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Toxoplasma Lysate Antigen (TLA144)-Ig8 Antigenic Component: Binding and Induction of Cytotoxic Cell Activity in Mouse Spleen Cells

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ABSTRACT

The antigenic component TLA-Ig8 purified from *Toxoplasma gondii*-lysate antigen(TLA-144) using anti-TLA-144 and anti-TLA-Ig8 monoclonal antibodies exhibited significant anti-tumor activity in methylcholanthrene tumor cell-bearing BALB/c mice. Binding activity of labelled TLA-Ig8 antigenic component to adherent cells was significantly higher relative to non-adherent cells, and reached a plateau approximately 30 minutes post-incubation. Binding reaching a saturation point early on facilitates the attachment of the antigen to the surface membrane receptor of adherent cells enhancing antigen processing through phagocytosis and thus inducing cytotoxic activity. Analysis of specific macrophage cell surface antigen revealed a molecule of molecular weight 80 kilodaltons. Our results suggest TLA-Ig8 antigenic component to be a tachyzoite surface antigen important in the induction of cytotoxic cell activity.

INTRODUCTION

In vivo and in vitro culture studies have shown the important role of cell-mediated immunity in preventing the establishment of *Toxoplasma gondii* (Jacob et al.,1954), and that cytokines such as IFN-gamma and *Toxoplasma* growth inhibitory factor (Toxo-GIF) have been identified as the major mediating factors (Kono et al.,1992; Sakurai et al.,1983; Omata et al.,1983; Suzuki et al.,1984,1990; Saito et al.,1986). Sensitization of mice with *Toxoplasma* lysate antigen (TLA-144) effected development of strong host resistance against *Plasmodium berghei* (Suzuki et al.,1987), *Babesia* spp. (Ogawa et al.,1985; Saito et al.,1985, 1987, 1990; Igarashi et al., 1990) and FerLV positive lymphoma cells (Yang et al.,1990). Likewise, administration of TLA-144 to allogeneic (S-180) and isogenic (MC) tumor cell bearing mice

showed a remarkable inhibition of tumor cell growth compared to the non-treated control (Saito et al.,1990; Miyahara et al.,1992a,b,c,). In vitro, TLA144 induces cytotoxic activity in mouse spleen cells, activates natural killer (NK) cells, and induce lymphokine activated killer (LAK) cells (Sharma et al.,1984; Igarashi et al., 1990; Miyahara et al., 1992b,c; Elizabeth et al., 1982). Saito et al.(1990) and Miyahara et al.(1992 b) suggested that this induction process must require contact with TLA sensitized lymphocytes and monocytes.

In our laboratory, anti-TLA144 monoclonal antibody (mAb) inhibited TLA-144 induced cytotoxic cell activity. We hypothesized then that the inhibition must have resulted from the binding of the mAb to the TLA antigenic determinant. Subsequently, we purified from TLA144, TLA-Ig8 antigenic component with a molecular weight of about 80 kilodaltons (Kda) consisting of two polypeptide chains bridged by S-S bond. In vitro, TLA-Ig8 antigenic component has demonstrated acceleration of the induction of cytotoxic cell activity of NK and LAK cells in mice sensitized with TLA-144. To date, there are no published reports of high molecular weight antigens obtained from *Toxoplasma gondii* that activate immunity. Thus, in the present study, we sought to determine the anti-tumor effect of TLA-Ig8 antigenic component, and to clarify, at least in part , its mechanism of action in vivo.

MATERIALS AND METHODS

***Mice and Parasites*:** Male and female BALB/c mice, 4-6 wks old bred at Animal Facility of the Department of Veterinary Physiology, Obihiro University were used in the study. *Toxoplasma gondii* (RH strain) was obtained from Hela cells in vitro culture system (Valkoun et al., 1983). Hela cells were cultured in Dulbecco's modified MEM+10% heat inactivated fetal calf serum (FCS) monolayer. Tachyzoites obtained from mouse peritoneum two days post-inoculation (PI) were washed with Hank's balanced salt solution (HBSS) supplemented with 5u/ml heparin and 60μg/ml of kanamycin sulfate. Tachyzoites were separated from dead Hela cells through density gradient centrifugation using Percoll (Pharmacia, Uppsala, Sweden) and tachyzoites were kept at -80℃ until use.

***TLA-144 Preparation*:** TLA-144 was prepared following the methods of Saito et al.(1990) and Sakurai et al.(1983). Mouse ascite containing tachyzoites obtained from mice two days PI was centrifuged and tachyzoites were washed with HBSS, three times at 750x g for 10 min at 4℃, each time. The sample was resuspended in distilled water, then frozen (-80℃) and thawed (37℃), three times, and ultrasonicated at 100W five times at an interval of 30 sec. After ultracentrifugation at 144,000xg for 120 min at 4℃, the supernatant designated as TLA-144 was freeze dried and then kept in -20 ℃ until use. Total protein was determined using Bradford method (1976) with bovine serum albumin (BSA) as the standard.

Preparation of Monoclonal Antibodies: Monoclonal antibodies (mAb) against TLA-144 were prepared following the method of Oi et al.(1980). TLA-144 (100μg/mouse) was injected intravenously, and three days post-injection, the spleen was aseptically removed and processed for screening positive hybridomas. IgG secreted by specific clones were purified using TSK-gel Protein A650 affinity column.

Preparation of TLA144 and TLA-Ig8 antigenic component: The separation of TLA-Ig8 antigenic component was carried out using ion-exchange chromatography on DEAE-Toyopearl 650M. Elution buffers A (20 mM Tris-HCl + 2mM EDTA + 6M Urea, pH 7.5) and B (buffer A + 1M NH_4Cl) with linear gradient of 0.0-0.2M NH_4Cl in solution B were used. Fractions were applied on nitrocellulose membrane, and those that reacted with anti-TLA-Ig8 mAb were pooled. Pooled fractions were assayed by chromatofocusing on PBE 94 column (Pharmacia, Uppsala, Sweden) at pH gradient 9.4-6.0 using a mixture of Polybuffer 96 and 6M Urea in 0.025M ethanolamine-acetic acid (pH9.4). Fractions obtained were dot blotted and reacted with anti-TLA-Ig8 mAb. Positive fractions were subjected to reverse phase chromatography on Capcell Pak C18 300A (Shiseido, Tokyo, Japan) and TSK-gel Phenyl 5pw-RP (TOSOH, Tokyo, Japan) using CCPM Pump unit, an SC8010 Data Processor and UV8010 detector (TOSOH, Tokyo, Japan). Eluted positive fractions were then subjected to SDS-PAGE (Laemmli, 1970) to determined molecular weights.

Effect of TLA-144 and TLA-Ig8 antigenic component against tumor cell growth in vivo: In vitro cultured MC tumor cells in HBSS ($5x10^5$ cells/ml) were intradermally injected into the back of 5 wks old male mice (5 mice /Groups A, B and C). Seven days after, each mouse in Groups B and C was intramuscularly injected with 100μg TLA-144, and 100μg TLA-Ig8 antigenic component, respectively. One week later, the same mice were injected similar doses of antigens. Group A mice served as the non-treated control. The inhibitory effect of TLA-144 and TLA-Ig8 antigenic component was noted on every day by measuring the length (a) and width (b) of tumor growth. Tumor size was assessed by multiplying tumor growth length with width.

Preparation on mouse spleen adherent and nonadherent cells: Collagenase (100u/ml) was injected into mouse spleen dissected aseptically. Spleen was cut into small pieces and after 30 min, the tissues were pressed through a stainless steel mesh with a disposable gasket. The cells were transferred into a 50 ml centrifuge tube, resuspended in cold RPMI 1640 + 0.83% ammonium chloride solution (37℃) centrifuged (200 xg,10 min,4℃), and then washed with RPMI 1640 twice. Cell suspension in a plastic dish was incubated overnight at 37℃. Nonadherent cells were removed by adding warm RPMI 1640 to culture dish, and adherent cells were washed with 0.2% EDTA-PBS. Adherent cells were recovered from the culture dish

using a rubber policeman and washed through centrifugation.

Binding Assay: Fifty mM borate buffer solution was mixed with TLA-Ig8 antigenic component as substrate and Bolton and Hunter reagent (250μCi), to which ^{125}I-BHR without benzene was added. The reaction was allowed to proceed for three hrs at 4℃ with shaking. Thereafter, 100 mM ethanolamine was added to the reaction mixture for one hr, 4℃ with shaking. Free ^{125}I-BHR was separated by gel filtration with Sephadex G15 column(Pharmacia, Uppsala, Sweden) in phosphate buffer saline (PBS) + 5% BSA. Every one μl of fraction collected was checked for radioactivity using agamma scintillation counter (Packard Auto Gamma 5650, United Technology, Packard,U.S.A.). The fraction that showed the most radioactivity was recovered and used as the ^{125}I-BHR-labelled TLA-Ig8 antigenic component.

A. Specific binding assay. To each tube containing 3-5x10^6 Hela cells /ml or 3-5x10^6 adherent cells/ml,100, 500, 1000, 5000 and 10000 ng of labelled TLA-Ig8 antigenic component were added and were incubated at 4℃ for two hrs. Each reaction mixture was washed twice with 5% BSA-RPMI 1640 and once with PBS-Tween 20, at 4500xg, 3 min at 4℃. Binding was measured using a gamma scintillation counter. For the control, non-specific binding of labelled antigenic component to Hela cells was measured. Time course of binding of labelled TLA-Ig8 antigenic component to adherent cells at 1000 ng or added with non-labelled TLA-Ig8 antigenic component at 500 ng/tube was monitored at 5, 15, 30, 60, 120 min post- incubation.

B. Binding rate as influenced by increase in the concentration of labelled antigen. Spleen cells were incubated with 100-10000 ng of labelled TLA-Ig8 antigenic component at 4℃ for two hrs. Cells were washed and radioactivity was measured as described above. For the control, Hela cells were used in determining non-specific binding.

C. Blocking and reversibility of binding. Reversibility of specific binding of TLA-Ig8 antigenic component to adherent cell binding site was assayed by incubating ^{125}I-labelled TLA-Ig8 antigenic component (4000, 6000, 8000 ng) with 3-5x10^6 adherent cells/ml for two hrs at 4℃. Reversibility of binding was based on the decreased rate of specific binding.

Molecular weight analysis of TLA-Ig8 antigen component present on mouse spleen adherent cells: Mouse spleen macrophages (1x10^5 cells/ml) were incubated with TLA-Ig8 antigenic component at 37℃, 4℃ for 2hrs. Each reaction mixture was centrifuged three times at 200xg, 4℃ for 3 min, each time. To obtained the lysate protein from macrophage surface membrane, cell pellet was mixed with lysate buffer (10mM Triethanolamine, 50mM NaCl,1mM EDTA,1% Digitonin, 1mM PMSF) for 2 hrs, at 4℃ or 37 ℃. The lysate was then centrifuged at 26000xg, 4℃ for 5 min. Supernatant obtained was collected and examined using SDS-PAGE and Western blotting (Towbin et al., 1979). Membranes were reacted with anti TLA-Ig8 mAb, and peroxidase labelled anti-mouse IgG. Western blot kit (Amersham Inter-

national, Amersham, Japan) was used in the visualization of bands.

RESULTS AND DISCUSSION

The anti-tumor effect of TLA-Ig8 antigenic component against MC tumor cells in vivo is summarized in Figure 1. Mean tumor size in mice at 28 days post-MC tumor cell inoculation were 445±86.0 mm^2 (non-treated control), 248.3±54.0 mm^2 (TLA-144), and 196.6±100.0 mm^2 (TLA-Ig8 antigenic component) treated groups. The anti-tumor suppressive effect of both TLA-144 and TLA-Ig8 antigenic component is significant compared to the control ($p<0.01$). Johnson (1990) has grouped antigens of *Toxoplasma gondii* as surface, excretory and secretory. Several authors have shown TLA-144 to function as an immunoactivator and induces anti-tumor activity in vivo (Denkers et al.,1993; Saito et al.,1989; Miyahara et al., 1992a,b,c; Sakurai et al.,1983). The marked anti-tumor effect noted by us in the present study cannot possibly be attributed to the purified p30 surface antigen (Denkers et al., 1993) because of the difference in the isolation of the TLA-Ig8 employed (i.e. using mAb). In a related study TLA-Ig8 antigenic component induced cytotoxicity in mouse splenocytes killing non-specific targets in vitro (data not shown). Our findings suggest the importance of TLA-Ig8 antigenic component in cell mediated immunity in effecting to anti-tumor activity.

Binding activity of labelled TLA-Ig8 antigenic component to adherent cells was significantly higher at 1656± 92.8 cpm compared to 1002 ± 96.8 cpm for nonadherent cells (Figure 2). Likewise, the binding of labelled TLA-Ig8

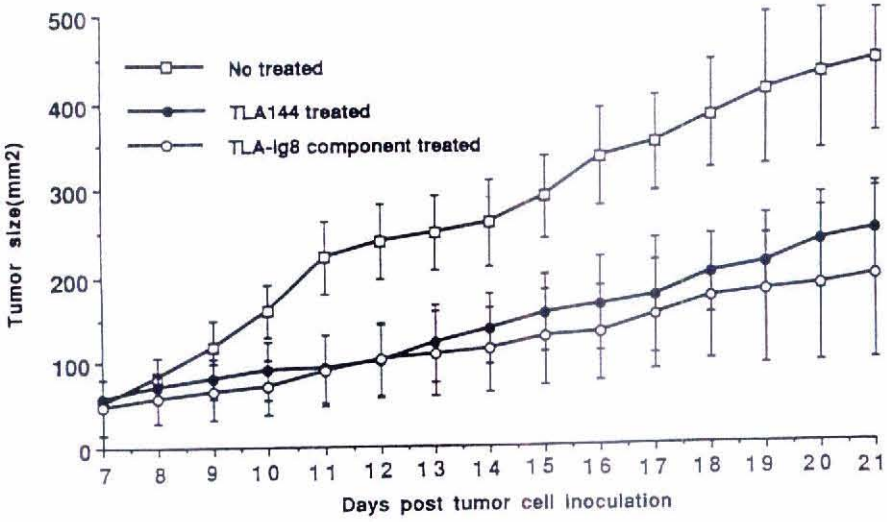

Figure 1. Anti-tumor activity of TLA (–●–), TLA-Ig8 antigenic component (–○–), and control (–□–); in methylcholanthrene-tumor cel l bearing mice. Each point represents mean valune ± S. D. of tumor size(mm^2) of 5 mice.

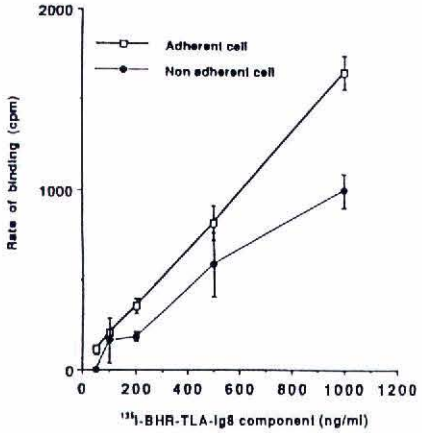

Figure 2. Binding activity of ^{125}I-TLA-Ig8 antigenic component to mouse spleen adherent (–□–) and nonadherent cells (–●–). Each point represents mean value ± S. D. of triplicate assay.

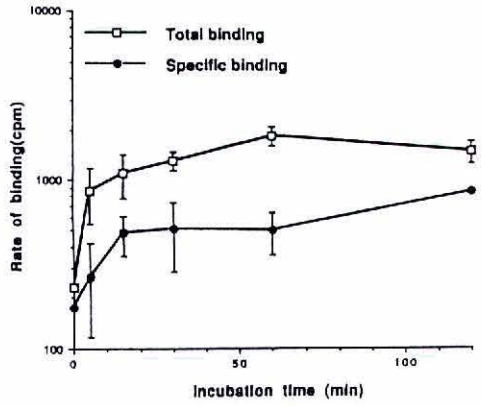

Figure 3. Time course of binding of ^{125}I-TLA-Ig8 antigenic component binding to mouse spleen adherent cells. Each point represents mean value ± S.D. of triplicate assay.

antigenic component to adherent cells started to reach a plateau at 30 min PI (Figure 3). Binding reaching a saturation point early on facilitates the attachment of the antigen to the surface membrane receptor of adherent cells, consequently, enhancing antigen processing through phagocytosis, thus inducing cytotoxic activity. TLA-Ig8 antigenic component possessing saturability and ligand specificity lend support to our hypothesis. Saito et al.(1989) noted the gradual loss of induction of cytotoxic activity in TLA-144 sensitized spleen cells following a serial removal of adherent cells.

As shown in Figure 4, specific binding of labelled TLA-Ig8 antigenic component to adherent cells increased with increased concentrations of

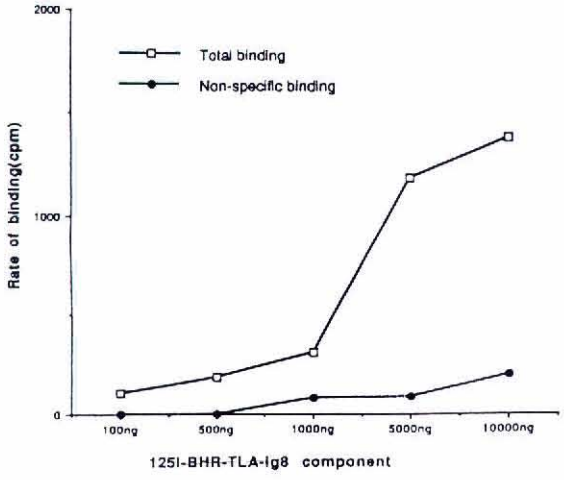

Figure 4. Concentration dependent binding of ^{125}I-TLA-Ig8 antigenic component to mouse spleen adherent cells.

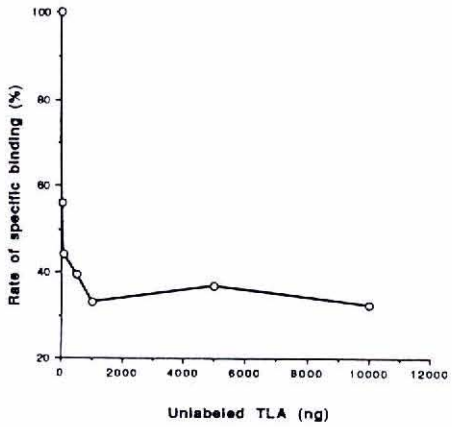

Figure 5. Specific binding of ^{125}I-BHR-TLA-Ig8 antigenic component to mouse spleen adherent cells.

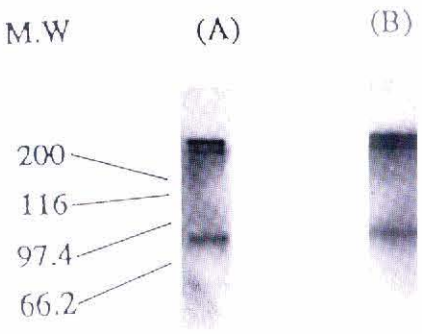

Figure 6. Effect of reaction temperature of TLA-Ig8 component binding to mouse spleen adherent cells. After antigen binding on antigen presenting cells, lysate was obtained and processed to western blot analysis. The band, molecular weight about 80kDa, was reacted with mAb (TLA-Ig8 antibody). (A) 4℃ and (B) 37℃.

labelled materials. Nonspecific binding to Hela cells at 1000 ng was 199 cpm compared to 1392 cpm for adherent cells, and binding plateau was reached at 5000 ng. Scatchard plot analysis suggests the presence of both low and high affinity sites for TLA-Ig8 antigenic component on adherent cells surface (data not shown). The addition of unlabelled TLA-Ig8 antigenic component effected a decrease in the rate of specific binding (Figure 5), and clearly demonstrates reversibility of binding activity.

Analysis of the molecular weight of the antigen present on macrophage cell surface after incubation with specific TLA-Ig8 antigenic component at 4℃ and 37℃ revealed specific antigen of molecular weight 80Kda, reactive with anti-TLA-Ig8 mAb (Figure 6). Our findings demonstrated TLA-Ig8 antigenic component to be a tachyzoites surface antigen, and we further suppose that TLA-Ig8 antigenic component does not contain p30 antigen (Denkers et al.,1993) but rather an antigen that is important in the induction of cytotoxic activity. Toxic shock syndrome toxin (TSST-1) binds major histocompatibility complex murine Class II molecules and stimulates T-cells, but it is not processed in the binding sites of antigen presenting cells, which is unlike our findings with TLA-Ig8 antigenic component. Whether the mechanisms controlling or influencing TLA-Ig8 antigenic component binding to antigen presenting cells is similar to that of TSST-1, is something that warrants further experimentations in vitro.

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