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ANTITUMOR EFFECT OF INTRALESIONAL INJECTION WITH FORMALIN-FIXED *TOXOPLASMA GONDII* ORGANISMS ON LEWIS LUNG CARCINOMA IN *TOXOPLASMA*-INFECTED MICE

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SUMMARY

The antitumor effect of formalin-fixed *Toxoplasma* organisms (f-Tp) as an immunostimulant was examined in *Toxoplasma*-infected female C57BL/6 mice using a syngeneic Lewis lung carcinoma (3LL). *Toxoplasma*-infected mice, intradermally inoculated with the tumor cells mixed with 10^5, 10^6 or 10^7 f-Tp, developed a marked antitumor effect, inhibition of tumor growth and prolongation of life-span, in direct relation to the strength of the delayed-type hypersensitivity (DTH) reaction induced by f-Tp. The antitumor effect could also be observed even if an intralesional injection with f-Tp was performed 1, 3 or 5 days after the tumor inoculation. In a control, the injection with 2.5×10^6 live *Mycobacterium bovis* (BCG) in BCG-sensitized mice induced a significant antitumor effect, only when the BCG was injected in the mixture of tumor cells. These results demonstrate that the injection with f-Tp can induce a potent antitumor activity in mice with *Toxoplasma* infection.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite, and latent infections with *T. gondii* are present in humans in high percentages throughout the world [11]. It was reported that the development of both spontaneous mammary tumors and leukemia is suppressed in *Toxoplasma*-infected C3H/He and AKR mice, respectively [4]. The peritoneal macrophages are activated in infected mice, and the macrophages show cytostatic effect on various tumor cells in vitro [7]. In infected humans, tuberculin-type DTH reaction can be induced by an injection with *Toxoplasma* antigens

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[3]. These facts suggest the possibility that *Toxoplasma* antigens could be useful as a stimulant of the cellular immune responses in humans with *Toxoplasma* infection, and be available for cancer immunotherapy, like BCG [9,10] and its active components [5,16]. In addition, it may be possible that new immunostimulating molecules could be found in *Toxoplasma* organisms, since *T. gondii* is an eukaryote, not a prokaryote such as BCG. In the present study, the antitumor activity of f-Tp was examined in an animal model using *Toxoplasma*-infected C57BL/6 mice and a syngeneic tumor, 3LL.

MATERIALS AND METHODS

Materials

Inbred female C57BL/6 mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan), and were 2 months old at the beginning of the experiments. 3LL, a malignant metastasizing tumor, was maintained and transplanted intramuscularly every 10 days in syngeneic C57BL/6 mice. For the preparation of f-Tp, outbred ddY mice, obtained from the same breeder, were inoculated with the virulent RH strain of *T. gondii*, and 3 days later, tachyzoites were obtained from the peritoneal fluid of the mice. The harvested organisms were washed three times in phosphate-buffered saline (PBS), centrifuged at 700 × *g* for 5 min, and resuspended in 1% formalin in PBS. Before usage for experiments, the fixed organisms were washed by centrifugation and resuspended in Hanks' balanced solution (HBSS). BCG was obtained from Nippon BCG Seizo Ltd. (Tokyo, Japan).

Antitumor test

C57BL/6 mice were injected subcutaneously in the back with 2×10^3 bradyzoites of the avirulent Fukaya strain of *T. gondii* or 5×10^7 live BCG 2 weeks before 3LL inoculation. The bradyzoites of *T. gondii* were obtained from the brains of chronically infected C57BL/6 mice by treatment with 0.25% trypsin as described elsewhere [13]. 3LL cells were suspended in HBSS, and 2×10^5 cells in a volume of 0.02 ml were implanted intradermally in the footpads of the C57BL/6 mice infected with *T. gondii* or BCG. In testing for the antitumor effect of f-Tp, *Toxoplasma*-infected mice were injected intradermally with 10^5, 10^6 or 10^7 f-Tp at the site of the tumor inoculation with, or on various days after the tumor challenge. As a control, BCG-sensitized mice were injected with 2.5×10^6 live BCG in the same way as with f-Tp. Tumor sizes were recorded as the increase of footpad thickness and measured regularly at intervals of 5 days. Each experimental group was composed of 5 or 6 mice.

Assay of DTH reactions against f-Tp and BCG

Thickness of the hind feet was measured with a dial thickness gauge

(Peacock Model G, Ikeda Rika Ltd., Tokyo, Japan) just before, and 24 h after the injection with f-Tp and BCG. Increase in footpad thickness was calculated as the difference between the thickness before, and 24 h after injection.

RESULTS AND DISCUSSION

Toxoplasma-infected mice were injected in the footpad with 2×10^5 3LL cells with or without 10^5, 10^6 or 10^7 f-Tp. The infected mice developed a DTH reaction against f-Tp after the inoculation of a mixture of f-Tp and 3LL cells. The antitumor effect was observed in direct relation to the strength of the DTH in the infected mice (Table 1, Fig. 1). In mice injected with 10^7 f-Tp, in which the strongest DTH reaction was induced, growth of the implanted tumor did not occur, the tumor rejection ratio being 4/5. With a decrease in the dose of f-Tp, both the strength of the DTH reaction and the antitumor effect became weaker. In uninfected mice, no antitumor effect was induced by the f-Tp injection except one mouse rejected the tumor in the group of 10^7 injection, in which a non-specific immediate type inflammation occurred after injection with a mixture of tumor cells and f-Tp. These results indicate that the development of the

TABLE 1

ANTITUMOR EFFECT (LIFE-SPAN PROLONGATION) OF VARIOUS DOSES OF f-Tp ON 3LL

Toxoplasma infection[a]	Dose of f-Tp[b]	Footpad swelling (mm)[c]	Survival (days)[d]	Survival ratio[e]
-	0	0.02 ± 0.01	30.0 ± 1.9	0/5
-	10^5	0.02 ± 0.01	30.8 ± 1.5	0/5
-	10^6	0.03 ± 0.01	30.8 ± 0.4	0/5
-	10^7	0.42 ± 0.07	35.3 ± 5.7	1/5
+	0	0.03 ± 0.01	33.2 ± 1.6	0/5
+	10^5	0.17 ± 0.02	44.8 ± 3.9[f]	0/5
+	10^6	0.48 ± 0.03	50.3 ± 3.3[g]	1/5
+	10^7	0.94 ± 0.08	56	4/5

[a] Mice were injected subcutaneously with 2×10^3 bradyzoites of *T. gondii* 2 weeks before 3LL challenge.
[b] Mice were inoculated with 2×10^5 3LL cells into footpad with or without various doses of f-Tp.
[c] Swellings 24 h after tumor challenge, mean ± S.E. of 5 mice.
[d] Mean ± S.E. of the mice which died with growing tumors.
[e] On 90 days after tumor challenge.
[f] Significantly different from the control mice, uninfected and inoculated with 3LL only, at $P < 0.01$ by Student *t*-test.
[g] $P < 0.001$.

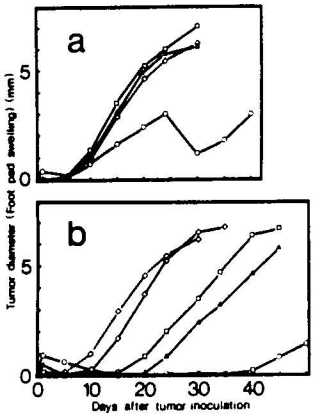

Fig. 1. Antitumor effect (tumor growth inhibition) of various doses of formalin-fixed *Toxoplasma* on 3LL. Mice were uninfected (a) or infected (b) with *T. gondii* 2 weeks before tumor inoculation. They were inoculated with 2×10^5 tumor cells with 10^5 (□), 10^6 (△), or 10^7 (○), or without (◇) f-Tp. The broken line shows the control tumor growth in normal mice injected with tumor cells only.

TABLE 2

ANTITUMOR EFFECT OF f-Tp ON 3LL AT INTRALESIONAL INJECTION WITH OR 1 DAY AFTER TUMOR CHALLENGE

Infected with[a]	Injected with f-Tp or BCG on day[b]	Footpad swelling (mm)[c]	Survival (days)[d]	Survival ratio[e]
—	—	0.02 ± 0.01	29.0 ± 1.1	0/6
—	0 (f-Tp)	0.34 ± 0.03	31.0 ± 0.7	1/5
—	1 (f-Tp)	0.21 ± 0.05	28.0 ± 1.1	0/5
Tp	0 (f-Tp)	0.91 ± 0.06		5/5
Tp	1 (f-Tp)	0.67 ± 0.09	42.0 ± 1.1[f]	2/6
—	0 (BCG)	0.46 ± 0.06	31.4 ± 2.1	0/5
—	1 (BCG)	0.41 ± 0.06	29.6 ± 3.1	0/5
BCG	0 (BCG)	1.14 ± 0.12	47	4/5
BCG	1 (BCG)	0.94 ± 0.11	34.4 ± 4.3	0/5

[a]Mice were injected subcutaneously with 2×10^3 bradyzoites of *T. gondii* or 5×10^7 live BCG 2 weeks before 3LL challenge.
[b]Mice were inoculated with 2×10^5 3LL cells into footpad on day 0. They received intralesional injection with 10^7 f-Tp or 2.5×10^6 live BCG on day 0 or day 1.
[c]Swellings 24 h after the injection with f-Tp or BCG, mean ± S.E. of 5 or 6 mice. In a control group inoculated with 3LL only, the swelling 24 h after tumor challenge is indicated.
[d]Mean ± S.E. of the mice which died with growing tumors.
[e]On 90 days after tumor challenge.
[f]Significantly different from the control mice, uninfected and inoculated with 3LL only, at $P < 0.001$.

TABLE 3

ANTITUMOR EFFECT OF f-Tp ON 3LL AT INTRALESIONAL INJECTION ON VARIOUS DAYS AFTER TUMOR CHALLENGE IN *TOXOPLASMA*-INFECTED MICE

Infected with[a]	Injected with f-Tp or BCG on day[b]	Tumor diameter on day 20 (mm)[c]	Survival (days)[d]
—	—	7.59 ± 0.12	25.2 ± 1.6
Tp	1 (f-Tp)	2.11 ± 0.44[e]	37.6 ± 2.4[f]
Tp	3 (f-Tp)	4.61 ± 0.35[e]	32.2 ± 1.3[g]
Tp	5 (f-Tp)	4.83 ± 0.38[e]	31.2 ± 1.8[g]
BCG	1 (BCG)	6.68 ± 0.67	28.8 ± 2.9
BCG	3 (BCG)	6.99 ± 0.51	30.8 ± 1.8
BCG	5 (BCG)	7.85 ± 0.65	27.6 ± 2.1

[a] Mice were injected subcutaneously with 2×10^3 bradyzoites of *T. gondii* or 5×10^7 live BCG 2 weeks before tumor challenge.
[b] Mice were inoculated with 2×10^5 3LL cells into footpad on day 0. They received intralesional injection with 10^7 f-Tp or 2.5×10^6 live BCG 1, 3 or 5 days after tumor challenge.
[c] Mean ± S.E. of 5 or 6 mice.
[d] All mice died with growing tumors.
[e] Significantly different from the control mice, uninfected and inoculated with 3LL only, at $P < 0.001$.
[f] $P < 0.01$.
[g] $P < 0.05$.

DTH reaction to f-Tp at the site of tumor inoculation induces a marked antitumor activity on 3LL.

It was reported that antitumor effects were developed by an induction of DTH reactions against various stimulants at the site of tumors; i.e. BCG [1,18], *Corynebacterium parvum* [14], *Listeria monocytogenes* [17] and 2,4-dinitrochlorobenzene [6]. An accumulation of cells at the site of the DTH against f-Tp occurs with a specificity for *Toxoplasma* antigens. However, it is readily conceivable that the accumulated cells have no specificity, and attack foreign substances for the host, including tumor cells. In a histological study, a large number of macrophage-like cells were found in the site of the DTH reaction against f-Tp (data not shown). Krahenbuhl and Remington reported that peritoneal macrophages are activated in *Toxoplasma*-infected mice during the course of infection, and the cells have cytostatic activity on various tumor cells in vitro [7]. Therefore, it is supposed that the effector cells of antitumor activity in the DTH reaction against f-Tp are activated macrophages, like in the cases of BCG [13] and *C. parvum* [14], since the marcophages usually do not have specificity in attacking foreign substances except in collaboration with specific antibodies [15]. A participation of polymorphonuclear leukocytes in the anti-

tumor activity induced by f-Tp is also conceivable. It is known that the leukocytes have antitumor activity [2], and they were also observed in the site of the DTH reaction against f-Tp (data not shown).

Next, we examined whether an antitumor effect could be induced in the infected mice even if an intralesional injection with 10^7 f-Tp was performed on various days after the inoculation with 3LL. As a control, BCG-sensitized mice were injected with 2.5×10^6 live BCG in the same manner as with f-Tp. As shown in Table 2, when *Toxoplasma*-infected mice were injected with f-Tp 1 day after the tumor inoculation, some mice completely rejected the growth of the implanted tumor; the tumor rejection ratio being 2/6. In uninfected mice, no antitumor effect was induced by the intralesional f-Tp injection 1 day after the tumor challenge. This indicates that the development of DTH reaction to f-Tp at the site of the tumor can cause antitumor activity on 3LL, even if the DTH reaction is induced after the tumor challenge. Both a significant inhibition of tumor growth and prolongation of life-span were observed even when the f-Tp injection was performed 3 days, or 5 days after the tumor inoculation in *Toxoplasma*-infected mice (Table 3).

By contrast, in the BCG groups, a significant antitumor effect was shown only when BCG-sensitized mice received an inoculation of BCG mixed with tumor cells (Tables 2 and 3). Antitumor activity was induced only in BCG-sensitized mice, but not in the unsensitized mice. This suggests that the development of the DTH reaction against BCG causes the antitumor effect. However, when an injection with BCG was performed 1 day after the tumor challenge, no antitumor effect was observed in the BCG-sensitized mice, although BCG-specific DTH occurred in the mice (Table 2). The intralesional injection with BCG on either the third or fifth day after the tumor inoculation also induced no antitumor effect (Table 3).

Lagrange and Thickstun also reported that an intralesional injection with live BCG on various days after tumor inoculation was much less effective than the injection in a mixture of tumor cells [8]. Reasons for the difference between the antitumor effects of the DTH reactions against f-Tp and BCG are not clear. However, it may be possible that a population or activity of the accumulated cells, or the duration of the active immune responses are different between DTH reactions to f-Tp and BCG in the infected mice, respectively. This possibility is supported by the findings of the previous workers [8] that the local antitumor effect of DTH reactions on 3LL was different between the stimulants of the DTH, even though similar footpad swellings were observed in the responses. A large difference in the structure of the surface membranes between *T. gondii*, an eukaryote, and BCG, a prokaryote, will also support the possibility of different activity of the accumulated cells, because the surface membrane is the major component to stimulate immune responses.

The present study demonstrated that a potent antitumor effect was induced by injection with f-Tp in *Toxoplasma*-infected mice. As mentioned

previously, a latent infection with *T. gondii* is present among humans in high percentages throughout the world [11]. These facts suggest a possibility that injections with f-Tp might be applied in cancer immunotherapy in humans with *Toxoplasma* infection. The effect of f-Tp on tumors other than 3LL is now under study in animal models.

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