EXPERIMENTS ON MOUSE PASSAGE OF THE CYST TYPE STRAIN OF *TOXOPLASMA GONDII* FOR ENHANCEMENT OF VIRULENCE

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It is well known that there is a wide range of variation in the virulence of *Toxoplasma gondii*⁴⁾. Some strain of this organism always cause severe fatal infections in mice since the first generation of their isolation, and maintain their virulence for a long time. Other strains indicate a relatively low virulence at first and then a high virulence after several generations of mouse passage⁵⁾ (an RH type strain).

On the contrary, there are some strains which maintain the characteristics of the low virulent strain; that is, a virulence too low to kill a mouse in spite of repeated inoculation^{3,7)} (a cyst type strain). Moreover, it has sometimes been said that there are many strains which disappear from tissues and organs before they show adaptation to mice. It is not clearly understood, however, whether the RH type strain and the cyst type strain are convertible to each other or absolutely independent from each other.

Accordingly, a clarification of these points may give the answer to a problem on the significance of the cyst type strains which are sometimes detected from the brain or muscle of apparently healthy animals.

Under these circumstances, the authors performed experiments on the cyst type strain²⁾ which had been isolated from healthy swine and retained its virulence in spite of repeated mouse passages.

MATERIALS AND METHODS

Strains used: The S-273²⁾ and the Beverley strain were used. The former had been isolated from a clinically healthy pig having given a positive skin test. Both strains were maintained at this laboratory by the intracerebral inoculation of mice.

For the first injection of the series, the mouse brain which contained many cysts was employed. It was ground, emulsified with physiological saline, and filtered through a coarse net. The resulting fluid which contained many *Toxoplasma* cysts, was inoculated into mice by the peritoneal route. After that, serial passages were performed by using the peritoneal washings collected on the fourth day after inoculation. In each case, 2.5 mg of cortisone acetate was administered intramuscularly at the time of inoculation.

Enhancement of the virulence was estimated from the time interval between inoculation and death and the multiplication of parasites in the peritoneal washings at the time of passage. Sometimes, the invasiveness and multiplication of the strains were examined by the tissue culture method with HeLa, FL, and MS cells.

EXPERIMENTS

1. Death of mice by the passage

A total of 4 experiments (A, B, C, and D) with the S-273 strain and 2 experiments (E and F) with the Beverley strain were carried out using 2 mice for each generation of passage, as indicated in Table 1.

Table 1. Increase in Virulence of *Toxoplasma gondii* by Serial Passage in Mice

Strain	Experimental group	No. of mouse passages														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
S-273	A	○	○	○	○	○	○	○	●	●	●	●	●	●	●	●
		○	○	○	○	○	○	○	●	●	●	●	●	●	●	●
	B	○	○	○	○	○	○	○	○	○	○					
		○	○	○	○	○	○	○	○	○	○					
	C*	○	●	△	○	○	○	○	○	○	○					
		○	●	●	○	○	○	○	○	○	○					
	D	△	●	○	○	○	○	○	○	○	○					
		●	●	○	○	○	○	○	○	○	○					
Beverley	E	●	●	●	●	●	●	●	●	●	●					
		●	●	●	●	●	●	●	●	●	●					
	F*	●	●	●	○	○	○	○	○	○	○					
		●	●	●	○	○	○	○	○	○	○					

Remarks: One ml of peritoneal washings was subinoculated by the intraperitoneal route at the same time with intramuscular injection with 2.5 mg of cortisone acetate.

* Administration of cortisone acetate was omitted.

● Death of mouse by toxoplasmosis. △ Death of mouse by other causes.

○ Toxoplasma cysts were detected from mouse surviving.

In experiment A, a heavy increase was observed in virulence after the lapse of the 7th generation of passage, and afterward death always occurred (the resultant strain being hereafter referred to as the modified S-273 strain). In the other experiments, the organisms disappeared from tissues and organs after 2 or 3 mouse passages, except in experiment B in which the presence of the organisms was not examined.

On the contrary, the Beverley strain cause death among the inoculated mice since the first passage and maintained this characteristic virulence for a long time (the resultant strain being hereafter referred to as the modified Beverley strain). In experiment F, however, the organisms disappeared from the mouse body after 3 blind passages, although cortisone acetate was not administered in this case.

2. Increase in virulence after mouse adaptation

As indicated in Table 2, the mean number of survival day of the mice inoculated with the modified S-273 strain was 6.6 and 5.2 when the strain had passed the 8~16th and the 64th generation of passage in mice, respectively. Eventually, all the mice tested succumbed, without exception.

With respect to the modified Beverley strain, enhancement was also observed in its virulence following the mouse adaptation. During the first to 5th generation of mouse passage, the number of survival days fluctuated from 5 to 24, and some of the mice inoculated did not die. The mean survival days was estimated to be 7.7 for the first 10 generation. Afterward, it was shortened to 5.0, on the average, after 40 serial passages had been accomplished in mice.

Intramuscular injection of cortisone acetate exerted no marked effect on the length

Table 2. Shortening of Survival Days by Serial Passage

1) S-273 Strain

No. of passages	1~7	8~16	17~26	27~33	34~43 (1~10)*	44~53 (11~20)*	54~63 (21~30)*	64~73 (31~40)*
Survived	→	6.6 →	6.3 →	6.0 →	6.1 →	5.7 →	5.7 →	5.2 →
Mean survival days		(5~8)	(5~8)	(5~11)	(5~10)	(5~8)	(4~10)	(3~8)
			(1~9)*	(10~13)*				
			→	6.4 →	5.5			

2) Beverley Strain

No. of passages	1~5	6~10	11~20 (1~10)*	21~30 (11~20)*	31~40 (21~30)*	41~50 (31~40)*
Mean survival days	9.7 →	5.7 →	6.7 →	5.4 →	5.6 →	5.0 →
	(5~24)	(5~7)	(6~8)	(4~7)	(4~8)	(4~7)

()* No. of mouse passages without injection with cortisone acetate.

() Fluctuation of survival days of mice.

→ Serial passage with administration of cortisone acetate.

----- Serial passage without administration of cortisone acetate.

of survival after the establishment of an increased virulence.

3. Multiplication of organisms in mice

Multiplication of the parasites in the mouse body was estimated from the number of organisms in the peritoneal washings harvested on the 4th day of passage.

In the case of the S-273 strain, no countable organisms were detected at first by the hemocytometer. Accordingly, serial blind passages were carried out for a while. Afterwards, the proliferative form of the organism appeared at the rate of tens of thousands per ml. Then it increased gradually in number with the progress in mouse passage. However, especially in the early stage of passage, the multiplication of the organisms decreased temporarily, when the administration of cortisone acetate was

Fig. 1. Multiplication of Organisms in Mice

* Number of organisms in peritoneal washings on the 4th day after inoculation.

—○— Mouse group treated with cortisone acetate.

- - -○- - - Mouse group not treated with cortisone acetate.

omitted, as indicated in Fig. 1.

In the case of the Beverley strain, the organisms were demonstrated since the first mouse passage, showing the same tendency as those of the S-273 strain.

Now, by serial passages through mice these modified strains have reached the 150th or more advanced generation. The multiplication, however, became slow among the organisms which contained in the peritoneal washings were generally at the rate of less than five millions per ml, together with many body cells.

4. Invasion by and multiplication of the modified strains

Invasiveness into cells was compared between the modified S-273 strain and the RH strain. The results are shown in Table 3. From the table, it will be seen that the former indicated as good invasiveness as the latter, there being no significant difference between the two strains.

Prior to this experiment, the authors examined the modified S-273 strain for invasiveness at the initial mouse passage. However, they could not detect any cells which harbored the parasites, even 48 hours after inoculation. So, these results may suggest the establishment of an increased virulence by the serial passages in mice.

In spite of the good invasiveness of this strain into cells, the organisms of this strain were rather poorer in multiplication than those of the RH strain, as can be seen from Fig. 2. They seemed, however, to show a tendency to increase in number with

Table 3. Invasion of Cells by Modified S-273 Strain

Strain		S-273			RH		
Hours after inoculation		3	4	6	2	4	6
Rate of infection	HeLa cells	31.8	42.8	39.0	19.0	23.0	43.2
	MS cells	18.8	29.8	42.4	8.5	16.4	30.9
	FL cells	5.6	12.6	19.8	4.6	7.4	15.3

- Remarks: 1. No. of parasites inoculated: No. of cells $\div$ 1:3 for FL cells.
No. of parasites inoculated: No. of cells $\div$ 1:1 for HeLa and MS cells.
2. The organisms of the 16th passage in mice were employed.

Fig. 2. Multiplication of Organisms of Modified Strains in Tissue Culture

* From peritoneal fluid of 20th-passaged mice.

** From peritoneal fluid of 18th-passaged mice.

*** From peritoneal fluid of 35th-passaged mice.

Table 4. Virulence for Mice of the Modified Strain

Strain	No. of passages	No. of organisms inoculated				
		10 ¹	10 ²	10 ³	10 ⁴	10 ⁵
S-273	52 (19)*			● ₇ ● ₇ ● ₇ ● ₈	● ₆ ● ₆ ● ₆	● ₆ ● ₆ ● ₆ ● ₅
	77 (44)*	● ₈ ● ₈	● ₇ ● ₈	● ₇ ● ₈	● ₆	
Beverley	56 (46)*	● ₉ ● ₁₂	● ₅ ● ₉	● ₇ ● ₇	● ₇ ● ₆	● ₆ ● ₆

●₇ Death of mouse on the 7th day after inoculation.

()* No. of passages in mice without injection of cortisone acetate.

the progress in mouse passage.

Table 4 indicates the virulence of these modified strains for the mouse. Judging from the data given in this table, it is clear that these strains have come to progress a virulence nearly equal to that of the RH strain.

DISCUSSION

It seems that there may be many factors by which the virulence of *Toxoplasma* is affected. One of these factors may be related to the susceptibility of the experimental animal. Up to date, no more susceptible experimental animals have been detected other than mice. Professor TSUNEMATSU⁶⁾, of the Institute for Infectious Diseases, University of Tokyo, however, has recently detected several pet birds which showed a susceptibility higher than, or the same as, that of the mouse for *Toxoplasma*. Then experiments are generally conducted by carrying out serial passages in mice to bring about an increase to the virulence of *Toxoplasma*.

The S-273 strain usually killed no mice and caused the cyst formation only in the mouse brain. According to HANAKI et al.³⁾ however, this strain produced proliferative-form organisms in the peritoneal cavity of mice injected intramuscularly with cortisone acetate, and it could be passage serially from mouse to mouse by the peritoneal route at 4 days' intervals. They reported, however, that an increase had never occurred in the virulence of the strain or in the numbers of cyst formed even after the 20th mouse passage.

Fortunately, the present authors succeeded in increasing the virulence of this strain

Table 5. Effect of Cortisone Acetate on Serial Passage

Strain	Dose of cortisone acetate per mouse	No. of mouse passages									
		1	2	3	4	5	6	7	8	9	10
Beverley	5.0 mg	△	●	○	○	○	○	○	○	○	○
		●	△	○	○	○	○	○	○	○	○
	2.5	●	●	○	○	○	○	○	○	○	○
		●	●	○	○	○	○	○	○	○	○
	0.5	●	○	○	○	○	○	○	○	○	○
		●	○	○	○	○	○	○	○	○	○
	0	●	○	○	○	○	○	○	○	○	○
		●	○	○	○	○	○	○	○	○	○
		△	○	○	○	○	○	○	○	○	○

Remarks: All mice were sacrificed for detection of cysts in the brain on the 30th day after inoculation.

● Mouse harboring *Toxoplasma* cysts.

△ Mouse which died from some other cause than *Toxoplasma gondii*.

in one experiment out of many that they had performed. In this case, two factors should be taken into consideration, though there may naturally be many complicated factors which have an effect on the enhancement of virulence. One of them is cortisone acetate. It has already been demonstrated that the administration of this drug induces the production of proliferative-form parasites. In the present experiments, however, this drug failed to enhance the virulence of the strain, as indicated in Table 5. It is suggested that a dose of more than 2.5 mg per mouse may be necessary to increase the virulence of the cyst type strain.

The other factor, that the present authors are confident of, is the age of the cyst which is used as initial material. Data concerning this point are listed in Table 6.

Table 6. Age of Starting Material and Enhancement of Virulence

Strain	*Age of cyst	Injection of cortisone	Enhancement of virulence	Remarks
S-273	36 days	+	+	Death of mice since the 8th passage
	196	—	—	Cyst positive to the 3rd passage
	221	+	—	N.T.
	272	—	—	Cyst positive to the 2nd passage
Beverley	92	+	+	Death of mice since the 1st passage
	166	—	—	Cyst positive to the 3rd passage

* Days after infection of mouse from which starting material (*Toxoplasma* cyst) was obtained.

Pertaining to the S-273 strain, a high virulent progeny was liberated from a cyst 36 days of age, but was not from one 221 days of age. The organisms from a cyst 196 days of age were possibly delivered to the 3rd generation, and those from a cyst 272 days of age only to the 2nd generation, though cortisone acetate was not used in these cases.

As for the Beverley strain, a similar tendency was observed.

In the present work, the number of cysts contained in the initial material was not exactly calculated. So, it is necessary to carry out re-examination under more strict condition.

At any rate, it has been demonstrated that such high virulent organisms as those of the RH strain can be induced from those of a low virulent strain, such as the S-273 strain, under some experimental conditions. Accordingly, it is clear that the each strains of *T. gondii* are originally identical with one another, at least from the viewpoint of the virulence.

Up to date, no antigenic comparison was possible between the cyst type strains and the high virulent one, because it was difficult to harvest a sufficient amount of proliferative-form organisms of the low virulent strain for preparation of the antigen. So any reciprocal examination necessary for antigenic analysis had never been conducted prior to the present investigation. These data presented in this paper may suggest a possibility of inducing proliferative-form organisms from those of a low virulent strain.

SUMMARY

Experiments were carried out to increase the virulence of a *Toxoplasma* strain. The results are summarized as follows.

1. The S-273 strain, which had been isolated from a clinically healthy pig, generally caused only cyst formation when inoculated into mice. It became to show a high

virulence, like the RH strain, when it had undergone the eighth serial passage in mice intramuscularly injected with cortisone acetate. The Beverley strain also changed in such way after the first passage that it became as virulent as the RH strain.

2. The S-273 strain caused death regularly among the mice of one passage level 4 or 5 days after inoculation when it had undergone 20~30 mouse passages as did the RH strain. Shortening of the survival period was observed in accordance with the progress in mouse passage.

3. Parasites of the S-273 strain exhibited poorer multiplication in the mouse body, as well as in tissue culture than those of the RH strain.

4. Modification of the virulence occurred in the S-273 and Beverley strains in only one each of the experiments performed.

5. Cortisone acetate seemed to be one of the most important factors to cause such modification of the virulence. Some experiments suggested, however, that there might presumably be some factors other than this chemical, which were related to the qualities such as age and number, of cysts in the starting material.

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REFERENCES

- 1) BEVERLEY, J. K. A. (1959): *Nature*, **183**, 1348~1349.
- 2) HANAKI, T., SATO, U., NISHIMURA, Y. and NOBUTO, K. (1961): *Jap. J. vet. Sci.*, **23**, 509.
- 3) HANAKI, T., SATO, U., NISHIMURA, Y. and NOBUTO, K. (1962): *Jap. J. vet. Sci.*, **24**, 370.
- 4) JACOBS, L. (1956): *Ann. N.Y. Acad. Sci.*, **64**, Art. 2, 168~170.
- 5) SHIMIZU, K. and TAKAGAKI, Y. (1959): *Jap. J. vet. Res.*, **7**, 95~103.
- 6) TSUNEMATSU, Y. (1964): *Rep. Res. Minist. Educ. (Japan), Agric. (I)*, 100.
- 7) TSUNEMATSU, Y. (1965): *Rep. Res. Minist. Educ. (Japan), Agric. (I)*, 228.

マウス継代によるチスト型弱毒トキソプラズマ株 の毒力増強試験

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花木らが無症状感染豚から分離した S-273 株と Beverley 株のマウス脳内チストを出発材料として、コートンの筋肉内注射と同時に、4 日間隔でマウス腹腔内注射による盲継代を行なった。その結果、前者では 8 代目から、後者では初代から、マウスをたおす毒力を獲得し、継代の進行とともに、増殖型虫体の増数も顕著となった。その後コートン投与を中止しても、マウスの斃死日数に影響なく、ほぼ 4～6 日間で例外なくこれをたおすが、マウス腹水中の虫体数、組織培養による虫体増数は、RH 株に比べ

てはるかに不良であった。マウス継代 150 代以上に及ぶ今日においてもこの性状はほとんど変わらない。上記の毒力増強試験は、出発材料であるチストのマウス感染後の日令を変えて、S-273 株については 4 回、Beverley 株については 2 回実施したが、強毒化がみられたのは、双方ただ 1 回に過ぎなかった。

以上の成績からみて、チスト型弱毒株から RH 型強毒株への移行が起こり得ることは、明らかであるが、これに関与する因子は、はなはだ複雑のようである。