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CTL induction by DNA vaccine with *Toxoplasma gondii*-HSP70 gene

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ABSTRACT

Toxoplasma gondii-derived heat shock protein 70 (*T.g.*HSP70) is a tachyzoite-specific virulent molecule. The DNA vaccine with *T.g.*HSP70 gene targeting peripheral epidermal or dermal dendritic cells (DC) induces in vivo DC maturation and successive early Th1 polarization at the draining lymph nodes (dLN) of C57BL/6 mice. In the present study, induction of cytotoxic T lymphocytes (CTL) has been explored. The CTL specific for a syngeneic DC line, DC2.4, either transfected with *T. g.*HSP70 gene or pulsed with recombinant *T. g.*HSP70 are induced in the spleen of the vaccinated mice. This CTL lyses *T. gondii*-infected, but not uninfected, DC2.4. Both $CD8^+$ and $CD4^+$ CTL are induced by the vaccine, and Fas/Fas ligand-mediated cytolysis dominantly participates in their CTL activities. Adoptive transfer experiments reveal that the vaccine-induced $CD8^+$ or $CD4^+$ T cells possess a protective role for toxoplasmosis at both acute and chronic phases of infection.

1. Introduction

Toxoplasma gondii is a common intracellular protozoan which causes worldwide parasitic infectious diseases, toxoplasmosis, in humans and animals [1]. In intermediate hosts such as humans and mice, per orally (p.o.) infected encysted *T. gondii* bradyzoites or sporozoites in sporulated oocysts undergo stage conversion into rapidly dividing tachyzoites that are responsible for acute toxoplasmosis in immunocompromised individuals and fetuses infected within the uterus [1,2]. Induction of cell-mediated protective immunity against *T. gondii*-infection has been attempted by vaccines using attenuated parasite or DNA vaccine encoding potential genes either alone or in combination with varying degrees of success [3–5].

DNA vaccine targeting dendritic cells (DC), i.e., professional antigen presenting cells (APC), is thought to be the most promising approach to activate T cell-mediated immunity required for intracellular microbial infections [6]. Among various vaccine candidates of *T. gondii* genes, we have developed the DNA vaccine encoding *T. gondii*-derived heat shock protein 70 (*T.g.*HSP70) gene targeting peripheral epidermal or dermal DC to elicit cell-mediated protective immunity [7–9]. *T.g.*HSP70 stimulates in vitro differentiation of peripheral immature DC into mature DC both in humans and mice [10,11], and DNA vaccine with *T.g.*HSP70 gene induces in vivo migration/maturation of peripheral skin-derived DC to the draining lymph nodes (dLN) where DC produces IL-12 and successively induces early Th1 polarization in mice [9]. However, induction of cytotoxic T lymphocytes (CTL) by the *T.g.*HSP70 gene vaccine has not yet been analyzed.

We previously reported the existence of CTL specific for *T. gondii*-infected cells in a patient with chronic toxoplasmosis [12–14] and, more importantly, that HSP71 (i.e., a human HSP70) was recognized as an antigenic epitope by *T. gondii*-infected cell-specific CTL line obtained from a patient with chronic toxoplasmosis [15]. Although critical roles of $CD8^+$ T cells in controlling both acute and chronic *T. gondii* infection have been extensively explored in murine experimental models [16–21], the report of the CTL induction by the vaccine is few [22–25]. Thus, in the present study, we have analyzed the effect of *T.g.*HSP70 gene vaccine on CTL induction and have revealed the existence of both $CD8^+$ and $CD4^+$ CTL specific for *T. gondii*-infected DC in the vaccinated mice. By adoptively transferring the $CD8^+$ or $CD4^+$ CTL from the vaccinated mice into naïve mice, the roles of vaccine-induced CTL against toxoplasmosis have been evaluated.

2. Material and methods

2.1. Mice and T. gondii

Eight-week-old wild type (WT) female C57BL/6 (B6) mice (a susceptible strain against *T. gondii* infection) were purchased from SLC (Hamamatsu, Japan). Mice were used at the age of 8 weeks. Tachyzoites of *T. gondii* RH strain were maintained in vitro by culturing in a human B lymphoma ARH_{77} cells (American Type Culture Collection [ATCC]) as hosts. Cysts of an avirulent *T. gondii* Fukaya strain were obtained from the brain of chronically infected mice as previously described [26]. Mice were per orally (p.o.) infected with 500 μl of the brain homogenates adjusted to contain 10 cysts by using a needle with a round head. All animal procedures used in this study complied with guidelines set by the Animal Care and Use Committee of Chiba University.

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2.2. Plasmid construction

*T.g.*HSP70 cDNA was amplified from the genomic DNA (gDNA) of *T. gondii* tachyzoites of RH strain by polymerase chain reaction (PCR) and inserted into eukaryotic expression plasmid vector $pME18_{100}$ (a gift from Dr. K. Maruyama, Medical Research Institute, Tokyo University) as previously described [7–9]. As a control, empty $pME18_{100}$ plasmid was used.

2.3. Gene gun vaccine

The procedure of gene gun vaccine has been previously described [7–9]. Briefly, plasmid with or without encoding *T.g.HSP70* gene was affixed to gold particles (1.6 μm diameter) at a rate of 2 μg of DNA per 1 mg of gold by the addition of 1 M $CaCl_2$ in the presence of 0.05 M spermidine. Then, the plasmid DNA-coated gold particles were loaded onto Gold-Coat tubing in the presence of polyvinylpyrrolidone (360,000 MW). Plasmid DNA-coated gold particles were accelerated into the shaved abdomen of mice using a Helios Gene Gun (Bio-Rad., Tokyo, Japan) at a helium discharge pressure of 400 psi. Mice were vaccinated twice at a 2-week interval with 2 μg of DNA each time as previously described [8,9]. Three to five mice were used for each experimental group.

2.4. Generation of T.g.HSP70-transfected DC line

DC2.4 (a murine DC line with B6 background) was provided by Dr. Kenneth L. Rock (Dana-Farber Cancer Institute, U.S.A.) and has been maintained in vitro in RPMI 1640 medium (Gibco by Life Technology, Japan) supplemented with 5% fetal bovine serum (FBS) and antibiotics (Penicillin and Streptomycin) at 37 °C in a 5% CO_2 incubator. Five million DC2.4 cells were suspended in 0.5 ml HEPES-buffered saline and transferred into a 4 mm electroporation cuvette on ice. Then, DC2.4 cells were transfected at a dose of 3 μg of $pME18_{100}$ plasmid with or without encoding *T.g.*HSP70 gene by using the Gene Pulser electroporation apparatus (Bio-Rad Laboratories, Tokyo, Japan). Forty-eight hours after transfection, 400 μg/ml G418 (Wako Chemicals, Osaka, Japan) was added to the medium for neo-gene selection and the existence of *T. g.*HSP70 gene in DC2.4 cells was confirmed by PCR.

2.5. CTL assay

Mice were euthanized at 1 week after the last vaccine with or without *T.g.*HSP70 gene. After deleting red blood cells from splenocytes with Gey's solution, $CD4^+$ or $CD8^+$ cells were fractionated from splenocytes by Vario magnetically activated cell sorting (MACS) separator system (Miltenyi Biotec, Auburn, CA, USA) with microbeads-conjugated anti-CD4 (clone GK1.5) or anti-CD8 (clone 53–6.7) monoclonal antibody (mAb) (Miltenyi Biotec) according to the company's instructions. Purity of the recovered cells was >93% according to flow cytometry (Becton Dickinson, Tokyo, Japan). Whole splenocytes or fractionated $CD4^+$ or $CD8^+$ splenocytes were used as effector cells. As target cells, DC2.4 cells, either uninfected, infected with tachyzoites of *T. gondii* RH strain, transfected with $pME18_{100}$ plasmid with or without encoding *T. g.*HSP70 gene, or pulsed with recombinant (r) *T. g.*HSP70 protein were used. *T. gondii*-infected cells were prepared as previously described [12,14]. Preparation of r *T. g.*HSP70 protein was previously described [27]. $CD11c^+$ cells isolated from the dLN (inguinal and axillary LN) of the vaccinated mice were also used as target cells. The effector cells (E) were co-cultured with 5×10^3 target cells (T) at the 40:1 or 20:1 E/T ratio in a 96-well u-bottom plate by using the assay medium (i.e., RPMI 1640 medium supplemented with 1% bovine serum albumin and antibiotics) at 37 °C in a 5% CO_2 incubator. After co-culturing E/T for 12 h, 100 μl of culture supernatant was collected from each well and cell mediated cytotoxicities were measured by using a LDH cytotoxicity detection kit according to the company's instructions (Takara Bio Inc., Otsu, Japan). Spontaneous or maximum LDH release from target cells was measured by incubating target cells in medium alone or in the medium containing 2% Triton X-100, respectively. Experiments were performed in triplicate and repeated at least three times. The percentage of cell mediated cytotoxicity is determined by calculating the average absorbance (492 nm) of the triplicates and subtracting the background. These values are substituted into the following equation: Cytotoxicity (%) = {[culture of effector plus target cells]–[culture of effector cell alone]–spontaneous release/maximum release–spontaneous release} × 100.

2.6. Adoptive transfer of splenocytes and quantifying T. gondii loads

One week after the last vaccine, $B220^+$, $CD8^+$, $CD4^+$, $CD11c^+$ and $CD11b^+$ cells were subsequently fractionated from splenocytes of the mice vaccinated with or without *T.g.HSP70* gene by using a microbeads-conjugated anti-$B220^+$, $CD8^+$, $CD4^+$, $CD11c^+$ or $CD11b^+$ mAb (all mAbs were purchased from Milteni Biotec). Total T cells were isolated by treating spleen cells with microbeads-conjugated both anti-CD8 and anti-CD4 mAbs. After washing in chilled PBS, each fraction of splenocytes from one mouse was transferred intravenously (i.v.) into one naïve B6 mouse.

One day after receiving the fraction of splenocytes from mice vaccinated with or without *T.g.*HSP70 gene, the recipient mice were p.o. infected with 10 *T. gondii* cysts of the Fukaya strain. At the acute phase of infection, the mice were euthanized at 1 week post infection (P.I.) and *T. gondii* abundance in the mesenteric LN (mLN) was evaluated by using a quantitative competitive-PCR targeting the tachyzoite-specific SAG1 gene as described [26]. At the chronic phase of infection, the mice received cell transfer twice (1 day before and 2 weeks P.I.) and was euthanized at 4 weeks P.I. A half brain of each mouse was triturated in 5 ml of PBS and cyst numbers in 100 μl of the brain suspension were counted microscopically at least three times/brain.

2.7. Reverse transcription-PCR

At 1 week after the last vaccine, total RNA was isolated from $CD4^+$ or $CD8^+$ splenocytes of mice vaccinated with or without encoding *T.g.*HSP70 gene and transcribed to cDNA by reverse transcription (RT), and then cDNA was used for real-time quantitative PCR using a TaqMan PCR system (Applied Biosystems, Tokyo, Japan). Primers and 6-carboxyfluorescein (FAM)-labeled probes (Applied Biosystems) specific for interferon gamma (IFN-γ), perforin, granzyme B, Fas ligand (FasL) were used. GAPDH was used as an internal control. The primer/probe sets were purchased from Applied Biosystems. We normalized each set of samples using the difference in threshold cycles (ΔC_T) between the sample gene and the internal control gene (GAPDH) as follows: $\Delta C_T = C_{T\ \text{sample}} - C_{T\ \text{GAPDH}}$. The calibrator sample ($\Delta C_{T\ \text{calibrator}}$) was assigned as the sample with the highest ΔC_T in each set. The relative scale of mRNA measurement is represented on the *y* axes of figures by the expression of log-transformed $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta C_{T\ \text{sample}\ (n)} - \Delta C_{T\ \text{calibrator}\ (n)}$. Each reaction was done in triplicate.

At the chronic phase of infection (4 weeks P.I.), RNA was isolated from a half brain of each mouse and transcribed to cDNA, and then mRNA expression of bradyzoite (i.e., resting stage of *T. gondii* zoite within cysts)-specific BAG1 gene was evaluated by the real-time quantitative PCR with the following primers/probe specific for BAG1: 5′-TGG GGT GGA GTC GGT CTT AAT-3′ (forward primer), 5′-GAT AAC GAT GGC TCC GTT GTC-3′ (reverse primer) and 5′-ATC GAC GAT ATG TTG TTC G-3′ (FAM-labeled probe). After normalizing the sample by using GAPDH, the relative scale of BAG1 mRNA expression is represented.

2.8. Flow cytometry analysis

Splenocytes of mice vaccinated with or without *T.g.*HSP70 gene were stained at 1 week after the last vaccine with fluorescein isothiocyanate (FITC)-conjugated either anti-CD4 (clone GK1.5) or anti-CD8 (53–6.7)