FOLIA PARASITOLOGICA (PRAHA) 18: 295–302, 1971.

KINETICS OF APPEARANCE OF CYSTS AND ANTIBODIES IN EXPERIMENTAL MOUSE TOXOPLASMOSIS

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Abstract. The authors studied production of complement fixing antibodies in relation to the formation of cysts of *Toxoplasma gondii*. White mice were infected with 10 or 20 cysts of Beverley strain. Traces of antibodies appeared in 2 weeks, reaching highest titres after 6–8 weeks. Cysts in the mouse brain appeared 3 weeks after the infection and reached the highest number in 6–8 weeks.

Low virulent cyst forming strains of *Toxoplasma gondii* represent a form of the parasite which is well adapted to the host. The cystogeneous strains seem to be the most frequent form in which the parasites circulate under natural conditions in populations of animal hosts. As far as the formation of natural foci of infection is concerned, Galuzo and Krivkova (1968) attach special importance to avirulent toxoplasma strains which in due course of evolution became adapted to a wide range of hosts. The term Beverley strain has been widely accepted for cystogeneous strains.

In the literature, several studies on location, distribution and persistence of cysts in various organs of experimental animals can be found. Lainson (1958, 1959) described cysts which survived for 5 years in the tissue of infected animals. Nakayama and Matsubayashi (1961) observed proliferative forms of Beverley strain in the peritoneal cavity of mice 3–4 days after intra-peritoneal inoculation. Takasu (1966) and Ito and Tsunoda (1968) detected cysts 7 and 8 to 10 days after inoculation. Savina and Zasukhin (1968) found first cysts in mouse brains after 9 days. After one month their number started to decrease.

Performing serological tests for human toxoplasmosis, it can be found that in connection with a typical disease there are usually high titres of serum antibodies, even in cases when the strain isolated from biopsy or necropsy material appears to have low virulence for mice. So, low virulent strains are able to provoke production of high antibody titres. Rommel and Müller (1963) studied kinetics of complement fixation test (CFT) in mice inoculated with a dog strain. Positive tests appeared in 14 days by when the symptoms of disease had disappeared. After 35–40 days the titres dropped to the value of 640 and then varied between 80 and 2560. Dye test titres had corresponding kinetics. Werner and Pichl (1969) state that dye test titres do not indicate the number of cysts in the host's organism.

In the following experiments with mice we studied the quantitative aspects of ki-

netics of cyst occurrence and production of complement fixing antibodies. To determine cysts we preferred the brain to other organs because they are more numerous and easier to detect in this organ.

MATERIAL AND METHODS

Two experiments were performed. In the first one we used an infectious dose of 20 cysts, whil in the second one of 10 cysts. A cystogeneous strain isolated from a hare was employed. This strain was given to us by dr. Rašín. It has been maintained in our laboratory for last 10 years by passaging it in mice every 2 to 5 months. Cysts for the experiment were obtained from brains of mice infected approximately two months earlier. The sterile brain was homogenized in physiological saline and cysts were counted in fresh unstained wet films at magnification of 100 ×. The concentration of cysts in the suspension was standardized by diluting with physiological saline so that the fixed infectious dose was contained in 0.2 ml of the liquid. Mice for the experiment were placed at random into plastic cages (35 × 25 × 25 cm) with metal cover, each containing 20 —30 individuals (females). The animals were fed with standard tablet diet ad libitum. In the first experiment, the infecting dose of 20 cysts was inoculated intraperitoneally into each of 120 mice. In the second experiment 174 mice were inoculated. The mice showed first disease symptoms after 10—14 days. In the first experiment 88 mice (= 73 %) gradually died; in the second one 114 (= 66 %), out of which 58 (= 33 %) died in 14 days and additional 36 (= 21 %) in one month. However, these data do not correspond to the exact mortality rate, because soon after the beginning of the experiments, a certain number of mice were removed for examination. Of the surviving mice 5 individuals were taken at a time for bleeding which was performed by puncture of retrobulbar venous plexus. After having killed the animals we examined the brain. Sera were tested by CFT with toxoplasma antigens. Sera were inactivated at 56 °C for 30 min. and diluted in doubling dilution series starting at a dilution of 1 : 1 (undiluted), 1 : 2, 1 : 5, 1 : 10, 1 : 20 etc. For the details we refer to preceeding publications (Jíra and Bozděch 1960, Bozděch and Jíra 1969). Examination of the brain was done by placing the whole right hemisphere, in parts, between two glass slides. Cysts were counted microscopically at magnification of 100 or 200 ×.

RESULTS

Results of experiment 1 are given in table 1. There were seven samplings — 2, 4, 6, 8, 12, 20 and 28 weeks after infection. Antibodies in low titres were already present at the first sampling. The highest ascertained titre was 640 — in four of the five mice after 8 weeks. After 28 weeks there were only 2 surviving mice each with a titre of 80. Cysts were detected after four weeks. The highest number of cysts found in one hemisphere was 419 (after 4 weeks).

Results of experiment 2 are given in table 2. There were 9 samplings — 1, 2, 3, 4, 6, 8, 12, 20 and 36 weeks after infection. Traces of antibodies in titres below 20 were ascertained 14 days after infection. The highest ascertained CF titre was 2560 in one mouse out of five after six weeks. After 36 weeks there remained only three mice whose titres were 320 or less. Cysts were detected after 3 weeks. The highest number of cysts in one hemisphere was 1160 after 6 weeks.

The results of both experiments were subjected to biometrical analysis. Geometric means were calculated from the number of cysts and titres of antibodies in samplings with positive values from all five animals. There was a considerably high range of the number of cysts. For instance, in the Vth sampling of experiment 2 the maximum number of cysts was 1160, while the minimum was 93. We applied here logarithmic transformation of measured values, because the number of cysts is compared with titres of antibodies; for this the geometric mean and logarithmic transformation represent an advantageous method. For comparative purposes, we have expressed both experiments in logarithmic transformation in the form of graphs (Figs. 1 and 2). The signi-

ficance of difference between separate samplings in both experiments were evaluated by analysis of variance as well as by Duncan's range test. Numerical operations were performed by an electronic computer according to a programme fixed by the Mathematical Centre. Results of these tests are given at tables 3 and 4 in a way which is usual in the Duncan's test. In experiment 1, the number of cysts did not differ in samplings VI, V and II at 1 per cent significance level, while samplings III and IV differed from one another. Results of serological examination of samplings V, III and IV differed at 5 per cent significance level. In experiment 2, the number of cysts and the result of serological examinations of sampling V differed at 5 per cent significance level.

Table 1. Experiment 1 — number of cysts of *Toxoplasma gondii* in mouse brains (right hemisphere) and titres of complement fixing antibodies. Infectious dose 20 cysts.

I Sampling: 2 weeks			II Sampling: 4 weeks		
Mouse number	Number of cysts	CF titre	Mouse number	Number of cysts	CF titre
1	0	negative	6	176	80
2	0	negative	7	69	10
3	0	1	8	79	10
4	0	10	9	419	40
5	0	negative	10	18	80
III Sampling: 6 weeks			**IV Sampling: 8 weeks**		
Mouse number	Number of cysts	CF titre	Mouse number	Number of cysts	CF titre
11	143	640	16	251	640
12	305	640	17	236	640
13	21	80	18	245	640
14	201	80	19	32	320
15	158	320	20	221	640
V Sampling: 12 weeks			**VI Sampling: 20 weeks**		
Mouse number	Number of cysts	CF titre	Mouse number	Number of cysts	CF titre
21	152	320	26	58	80
22	28	160	27	10	40
23	134	160	28	6	160
24	138	160	29	10	40
25	16	320	30	16	80

VII Sampling: 28 weeks		
Mouse number	Number of cysts	CF titre
31	342	80
32	44	80

Table 2. Experiment 2 — number of cysts of *Toxoplasma gondii* in mouse brains (right hemisphere) and titres of complement fixing antibodies. Infectious dose 10 cysts.

I Sampling: 1 week			II Sampling: 2 weeks		
Mouse number	Number of cysts	CF titre	Mouse number	Number of cysts	CF titre
1	0	negative	6	0	negative
2	0	negative	7	0	20
3	0	negative	8	0	2
4	1	negative	9	0	10
5	0	negative	10	0	negative
III Sampling: 3 weeks			**IV Sampling: 4 weeks**		
Mouse number	Number of cysts	CF titre	Mouse number	Number of cysts	CF titre
11	178	1280	16	95	320
12	146	160	17	114	320
13	42	320	18	61	160
14	23	320	19	28	160
15	16	320	20	36	160
V Sampling: 6 weeks			**VI Sampling: 8 weeks**		
Mouse number	Number of cysts	CF titre	Mouse number	Number of cysts	CF titre
21	210	2560	26	11	640
22	470	1280	27	26	320
23	93	1280	28	24	160
24	383	1280	29	36	1280
25	1160	320	30	18	160
VII Sampling: 12 weeks			**VIII Sampling: 20 weeks**		
Mouse number	Number of cysts	CF titre	Mouse number	Number of cysts	CF titre
31	12	160	36	432	320
32	512	160	37	11	80
33	9	80	38	15	80
34	750	640	39	26	160
35	33	320	40	245	640

IX Sampling: 36 weeks		
Mouse number	Number of cysts	CF titre
41	6	80
42	178	80
43	39	320

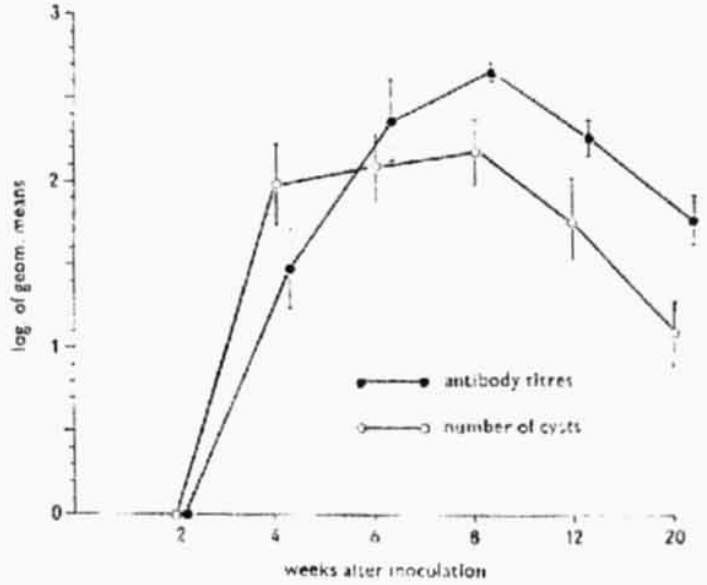

Fig. 1. Kinetics in appearance of cysts and complement fixing antibodies. Infectious dose 20 cysts. Each point represents the geometrical mean of five mice. Vertical lines indicate standard deviation.

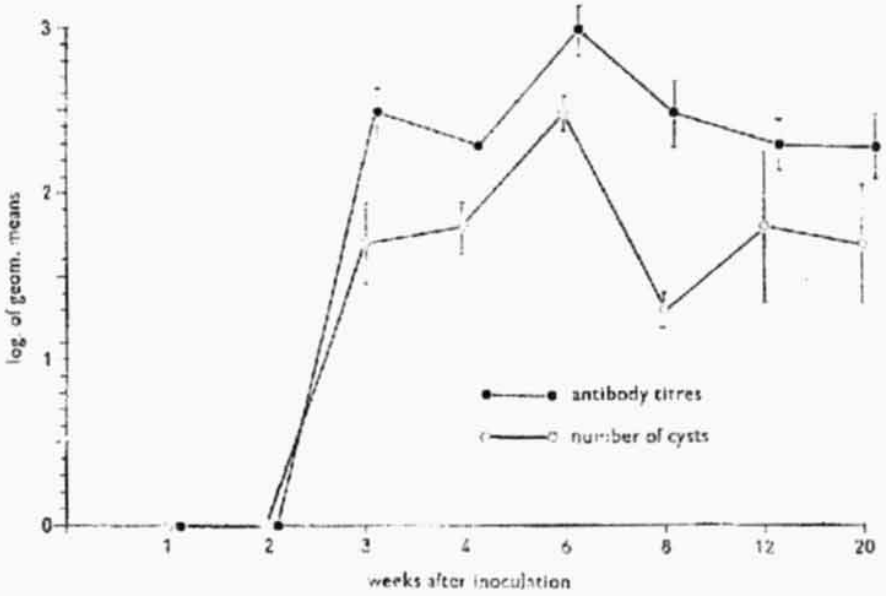

Fig. 2. Kinetics in appearance of cysts and complement fixing antibodies. Infectious dose 10 cysts. Each point represents the geometrical mean of five mice. Vertical lines indicate standard deviation.

Table 3. Experiment 1—infectious dose 20 cysts. Any two means underscored by the full line are not different from one another at the 1 per cent significance level, by the dotted line at the 5 per cent significance level.

Sampling number	VI	V	II	III	IV
Mean number of cysts	14	66	94	127	160
Sampling number	II	VI	V	III	IV
Mean number of titres	34	68	211	243	557

Table 4. Experiment 2 — infectious dose 10 cysts. The legend see Table 3.

Sampling number	VI	III	VIII	IV	VII	V
Mean number of cysts	21	53	54	58	67	333
Sampling number	VIII	IV	VII	III	VI	V
Mean number of titres	184	211	212	367	368	1115

DISCUSSION

It is known that Beverley strains of *Toxoplasma gondii* have often, if not always, a certain degree of pathogenicity. After inoculation the mice show symptoms of disease and a certain percentage of animals die. Rommel and Müller (1963) observed in their experiments disappearance of clinical symptoms after 14 days. Werner and Egger (1968) investigated differences in the mortality rate of mice infected with several cystogeneous strains. After i. p. inoculation, the mortality rate varied between 95% and 0. The maximum was reached after 10 days. After oral infection the mortality rate was considerably higher. On the basis of our experiments in which three quarters of infected mice died, as well as on the basis of our experience with other cystogeneous strains, we come to the conclusion that the term "avirulent" is not identical with the term "cystogeneous". The phenomenon of real nonpathogenicity of a toxoplasma strain seems to be very exceptional.

Another aspect of the investigation of cystogeneous strains consists in differences in the size of cysts found during a longitudinal follow-up. Beverley (1958) observed fairly uniform growth of cysts in mouse brain up to about 10 weeks, followed by a great variation in the average of cyst volume. He assumes that in some mice mature cysts rupture, liberate free forms and small cysts of second generation are formed. Werner and Piehl (1969) demonstrated in their investigations a uniform developmental phase between the 6th and 8th month after infection together with a similarity of the mean values of antibody titre. Van Thiel (1966), on the basis of van der Waaij's experiments, doubts the rupture of cysts on account of findings that the growth curve of cysts in the brains of white mice inoculated with the low virulent Burk strain is a hyperbolic tangent. When periodic bursting would take place, the growth curve would not have been a curved line, but a jagged line.

Our observations indicate that individual cysts vary widely in size and tend to be clumped or aggregated together. In our case the diameter of cysts varied from 7.5×8.5 to 91×104 μ. Aggregates of small cysts appeared in the 8th and 12th weeks. Numerical representation of this observation consists in the range of the number of cysts in individual samplings, as well as in the variance which is higher than the mean. For the interpretation of series in which the variance was higher than the mean, several distributions were proposed. One of them is the negative binomial distribution (Weber 1967). The contagious distribution, the so called "Neyman distribution" (Neyman 1939), too, could be taken into consideration. Its name is derived from the distribution of probability in epidemics, but it meets a wider application in biology, particularly in entomology, microbiology and botany (Beall and Rescia 1953: Greig—Smith 1964). It is suitable for the distribution in which individuals tend to be clumped or aggre-

gated together, sampling of which gives an excess of blanks and high values compared with random expectation. The term "overdistribution" is being used here. On the basis of our experiments, we are not yet able to determine the characteristics of the distribution of cysts. Occurrence of individual animals with comparatively low and high number of cysts supports the hypothesis stating that in the group of five mice individual sampling is not homogeneous and thus the growth cycle in individual infection phase is not equal. Technically it is very difficult to obtain information on the number of cysts from a high number of mice in the same infection phase. It is also impossible to get successive data from the same animal. The main aspect of our experiment consisted in quantitative relations between the cyst formation in the brain and the production of antibodies. Due to technical difficulties we obtained data from one (right) hemisphere only. As a result of the first experiment it has been found that the number of cysts reaches statistically significant peak level after 6 and 8 weeks, the decrease starts after 12 weeks. The peak level of production of antibodies has been reached after 8 weeks followed by the decrease. In experiment 2, with a smaller infectious dose, we have come to the conclusion that both values reached the peak level after 6 weeks. Statistical differences between the samplings were not so significant here. In our opinion, it is necessary to extend the experiments to several cystogeneous strains of various degrees of virulence if one wants to come to more general conclusions. We also assume that the problem of the size of cysts will require further biometrical investigations.

We have ascertained a particular correlation between kinetics of antibody production and dynamics of cyst formation. The number of cysts in mouse brains may be considered as an indicator of antibody level and viceversa. However, we will not interpret this correlation as a causal one in the sense that cysts in brain tissue stimulate the antibody response. The previleged status of the brain seems to be result of the absence of lymphatic vessels, so that there is no pathway for transmission of an antigenic stimulus to a seat of response (Billingham and Silvers 1965). The effectiveness of the hypothetical "blood-brain" barrier for regulating the selective passage between blood and the central nervous system has been debated for a number of years (Weiser et al. 1969). Homografts are often well tolerated when grafted into the normal brain, presumably since no immune response is forthcoming (Humphrey and White 1970). A successful parasite can be regarded as a successful graft that does not stimulate a rejection response on the part of the host (Kagan 1967). It seems likely that in the case of toxoplasmosis the antibodies are produced in cells of extracerebral tissues and the low virulent toxoplasma parasite is repelled behind the blood-brain barrier where it can multiply easily. Moreover, the imune phenomenon like "molecular mimicry" or "eclipsed antigens" can here be taken into consideration.

Acknowledgement: The authors wish to express their thanks to Professor J. K. A. Beverley, University of Sheffield, for reading the manuscript of this paper.

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Received 6 November 1970.

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