Tropical Medicine, 10 (2) : 72~80, Sept. 1968

A Method for Separating Cysts of *Toxoplasma gondii* from the Infected Mouse Brains by Multi-layer Centrifugation with Gum Arabic Solution

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(Received for publication July 6, 1968)

Abstract

A multi-layer centrifugation technic with Gum arabic solution was examined with the object of purely separating cysts of *Toxoplasma gondii* from infected mouse brains.

This paper outlines the preparations of infected mouse brain emulsions and of the Gum arabic solution having a certain specific gravity, and the experimental results on the recovery rate of cysts, the removal rate of brain tissue and the infectivity of cysts are also reported. The results followed multi-layer centrifugation with Gum arabic solution with a sp. gravity of 1.050 and 1.070 at 1,000 G for 15 min. were quite satisfactory.

Introduction

Up to the present time, the peritoneal fluids or brains of mice infected with *Toxoplasma gondii* have been principally used in a variety of works on this parasite. It might be needless to state that use of the parasites purely separated from the host animal tissue would make innumerable contributions to the advance of knowledge in research of Toxoplasmosis.

Regarding the methods for separating the proliferative form of this parasite, reports were published by Behrens and Geissler (1954), Fulton and Spooner(1957), Westphal (1958), Tsunematsu (1960) and, Lycke and Lund (1964), whereas no report has been so far presented on the separation technic of the cyst form.

For preparing the pure suspension of proliferative forms from the infected animal ascites, Fulton and Spooner (1957) used a filtration method with a sintered

Contribution No. 522 from the Institute for Tropical Medicine, Nagasaki University

glass filter with a porosity of 15 to 40 microns. Westphal (1958) described a method of ultrasonic treatment, Tsunematsu (1960) reported a combined method of sonic oscillation for a short time followed by tryptic digestion and Lycke *et al.* (1964) compared both methods of filtration and centrifugation. It was, however, noticed that ultrasonic or sonic vibration of the parasites probably caused a cellular damage to them and trypsin treatment might exert some harmful influence to the parasites. Furthermore, it was observed that considerable amounts of particulate cell debris or minute fibrous components still remained in the parasite fraction prepared by any of methods described above. Accordingly, any method provided for the separation of proliferative forms could not be considered adequate for separating cysts and infected organ tissues.

In this experiment, attempts were first made to separate cysts and infected mouse brain tissues by differential centrifugation and by the digestion method with trypsin, although all had resulted in failure. Subsequently, the multi-layer centrifugation method with Gum arabic solution which had been recommended by Kimura *et al.* (1960) for the separation of leucocytes from mammalian blood, was examined and the most interesting results were obtained. The technic and results are briefly described in this paper and the detailed data will be reported elsewhere.

Materials and Methods

Materials

Toxoplasma parasites used were the Beverley strain and mice were approximately 20 gm in weight. Gum arabic powders (Japanese pharmacopoeia) is a product of Sanwa Kako Pharm. Co.

Methods

I. *Preparation of a cyst suspension (the initial sample) from the infected mouse brains*

Four or five infected mouse brains were collected from mice which had been inoculated intraperitoneally with 20 cysts of the Beverley strain in 0.2 ml of Hanks' balanced salt solution and sacrificed on the 30th day of infection. These brains, which were presumably harboring numbers of cysts by the time of their excision, were washed in saline to remove blood and weighed. Then, they were ground in a mortar and were made into a 1% brain emulsion by adding Hanks' solution. This emulsion was repeatedly spouted out of a pipette in a flask to liberate the cysts still remaining confined in tissue masses. It was further passed through two sheets of sterile gauze to remove rough tissue masses and fibrous debris from it.

The brain emulsion thus prepared was regarded as a cyst suspension in which numbers of cysts were evenly floating, and was called the initial sample in contrast with the final sample which was a suspension of the cysts separated by multi-layer centrifugation with Gum arabic solution. The entire procedure of this preparation could be done, when necessary,

aseptically.

A small portion of this sample was used for smear preparation, cyst counting and infectivity test by the methods described as follows.

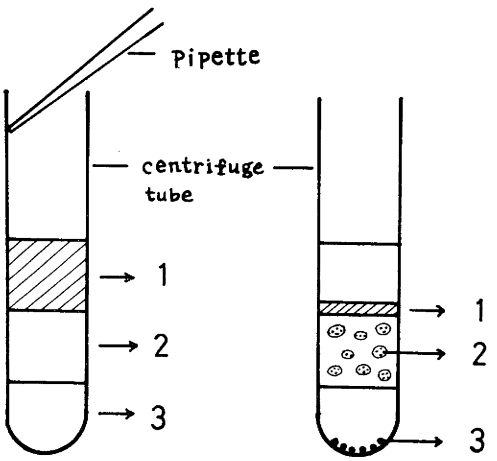
II. *Preparation of Gum arabic solution for cyst separation*

Preparation of Gum arabic solution for separating cysts from the initial sample was carried out as follows : Gum arabic powders of 2,8,16,24,32,40 & 48 gm were each dissolved in the flasks containing 100 ml saline by stirring them with glass rods in boiling water. The solution were centrifuged at 17,000 G for 30 min. to sediment undissolved dregs. Each supernatant while being kept at 15°C, was adjusted to the specific gravity of a designated degree by Böhme's hydrometer by adding saline to it and also the pH to 7.4 with saturated NaOH solution. Specific gravities of the solutions ranged from 1.010 to 1.130 for every 0.02 degrees. The solutions were autoclaved at 121°C for 15 min. and stored in a refrigerator until used.

III. *Fractional separation of cysts from the initial sample by multi-layer centrifugation*

Siliconized glass centrifuge tubes of 0.6 ml in capacity, 1.0 cm in inner diameter and 11.0 cm in height were used for the fractional separation of cysts from the initial sample by multi-layer centrifugation with Gum arabic solution. In a preliminary examination, it was recognized that a double-layer of Gum arabic solutions with a sp. gravity of 1.050 and 1.070 was most effectively applicable to this method. Therefore, only the results

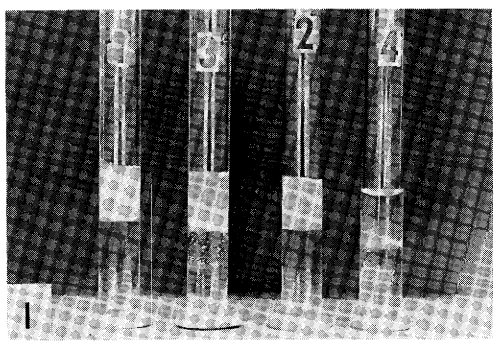
Fig. 1. Schematic illustration of a multi-layer centrifugation of cysts with Gum arabic solution



left : before centrifugation
(1) : Brain emulsion (initial sample)
(2) : Solution "A" (Gum arabic solution of sp. gravity 1.050)
(3) : Solution "B" (Gum arabic solution of sp. gravity 1.070)

right : after centrifugatcn
(1) : Thin layer of compressed brain tissues
(2) : Brain tissue masses floating in solution "A"
(3) : Cyst fraction sedimented

Photo. 1 : Multi-layer columns before (1 and 2) and after (3 and 4) centrifugation in the Gum arabic method (see Fig. 1)
Column 1 and 3 : 10% brain emulsion was used.
Column 2 and 4 : 1% brain emulsion was used.



with both of these solutions are described in this paper. In the following description, the solutions with a sp. gravity of 1.050 and 1.070 were expressed as solutions "A" and "B".

A double-layer, in which the heavier solution "B" formed a bottom layer and "A" a top layer, was prepared in a centrifuge tube by pouring each 1.0 ml of both solutions into it and 1.0 ml of the initial sample was further layered on it. The pouring of these solutions into a tube was done by dropping them with care along the inner wall of the tube with a pipette in order to form clear boundary lines between the layers.

The layered solutions in a tube were spun at 1,000 G for 15 min. by a horizontal head centrifuge. The initial sample was distinctly separated by this centrifugation into three portions as illustrated in Fig. 1 and Photo. 1 ; 1) a thin layer composed of compressed brain tissues at the junction between "A" and the brain emulsion, 2) fine tissue masses floating in "A" and 3) a cyst fraction sedimented on the bottom of tube. The supernatant fluid was carefully removed by a pipette and the sediment containing cysts was washed twice with Hanks' solution by spinning at 50 G for 5 min. The final sediment was resuspended in 1.0 ml of Hanks' solution and this was the final sample in which cysts were withdrawn from the initial sample.

IV. *Examination of the cysts collected*

The final sample was made up with Hanks' solution to the same volume to that the initial sample used so that the estimations of the recovery rate of cysts and the amount of admixed brain tissue debris might be readily made. The infectivity of the cysts withdrawn into the final sample was likewise examined by the mouse inoculation test.

1) Recovery rate of cysts : This rate was obtained by the ratio of total cyst numbers in the final and initial samples. In practice, it could, however, be readily obtained from the total numbers of cysts counted in 0.1 ml of samples with a microscope.

2) Removal rate of brain tissue (Estimation of the amount of admixed brain tissue components in the final sample) :

The initial sample was regarded as a mouse brain emulsion of high concentration containing numerous cysts. Accordingly, an important problem was the amount of brain tissue components remaining unseparated in the final sample by this technic. First, the removal rate of brain tissue was roughly estimated by comparing the quantity of tissue debris in both the initial and final samples on microscopical preparations. Next, turbidimetrical measurement at 580 mμ was done of the final samples by the Shimazu, Bausch and Lomb Colorimeter, Type Spectronic 20. The concentration of admixed brain components in the samples was estimated from the standard transmittance curve which was previously made for the determining the concentrations of mouse brain emulsions, although the concentrations thus determined might not indicate the correct ones of brain tissue components in the final samples because numbers of cysts co-existed in them.

3) Infectivity test of the cysts collected in the final sample :

Each approximately 180 cysts taken from the initial and final samples were inoculated intraperitoneally into a mouse and the brains of the inoculated mice were examined for cysts on the 30th day after inoculation. In this experiment, cysts collected by this method were likewise examined for their survival, when preserved in Gum arabic solution of sp. gravity 1.070 at 5°C.

Results

I. *Examination on applicability of some solutions to the separation method*

Copper sulfate, sucrose and Gum arabic solutions in concentrations ranging from sp. gravity of 1.010 to 1.130 were preliminarily examined for comparing their applicability to the separation of cysts. With Copper sulfate solution, no achievement was obtained in separating cysts. On the other hand, cysts were sufficiently separated and collected on the bottom of the tube by multi-layer centrifugation with sucrose solutions of sp. gravity 1.070 and 1.090 at 650 G for 15 min. The recovery rate of cysts, however, was found usually lower than that in the Gum arabic method and a considerable amount of admixed brain tissue debris was observed in the cyst fraction. Whereas, in the multi-layer centrifugation method at 1,000 G for 15 min. with Gum arabic solutions of sp. gravity 1.050 and 1.070, the recovery rate of cysts was very high and the amount of admixed brain tissue debris was little.

II. *Recovery rate of cysts in the Gum arabic method*

Attempts were first made to find out the differences brought forth in the recovery rate of cysts when different concentrations of infected mouse brain emulsions were used. As shown in Table 1, 1% brain emulsion was found to give a higher recovery rate of cysts than 5 or 10% did, and 92.3% in the average of 10 experiments.

Table 1. Recovery rates of cysts given by the Gum arabic method when 1, 5 and 10% emulsions of the infected brains were used as initial samples

	Recovery rate of cysts				
Conc. of brain emulsion	10%	5 %	1 %		
	%	%	*	**	%
Exp. 1	40	60	80	80	100
2	44	50	45	40	89
3	50	72	135	120	90
4	45	62	175	170	97
5	33	45	300	270	90
6	43	70	345	310	90
7	34	54	115	100	87
8	52	62	175	170	97
9	40	58	245	220	90
10	46	64	260	250	96
	33~52% (range)	45~72% (range)	1875	1730	92.3% (average)

* No. of cysts in an initial sample
** No. of cysts in a final sample

III. *Removal rate of brain tissue (Estimation of admixed brain tissue components in the final sample)*

The final samples were, in general, a little turbid or nearly transparent as water. It was understood from Photos. 2 and 3 that brain tissue debris became very scanty in the final samples. By turbidimetrical measurement, the concen-

Photo. 2 : A cyst in 10% brain emulsion before centrifugation, 400X.

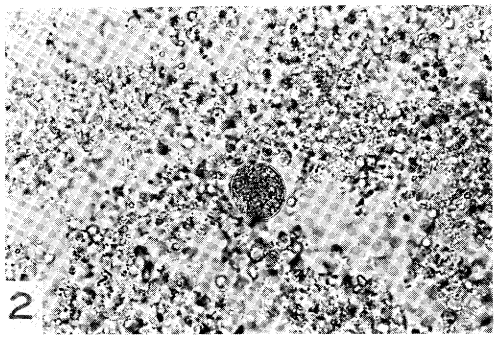
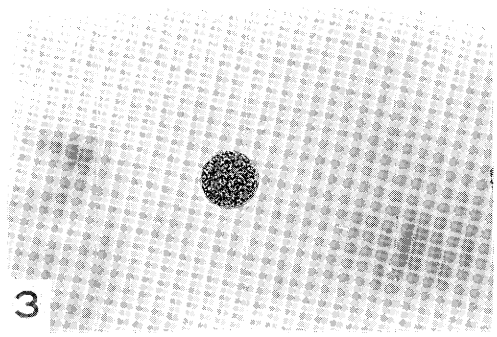


Photo. 3: A cyst separated from 10% brain emulsion by the Gum arabic method, 400X.



tration of the final sample derived from 1% emulsion of the infected brains was found to be equivalent to less than 0.01% brain emulsion and that derived from 10% emulsion was equivalent to 0.2 to 0.3% brain emulsion. From these findings, it could be considered that it might be possible to obtain a 99% removal rate of brain tissue, when 1% brain emulsion was used as an initial sample.

IV. *Infectivity test for mice of the cysts separated by the Gum arabic method*

Approximately 180 cysts respectively taken from the initial and final samples were inoculated into each of 5 mice in a group. As shown in Table 2, cysts were detectable in the brains of all mice in both groups on the 30th day after inoculation, namely, it was confirmed that the infectivity of the cysts collected by this method was kept unchanged during the course of this separation method.

In another experiment, cysts collected by our method were preserved further at 5°C in Gum arabic solution of sp. gravity 1.070 for the survival test of them. Table 2 also showed that the cysts were capable of survival as long as 35 days without losing their infectivity for mice.

Table 2. Infectivity test by the mouse inoculation method of the cysts collected by the Gum arabic method and ones further preserved in Gum arabic solution, sp. gravity of 1.070 at 5°C

Time examined	No. of mice inoculated	No. of mice cyst-positive
Before centrifugation (initial sample)	5	5
After centrifugation (final sample)	5	5
7 days after separation	3	3
14 〃	3	3
21 〃	3	3
35 〃	3	3

Note ; Mice were intraperitoneally inoculated with 180 cysts and examined for cysts in the brains 30 days after inoculation.

Discussion

There have been several studies reported on the separation method of proliferative forms of *Toxoplasma gondii* as here described, however, no previous report has described the pure separation of cysts from the infected animal organs. In

1967, the Gum arabic method for the separation of cysts was reported by the authors at the protozoologist meeting in Japan. Very recently, a similar technic to that described in this paper was stated by Iseki *et al.* (1968). Studies on biological characters of cysts with use of the purely separated ones from the infected organs would be expected to contribute invaluably knowledge in medical research of Toxoplasmosis.

As to methods for the separation and collection of animal cells from other tissue components by the multi-layer centrifugation technic, few reports have so far been published in which Gum arabic, sucrose, egg albumine, dried blood plasma or polyvinylpyrrolidone was used for a multi-layer solution. In this study, Gum arabic solution was principally examined as recommended by Kimura *et al.* (1960) for the separation of leucocytes from other blood cells.

It was demonstrated that multi-layer centrifugation with sucrose solution was likewise applicable to the cyst separation. With sucrose, however, the recovery rate of cysts was lower and the removal rate of brain tissue from the initial brain emulsion was higher than with Gum arabic. Since it was clearly shown in Table 3 that the viscosity of Gum arabic solution was much higher than that of sucrose solution at the same sp. gravity, the viscosity of the solution might probably play a significant role in the course of the cyst separation similarly as the sp. gravity.

Generally speaking, the recovery rate of cysts and the removal rate of brain tissue from the initial sample showed a contradictory tendency towards each other. Accordingly, an attempt to raise the recovery rate of cysts and to minimize the amount of admixed tissue components should be performed by means of selecting the kind and the sp. gravity of the solution and determining the G-value and time of centrifugation. The multi-layer centrifugation method with Gum arabic solutions of sp. gravity 1.050 and 1.070 at 1,000 G for 15 min. could be considered a satisfactory one to meet the above-mentioned requirements to the maximum extent.

Table 3. Comparison of the viscosity of Gum arabic and sucrose solutions

Solutions	Sp. gravity	Viscosity
Sucrose	1.080	2.2
	1.100	>7
Gum arabic	1.050	5.5
	1.070	>7

Viscosity was measured by Hess' viscosimeter at 15°C of solution temperature.

In this Gum arabic method, when 1% emulsions of the infected brains were used as an initial sample, it gave a higher value in the removal rate of brain tissue as well as in the recovery rate of cysts than 10% did. From the results on the recovery rate of cysts, it was understood that a brain emulsion of high concentration would obstruct the sedimentation of cysts during centrifugation. Although it would be impossible to completely exclude any admixture of brain tissue components in the cyst fraction, it was reliably noticlable that the removal rate was approximately 99% in the experiment

done with 1% emulsions. The facts that this simple technic could be done with an aseptic operation and did not affect the infectivity of cysts suggested a broad application of this technic to the future works of cysts.

Summary

A simple technic for separating cysts from the infected mouse brains by multi-layer centrifugation with Gum arabic solution was examined and the following results were obtained.

1. The Gum arabic method as compared with the sucrose method, gave a high recovery rate of cysts as well as a high removal rate of brain tissue components from the initial brain emulsion.

2. Satisfactory results were obtained by multi-layer centrifugation with Gum arabic solutions of sp. gravity 1.050 and 1.070 at 1,000 G for 15 min.

3. When 1% emulsion of the infected brains was used as an initial sample, it was demonstrated that the recovery rate of cysts averaged 92.3% and the removal rate of brain tissue was approximately 99% by turbidimetrical measurement.

4. Cysts separated from the infected brains by this technic demonstrated the unchanged infectivity for mice.

Acknowledgement

The authors wish to express their sincere appreciation to Dr. T. Shozawa of this institute for his kind help in taking microphotographs of specimens and also to Dr. J. D. Kirshbaum, Atomic Bomb Casualty Commission in Nagasaki, for his favorable aid in composing English sentences.

The major part of this experiment was reported at the lst annual meeting of the Japanese Society of Protozoology held in Tokyo on December 3, 1967.

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アラビアゴム重層遠心沈澱による *Toxoplasma gondii* 感染マウス脳からのシストの分離法について

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摘　　要

著者らはアラビアゴム重層法を用いて ***Toxoplasma gondii*** の Beverley 株感染マウスの脳からシストを分離する実験を行ない，以下の成績を得た．

1. アラビアゴム重層法は，蔗糖重層法と比較して分離前の脳乳剤から高いシスト回収率と高度の脳組織除去率が得られた．

2. 比重，1.050および1.070のアラビアゴム分離液を用い，1,000G15分間の遠心沈澱によって満足すべき結果が得られた．

3. 1％脳乳剤をアラビアゴム重層法によって遠心沈澱し，平均92.3％のシスト回収率と約99％の脳組織除去率（比濁法により測定）を得た．

4. 本法によって分離されたシストはマウスに対する感染性に著明な変状が認められなかった．

本法は無菌的操作も容易であり，分離術式も簡便であることから今後感染マウスの脳からシストを分離する実験に広く応用できるものと考えられる．