Characteristics of Cytotoxic Cells Induced by *Toxoplasma* Lysate Antigen in Mouse Spleen

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ABSTRACT. Spleen cells from *Toxoplasma* lysate antigen (TLA)-sensitized BALB/c mice showed the strong cytotoxic activity against both natural killer (NK)-sensitive cells (YAC-1 and RL♂-1) and NK-insensitive cells (P-815), when incubated with TLA or recombinant human IL-2 (rhIL-2). The increment of TLA concentration in culture medium increased the cytotoxic activity. Treatment of effector cells; spleen cells from TLA-sensitized mice incubated with TLA, with anti-asialo GM1 or anti-Thy-1 plus complement inhibited the cytotoxic activity of effector cells, whereas treatment with anti-mouse Lyt-2.2 serum plus complement had no effect on the cytotoxic activity. Treatment of spleen cells from TLA-sensitized mice with anti-asialo GM1 and/or anti-Thy-1 plus complement inhibited cytotoxic activities of effector cells. These results suggested that spleen cells sensitized with TLA both *in vivo* and *in vitro* were asialo GM1 positive and Thy-1 positive, and the majority of cytotoxic cells induced by TLA were similar to lymphokine-activated killer (LAK) cells induced by IL-2.—KEY WORDS: cytotoxic activity, mouse, phenotype, spleen cell, *Toxoplasma* lysate antigen.

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It has been reported that mice treated with *Toxoplasma* lysate antigen (TLA) and infected with *Plasmodium berghei* or *Babesia rodhaini* are capable of surviving infections that would otherwise be fatal [8, 18, 19, 23, 25]. It was revealed that interferon-gamma (IFN-gamma) and other lymphokines (LKs) were present in the serum of TLA-sensitized animals [9, 20]. The number of T cells and natural killer (NK) cells increased in the spleen, liver and peripheral blood of both TLA-sensitized and unsensitized mice on day 10 after *Babesia* infection, although numbers were much higher in the TLA-sensitized mice [8]. Incubation of TLA-sensitized mouse spleen cells with TLA *in vitro* causes induction of nonspecific cytotoxic cells that are capable of lysing target cells [8, 14].

The growth of allograftable Sarcoma-180 (S-180) tumors and isograftable tumors (Meth A and 20-methylcholanthrene (MC)-induced tumor) in BALB/c mice and the growth of MC-autoinduced tumor in rats were strongly inhibited after administration of TLA [14, 15, 25]. Furthermore, the histological analysis of MC-induced tumor tissue showed sporadic numbers of large Thy-1 positive granular cells in TLA-treated rats [15]. These observations indicate that TLA is a biological response modifier and that cytotoxic cells may be involved in the mechanism of anti-tumor development during nonspecific stimulation of the body's immunoprophylactic system.

In this study, ^{51}Cr release assay was used to investigate the anti-tumor effects of TLA-induced cytotoxic cells *in vitro*.

MATERIALS AND METHODS

Experimental animals: Inbred male BALB/c mice were reared and maintained in the Department of Veterinary Physiology and Protozoan Immunology, Obihiro University and 4–8 week old mice were used in this experiment.

Preparation of TLA and recombinant human interleukin 2 (rhIL-2): TLA was prepared according to the method described previously [7, 21, 22]. In brief, after centrifugation of crude antigen solution at 144,000 × g for 120 min, the supernatant was used as TLA preparation throughout the experiment. The rhIL-2 (Ajinomoto Co., Inc., Tokyo, Japan) was diluted to 1×10^5 units/m*l* with RPMI-1640 (Flow Laboratories, U.K.) at the time of during use.

Sensitization of mice with TLA: Mice were sensitized by two intramuscular injections of 30 μg of TLA in physiological saline at 2-week interval and used as TLA-sensitized mice four weeks after the first TLA injection.

Preparation of spleen cells: Spleen cells were prepared according to the method described previously [8, 14]. In brief, spleens from TLA-sensitized or unsensitized mice were ground and suspended in Hank's balanced salt solution (HBSS).

The spleen cells were collected from the crude suspension with Conray-Ficoll [26].

For cultivation of spleen cells, cell suspensions prepared were resuspended in RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (FCS; Flow Laboratories, Australia) containing 30 μg of TLA or 1,000 units of rhIL-2/m*l*, or 10% FCS-RPMI alone to a final cell density of 3.0×10^6 cells/m*l*. The suspension was incubated in a culture flask for 6 days. After incubation, each cultivated spleen cells were used as effector cells.

Cytotoxicity test: Cytotoxic function was examined with a specific ^{51}Cr release assay [11]. P-815 mastocytoma cell line, YAC-1 thymoma cell line and RL♂-1 leukemia cell line were supplied by Dr. Tsuneo Kamiyama, Department of Animal Epidemiology, National Institute of Health, Japan, and used as target cells. P-815 cell line was resistant to NK cells, whereas YAC-1 and RL♂-1 were sensitive to NK cells.

Target cells were prepared according to the method described previously [8, 14].

Preparation of anti Thy-1 serum: Golub's methods [3] was used to prepare anti-mouse Thy-1 serum. The cytotoxicity of this serum was tested by the method of Barker *et al.* [1]. This anti-mouse Thy-1 serum (1:4–1:32 dilution) killed more than 90% of mouse thymocytes and 30 to 40% of splenocytes.

Treatment of spleen cells with antibody (asialo GM1 and Thy-1) plus complement: Spleen cells prepared from TLA-sensitized or unsensitized mice were used as original cells. The suspension of original cells was divided into 3 groups—no treatment, treated with rabbit antiserum against asialo GM1 antigen (Wako Pure Chemi. Ind., Tokyo, Japan) and treated with anti-mouse Thy-1 serum, respectively. Original cells (3.0×10^7 cells/0.3 m*l*) were added to anti-mouse Thy-1 serum (1:4 or 1:8 dilution with 10% FCS-RPMI) and anti-asialo GM1 serum (1:60 dilution with 10% FCS-RPMI), and incubated with a 1:8 dilution of the low toxicity rabbit complement (Cedarlane Lab., Hornby, Ontario, Canada) at 37°C for 60 min. Spleen cells from TLA-sensitized or unsensitized mice were incubated with or without TLA for 6 days and used as effector cells.

The effector cells were treated with the same antibody plus complement as the original cells.

Treatment of effector cells with anti-Lyt-2.2 plus complement: The effector cells, incubated with TLA, were divided into two groups. The original cells (3.5×10^6 cells / 0.35 m*l*) were added to anti-Lyt-2.2 monoclonal antibody (1:60 dilution with 10%-FCS-RPMI) (Cedarlane Lab., Hornby, Ontario, Canada) and incubated with 0.35 m*l* of a 1:8 dilution of the low toxicity rabbit complement at 37°C for 60 min.

Two-color indirect immunofluoresence of effector cells: The smeared effector cells were washed with PBS, incubated with a 1:100 dilution of rabbit antiserum against asialo GM1 antigen at 37°C for 45 min and washed twice with PBS. For two-color indirect immunofluoresence, it was incubated with a 1:60 dilution tetramethyl rhodamine isothiocyanate conjugated goat anti-rabbit IgG (Immunotech, France) at 37°C for 30 min and washed twice with PBS. And then, it was incubated with a 1:10 dilution of rat anti-mouse T cell antibody (Biosys, France) at 37°C for 60 min and incubated with fluorescein-isothiocyanate conjugated goat anti-rat IgG (Kirkegaard & laboratories Inc., U.S.A.) at 37°C for 60 min. The stained smear was washed with PBS, and examined by using immunofluoroscopy.

RESULTS

Effect of dose of TLA in vitro against cytotoxic activity: Cytotoxic activity against P-815 target cell of spleen cells from TLA-sensitized mice incubated with medium containing 10, 30, 50, and 100 μg/m*l* TLA, increased significantly, compared with spleen cells from TLA-sensitized mice incubated with medium alone (TM) ($p<0.02$) (Fig. 1). Cytotoxic activity against RL♂-1 target cell of spleen cells from TLA-sensitized mice incubated with medium containing 30, 50, and 100 μg/m*l* TLA, increased significantly compared with TM ($p<0.02$).

The cytotoxic activity against P-815 target cell of spleen cells from TLA-sensitized mice incubated with 30, 50, and 100 μg/m*l* TLA, was significantly higher than that of spleen cells from unsensitized mice incubated with the same dose of TLA ($p<0.02$). The cytotoxic activity against RL♂-1 target cell of spleen cells from TLA-sensitized mice incubated with 50 and 100 μg/m*l* TLA, was significantly higher than that of spleen cells from unsensitized mice ($p<0.02$).

Effect of addition of TLA or rhIL-2 in in vitro culture on cytotoxic activity: Cytotoxic activity against P-815 of spleen cells from TLA-sensitized mice incubated with TLA (TT) or rhIL-2 (TI) was higher than that of TM ($p<0.002$) (Table 1). The

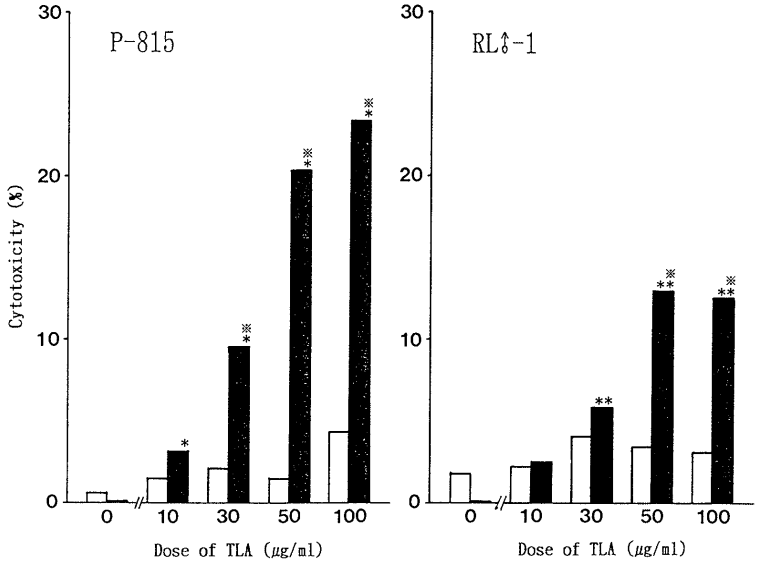

Fig. 1. Effect of dose of TLA *in vitro* against cytotoxic activity of effector cells. □: unsensitized mice; ■: TLA-sensitized mice. The ratio of effector to target cells (E/T ratio) was 25:1. Significant difference from TM, *, $p<0.02$; **, $p<0.005$. Significant difference from corresponding group of spleen cells from unsensitized mice incubated with the same dose of TLA, ※, $p<0.02$.

Table 1. Comparison of cytotoxic activities induced by TLA or rhIL-2 *in vitro*

Effector cells		Cytotoxicity (%)[1]	
Spleen cells	Incubated	Target cells	
from	with	P-815	YAC-1
Unsensitized control mice	Medium (CM)	2.0±0.4	1.6±0.4
	TLA (CT)	1.9±0.8	3.6±2.8
	rhIL-2 (CI)	13.6±2.1[a)]	10.2±2.3[a)]
TLA-sensitized mice	Medium (TM)	0.2±0.5	0.0±0.5
	TLA (TT)	20.7±0.9[c)]	19.8±0.8[b)]
	rhIL-2 (TI)	17.3±2.1[c)]	19.5±3.5[b)]

1) Cytotoxicity (%)=100×.

$$\frac{\text{Experimental release of } {}^{51}\text{Cr (cpm)} - \text{Nonspecific release of } {}^{51}\text{Cr(cpm)}}{\text{Total release of } {}^{51}\text{Cr(cpm)} - \text{Nonspecific release of } {}^{51}\text{Cr(cpm)}}$$

Data represent the mean±SE for triplicate cultures. E/T ratio was 25: 1.

a) Significantly different compared with CM at $p<0.05$.

b, c) Significantly different compared with TM at $p<0.01$, $p<0.002$, respectively.

cytotoxic activity against YAC-1 of TT or TI was higher than that of TM ($p<0.01$).

The cytotoxic activity of spleen cells from unsensitized mice incubated with rhIL-2 (CI) was higher than that of CM against P-815 and YAC-1 ($p<0.05$). The cytotoxic activity of TI was higher than that of CI, but was not statistically significant.

Effect of treatment of TLA-induced effector cells with anti-asialo GM1 or anti-Thy-1 plus complement on cytotoxic activity: Treatment of TT effector cells

Table 2. Treatments of TLA-induced effector cells with anti-asialo GM1 or anti-Thy-1 serum plus complement on cytotoxic activity

Effector cells		Treatment of effector cells	Cytotoxicity (%)[1]	
			Target cells	
In vivo sensitization	*In vitro* stimulation with TLA		P-815	YAC-1
No	No	No (CM)	5.3±0.8	2.7±0.3
	Yes	No (CT)	11.0±1.9	4.0±0.6
	Yes	Anti-asialoGM1+C[2]	9.4±0.8	2.8±1.1
	Yes	Anti-Thy-1+C	7.6±1.5	2.5±1.3
Yes	No	No (TM)	3.6±1.4	3.1±0.9
	Yes	No (TT)	21.8±1.2	9.3±0.4
	Yes	Anti-asialoGM1+C	16.4±0.3[a]	7.2±0.6[a]
	Yes	Anti-Thy-1+C	6.4±1.0[b]	4.6±0.4[b]

1) See footnote 1) in Table 1. E/T ratio was 25: 1.
2) Complement.
a, b) Significantly different compared with TT at $p<0.05$, $p<0.005$, respectively.

Table 3. Treatment of TLA-induced effector cells with anti-Lyt-2.2 serum plus complement on cytotoxic activity

Effector cells		Treatment of effector cells	Cytotoxicity (%)[1]	
			Target cells	
In vivo sensitization	*In vitro* stimulation with TLA		P-815	YAC-1
No	No	No (CM)	−2.6±0.3	−1.2±0.3
	Yes	No (CT)	−1.8±0.7	0.1±0.4
	Yes	Anti-Lyt-2. 2+C[2]	−1.6±0.2	2.8±0.1
Yes	No	No (TM)	−6.5±0.5	−1.3±0.2
	Yes	No (TT)	27.7±3.6	25.1±4.9
	Yes	Anti-Lyt-2. 2+C	19.1±3.5	26.9±2.6

1) See footnote 1) in Table 1. E/T ratio was 20: 1.
2) Complement.

with anti-asialo GM1 or anti-Thy-1 plus complement inhibited cytotoxic activity against P-815 compared with untreated TT ($p<0.05$ and $p<0.005$, respectively) (Table 2). The rates of inhibition of TT treated with anti-asialo GM1 and anti-Thy-1 plus complement were 24.8% and 70.6%, respectively. Treatment of TT with anti-asialo GM1 or anti-Thy-1 plus complement inhibited cytotoxic activity against YAC-1 compared with untreated TT, the rates of inhibition of TT treated with anti-asialo GM1 and anti-Thy-1 plus complement were 22.6% ($p<0.05$) and 50.5% ($p<0.005$) respectively.

Effect of treatment of TLA-induced effector cells with anti-Lyt-2.2 plus complement on cytotoxic activity: Treatment of TT effector cells with anti-Lyt-2.2 plus complement did not inhibit cytotoxic activity against both target cells compared with untreated TT (Table 3). Treatment of TT with anti-Lyt-2.2 plus complement showed slightly high inhibition of cytotoxic activity against P-815 compared with untreated TT, but this was not significant.

Effect of treatment of TLA-induced original cells with anti-asialo GM1 and/or anti-Thy-1 plus complement on cytotoxic activity of effector cells: Treatment of CT and TT original cells with anti-asialo GM1 and/or anti-Thy-1 plus complement inhibited cytotoxic activity of effector cells against P-815 compared with untreated CT and TT ($p<0.01$ and $p<0.001$, respectively) (Table 4). The rates of inhibition of TT original cells treated with anti-asialo GM1, anti-Thy-1 and both plus complement were 95.8%, 91.9%, 96.1%, respectively. Treatment of CT and TT original cells with anti-asialo GM1 and/or anti-Thy-1 plus complement inhibited cytotoxic activity of effector cells against YAC-1 compared

Table 4. Treatments of TLA-induced original cells with anti-asialo GM1 and/or anti-Thy-1 serum plus complement on cytotoxic activity[1)]

In vivo sensitization	Treatment of original cells	*In vitro* stimulation with TLA	Cytotoxicity (%)[1)] Target cells P-815 (%)	Target cells YAC-1 (%)
No	No	No (CM)	2.9±0.1	3.7±0.9
	No	Yes (CT)	7.4±0.4	7.2±0.8
	Anti-asialoGM1+C[2)]	Yes	1.4±0.2[a)]	1.2±0.4[a)]
	Anti-Thy-1+C	Yes	0.0±0.6[a)]	1.5±0.7[a)]
	Anti-asialoGM1+ Anti-Thy-1+C	Yes	0.7±0.3[a)]	0.0±0.7[a)]
Yes	No	No (TM)	4.9±0.2	6.1±0.3
	No	Yes (TT)	33.5±2.0	31.8±1.2
	Anti-asialoGM1+C	Yes	1.4±0.7[b)]	2.4±0.8[b)]
	Anti-Thy-1+C	Yes	2.7±0.2[b)]	3.0±0.3[b)]
	Anti-asialoGM1 + Anti-Thy-1+C	Yes	1.3±0.1[b)]	2.2±0.3[b)]

1) See footnote 1) in Table 1. E/T ratio was 20: 1.
2) Complement.
a) Significantly different compared with CT at $p<0.01$.
b) Significantly different compared with TT at $p<0.001$.

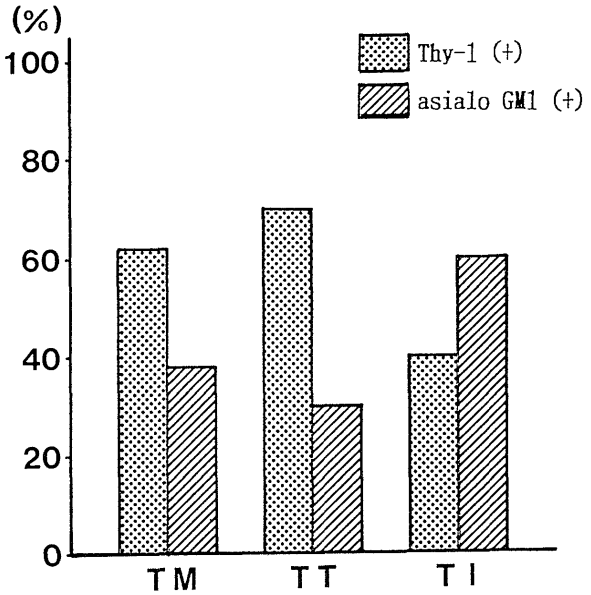

Fig. 2. Percentage of phenotypes of effector cells in TLA-sensitized mice. TM: spleen cells from TLA-sensitized mice incubated with medium alone; TT: spleen cells from TLA-sensitized mice incubated with TLA; TI: spleen cells from TLA-sensitized mice incubated with rhIL-2.

with untreated CT and TT ($p<0.01$ and $p<0.001$, respectively). The rates of inhibition of TT original cells treated with anti-asialo GM1, anti-Thy-1 and both plus complement were 92.5%, 90.6%, 93.1%, respectively.

Phenotypes of effector cells: Analysis of phenotypes of TT effector cells showed that 70% of cells were Thy-1 positive and 30% of cells were asialo GM1 positive (Fig. 2). But in TI, 40% were Thy-1 positive and 60% were asialo GM1 positive. TT had a greater number of Thy-1 positive cells than asialo GM1 positive cells, compared with TM. TI had a smaller number of Thy-1 positive cells than asialo GM1 positive cells, compared with TM. The ratio of Thy-1 positive cells to asialo GM1 positive cells appeared to increase after incubation with TLA and decrease after incubation with rhIL-2. In all groups, no effector cells were found to contain both antigens.

DISCUSSION

Spleen cells from TLA-sensitized mice incubated with TLA showed strong cytotoxic activity against NK-nonsensitive cells (P-815) and NK-sensitive cells (RL♂-1). The degree of cytotoxic activity increased with increasing dose of TLA *in vitro*. Spleen cells from untreated mice incubated with TLA did not show any cytotoxicity. Spleen cells from TLA-sensitized mice incubated with IL-2 showed strong cytotoxic activity against NK-nonsensitive cells (P-815) and NK-sensitive cells (YAC-1), compared with TM. Spleen cells from unsensitized mice incubated with IL-2 showed cytotoxic activity, compared with CM. Original cells may, therefore, be differentiated from killer cells and can be induced *in*

vivo by administration of TLA, and these original cells may need stimulation by TLA or IL-2 to obtain cytotoxicity.

Treatment of TLA-cultured spleen cells from TLA-sensitized mice (TT) with anti-asialo GM1 plus complement decreased cytotoxicity against NK-sensitive target cells slightly. It has been reported that antigen extracted from *Toxoplasma gondii* (Tp) was able to activate human NK cells *in vitro* [24], and Tp antigen or *Toxoplasma* infection was able to activate NK cells of spleen cells and peritoneal infiltrated cells in mice [6]. The number of NK cells increased in the spleen, liver and blood in TLA-sensitized mice on day 10 after *Babesia* infection [8]. Accordingly, it can be suggested that administration of TLA *in vivo* and *in vitro* is able to activate NK cells or NK-like cells.

Spleen cells from mice administered with TLA *in vivo* and *in vitro* also showed strong cytotoxicity against NK-nonsensitive cells. This cytotoxicity was inhibited by treatment with anti-Thy-1 and anti-asialo GM1 plus complement ($p<0.05$), but did not change upon treatment with anti-Lyt-2.2 plus complement. The ratio of Thy-1 positive cells to asialo GM1 positive cells in TLA-induced cytotoxic cells increase, but decreased in IL-2-induced lymphokine-activated killer cells, compared with TM. Furthermore, scattered large Thy-1 positive granular cells were observed in tumor tissue in TLA-treated rat [15]. It is suggested that TLA-induced cytotoxic cells contained not only NK or NK-like cells but also other killer cells, Thy-1 positive killer cells having strong cytotoxic activity against NK-nonsensitive cells. The lymphokine-activated killer (LAK) cells were induced by IL-2, exhibiting strong nonspecific cytotoxicity against a variety of tumor cells [4, 27]. LAK cells contain at least two cell types; one is assigned as asialo GM1 positive LAK of NK cell type and the other as Thy-1 rich, Lyt-2 positive, asialo GM1 negative LAK of T cell type [2, 12, 13, 16, 28]. A recent study showed that phenotypes of splenic LAK cells induce by *Nocardia rubra* cell wall skeleton and IL-2 were Thy-1.2 and asialo GM1 positive and Lyt-1.1 and Lyt-2.1 negative [10].

TLA can not induce cytotoxic cells from TLA-unsensitized spleen cells. IL-2, however, appears to induce LAK cells from TLA-unsensitized spleen cells. This finding may indicate different mechanism of induction of cytotoxic cells by TLA and IL-2.

It has been reported that precursor cells of IL-2-induced LAK were non-marker cells; asialo GM1 negative and Thy-1 negative [5, 17]. These precursor cells come from two cell types, Thy-1 positive and negative, and during differentiation of LAK cells, Thy-1 negative cells become Thy-1 positive cells [12, 29]. In this study, when original cells from TLA-induced cytotoxic cells were treated with anti-asialo GM1 and/or anti-Thy-1 plus complement, cytotoxic activity was inhibited if the original cells were incubated with TLA. Thy-1 positive cells and asialo GM1 positive cells increased in spleen in TLA-sensitized mice [8]. It can be suggested that: a) original cells of cytotoxic cells are induced by administration of TLA *in vivo*; b) original cells are not only asialo GM1 positive but also Thy-1 positive; c) stimulation with TLA causes original cells to differentiate into cytotoxic cells of two cell types, asialo GM1 positive and Thy-1 positive which are similar to IL-2-induced LAK cells.

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