITC-conjugated goat anti-mouse Igs for another 30 minutes on ice.

*CTL assay*

A CTL assay was performed as described previously (Yano *et al.*, 1989; Aosai *et al.*,, 1991). To test the effects of IFN-$\gamma$ on CTL activity, target cells were preincubated with various concentration (0.6 ng/ml, 6 ng/ml and 60 ng/ml) of rIFN-$\gamma$ for 24 hours. The preincubation time of target cells with rIFN-$\gamma$ was determined following the data previous described (Yang *et al.*, 1995). The same concentration of rIFN-$\gamma$ was included in cultures through the $^{51}$Cr-labeling and CTL assay. $5\times10^3$ $^{51}$Cr-labeled target cells (either rIFN-$\gamma$ treated or untreated) were cultured with the effector CTL at the indicated effector/target ratio in a round-bottomed 96-well plate at a total volume of 200 $\mu$l per well. After 4 hours incubation, 100 $\mu$l of each supernatant fraction was collected and the percent specific lysis was determined using the equation: [(experimental release – spontaneous release) / (maximal release – spontaneous release)]×100. Experiments were performed in duplicate or triplicate.

## Results

*IFN-$\gamma$ production by T.gondii-infected cell-specific CD8$^+$ CTL or CD4$^+$ CTL*

As IFN-$\gamma$ has been shown to be a major protective cytokine in immunity to *T.gondii*, IFN-$\gamma$ production by *T.gondii*-infected cell-specific CD8$^+$ CTL or CD4$^+$ CTL line was comparatively examined. Both CD8$^+$ CTL and CD4$^+$ CTL lines produced a high level of IFN-$\gamma$ when cultured with *T.gondii*-infected B lymphoma (ARH or EBV-Ya cells) or melanoma (P36 cells) respectively (Fig. 1). The IFN-$\gamma$ production from CD8$^+$ CTL or CD4$^+$ CTL was equal and was antigen-specific, because uninfected B lymphoma or melanoma failed to stimulate CTL for production of IFN-$\gamma$. Although CD4$^+$ CTL by itself

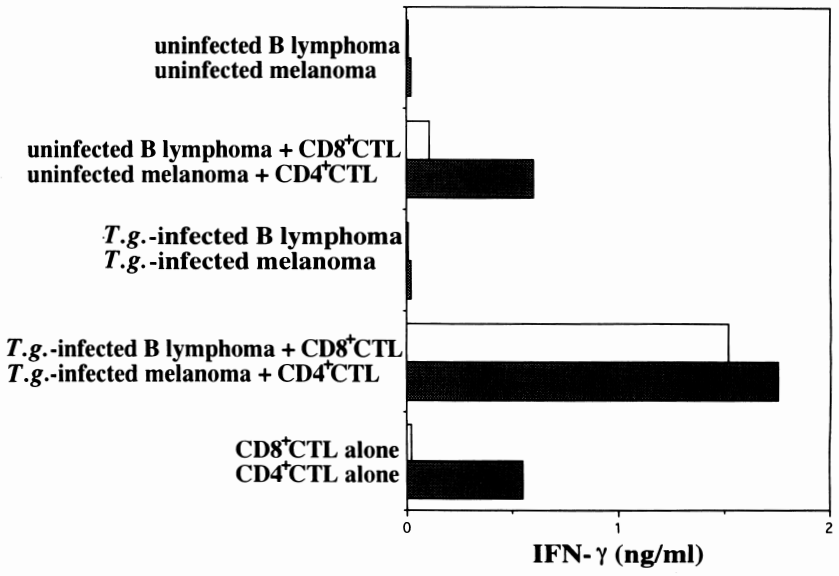


Fig. 1 Interferon-$\gamma$ production of *T.gondii*-infected cell-specific CD8$^+$ or CD4$^+$ CTL. CD8$^+$ CTL (open column) was cultured with either *T.gondii*-infected or uninfected B lymphoma (ARH or EBV-Ya cells). CD4$^+$ CTL (solid column) was cultured with either *T.gondii*-infected or uninfected melanoma (P36 cells). After the culture at 37°C for 48 hours, supernatants were harvested and assayed for IFN-$\gamma$ production by ELISA. Supernatants of either stimulator alone or CTL alone were tested as controls.

secreted low level of IFN-γ, the amount of IFN-γ production was enhanced by antigen-specific stimulation. Neither the culture with $CD8^+$ CTL alone nor the culture with the stimulator alone produced IFN-γ.

*Effects of IFN-γ on HLA molecule expression of B lymphoma and melanoma cells*

*T.gondii*-infected B lymphoma (ARH or EBV-Ya cells) or melanoma (P36 cells) was cultured in the absence or in the presence of 60 ng/ml human rIFN-γ for 24 hours and the effects of IFN-γ treatment on the HLA molecule expression were comparatively analysed. The treatment with rIFN-γ up-regulated the surface expression of HLA-DR molecules of melanoma, whereas the expression of HLA-DR molecules of B lymphoma was not affected (Fig. 2B and D). The expression level of HLA class I molecules of both lines was not augmented by the treatment with rIFN-γ (Fig. 2A and C). The expression levels of HLA-A,B,C and HLA-DR molecules by both *T.gondii*-infected and uninfected B lymphoma or melanoma were similar (data not shown).

*Enhancement of $CD4^+$ CTL-mediated but not $CD8^+$ CTL-mediated lysis against T.gondii-infected target cells by IFN-γ*

*T.gondii*-infected B lymphoma (ARH or EBV-Ya cells) or melanoma (P36 cells) was treated with various concentration of rIFN-γ for 24 hours and was used as a target of the CTL assay for $CD8^+$ CTL or $CD4^+$ CTL to examine the effects of rIFN-γ on the targeting activities. $CD4^+$ CTL showed enhanced cytotoxicity against *T.gondii*-infected P36 cells that were co-cultured with rIFN-γ. The CTL activity against *T.gondii*-infected P36 cells was enhanced by rIFN-γ dose-dependently. On the other hand, the lytic activity of $CD8^+$ CTL against rIFN-γ treated B lymphoma was not augmented (Fig. 3). Thus, the

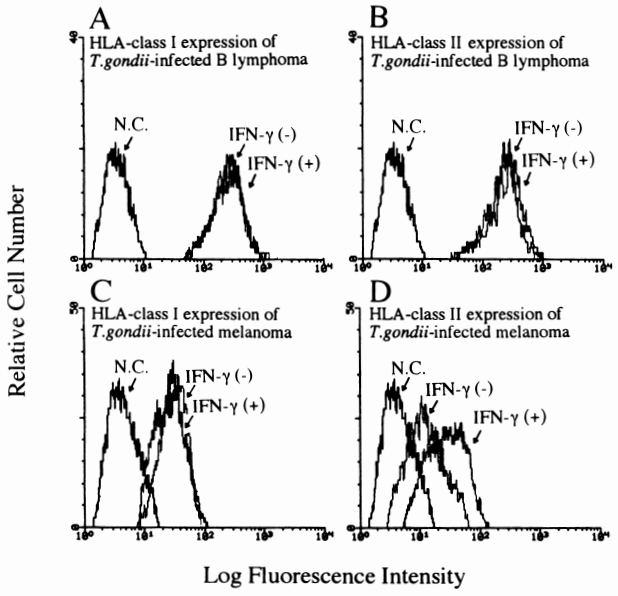


Fig. 2 Effects of IFN-γ on HLA molecule expression of B lymphoma or melanoma cells. *T.gondii*-infected B lymphoma (EBV-Ya cells) or lymphoma (P36 cells) were incubated with or without 60 ng/ml of human rIFN-γ for 24 hours. After incubation, the cells were harvested and stained with either anti-HLA-A, B, C mAb (A and C) or anti-HLA-DR mAb (B and D) followed by a staining with second-layer FITC-conjugated goat anti-mouse immunoglobulins (Igs). Samples stained only with FITC-conjugated goat anti-mouse Igs were used as negative control (N.C.). The intensity of FITC was analysed by FACScan.

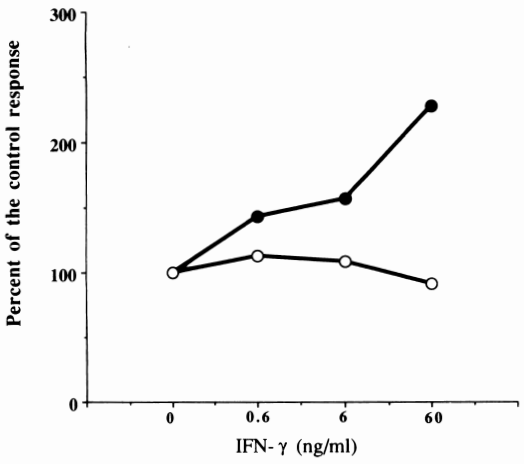


Fig. 3 Dose-dependent enhancement of $CD4^+$, but not $CD8^+$ CTL lysis against IFN-γ treated *T.gondii*-infected target cells. Lytic activity of $CD8^+$ CTL against IFN-γ treated *T.gondii*-infected B lymphoma (ARH or EBV-Ya cells) (open circle) and lytic activity of $CD4^+$ CTL against IFN-γ treated *T.gondii*-infected melanoma (P36 cells) (closed circle) were comparatively examined by an ordinary 4 hours CTL assay. Preparation of target cells was carried out as described in Materials and Methods. The effector to target ratio used for $CD8^+$ or $CD4^+$ CTL lysis was 15:1 or 30:1, respectively.

up-regulation of the surface HLA-DR expression of P36 cells by IFN-γ seemed to induce the augmentation of the targeting activities (i.e. antigen presentation) of *T.gondii*-infected P36 cells to $CD4^+$ CTL.

*Effects of IFN-γ on LFA-1 or ICAM-1 molecule expression of B lymphoma or melanoma cells*

To test whether some accessory molecules participate or not in different cytotoxicities of $CD8^+$ CTL and $CD4^+$ CTL against rIFN-γ treated *T.gondii*-infected target cells, the surface expression of either LFA-1α, LFA-1β or ICAM-1 molecule of B lymphoma (ARH or EBV-Ya cells) or melanoma (P36 cells) treated with or without rIFN-γ (60 ng/ml for 24 hours) was comparatively analysed. The treatment with rIFN-γ up-regulated the ICAM-1 expression of *T.gondii*-infected melanoma but not that of B lymphoma (Fig. 4C and F), suggesting the participation of the ICAM-1 molecules in the enhanced targeting activity of rIFN-γ treated *T.gondii*-infected P36 cells. The surface expression of LFA-1 molecules of either B lymphoma or melanoma line was not affected by the treatment with rIFN-γ (Fig. 4A, B, D and E). The expression levels of LFA-1α, LFA-1β or ICAM-1 molecules of both *T.gondii*-infected and uninfected B lymphoma or melanoma were similar (data not shown).

## Discussion

We have reported the existence of two types of *T.gondii*-infected cell-specific CTL induced from PBL of a patient with chronic toxoplasmosis. $CD8^+$ CTL lines generated by *in vitro* stimulation of the patient PBL with *T.gondii*-infected B lymphoma (ARH or EBV-Ya cells) recognize *T.gondii*-infected cell-specific antigens in association with HLA-A2 molecules (Yano *et al.*, 1989; Aosai *et al.*, 1994). On the other hand, $CD4^+$ CTL lines established from PBL of the same patient by *in vitro* stimulation with *T.gondii*-infected melanoma (P36 cells) recognize *T.gondii*-infected cell-specific antigens in association with HLA-DR molecules (Yang *et al.*, 1995). It is reasonable that *T.gondii*-infected ARH cells, which share only HLA-A2 and -Cw4 molecules with the patient, induced only $CD8^+$ CTL. However, when *T.gondii*-infected autologous B lymphoma, EBV-Ya cells, was used as APC, only $CD8^+$ CTL were induced (data not shown). Also we failed to induce HLA-class II restricted $CD4^+$ CTL when another *T.gondii*-infected B lymphoma (EBV-Wa cells) that shared only HLA-DR4 molecules with the patient was used as APC (data not shown). Thus, some preference in inducing $CD8^+$ CTL or $CD4^+$ CTL may exist according to the APC types, although further studies need to be done including a limiting dilution analysis to see the precursor frequencies of $CD8^+$ CTL or $CD4^+$ CTL by the stimulation with *T.gondii*-infected EBV-Ya cells or an analysis by using the HLA-class I and class II matched melanoma as APC. *T.gondii*-infected melanoma-specific $CD4^+$ CTL also lysed *T.gondii*-infected autologous B lymphoma EBV-Ya cells (Yang *et al.*, 1995), indicating that *T.gondii*-infected B lymphoma expressed *T.gondii* antigen in association with HLA-DR molecules which were recognizable by the *T.gondii*-infected melanoma-specific $CD4^+$ CTL. Together, it could be speculated that the number of HLA-DR molecules of *T.gondii*-infected B lymphoma presenting *T.gondii* epitopes was not enough to induce

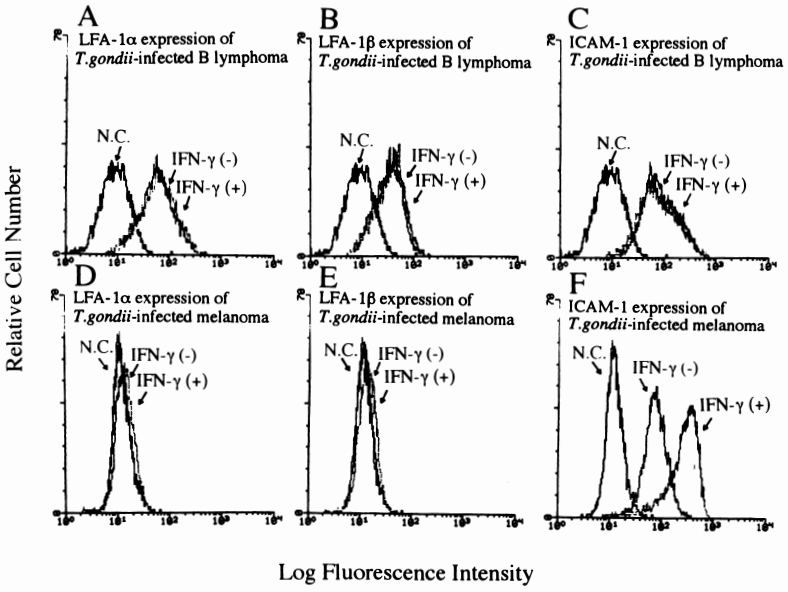


Fig. 4 Effects of IFN-γ on LFA-1 or ICAM-1 molecule expression of B lymphoma or melanoma cells. *T.gondii*-infected B lymphoma (EBV-Ya cells) or melanoma (P36 cells) were incubated with or without 60 ng/ml of human rIFN-γ for 24 hours. After incubation, the cells were harvested and stained with either anti-LFA-1α mAb (TS1/22.1.1.13) (A and D), anti-LFA-1β mAb (TS1/18.1.2.11.4) (B and E) or anti-ICAM-1 mAb (84.H.10) (C and F) followed by a staining with second-layer FITC-conjugated goat anti-mouse immunoglobulins (Igs). Samples stained only with FITC-conjugated goat anti-mouse Igs were used as negative control (N.C.). The intensity of FITC was analysed by FACScan.

$CD4^+$ CTL but was enough to be recognized by the induced $CD4^+$ CTL. Alternatively, B lymphoma may lack some accessory molecules or some types of lymphokines for inducing *T.gondii*-infected cell-specific $CD4^+$ CTL from their precursor cells.

IFN-γ has been reported to play a key role in protection against acute or reactivate chronic infection with *T.gondii*. As a major source of IFN-γ, $CD8^+$ CTL (Suzuki and Remington, 1990; Gazzinelli *et al.*, 1991; Denkers *et al.*, 1994) or NK cells (Sher *et al.*, 1993) have been reported. Although the synergistic participation of $CD4^+$ T cells to IFN-γ production by $CD8^+$ CTL (Gazzinelli *et al.*, 1991; Gazzinelli *et al.*, 1992; Kasper and Khan, 1993) or the contribution of $CD4^-CD8^-$ cells (Johnson *et al.*, 1993) have been also shown, adoptive cell transfer experiments have revealed that $CD8^+$ T cells but not $CD4^+$ T cells (even though equal quantity of IFN-γ was secreted by $CD4^+$ T cells), worked in protection probably through the production of IFN-γ (Suzuki and Remington, 1990; Khan *et al.*, 1994; Gazzinelli *et al.*, 1992). Thus, the IFN-γ produced by $CD8^+$ CTL has been thought to be a major cytokine that works in protection. We have herein reported that both $CD8^+$ CTL and $CD4^+$ CTL secreted equally high levels of IFN-γ when they were stimulated with *T.gondii*-infected B lymphoma or melanoma, respectively. Although $CD4^+$ CTL alone produced a small amount of IFN-γ, the amount of IFN-γ production was definitively augmented by culturing $CD4^+$ CTL with *T.gondii*-infected melanoma. The IFN-γ production by $CD8^+$ CTL or $CD4^+$ CTL was partially inhibited by the anti-HLA-class I mAb or anti-HLA-DR mAb, respectively (data not shown). The blocking effects of the anti-HLA-class I mAb or anti-HLA-DR mAb on the IFN-γ production by $CD8^+$ CTL or $CD4^+$ CTL was much less than those on the CTL activities against *T.gondii*-infected target cells. One may speculate that other mechanisms, such as antigen presentation of superantigens, in

IFN-γ production by $CD8^+$ CTL or $CD4^+$ CTL may participate (Denkers *et al.*, 1994).

The targeting activity of *T.gondii*-infected melanoma to $CD4^+$ CTL was easily enhanced by rIFN-γ, while that of *T.gondii*-infected B lymphoma to $CD8^+$ CTL was not enhanced. As the level of surface HLA-DR expression of *T.gondii*-infected melanoma (even after rIFN-γ treatment) was much less than that of *T.gondii*-infected B lymphoma, it could be speculated that enough numbers of surface HLA-DR molecules of *T.gondii*-infected melanoma expressed *T.gondii* epitopes. As we have hypothesised (Yang *et al.*, 1995), the up-regulation of HLA-DR molecule expression induced by IFN-γ (produced from likely $CD8^+$ CTL or NK at the initial stage) would induce the efficient binding of *T.gondii* peptide fragments to HLA-DR molecules in infected P36 cells that resulted in the efficient antigen presentation and the enhanced cytolysis of infected target cells by $CD4^+$ CTL. On the other hand, surface HLA-A, B, C or HLA-DR molecule expression of B lymphoma was not augmented by the treatment with exogenous human rIFN-γ. It seemed that B lymphoma cells had already expressed the maximal level of HLA molecules (both class I and class II) on their cell surface so that no augmenting effects of HLA expression was detected by rIFN-γ treatment.

The treatment with rIFN-γ augmented the surface ICAM-1 expression of *T.gondii*-infected P36 cells but not that of *T.gondii*-infected B lymphoma cells. The importance of ICAM-1 molecule expression on the target cells in the cytotoxicity has been reported (Dohlsten *et al.*, 1991, Nowak *et al.*, 1991, Chong *et al.*, 1994). Thus, the up-regulation of surface ICAM-1 of *T.gondii*-infected P36 cells by the treatment with rIFN-γ would suggest the contribution of ICAM-1 in the enhanced targeting activity of infected P36 cells treated with rIFN-γ.

We have also analysed the direct binding of human rIFN-γ to *T.gondii* tachyzoites, *T.gondii*-infected B lymphoma (ARH and EBV-Ya cells) or *T.gondii*-infected melanoma (P36 cells) by using a biotinylated human rIFN-γ followed by a staining with streptavidin-FITC. Human rIFN-γ directly bound to the surface of both *T.gondii*-infected melanoma (Yang *et al.*, 1995) and B lymphoma (data not shown) but not to the surface of *T.gondii* tachyzoites. Also the surface expression of IFN-γ receptors on both *T.gondii*-infected melanoma (Yang *et al.*, 1995) and B lymphoma (data not shown) but not on *T.gondii* tachyzoites was confirmed by a staining with anti-IFN-γ receptor mAb. Thus, the parasiticidal activity of IFN-γ in host resistance to *T.gondii*-infection was indirect. The indirect function of IFN-γ in the protection would be agreed with the hypothesis that the extracellular *T.gondii* tachyzoites released from the *T.gondii*-infected cells by a CTL attack would be transmitted to and destroyed by activated phagocytes under the influence of inflammatory stimuli and T-cell lymphokines (Kaufmann, 1988). Recently, Yong *et al.* (1994) reported that 5-lipoxygenase (e.g., leukotriene[LT] $B_4$, $LTC_4$) arachidonic acid products were released from macrophages by the treatment with IFN-γ and the released LTB4 induced intracellular killing of ingested *T.gondii* by non-IFN-γ treated macrophages. More precise mechanisms of anti-*T.gondii* prophylactic immunity and immunopathogenesis of IFN-γ, $CD8^+$ CTL and $CD4^+$ CTL in *T.gondii* infection remain to be elucidated.

**Acknowledgement**

The authors thank Daiichi Pharm. Co., Tokyo, Shionogi Pharm. Co., Osaka and Ohtsuka Pharm. Co., Ohsaka for their generous gifts of human rIFN-γ and ELISA assay plates.

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