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ATTENUATION OF THE VIRULENT RH STRAIN OF *TOXOPLASMA GONDII* BY PASSAGES IN MICE IMMUNIZED WITH *TOXOPLASMA* LYSATE ANTIGENS

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SUMMARY The virulent RH strain of *Toxoplasma gondii* was attenuated after a few passages or just one long passage in mice immunized twice with a four-week interval between immunizations with an emulsion of *Toxoplasma* lysate antigens and complete Freund's adjuvant. Three avirulent strains, RH-cyst III, IV and VIII were established from the RH strain. The RH-cyst III strain was effective for vaccination against challenge with the original, virulent RH strain. The attenuation of *T. gondii* is an expression of the innate attributes of this parasite necesary to maintain its parasitic life cycle in nature.

INTRODUCTION

High virulence of *T. gondii* can be induced by rapid passages in mice (Jacobs, 1953, Jacobs and Melton, 1954), as in the case of the RH strain, whose virulence was established by three passages in mice (Sabin, 1941). The highly virulent RH strain, which is maintained in laboratories by inoculation into mice twice a week, is too virulent to be successful in nature as a parasite and is also unsuitable for demonstrating resistance of mice immunized with killed *Toxoplasma* (Krahenbuhl et al., 1972). Cats infected with tachyzoites discharge fewer oocysts and only after a longer time than those infected with low virulent *Toxoplasma* cysts (Dubey and Frenkel, 1976). Thus, highly virulent *Toxoplasma* tachyzoites can be maintained in laboratories but not in nature. If a parasite is to be successful in nature, it must not increase its pathogenicity for a given host; avirulence is a necessary attribute (Lainson, 1955). Virulent strains of *T. gondii* must possess an innate ability to decrease virulence to produce cyst forms.

Attempts to decrease the virulence of *T. gondii* have not been successful (MacFarlane and Ruchman, 1948; Jacobs and Melton 1954; Chang and Gabrielson, 1984). *Toxoplasma* passaged 53 times in mice immunized with *Toxoplasma* lysate antigens without complete Freund's adjuvant (CFA) showed a decreased growth rate when inoculated into immune mice or even normal mice (Yano et al., in press). About half the mice immunized with *Toxoplasma* lysate antigens and CFA twice with a 4-week interval between immunizations were protected against challenge with 1×10^3 tachyzoites of the RH

strain of *T. gondii*. Cyst forms were observed in the brain of surviving mice (Yano and Nakabayashi, 1984). Mice immunized with *Toxoplasma* lysate antigens and CFA showed the highest resistance to challenge with the RH strain except those vaccinated with avirulent strains of *T. gondii*. In this study, we tried to attenuate the virulent RH strain of *T. gondii* by its passage in mice immunized with an emulsion of *Toxoplasma* lysate antigens and CFA.

MATERIALS AND METHODS

Toxoplasma strains and mice

The virulent RH strain (Sabin, 1941) and the avirulent S-273 strain (Nakayama, 1967) of *T. gondii*, which have been maintained in this laboratory by serial passage in mice, were used. Female albino (ddY) mice of 5–6 weeks old, purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, were used for experiments.

Preparation of *Toxoplasma* lysate antigens and immunization

Mice were inoculated intraperitoneally(i.p.) with 5×10^5 tachyzoites of the virulent RH strain of *T. gondii*. Peritoneal exudates (PE) were collected four days later by paracentesis with 2 ml of Alsever's solution and then used as *Toxoplasma* lysate antigens (ToxoPE) after killing the tachyzoites present by freeze-thawing twice.

Mice were immunized i.p. with 0.4 ml of an emulsion of ToxoPE and complete Freund's adjuvant (CFA) (DIFCO, USA) twice with a 4-week interval between immunizations (Yano and Nakabayashi, 1984).

Attenuation of the virulent RH strain of *T. gondii*

The immune mice were challenged i.p. with 1×10^3 tachyzoites of *T. gondii* one week after the second immunization. Small doses of *Toxoplasma* cysts in brain homogenates of some surviving mice were passaged further in immune mice and normal mice to check the virulence. All passages were for more than 4 weeks. Three of eight attempts to attenuate the RH strain were successful and the resultant attenuated strains were named RH-cyst III, IV and VIII.

Comparison of the biological characteristics of the RH-cyst III strain and the avirluent S-273 strain.

Mice were infected with 4 cysts of the RH-cyst III (6th passage) or the S-273 strain and the numbers of cysts per brain and indirect latex agglutination (ILA) titers were examined.

The effect of vaccination with the RH-cyst III strain against the original virulent RH tachyzoites was examined. Mice vaccinated with 4 cysts of the RH-cyst III (6th passage) or the S-273 strain 4, 6 and 8 weeks previously, were challenged with 1×10^3, 1×10^6, and 1×10^7 tachyzoites of the RH strain. The survival of mice during the next 8 weeks was determined.

Enumeration of cysts in mice and titration of *Toxoplasma* antibodies

The brain containing the olfactory lobe, cerebrum and cerebellum was removed from a mouse and emulsified with Alsever's solution in a total volume of 1.5 ml in a 2.5 ml disposable syringe by passing it three times each through a 23G needle and then a 27G needle. Cysts found in 50 μl of the brain homogenate were counted under a Normarski's interference microscope and the number was multiplied by 30 to calculate the total number of cysts per mouse brain.

An indirect latex agglutination (ILA) test (Toxo test MT, Eiken Co., Tokyo) was used for assay of *Toxoplasma* antibodies as described by Kobayashi et al. (1977). Titers were expressed as $\log_2$ values of the reciprocal of the serum dilution.

RESULTS

Attenuation of the virulent RH strain of T. gondii

Cyst forms were observed in brain homogenates of some surviving mice challenged with 1×10^3 tachyzoites of the RH strain: 120 cysts per brain were found in a RH-cyst III mouse and 60 in a RH-cyst IV mouse. Inoculation of one RH-cyst III cyst (2nd passage) resulted in death of all three normal mice tested on day 9 and of one of four immune mice (Table 1). Three of four control mice died 10 days after 2nd inoculation of two RH-cyst IV cysts. Four cysts per mouse from a mouse at the second passage of RH-

TABLE 1. *Conditions for attenuation of the RH-cyst III, IV, and VIII strains.*

Passage	Infection dose per mouse	Duration (weeks)	Mortality (dead/total)	
			Immune mice	Control mice
RH-cyst III				
1st	1000 tachyzoites	8	5/10(14.4)[a]	10/10(8.0)
2nd	1 cyst	5	1/4 (21.0)	3/3 (9.0)
	10 cysts		4/4 (13.5)	n.d.
3rd	1 cyst	4	0/3	0/2
	4 cysts		0/2	0/2
4th	6 cysts	6	n.d.[b]	1/10(13.0)
	80 cysts		n.d.	1/2 (41.0)
5th	10 cysts	14	n.d.	0/8
	100 cysts		n.d.	0/2
6th	4 cysts	4	n.d.	0/90
7th	4 cysts	26	n.d.	0/3
8th	5 cysts	20	n.d.	0/12
	100 cysts		n.d.	1/8 (13.0)
RH-cyst IV				
1st	1000 tachyzoites	8	1/2 (12.0)	2/2 (7.5)
2nd	2 cysts	10	0/4	3/4 (10.0)
3rd	4 cysts	8	0/3	3/3 (16.3)
4th	4 cysts	25	0/2	0/6
	20 cysts		0/2	1/2 (11.0)
5th	5 cysts	16	n.d.	0/7
	100 cysts		n.d.	1/8 (12.0)
RH-cyst VIII				
1st	1000 tachyzoites	26	4/8 (14.5)	10/10(8.1)
2nd	0.4 m*l* of brain[c]	6	n.d.	0/2
3rd	8 cysts	10	n.d.	1/4 (12.0)
	80 cysts		n.d.	1/2 (9.0)
4th	5 cysts	20	n.d.	0/7
	100 cysts		n.d.	0/8

[a] Mean survival days of mice that died.
[b] Not determined.
[c] 0.4 m*l* of 1.5 m*l* of brain homogenate.

cyst III cysts or the 3rd passage of RH-cyst IV cysts were inoculated i.p. into control mice but did not cause death. Cysts in a surviving mouse (RH-cyst VIII) after inoculation of 1×10^3 tachyzoites were under patency after six months. Two control mice that survived against challenge with 0.4 ml of brain homogenate of a RH-cyst VIII mouse i.p. showed 60 and 240 cysts, respectively. No appreciable deaths of normal mice occured after i.p. inoculation of 100 cysts of the 5th passage of the RH-cyst III and IV strains, or the 4th passage of the RH-cyst VIII strain. The RH-cyst III, IV and VIII strains could be attenuated by passage in immune mice.

Cyst productions, 923 ± 312 cysts per brain

TABLE 2. *Comarison of numbers of cysts per brain found in mice at 3rd, 4th, and 6th passage of the RH-cyst III strain.*

Passage	Infection time	Infection dose per mouse	Number of cysts per brain	ILA titers
3rd	4 week	4 cysts	60	6
			180	5
	4	1	0[a]	4
			150	5
			98±83	5.0±0.8
4th	6	6	690	13
			960	12
			1350	12
			690	11
			923±312	12.0±0.8
6th	4	4	600	8
			660	7
			570	9
			900	8
			658±259	8.0±0.8
6th	6	4	1410	9
			1890	9
			1590	9
			870	10
			1440±428	9.3±0.5

[a] Cysts were recognized by inoculation.

TABLE 3. *Comparison of survivals of mice vaccinated with 4 cysts of the RH-cyst III strain or S-273 strain after intraperitoneal challenge of 1×10^3, 1×10^6, and 1×10^7 tachyzoites per mouse of the RH strain over a period of 8 week.*

Vaccination	Weeks[a]	Inoculation dose per mouse		
		1×10^3	1×10^6	1×10^7
Control	—	0/10[b] (8.1)[c]	0/10 (5.8)	0/10 (4.9)
RH-cyst III	4	9/10 (33.5)	9/10 (10.0)	5/6 (48.0)
	6	n.d.[d]	6/10 (33.5)	3/5 (9.0)
	8	8/10 (37.5)	7/10 (21.3)	n.d.
S-273	4	8/10 (32.0)	7/10 (15.3)	3/6 (18.3)
	6	n.d.	7/10 (8.3)	3/6 (9.0)
	8	6/10 (29.3)	8/10 (30.0)	n.d.

[a] Weeks after vaccination of 4 cysts.
[b] Number of mice surviving / number of mice challenged.
[c] Mean survival days of mice that died.
[d] Not determined.

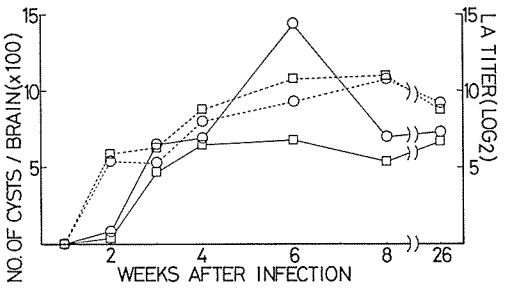

FIGURE 1. Comparison of cyst productivities and ILA titers in mice inoculated with 4 cysts of the RH-cyst III strain and the S-273 strain. Numbers of cysts per mouse brain are shown by continuous lines, and ILA titers by dotted lines. Symbols: ○, RH-cyst III; □, S-273. Values are means for four mice (SD are not included).

at the 4th passage and 658±259 or 1440±428 cysts at the 6th passage of the RH-cyst III strain were much greater than those 98±83 cysts at the 3rd passage (Table 2), 30 cysts at the 2nd passage, and 120 cysts at the 1st passage.

Comparison of the biological characteristics of the RH-cyst III strain and avirulent S-273 strain

Cyst production and the ILA titer of mice infected with 4 cysts of the RH-cyst III strain (6th passage) were compared with those of mice infected with 4 cysts of the avirulent S-273 strain of *T. gondii* (Fig. 1). Mice infected with the RH-cyst III strain showed higher cyst production and lower ILA titers than mice infected with the S-273 strain, but the differences were not significant ($p>0.1$: Cyst production in week 6).

The effect of vaccination with the RH-cyst III strain against challenge with tachyzoites of the original virulent RH strain of *T. gondii* was studied (Table 3).

Totals of 73% of the groups of mice vaccinated with the RH-cyst III and S-273 strain, respectively, survived challenge with 1×10^6 tachyzoites of the RH strain. Eight of 11 mice vaccinated with the RH-cyst III strain were even protected against challenge with 1×10^7 tachyzoites, whereas 6 of 12 mice vaccinated with the S-273 strain were protected. The mean survival days of fatal mice vaccinated with the RH-cyst III strain that died were 35.5 days after challenge with 1×10^3 tachyzoites, 26.0 days after challenge with 1×10^6 tachyzoites, and 22.0 days after challenge with 1×10^7 tachyzoites, while the respective values for mice that died after vaccination with the S-273 strain were 30.2, 16.4, and 13.7 days. Thus vaccincation with the RH-cyst III strain was more effective than that with the S-273 strain for protection against challenge with the virulent RH strain. Variation in the time for protection (4, 6 and 8 weeks previously) had no significant effect on survival after challenge.

DISCUSSION

The virulent RH strain of *T. gondii* was attenuated after a few passages or just one long passage in mice immunized twice with a four-week interval between immunizations with an emulsion of ToxoPE and CFA. Three points were important for achievement of attenuation: 1) use of CFA for immunization, 2) a passage duration of more than 4 weeks, and 3) a small inoculation dose in early passages. After attenuation, the RH-cyst III, IV and VIII strains were as stable as the avirulent S-273 strain of *T. gondii*. In mouse embryo cell cultures, the RH-cyst III strain forms large cyst-like bodies (more than 20 μ diameter) continuously for a long time, while these cyst-like bodies are not seen in cell cultures infected with RH strain (Omata et al., in preparation).

Attenuation of the RH strain by passage in immune mice is a similar phenomenon to loss of virulence of the RH strain after 16 week or longer in most mice vaccinated with the Beverley strain (Reikvan and Lorentzen-Styr, 1976) or the 273 strain (Yano et al., in press).

The attenuation of *Toxoplasma* can prob-

ably be explained by growth modification (Yano et al., in press). This growth modification may occur during long passages in mice immunized with ToxoPE and CFA, in which the whole immunological framework of the mice is highly activated against *Toxoplasma* by infection of the RH strain, as shown by increase in the ILA titers.

At the 4th and 6th passage in mice, the RH-cyst III strain was sufficiently attenuated, and produced many more cysts than at early passages, when it was virulent (Table 2). At the 6th passage in mice, the RH-cyst III strain produced more cysts in mice than the aviruelnt S-273 strain (Fig. 1). More cysts are produced in the brains of mice infected with *Toxoplasma* strains of lower virulence. Thus we conclude that the RH-cyst III strain does not have lower virulence than the S-273 strain. The ability of the RH strain to form cysts was recognized in vivo as well as in vitro (Shimada et al., 1974). The mean survival times of mice that died after vaccination with the RH-cyst III strain were longer than those of mice that died after vaccination with the S-273 strain (Table 3).

It is noteworthy that mice vaccinated with the RH-cyst III strain did not show complete resistance to challenge with the original virulent RH strain. This phenomenon could probably be explained by the presence of a specific antigen in tachyzoites (Lunde and Jacobs, 1983). Probably, the slower growth also decreased the antigenic stimulation of mice.

Mice chronically infected with an avirulent *Toxoplasma* such as the RH-cyst III strain, have disturbed mental conditions (Piekarski, 1980) making it easier for cats to catch them. *Toxoplasma* cysts are more differentiated facultative forms than *Toxoplasma* tachyzoites (Dubey and Frenkel, 1976). Hence, cats infected with the avirulent RH-cyst III strain could discharge many more oocysts and survive as a source of infection. The attenuation of the virulent RH strain by a few passages in mice immunized with Toxo-PE and CFA is an expression of the innate attributes of this parasite necessry for maintenance of its parasitic life cycle in nature.

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REFERENCES

Chang, G., Gabrielson, D. A. 1984. *Toxoplasma gondii:* Growth in ovine fetal kidney cell cultures. Exp. Parasitol. 57: 81–85.

Dubey, J. P., Frenkel, J. K. 1976. Feline toxoplasmosis from acutely infected mice and the development of *Toxoplasma* cysts. J. Protozool. 23: 537–546.

Jacobs, L. 1953. The biology of toxoplasma. Am. J. Trop. Med. Hyg. 2: 365–389.

Jacobs, L., Melton, M. L. 1954. Modification in virulence of a strain of *Toxoplasma gondii* by passage in various hosts. Am. J. Trop. Med. Hyg. 3: 447–457.

Kobayashi, A., Hirai, N., Suzuki, Y., Nishikawa, H., Watanabe, N. 1977. Evaluation of a commercial *Toxoplasma* latex agglutination test. Jpn. J. Parasitol. 26: 175–180.

Krahenbuhl, J. L., Ruskin, J., Remington, J. S. 1972. The use of killed vaccines in immunization against an intracellular parasite: *Toxoplasma gondii*. J. Immunol. 108: 425–431.

Lainson, R. 1955. Toxoplasmosis in England II. Variation factors in the pathogenesis of *Toxoplasma* infections: The sudden increase in virulence of a strain after passage in multimammate rats and canaries. Ann. Trop. Med. Parasitol. 49: 397–416.

Lunde, M. N., Jacobs, L. 1983. Antigenic differences between endozoites and cystozoites of *Toxoplasma gondii*. J. Parasitol. 69: 806–808.

MacFarlane, J. O., Ruchman, I. 1948. Cultivation of *Toxoplasma* in the developing chick em-

bryo. Proc. Soc. Exp. Biol. Med. 67: 1–4.
Nakayama, I. 1967. A method of detection of *Toxoplasma* infection in man. Jpn. J. Parasitol. 16: 381–388.
Omata, Y., Nishimoto, N., Ramos, M.I.A., Yano, K., Nakabayashi, T. Virulence of *Toxoplasma gondii* (RH-cyst III strain) cultured in mouse embryo cells. In preparation.
Piekarski, G. 1980. Behavior alteration caused by parasitic infection. Proceedings of the 3rd Japanese-German Cooperation Symposium on Protozoan Diseases. P. 40.
Reikvan, A., Lorentzen-Styr, A. M. 1976. Virulence of different strains of *Toxoplasma gondii* and host response. Nature 261: 508–509.
Sabin, A. B. 1941. Toxoplasmic encephalitis in children. J. Am. Med. Assoc. 116: 801–807.
Shimada, K., O'Connor, R. G., Yoneda, C. 1974. Cyst formation by *Toxoplasma gondii* (RH strain) in vitro. Arch. Ophthalmol. 92: 496–500.
Yano, K., Nakabayashi, T. 1984. Delayed secondary immune response in mice immunized with *Toxoplasma* antigens and its effect for protection against *Toxoplasma gondii*. Zentralbl. Bacteriol. [Orig. A] 256: 381–389.
Yano, K., Nakabayashi, T., Inoki, S. 1986. Modification of growth rate of *Toxoplasma gondii* by continuous passage in immune mice. Zentralbl. Bacteriol [Orig. A] In press.