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A METHOD OF DETECTION OF *TOXOPLASMA* INFECTION IN MAN

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The diagonosis of human toxoplasmosis is mainly based upon the immunological tests and clinical symptoms of the patients. The detection of the parasite, however, is desirable to make the diagnosis more reliable, because the symptoms are not always specific and immunological tests are often positive among healthy people.

The usual method of detection of the parasite from human being is to inoculate with the suspected human materials into mice such as spinal fluid and tissues obtained by biopsy or surgical operation. This method can be expected to be effective when the materials are taken from acute, fulminant cases in which the parasite is highly virulent and is growing rapidly. Most of the human toxoplasmosis is, however, found in the chronic stage of infection. The parasite in the material taken from chronic cases, if any, is a few in number. If a strain is highly virulent, it can grow in mice actively even if the number of the parasite is small and the test will give a positive result. If the strain is of low virulence, it will produce only a small number of parasite in mice inoculated and may escape the detection by microscopic examination, giving a negative result. The routine serial transmission of a low virulent strain through mice suggests that trophozoites and cysts growing in mice are so few in number that they are often missed by the microscopic examinations.

The purpose of the present study is to detect a very light infection of *Toxoplasma* in mice which are inoculated with human material and to make a contribution to the diagnosis of human toxoplasmosis.

Materials and Methods

Seven strains of *Toxoplasma* were used in this study. They are as follows.

S-273 and S-31 strains. Both were isolated from pigs in Japan by Nobuto *et al.* are almost avirulent to mice. They are kept by serial intraperitoneal transplantation of cysts through mice. Trophozoites rarely appear in the peritoneal cavity and very few cysts are produced in the brain. Mice do not die of the infection. These two strains are tentatively called avirulent strain.

Beverley strain was isolated from a rabbit in England and was sent to our laboratory from late Dr. Eyles. C-37 strain was isolated from a chicken in U. S. A. and was sent to our laboratory from Dr. Jacobs. The two strains are virulent to mice to some extent. More than a half of the animals which were inoculated intraperitoneally with cysts of these strains succumb to the infection within 3 weeks. Those survived the infection usually have a large number of cysts in their brains. These are tentatively a low virulent strain.

RH, SM and KM strains. RH is a famous strain isolated from a child in U. S. A.. SM and KM strains were isolated from children in Japan by Nakayama *et al.* (1966). These three strains are highly virulent to mice; all mice inoculated with trophozoites of these strains die of the infec-

tion within a week. SM and KM strains seem to have a higher virulence to mice than RH strain.

It is quite natural to conjecture that the number of parasite contained in human material to be examined is usually very small. The problem is how to detect the infection when such an extremely small number as 1 or 2 trophozoites are transmitted into mice and subsequent growth of the parasite is not active. This circumstance must happen very often when the trial of *Toxoplasma* isolation from human being is made. The direct examination of mouse tissue will miss the parasite very often in these cases and the result will turn out negative. To detect the infection of this kind, hemoaggulutination test (HA-test) with the mouse serum and challenge inoculation of a high virulent strain into the mouse were tried. The HA-test was carried out after Hanaki-Nobuto-Sato's (1963) method using lyophilized sensitized red cells. The mouse blood was absorbed in a piece of filter paper which was later infused in a phosphate buffer saline (pH 7.2) for 1 hour. This serum buffer solution was tested for HA. The challenge inoculation was made with a highly virulent strain which kills all non-immune mice within a week. Usually RH-strain was used for this purpose. Mouse blood for HA-test was taken just before the challenge inoculation which was made one month or more after the experimental inoculation of low virulent or avirulent *Toxoplasma* strains.

For the experimental inoculation, it was necessary to obtain an inoculum containing a small number of trophozoite. For this purpose, one small drop of trophozoite suspension was put on a small piece of broken cover slip. The number of trophozoite in the drop was counted under microscope and this piece of cover slip was inserted into the abdominal cavity of a mouse by opening the abdominal wall. By this procedure, even 1 or 2 trophozoites successfully inoculated into mice. To get the trophozoite suspension, the peritoneal fluid of an infected mouse was diluted with normal saline. In cases of avirulent strains such as S-273 or S-31, however, trophozoites rarely appear in the peritoneal cavity. In these cases, cortizone in amount of 2.5 mg, was injected just after the inoculation of cysts. Two additional injections of cortizone were made with an interval of 3 days. A number of trophozoites appeared in the peritoneal cavity following the injections and they were harvested for the inoculation.

Results

1) Resistance of mice chronically infected with a low virulent or avirulent strain to the challenge inoculation of highly virulent strains.

Mice were inoculated with cysts of Beverley, C-37, S-273 or S-31 strain. In this case, the number of organisms inoculated must be very large, because one cyst contains innumerable organisms. Mice died often by the infection of Beverley or C-37, as was stated above, but rarely died of infection with S-273 or S-31 strain. Those which survived the infection for more than 1 month or more were challenged with a strain of high virulence. About 100,000 trophozoites were inoculated for the challenge. The result are shown in Table 1 and 2.

Among 25 mice previously inoculated with Beverley strain, 7 died within 4 weeks

Table 1 Resistance of mice which have been infectetd with a low virulent or avirulent strain for more than one month to a challenge with high virulent strains

Mice challenged with:	Mice chronically infected with:			
	Beverley	C-37	S-273	S-31
RH	4/9*	1/5	0/8	0/8
SM	2/8	0/5	0/8	0/8
KM	1/8	0/5	1/8	0/8
Total	7/25	1/15	1/24	0/24

* No. of mice died/ No. of mice challenged

Table 2 Titer of HA-test and days of survival of mice which had been infected with a low virulent or avirulent strain and died after challenge

Mice chronically infected with :	HA titer of chron. infect. mice	Mice challenged with :	Days of survival after challenge	Trophozoites in abdominal cavity after challenge	Cysts in brain
Beverley	256	RH	10	a few	(±±±)
	1024		10	a few	(±±)
	1024		11	none	(±±)
	(−)		28	none	(±±)
	(−)	SM	9	a few	(±±±)
	(−)		11	a few	(±±)
	256	KM	23	none	(±±)
C-37	64	RH	4	a few	(±)
S-273	256	KM	25	none	(−)

Presence or absence of *Toxoplasma* organisms is also indicated.

after the challenge with RH, SM or KM strain (Table 1). A few or no trophozoites were found in the mice after the challenge (Table 2). The period of their survival was much longer than in cases of control mice which died within a few days after the inoculation of the same inoculum as the challenge. A large number of trophozoites were found in the peritoneal cavity of control mice before death. It is hardly believable, therefore, that these Beverley-infected mice died of the challenge with a highly virulent strain. The death of these mice must be caused by Beverley infection rather than by the challenge. In our experience, mice inoculated with cysts of Beverley strain die of the infection one by one during a prolonged period ranging from one to two months, although some of them can survive the infection completely.

In case of C-37 infection, one out of 15 mice died after the challenge (Table 1). The virulence of this strain is similar to Beverley strain and the death must be due to C-37 infection rather than to the challenge. That is to say, trophozoites appearing in the peritoneal cavity were very few in number (Table 2).

By the infection of S-273 strain, one of 24 mice died after the challenge (Table 1). The death could not be attributed to the challenge because no trophozoites could be found in the peritoneal cavity and the days of survival attained 25 days after the challenge (Table 2). On the other hand, S-273 infection may not be cause of this death, because this strain is almost avirulent to mice: mice rarely succumb to its infection. The death should be regarded as circumstantial.

By the infection of S-31 strain, none of the mice died after the challenge (Table 1).

All of these findings indicated that mice chronically infected with a low virulent or avirulent strain survived the challenge inoculation of a large dose of a high virulent strain.

The presence or absence of trophozoites in the peritoneal cavity is shown in the table 2. In any case, they are very few in number. They must be trophozoites of the strain used for the challenge, because in the chronic stage of infection of Beverly or avirulent strain, trophozoites cannot be found in the peritoneal cavity. On the other hand, cysts found in the brain of these mice must be produced by the low virulent or avirulent strain and not by the high virulent strain used for the challenge, because the period of survival after the challenge was too short to produce cysts.

It is noteworthy that HA-titer was negative in a few case of Beverley infection, in spite of a large number of cysts were found in the brain. It is also important to

Table 3 Comparison of the cyst productivity between Beverley and S-273 strains

Periods between infect. and exam.	Beverley			S-273		
	No. of mice exam.	No. of cysts inoculated	Average no. of cysts produced in brain	No. of mice exam.	No. of cyst inoculated	Average no. of cysts produced in brain
3 wks.	3	70	2,868	3	60	100(38-200)*
4 〃	3	60	2,617	4	60	170(18-620)
6 〃	5	25	1,443	4	60	60(34-108)
9 〃	3	70	2,215	5	10	118(46-220)
13 〃	3	45	818	5	15	108(55-146)
18 〃	3	80	1,031	5	60	61(10-200)
25 〃	2	40	299	2	15	62(16-108)
1 year	—	—	—	1	60	10

( )* range

note that no cysts were found in the brain of the mouse which had been infected with S-273 strain and survived the challenge with a high virlent strain (Table 2).

As is shown in the table 1, 9 out of 88 mice died after the challenge. As already stated, their death shold not be attributed to the challenge. The remaining 79 mice were sacrificed one month or more after the challenge and their brain suspensions were subinculated into clean mice. Organisms of high virulence were isolated from 54 out of these 79 mice. The finding indicated that the high virulent strain could survive in these mice which had developed immunity by the previous infection of low virulent or avirulent strain. The immunity was strong enough to protect the host from death, but was not able to eradicate the organisms of challenge inoculation.

2) Cyst productivity of low virulent and avirulent strains in mice.

It was suggested in the experiment stated above that Beverley strain produced far more cysts than S-273 strain. An experiment was carried out to compare the ability of cyst-production between these two strains. About 40-70 cysts of each strain were inoculated intraperitoneally into mice. They were examined during a period from 3 weeks to one year after the inoculation. The telencephalon of each mouse was cut into equal two parts at the longi tudinal fissure and number of cysts in the cerebral hemisphere was counted by the examination of direct smears. The number obtained was doubled to estimate the total number of cysts in the total telencephalon. The results are shown in the Table 3. The number of cysts produced had no correlation with the number of cysts administered. It was clearly demonstrated, however, that the Beverley strain produced far more cysts than S-273 strain throughout the examination period. Thus the productivity of cyst is different according to individual strains. Generally speaking, a strain of higher virulence produces far more cysts than it of lower virulence or avirulent one, provided the host can survive the infection for a period of time which is necessary for the parasite to produce cyst. The problem is how to detect the infection of these strains in man which produces only a small number of cysts when inoculated into mice. Human materials, spinal fluid or tissues to be examined cannot be expected to contain a large number of organisms, especially in case of avirulent strains. An experiment was carried out to determine whether the mice inoculated with a small number of trophozoite can survive the challenge inoculation of a high virulent strain, just as in cases of the experiment stated above in which a large number of organisms of a low virulent or avirulent strain had been inoculated.

3) Resistance of mice which had been inoculated with a small number of low virulent organisms to a challenge inoculation of a high virulent strain.

Mice were divided into 3 groups. The

first group was inoculated with 1,000, the second group with 100 and the third group with only 1 trophozoite of S-273 strain (Table 4). One month after the inoculation, each group was challenged with 100,000 trophozoites of RH, SM and KM strains, respectively. None of the mice of the first and second group died of the challenge. In the third group, 4 out of 36 mice died after the challenge. All control mice which were inoculated with the same number of high virulent trophozoites died within 8 days after the challenge. Mice which survived the challenge were examined for the presence of cysts in the brain. As a routine procedure, six fresh smears were made from one brain and examined microscopically. Cysts were found from 27 out of 48 mice previously inoculated with 1,000 or 100 trophozoites. In the remaining 21 mice of these two groups, cyst was not found, but they must have been infected with S-273 strain as they could survive the challenge. HA-test turned out to be negative in 3 mice of these two groups.

Table 4 Resistance of mice infected with S-273 trophozoites for 1 month to the challenge with 1×10^5 trophozoites of highly virulent strains

Strains used for challenge	Mice chronically infected with the following numbers of trophozoites:		
	1,000	100	1
RH	0/8*	0/8	1/14
SM	0/8	0/8	1/9
KM	0/8	0/8	2/13
Total	0/24	0/24	4/36

* No. of mice died/No. of mice challenged

In the third group previously inoculated with only one trophozoite, cyst was not found in 22 of 32 mice which survived the challenge. It can be concluded that these 22 mice were also infected with S-273 strain as they could survive the challenge. In the third group, 4 died after the challenge and in 3 of them HA-test was negative and a huge number of trophozoites were found in the peritoneal cavity when they died 5-7 days after the challenge (Table 5), just as was the case in the control mice. It is highly probable from these results that the trophozoite of S-273 strain inoculated previously did not grow in these 3 mice. Remaining one mouse gave positive HA-test and died 19 days after the challenge. Trophozoite could not be found in the peritoneal cavity. The death of this mouse, therefore, cannot be attributed to the challenge and the cause of the death was not determined.

Table 5 HA-titer and days of survival of mice inoculated with only one trophozoite of S-273 and died after the challenge

Mice no.	HA titer at 1 month after inocul.	Strains used for challenge	Days of survival after challenge	Trophozoites in peritoneal cavity
1	negative	RH	7	(卅)
2	〃	KM	7	(卅)
3	〃	〃	5	(卅)
4	1 : 64	SM	19	(−)

Presence or absence of trophozoites in the peritoneal cavity is also indicated.

The titer of HA-test had no correlation with the number of trophozoite inoculated previously (Table 6). Some of the HA-test negative mice could survive the challenge. But all the mice succumbed to the challenge gave negative HA titer.

Table 6 HA-titer obtained 1 month after the inoculation with a small number of S-273 trophozoites

No. of trophozoites inoculated	No. of mice exam.	HA titer				
		negat.	1:64	1:256	1:1024	1:4096
1,000	24	2	13	4	4	1
100	14	1	4	5	10	4
1	33	3	11	7	10	2
Total	81	6	28	16	24	7

Discussion

When animals survived the acute stage of *Toxoplasma* infection, they develop an immunity which is evidenced by a subsequent challenge inoculation of a high vir-

ulent strain. Thus, a number of investigators such as Weinman (1943), Beattie (1963), Ruchman & Johansman (1948), Wolf *et al.* (1940), and Jacobs & Melton (1955) all reported the protection from death or prolongation of survival period after the challenge. According to Ueda (1950), the prolongation of the survival time after challenge becomes manifest already two weeks after the Beverley strain inoculation and the protection from death can be recognized after 4 weeks. Nakayama (1964) also reported that by the Beverley strain inoculation into mice, some evidence of protection appeared at about 2 weeks and increased to what can be considered as an effective immunity by one month. Frenkel (1952, 1956) inoculated the low virulent BDA strain into mice. Three mice thereafter were challenged every day with the high virulent CJ strain. During the first week, mice succumbed to the challenge within 3-4 days just as the control mice. The survival time gradually increased thereafter and after the 14 days of vaccination none of the vaccinated mice died of the challenge. When mice were challenged with RH strain 6-12 weeks after the vaccination with BDA strain, some of them succumbed to the challenge. According to these findings, Frenkel expressed an opinion that BDA strain developed only a partial cross immunity to RH strain. Stahl & Akao (1964) also recognized some degree of protection 2 weeks after the Beverley inoculation and it was in evidence even after 7 months. According to them, some of the mice previously infected with Beverley strain for more than one month died within one or two weeks after the challenge. It was not made clear, however, whether the death was due to the challenge or to the Beverley infection which had persisted for more than one month. Ueda (1950) reported gradual decrease in number of surviving mice after the Beverley inoculation during a period of one month after the inoculation. In our experience it is also true some of the Beverley-infected mice often succumb to the infection much later.

In the present study, it was demonstrated that the death of Beverley-infected immune mice after the challenge was due to the Beverley infection and not to the challenge (Table 1 and 2). That is to say, very few or no trophozoites were found in the mice died after the challenge, but a large number of cysts were found in the brains. It is not unreasonable to determine from the course of infection of the high and low virulent strains that the trophozoites found in the peritoneal cavity were of the high virulent strain and the cysts in the brain were of Beverley strain.

The previous inoculation of a low virulent or avirulent strain of *Toxoplasma* protected the animals from death when they were challenged with a high virulent strain with an interval of one month. This high immunity was developed not only by the inoculation of a large number of low virulent or avirulent organisms but also of a few or only one living trophozoite.

The diagnosis of human toxoplasmosis is often very difficult. The immunological tests of suspected cases are not always conclusive due to high percentage of positive titers among healthy people or to the negative titers of parasite-positive cases. Saram *et al.* (1962) also expressed a similar opinion as to the immunological tests on toxoplasmosis. The isolation of the parasite from human being is much more difficult, especially when a low virulent or avirulent strain is involved. These strains produce rather a small number of parasite in the host. Material to be examined, therefore, would contain, if any, only a few parasites. When the material is inoculated into mice, the organisms growing subsequently will be so few in number that they may be readily overlooked by the microscopic examinations. Serial subinoculation of these avirulent strains do not increase virulence to mice, producing only a few cysts in

their brains. Even in these cases, however, challenge inoculation of a high virulent strain will substantiate the infection in mice and consequently the presence of *Toxoplasma* organisms in the human material examined.

On the basis of these circumstances, the following procedures are recommended as an additional method of laboratory diagnosis of human toxoplasmosis.

a) Human material is inoculated intraperitoneally into 5 mice. Those died within one month will be examined for the presence of trophozoite or cyst. Subinoculation of their brains, livers and spleens will be made.

b) Those survived more than one month are tested by dye test or HA-test.

c) They will be challenged intraperitoneally with less than 100,000 trophozoites of high virulent strain.

d) When these mice die within a week, showing a large number of trophozoites in the peritoneal cavity and no cyst in the brain, then the human material tested is considered to have had no parasite.

e) When these mice survive the challenge or at least their survival period is much prolonged as compared with controls, it can be determined that a protection is produced by an infection from the human material whether *Toxoplasma* organisms can be found by the subsequent microscopical examinations of the mice or not.

Conclusion

1) Mice previously infected with a low virulent or avirulent strain of *Toxoplasma* for more than one month did not succumb to a challenge with a high virulent strain.
2) Those mice died after the challenge had a large number of cysts in their brains and only a few or no trophozoites in the peritoneal cavity where the challenge inoculation was made. These findings indicated that the death was not due to the challenge but to the chronic infection of the low virulent strain.
3) The protection against the challenge was fully materialized by the previous inoculation of a very small number of trophozoites of a low virulent or avirulent strain.
4) It was suggested that the protection against challenge would be useful for the laboratory diagnosis of human toxoplasmosis. Routine procedures of this method are presented.

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ヒトのトキソプラスマ感染検出の方法について

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ヒトの材料からトキソプラスマ（以下Tと略記す）を検出する場合，材料をマウスに接種してマウスから虫体を証明する方法が一般に用いられている．マウスに弱毒性の虫体の場合，マウス脳に産生されるシスト数は頗る少なく検出は容易でない．そこで次の実験成績に基づいた検出の一方法を提唱する．本実験にはすべて ICR マウスを用い，弱毒株として Beverley，C—37，S—273，S—31 株，強毒株として RH，SM，KM 株を用いた．強毒株の接種をうけたマウスは接種後1週前後で死亡するが，予め弱毒株シストの接種をうけ1月以上経過したマウスは強毒株の攻撃接種をうけても死亡しなかつた．1コのシスト中に包蔵される虫数は1万コ以上の多数である．一般的にヒトからの材料中にこのような多数の虫体が含まれているとは考えられないので，弱毒 S—273 株の栄養型虫体1,000，100及び唯1コをマウス腹腔内に接種した．接種1月後に前記強毒株をそれぞれ10万コ宛攻撃接種したところ攻撃により死亡したマウスはなかつた．本成績に基づいて次の方法を具体的に提唱する．1) 材料を数匹のマウス腹腔内に接種し1月以内に死亡したものについては型の如く栄養型，シスト型虫体の検出につとめる．2) 1月以上生存したマウスについて免疫学的に血清抗体価を測定し参考にする．同時期に10万コ以下（3,000 コで充分と考える）の強毒株栄養型虫体を以て腹腔内に接種する．3) これらマウスの腹腔内に多数の虫体が認められ死亡し，脳にシストが認められなかつた場合，被検材料はT陰性とする．4) これらの攻撃接種をうけたマウスが1月以上生存するか，又は対照に比して明らかに延命した場合，T感染による防禦が認められたものとしてマウスからT虫体検出の有無に拘らず被検材料はT陽性とする．