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PROLIFERATION OF Toxoplasma gondii AND ITS CYST-FORMATION IN MOUSE BRAINS

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INDEXING WORDS

Toxoplasma; toxoplasmosis; mouse; cysts; ultrastructure

SYNOPSIS

The present paper describes studies of the proliferation of Toxoplasma gondii (S-273 strain, abbreviated to Tp), especially in relation to the formation, distribution and maturation of the cyst in ICR albino mouse brains by light and electron microscopy. The Tp with which the mouse was infected reached the brain on the 9th day and formed the cyst on the 12th day after inoculation. The cyst size was an average of 15 μm in diameter. On the 15th day the cyst diameter reached 30 μm, and thereafter increased gradually with the increase of the antibody titer of the Tp.

Under the transmission electron microscopic observation of the mouse brain on the 12th day after inoculation, the earliest stage of the cyst could be observed. The immature cyst consisted of a homogeneous clot which was electron less-dense, including a few trophozoites. There was a electron lucent space around this structure, which contained mitochondria, a filamentous substance, and other small bodies. However, there was no definite cyst wall around this substance.

The outer layer of the cyst recovered from the 1st month after inoculation could be observed as an electron-dense membranous structure, fusing precipitates derived from the host cell in some areas.

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A structure which could be fully recognized as the cyst wall was found in the brain in the 2nd month after inoculation. This cyst wall had electron-density entirely different from that of the homogeneous substance filling the space between the trophozoites in the cyst and covered the cyst with uniform thickness. In cysts containing polygonal shaped trophozoites, the outer space surrounding the cyst was often extremely narrow or hardly seen.

The cyst wall in the 3rd month after inoculation became more conspicuous and electron-dense. It consisted not of homogeneous material, but of a filamentous structure probably derived from the host cell membrane, which was buried in many parts of the cyst wall.

Most of the cysts in the 4th month after inoculation resembled those obtained from the 2nd and 3rd months after inoculation. Some of them were similar to the cysts observed on the 12th day after inoculation.

From the results obtained so far, it was suggested that the antibody production is related to the process of cyst maturation rather than its formation, and that the cyst wall originated from the secretion of the protozoal cell and host-cellular debris.

INTRODUCTION

Toxoplasma gondii (abbreviated to Tp), which was discovered in Ctenodactylus gondii by Nicolle and Manceaux in 1908, is a protozoan parasite infecting a wide range of animals like birds and mammals, including humans. Although this human infectious disease usually takes a subclinical form, it produces serious manifestations in some cases. Tp, therefore, has recently attracted attention of the investigators as a causative agent of the infectious disease called "zoonoses" common to both humans and animals.

The classification of this protozoa was not clear for a long time. At present, however, it has been settled as Isospora, since it was found to perform gamotogenesis in cats. In addition, its life cycle has been clarified by Hutchison and Work (1969), Work and Hutchison (1969), Siim *et al.*, (1969), Hutchison *et al.*, (1970), Sheffield and Melton (1970), Frenkel *et al.*, (1970) and Dubey *et al.*, (1970).

A number of papers have been reported on the distribution or ultrastructure of the cyst formed in a host with chronic infection (Nakayama and Matsubayashi, 1962 : Motomura and Jo, 1970 : Wanko *et al.*, 1962 : Matsubayashi and Akao, 1963 : Ghatak and Zimmerman, 1973). Matsubayashi and Akao (1963) have clarified the process of cyst-formation by using the transmission electron microscope. Ghatak and Zimmerman (1973) have also described the ultrastructure of the Tp cysts isolated from the naturally in-

fected human brain. In spite of their reports, the origin of the cyst wall is not yet clarified. There have been presented various theories concerning the origin of the cyst wall; it may be produced from the protozoal cell (Vander, 1959: Matsubayashi and Akao, 1966), from the host cell (Levine, 1961) or from components of both cells (Wanko *et al.*, 1962). At present, it is postulated that the cyst wall consists of components of protozoal origin (Carey *et al.*, 1968), but there is no clear confirmative evidence yet.

The present study was performed to learn on: (I) the movement of Tp trophozoite up to cyst-formation under a light microscope and serological and histopathological response in the host after the cyst-formation, and (II) the maturation process of the cyst by the transmission electron microscope (TEM).

MATERIALS AND METHODS

Toxoplasma gondii S-273 (known to be low virulence) strain which had been passed through ICR albino mice was used for this study. The infected mouse brains were homogenized in 10 ml of saline, and about 50 cysts were inoculated intraperitoneally.

Part I

Some of the mice infected with Tp were sacrificed at three day intervals after inoculation. The mouse brains were examined for cysts, and the diameters of the cysts were measured. The antibody production in the blood of the mice was examined by the indirect hemagglutination test using a kit (Hanaki, Shindo, Sato and Matsuno's method : Kaketsuken).

Part II

Considering the findings and results from Part I, TEM observations were performed on the specimens prepared on the 12th day after inoculation, the earliest detectable day of the cyst, and after 1, 2, 3 and 4 months, respectively. The brain tissues taken at each stage were cut into blocks of 4 to 5 mm in size, double-fixed with 2.5% glutalaldehyde (4 C, 1 hour) and 2% osmic acid (4 C, 1 hour), dehydrated in graded concentrations of alcohol, embedded in epoxy resin, and ultrathin-sectioned with Porter-Blum microtome (Ivan Sorvall). The pictures were taken at magnifications ranging from 2,000 to 15,000 times with a Hitachi HU 11-D type electron microscope.

RESULTS

Part I

Mice were examined of the inoculation with 50 cysts for manifestations such as change in body weight, roughing of the hair coat and the appearance of ascites. Ascites was found on the 8th day after inoculation and roughing of the hair coat on the 10th day. Ascites always appeared first, followed by the roughing of the hair coat, and each symptom disappeared within 2 or 3 days. Body-weight loss tended to occur. However, weight increased thereafter at almost the same rate as in the control group (Fig. 1.)

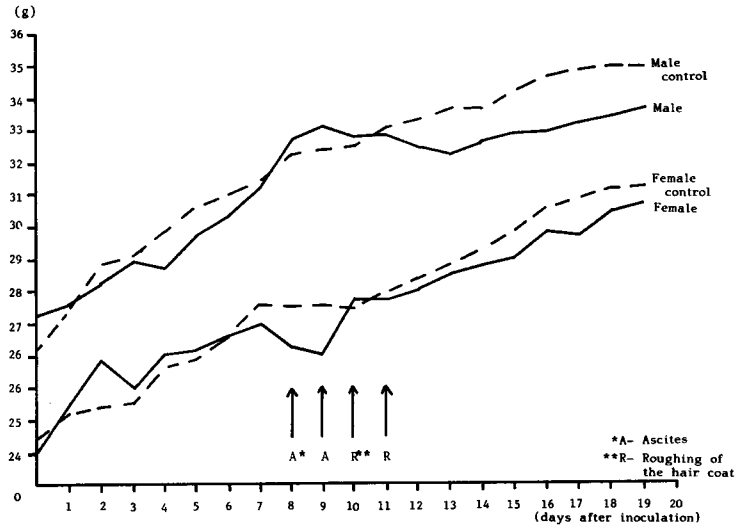


Fig. 1 Change of mouse weight after inoculation of Toxoplasma gondii

The cyst could be detected first on the 12th day after inoculation. The cyst size was an average of 15 μm in diameter. On the 15th day, the cyst diameter was 30 μm and thereafter increased gradually (Table 1).

The antibody in the blood was detected together with appearance of the cyst and showed a titer of 1 : 512 about 24 days after inoculation (Table 1).

Histopathological findings are shown in Fig. 2 and 3. The inflammatory reaction could first be detected on the 9th day after inoculation. This observation corresponded to the time when ascites and the roughing of the hair coat became manifest. The focus of infective Tp existence appeared as local gliosis distributed sporadically in the superficial layer of the brain as if the lesion had invaded from the meninges (Fig. 2). This kind of gliosis became noticeable from the 15th to 18th days after inoculation.

On the 24th day, the inflammatory reaction became still more remarkable; small round cells appeared as gliacytes infiltrating perivascularly (Fig. 3). These cells containing trophozoites were located not in the mid-

Table 1 The relationship between cyst appearance, its diameter and antibody titer after inoculation of Tp

No	Days after inoculation	Titer of Ab	Presence of cysts		Blind passage	
			+ or −	diameter	+ or −	Titer of Ab *
1	3	0	−	− (μm)	−	0
2	6	0	−	−	−	0
3	9	0	−	−	+	64
4	12	32	+	15	+	128
5	15	64	+	30	+	128
6	18	64	+	35	+	128
7	21	128	+	31.7	+	128
8	24	512	+	31	+	128
9	27	512	+	36.5	+	128
10	30	512	+	38.9	+	128
11	90	1024	+	48.5	+	128
	control	0	−	−	−	0

* 20 days after inoculation

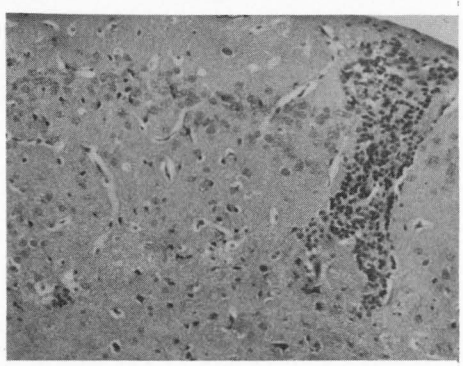


Fig. 2
The local gliosis distributing sporadically on the superficial layer of the brain had invaded from the meninges. 9 days after inoculation.

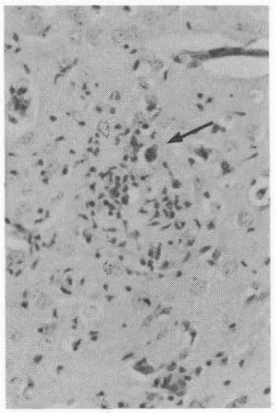


Fig. 3
Small round cells arrow appeared as gliacytes infiltrating perivascularyly. 24 days after inoculation.

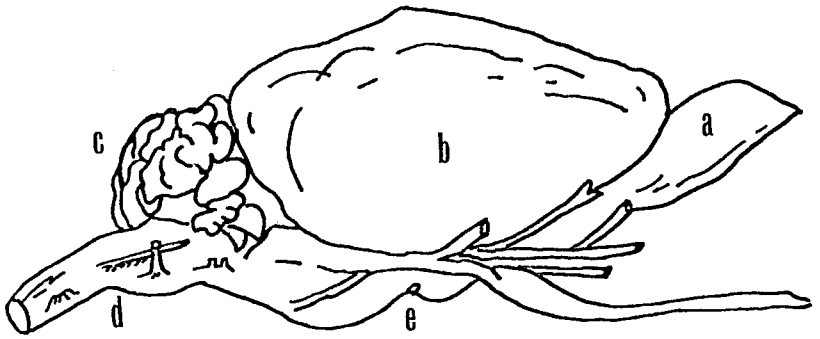
dle of the gliosis but in the peripheral area of the lesion, although the reason for this location is unknown.

Table 2 shows the comparison of number of cysts existing in the brain from 1 month and 3 months after inoculation. The number of cysts in 5mg brain tissues were determined in each part after dividing the brain into five parts: i.e., cerebrum, cerebellum, hypophysis, rhinencephalon, and

medulla oblongate.

The cerebrum showed the largest number of cysts at both the sub-acute and chronic stages, next were the rhinencephalon and cerebellum in that order. The hypophysis showed no cysts at the sub-acute stage and only a few at the chronic stage.

Table 2 Comparison of numbers of cysts existing in the brain from acute and chronic stages of the infection at each parts of mouse brain



	No of cysts / 5mg		Existence of cysts after blind passage		Diameter of cysts in each organ		Average weight of each organ (g)
	A	C	A	C	A	C	
a: Rhinencephalon	28	24	+	+	24.4	45.8	0.02
b: Cerebrum	78	79	+	+	38.9	48.5	0.25
c: Cerebellum	12.5	15.7	+	+	21.5	45.3	0.08
d: Medulla oblongata	9	11	+	+	20.5	37.3	0.13
e: Hypophysis	0	0.5	-	+	0	18.7	0.002

A: sub-acute (1 month after inoculation)
C: chronic (3 months after inoculation)

Part II

In this part, the morphology of the cyst in the acute stage (on the 12th day after inoculation) and the chronic stage (in month 1, 2, 3 and 4) were studied by TEM. The materials were collected from the cerebrum where the cysts had been detected in the greatest incidence in Part I of the study. The structure of the earliest stage of the cyst consisted of a homogenous substance and contained two or three trophozoites (Fig. 4 (R)).

Around the homogeneous clot which was electron less-dense, there was rather a rough space which contained mitochondria, a filamentous substance, and other small bodies. However, no structure was found to correspond to the cyst wall. Although the homogeneous substance was present among the trophozoites, no rough space was present around these cysts as shown in Fig. 4 (R). The mature adjacent cyst contained many trophozoites, many of which were compressed to be of polygonal shape (Fig. 4 (L)).

The most common shape of the cysts, observed frequently on the 12th day after inoculation, was globular and the trophozoites were scattered in disorder inside the cyst. No trophozoite of polygonal shape was detected in Fig. 5. The space between trophozoites, except for the outermost layer, was filled with a homogenous electron less-dense substance and the trophozoites in the cyst were separated by this substance from one another. The outermost layer was slightly electron dense in comparison with the inner layer and fused with the surrounding structure (Fig. 6). Thus, it is doubtful that the cyst wall was formed independently from the host cells.

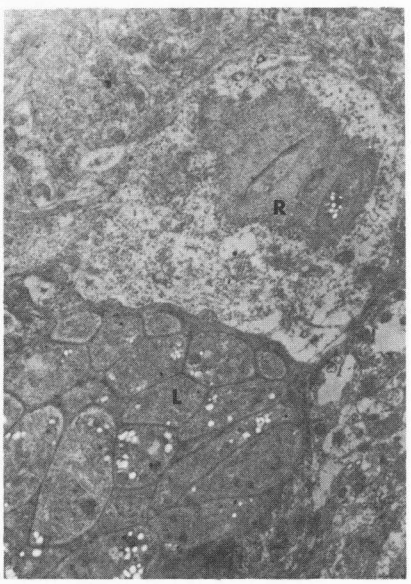


Fig. 4
The structure related to the earliest stage of cyst formation in the mouse brain on day 12 after inoculation. X 4,200 The structure in the right side (R) consisted of homogeneous substance and contained two or three trophozoites. Mitochondria and filamentous substance were presented around this structure, while the polygonal shape trophozoites in the structure were observed in the left side (L).

In the cytoplasm of the trophozoites in the cysts collected in the 1st month after inoculation, a crack seemed to have formed (Fig. 7). Observations indicated that vaculoes from the cross section were distributed partially rather than uniformly within the cytoplasm (Fig. 8). The electron dense part at the outer side of the cyst, which was extremely thin at this stage, became a clearer membranous structure (Fig. 8). In many places, this dense part still merged with cellular precipitates (Figs. 8, 9).

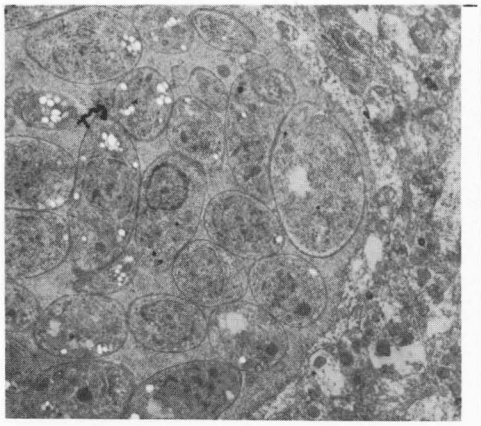


Fig. 5
The most common shape of the cysts observed frequently on day 12 after inoculation.
X 4,800

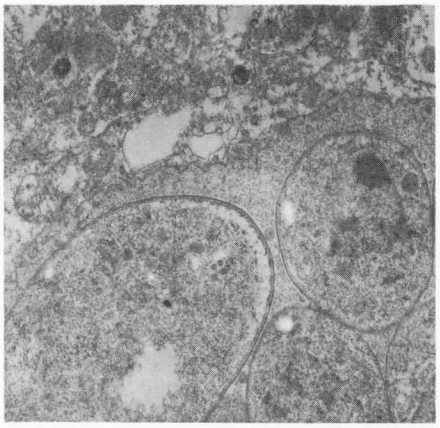


Fig. 6
The outermost layer was slightly electron dence as compared with the inner layer and fused with the surrounding structure. The high magnification of the cyst recovered from 12 days after inoculation. X 11,000

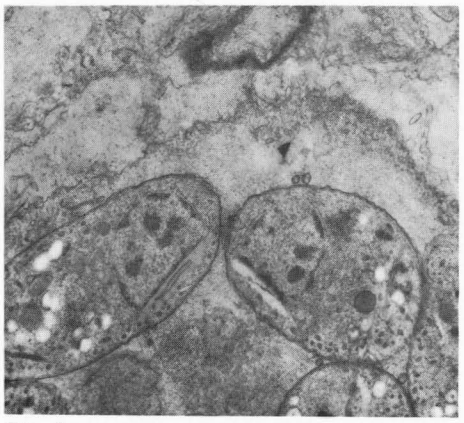


Fig. 7
Many parts in which outer side of the cyst fused with the cellular precipitates. One month after inoculation. X 7,900

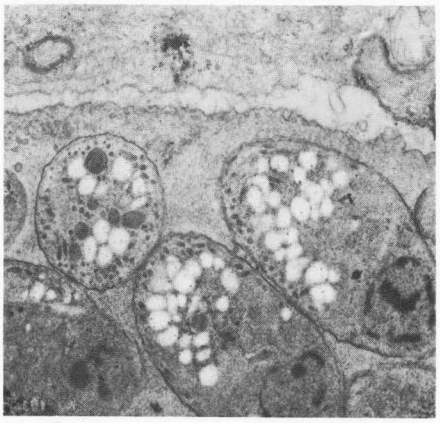


Fig. 8
The electron dense part at the outer side of the cyst, which was extremely thin at this stage, become clearly membranous structure. One month after inoculation. X 10,000

The trophozoites in the cyst in the 2nd month after inoculation manifested a large number of vacuoles. The polygonal shape of the trophozoites was also observed (Fig. 10).

From this stage, the structure which can be fully recognized as a cyst wall was formed at the outer side of the cyst (Fig. 10). This cyst wall with a uniform thickness had an entirely different electron-density

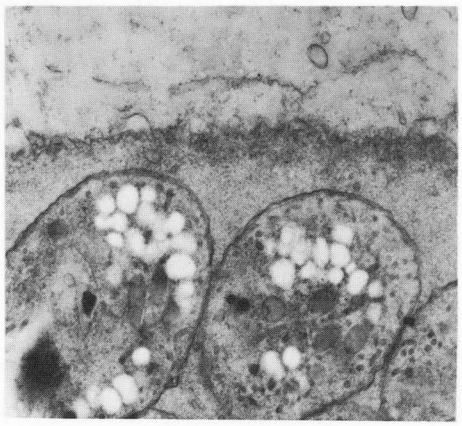


Fig. 9
In the space surrounding the cyst, there were filamentous substance and small body. One month after inoculation. X 15,000

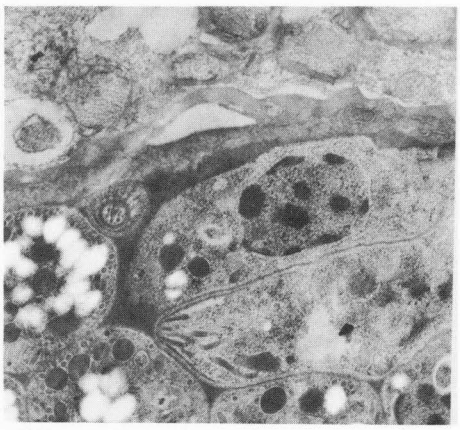


Fig. 10
From this stage the structure which can be fully recognized as a cyst wall was formed at the outer side of the cyst and covered with uniform thickness. Second month after inoculation. X 15,000

from that of homogenous substance filling the space among the trophozoites in the cyst. With the cyst containing the polygonal shaped trophozoites, the space surrounding the cyst was often extremely narrow or hardly seen. The vesicular substances were observed on the surface of trophozoites in the cysts recovered later than the 3rd month after inoculation. This change was noticed on various parts of the trophozoite. In some cross sections, vesicular substances were observed in 5 parts (Fig. 11). These structures seemed to be spaces which had been formed after swelling of the cell membrane or budding of the vacuole. Only 1 out of 8 cysts observed at this stage contained trophozoites of the polygonal shape. The cyst wall became more conspicuous and electron-dense. It had no homogenous material on the outer side of the wall. There were filamentous structures of various lengths which probably derived from the cell membrane of the host cell. This filamentous structure not only covered the outer surface but also was buried in many parts of the cyst wall (Fig. 12). The filamentous substance of this kind was never detected in the homogenous material filling the space between trophozoites, but in the space between the cyst and the host cell it was noted sporadically (Fig. 12).

The mice in the 4th month after inoculation revealed various types of cysts. Most of them resembled those obtained from the mouse brain in the 2nd and 3rd months.

The cysts which had other forms or structures are shown in Figs. 13, 14. Fig. 13 shows a form which appears similar to that in Fig. 4 (R).

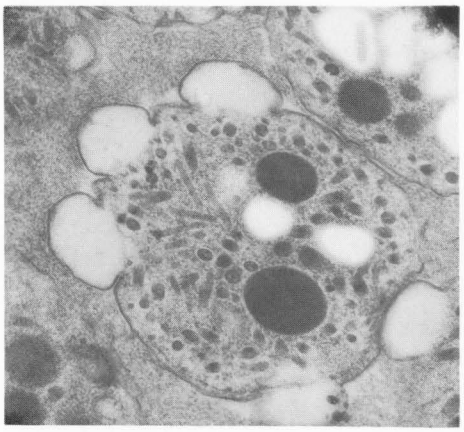


Fig. 11
The vesicular substance were observed on the surface of trophozoite. Third month after inoculation. X 26,000

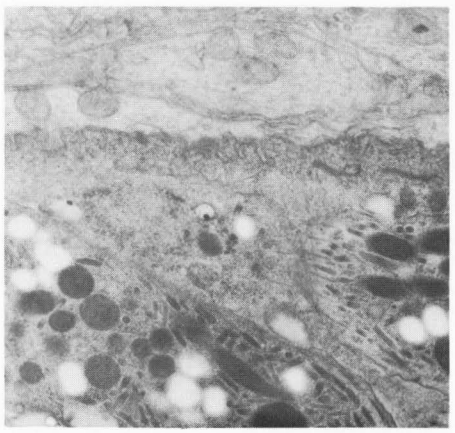


Fig. 12
The cyst wall become more conspicuous and electron denser but consisted of no homogeneous material. There were filamentous structures of various length and mitochondria which probably derived from the host cell. Third month after inoculation. X 15,000

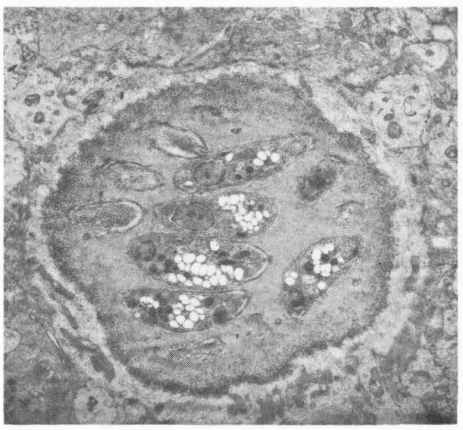


Fig. 13
Several trophozoites were wrapped by homogeneous clot spreading over wide area for this number. Four month after inoculation.
X 3,400

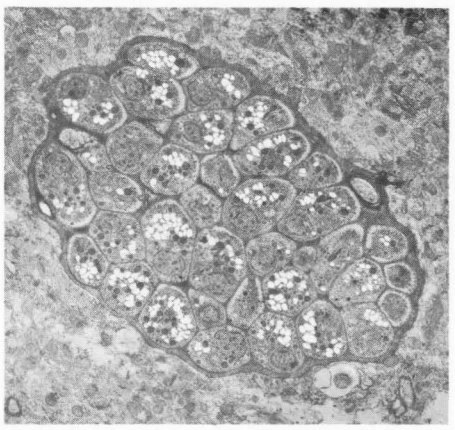


Fig. 14
The trophozoites showed the polygonal shape, and no distinct space surrounding them was observed. Four month after inoculation.
X 3,000

Here, 8 to 9 trophozoites were wrapped by a homogenous clot spreading over a wide area surrounding them. The cyst shown in Fig. 14 was also observed in the brain tissue in the 4th month after inoculation. Numerous trophozoites filled one cyst as if it were packed tightly within the cyst wall. The trophozoites were polygonal in shape, and even the cyst wall was distorted along the contour of the trophozoite. A distinct space between the cyst wall and the host cell was not seen.

DISCUSSION

Part I

The mechanism by which Tp forms cysts in the host cell was formerly thought to be related to the immunological mechanism : it was postulated that the cyst was formed in the brain where the antibody was limited in distribution (Tsunematsu 1961 : Jacobs 1967). At present, the cyst formation is thought to have nothing to do with the immunological mechanism but is regarded as a part of the life cycle of Tp.

The results of the present study also support the latter view. On the 12th day after inoculation when the antibody titer was only 1:32, the cyst had already been formed. Thus, is appears unreasonable to conclude that the cyst is formed under the direct influence of the antibody.

Although the cyst could not be detected in the brain of the mouse on the 9th day after inoculation, the blind passage of brain homogenate into other healthy mice could produce the cyst, which indicated that trophozoites had already reached the brain on the 9th day after inoculation. On the 12th day, when the cyst was first detected, it showed 15 μm in average diameter. On the 15th day, its size became 30 μm in diameter and thereafter increased gradually. It was presumed that once the trophozoite reached the brain on the 9th day and stayed there for a few days, it formed the cyst, which grew rapidly to about 30 μm in diameter on the 15th day. After the antibody titer had risen to a high level, the growth rate was reduced.

The pathological inflammatory reaction in the brain started to appear from the 9th day which corresponded to the day when ascites or rough hair -coat was noted and also to the day when infectious trophozoites could be collected in the brain. The inflammation began with gliosis in the part where a swollen trophozoite-like structure was found in the gliacytes. The gliosis became more distinct on the 15th to 18th days after inoculation and small round cells on the 24th day were considered to be gliacytes which also appeared around the vessels.

The cysts were not evenly distributed, but they were frequent in the cerebrum and rhinencephalon and scarce in the hypophysis. The highest distribution of cysts was in the cerebrum. This was in close agreement with the results reported by Nakayama and Matsubayashi (1962), Motomura (1970) and other workers. After examining cyst distribution in each division of the brain, cyst size and changes in number at the various intervals, Shingaki (1971) conclude that the increase of the incidence was greatest in the rhinencephalon and the lower layer of the cerebral corte in that

order and the number of cysts in the brain did not always increase with the progress of time but decreased rapidly in the 6th to the 7th week after inoculation.

According to Nakayama and Matsubayashi (1962), the difference in virulence between the cyst-forming strain and non cyst-forming strain depends solely on the rate of multiplication. They succeeded in inducing a higher incidence of cyst formation in mice being treated with a sulfa drug after inoculation of the RH strain. They attributed this success to the multiplication of Tp delayed by the sulfa drug and to the inability of Tp to destroy host cells. Similar study had been done by Stahl *et al.*, (1965) using 6-mercaptopurine. The S-273 strain in the present study allowed no recovery of trophozoites at any stage after inoculation. The authors observed that intramuscular injections of 0.1 ml of anti-antibody solution (Corton, Japan Merck-Banyu) to the mice 3 days before, on the day and 3 days after inoculation of Tp, made it possible to recover trophozoites from the abdominal cavity and delay the earliest stage of cyst formation. This indicates that the inhibition of the antibody effect on trophozoites resulted in an increased rate of multiplication in the host cell, which was destroyed to liberate trophozoites in the abdominal cavity and to delay cyst formation. Therefore, it can be concluded that the rate of multiplication of the trophozoite was related to the cyst formation and its antibody affected the process of cyst maturation rather that its formation.

Part II

Of greatest importance is the fact that the observance of a cyst at a certain stage does not always mean that the cyst was formed throughout the infection period up to the time of observation, because the number of cysts in the brains of mice increased or decreased during the infection (Fujita 1960 : Nakayama and Matsubayashi 1962 : Shingaki 1971). This means that the cysts collected from a brain 3 months after inoculation, for instance, may have been formed 1 or 2 months after inoculation. For this reason, it is meaningless to discuss one point in the development of the cyst at the various intervals of observation. However, the authors emphasized that the observation of many cysts, in spite of the limitation mentioned above, could elucidate the maturation process of the cyst to a certain extent.

The above limitation need not be applied in the case of the cysts obtained from the mouse brains on the 12th day after inoculation, because the mice at this stage had been injected with Tp 12 days before for the first time.

For this reason, according to the results of Part I, it is reasonable

to presume that all the cysts observed on the 12th day were newly formed. The structure as shown in Fig. 4 (R) is of significance since it can be thought to show specific features of the earliest cyst.

Matsubayashi and Ako (1963) observed a similar structure in the tissue cultured cells. It is not clear whether or not this sort of the structure observed in vitro can be formed in vivo. The rosette formation of Tp which was observed in a similar experiment using the RH strain, for instance, can not be noted in vivo. Ghatak and Zimmerman *et al.* (1973) and Powell *et al.*, (1978) reported that a structure of this kind existed in the brain tissue of a human infected naturally with Tp. It is unknown whether or not the structure is related to the amount of time elapsed after infection and virulence of the Tp strain.

As shown in Fig. 4 (R) the architecture within the host cell was damaged over a relatively wide area and in the middle of the area was the homogeneous substance containing several trophozoites inside. The area around the homogeneous substance was dotted with small particles, probably the precipitate of the host cell.

This indicates the possibility that this structure has grown for a short period without physical obstruction to the same size as that of the adjacent cyst. The results of Part I also support this possibility. The cyst as shown in Fig. 4 (L) is thought to have grown in this way. This newly formed cyst lead us to make a conclusion contrary to that of Matsubayashi and Akao (1963) who reported that tightness of trophozoites in the cyst is one of the indications of cyst maturation. The cyst which we observed had been rapidly growing to fill the space of the host cell. For this reason there was no space around the cyst, though such a space could be commonly observed in other cysts between their outermost surface and the host cell. The cyst filling the host cell in this way gathers around itself precipitates derived from the host cell, which will probably serve as initial materials of the cyst wall. Otherwise, the homogenous substance containing trophozoites was evidently different in density from the surrounding material, which indicates the possibility that the clot has its origin in a substance which comes from the trophozoites.

The cyst collected one month after inoculation (Fig. 9) showed a fairly wide space around it. As shown in Fig. 12 this finding suggests the mechanism by which the cyst wall accumulates filamentous material and granular substance in its outer membrane in the process of growth. The vesicular substance which is distinctive at this stage presumably corresponds to the bleb reported by Wanko *et al.*, (1962). The function of this substance and its physiological significance is not clarified as yet.

In Fig. 13, the cysts collected from the brain in the 4th month had

a form similar to that of the cyst presented in Fig. 4 (R). This is probably a cyst which was newly formed at this stage. This kind of new cyst formation has a tendency to occur at this stage (Beverley 1958 : Fujita 1960).

In conclusion, the S-273 strain in the present study has a low virulence in mice because of its slower rate of multiplication. The trophozoites which invade host cells first destroy the tissue cells around them and then produce a rather homogeneous clot using partly the resulting precipitates and partly materials which are presumably excreted by the trophozoite (Fig. 4 (R)). This homogeneous clot probably protects the Tp from surrounding host cell or antibodies in body fluid at the stage before the formation of the cyst wall. The trophozoite repeats cell divisions, expands the homogeneous clot and simultaneously accumulates or compresses precipitates in the space of the brain tissue of the host (Figs. 8, 9, and 13). Excessive multiplication of the trophozoites results in the repletion of the pre-existing space with trophozoites and induces polygonal changes, when the space between the cyst wall and the host cell disappears or becomes extremely narrow (Figs. 4 (L), 10).

A question which remains unresolved is whether or not the trophozoite repeatedly forms a space in the host tissue, accumulates the substance in the space which is derived from the host cell, and grows into maturity.

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