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With 1 plate and 1 figure in the text

Separation of *Isospora* (*Toxoplasma*) *gondii* cysts and cystozoites from mouse brain tissue by continuous density-gradient centrifugation

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SUMMARY

A simple, quick and reproducible method consisting of density-gradient centrifugation of homogenized infected mouse brain tissue on Percoll is described for the isolation and purification of cysts of *Isospora* (*Toxoplasma*) *gondii*. A 100% recovery of cysts, with 74·2% in a single fraction with a specific gravity of 1·056, was obtained by overlaying homogenates of infected mouse brains on a pre-formed Percoll gradient and centrifugation at low *g* forces. With this procedure recovery was independent of the age of the cysts. Titration of purified cystozoites showed there to be no loss of infectivity.

INTRODUCTION

In order to facilitate cloning of cysts and cystozoites of *Isospora* (*Toxoplasma*) *gondii* by micromanipulation a suitable method was sought for the separation of cysts from mouse brain tissue. So far two methods have been described. Nakabayashi & Motomura (1968) used discontinuous 2-step gradients of Gum Arabic or sucrose solutions. With Gum Arabic they were able to remove 99% of the brain material, with a recovery of cysts of more than 90%. However, they were only successful if they used small amounts, for example, a 1% brain emulsion, as the initial sample. Moreover, their procedure was very time consuming.

Pettersen (1979) used a rabbit anti-mouse immunoglobulin preparation to purify cystozoites from comminuted infected mouse brains that had been treated intact with a pepsin/HCl solution for 5 min at 37 °C. The method was rather laborious, and although the yield of cysts was not given, it may be presumed to have been low.

In this paper a quick method for the high yield separation of cysts and cystozoites from large amounts of brain tissue by centrifugation on a continuous density gradient of Percoll is described.

MATERIALS AND METHODS

Preparation of crude cyst suspensions

The pooled brains of 3 mice which had been inoculated intraperitoneally (i.p.) with the KB-strain of *I.* (*T.*) *gondii* (Overdulve, 1978) were homogenized with 7–10 complete strokes in 5 ml of 0·9 % NaCl in a Potter tube. Ten 10 μl droplets of the homogenate (25 %, w/v) were examined microscopically (40 × objective) and the average number of cysts per droplet, multiplied by the total volume of the homogenate gave the number of cysts in the brain suspension.

Purification of cysts

Cysts were separated from brain material by continuous density-gradient centrifugation using Percoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The gradient was made by centrifuging 30 ml of a 45 % (v/v) isotonic suspension of Percoll in phosphate-buffered saline (PBS), pH 7·2 for 20 min at 27 138 $\boldsymbol{g}$ in 50 ml polycarbonate tubes, using a Sorvall RC 2–B centrifuge with a fixed angle SS-34 rotor.

Two different methods of separation were performed. In procedure A, 5 ml of a brain suspension containing *I.*(*T.*)*gondii* cysts were mixed with a 45 % Percoll suspension prior to centrifugation in the Sorvall. In procedure B, 5 ml of a brain suspension were carefully overlaid on top of a pre-formed Percoll gradient and recentrifuged for 15 min at 1250 $\boldsymbol{g}$ in a Hereaus Christ 03400 centrifuge with a swinging bucket rotor.

A control tube with markerbeads (Pharmacia) was used to estimate the precise specific gravity (sp.gr.) of the gradient, and a total of 7 fractions could be identified.

All fractions, except fraction 3, were sucked up in 2 ml syringes through a long steel needle (Ø: 2 mm) inserted onto the bottom of the tube. Fraction 3 was collected in the same way in a 20 ml syringe. The number of cysts in each fraction was counted microscopically as described above. If needed for accurate counting, the fractions were concentrated by recentrifugation at 1250 $\boldsymbol{g}$ for 5 min and the pellets were resuspended in a small volume of PBS. Some cyst suspensions from fraction 3 were incubated with haemolysin (Tryon, Weidner, Larson & Hart, 1978) to examine whether a still higher degree of purification was feasible.

Infectivity of purified cysts and cystozoites

Cysts were freed from particles of Percoll by centrifugation in PBS for 10 min at 150 $\boldsymbol{g}$ and resuspension of the pellet in PBS. Cystozoites were liberated from the cysts by adding the resuspended pellet to 1 ml of pepsin/HCl at pH 1·3 for 5 min at 37 °C (Work, 1971). All glassware and centrifuge tubes were siliconized with siliclad (Clay Adams Inc., New York, USA). Purified cysts were inoculated i.p. into 2 groups each containing 6 mice, using inocula containing an average of 100 or 10 cysts, respectively. Six weeks post-infection (p.i.) brains were removed for microscopical examination for the presence of cysts. Cystozoites were titrated by i.p. inoculation in mice, using 10-fold serial dilutions in PBS to determine the ID_{63} (Overdulve & Antonisse, 1970). Five 10-fold dilutions, from 10^2 to 10^{-2} parasites/

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inoculum, and groups of 20 mice/dilution were used. At three weeks p.i. the sera of mice were diluted 1:16 (final dilutions) and were tested for the presence of specific antibodies by the Sabin–Feldman dye test (SFT) (Overdulve, 1978).

RESULTS

Recovery of purified cysts

In all experiments almost complete separation of cysts from mouse brain tissue was achieved (Pl. 1). Mouse brain tissue always layered at the top of the tube. A small ring of erythrocytes was found at the bottom of the tube.

The sp.gr. profiles of the Percoll gradients, generated by high-speed centrifugation, were typically flat, S-shaped and the middle portion of the tube, with an almost constant sp.gr. of 1·056, could be sampled as a single fraction (Fig. 1, fraction 3).

Table 1 shows concentrations and yields obtained in 5 separate experiments using procedure A and in 2 experiments using procedure B. With procedure A, a mean recovery of $64{\cdot}1 \pm 17{\cdot}2\,\%$ was obtained, with $32{\cdot}6 \pm 8{\cdot}3\,\%$ of the parasites in fraction 3, when cysts of 6–12-week-old infections were used, and a recovery of $99{\cdot}2 \pm 2{\cdot}2\,\%$, with $85{\cdot}5 \pm 10{\cdot}0\,\%$ of the parasites in fraction 3, when cysts of 23·5-week-old infections were used. Cystozoites were sometimes concentrated at one pole of the cyst after centrifugation.

With procedure B, a 100 % recovery was obtained, with $74{\cdot}2 \pm 12{\cdot}1\,\%$ of the parasites in fraction 3 (Fig. 1). There was no difference in the recovery rate between young and old infections and no effect of centrifugation on cyst morphology was seen. Further purification of intact cysts by lysis of the small amount of remaining brain material in fraction 3 with haemolysin proved impossible, because the haemolysin lysed the cyst walls. The infectivity of cystozoites excysted with haemolysin was not titrated but their survival for at least 1 h in haemolysin was confirmed when 3 mice, inoculated i.p. with these cystozoites, had cysts in their brains 6 weeks p.i.

Infectivity studies

Cysts and cystozoites used for titration were obtained from fraction 3 according to procedure B.

(i) *Cysts*

All mice inoculated with either 100 or 10 intact cysts showed cysts in their brains 6 weeks p.i.

(ii) *Cystozoites*

The results of the titration of purified cystozoites are given in Table 2. In the dilution containing approximately 1 cystozoite/inoculum the actual mean number of inoculated parasites, as counted with a Turck haemocytometer, was $\bar{x} = 1{\cdot}003 \pm 0{\cdot}137$ ($\bar{x} \pm 2$ S.E.). The number of infective parasites/inoculum (μ_0) in the same dilution, estimated by titration, lay, with a same confidence of 95 %, between

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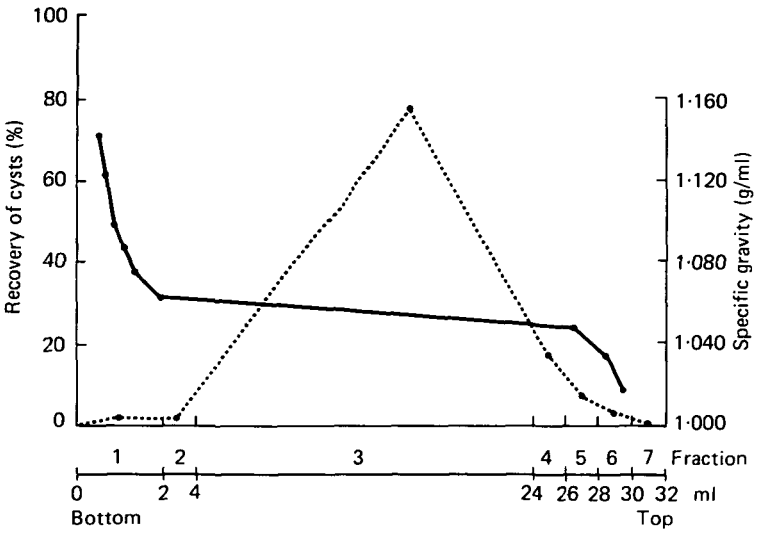

Fig. 1. Fractionation of homogenate of infected mouse brains by density-gradient centrifugation on Percoll for 15 min at 1250 *g* in a Hereaus Christ 03400 centrifuge with a swinging bucket rotor. Five ml of homogenate in 0·9% NaCl were laid on top of a pre-formed gradient of 30 ml of a 45% (v/v) isotonic suspension of Percoll in PBS, pH 7·2. Percoll gradients were made by centrifugation for 20 min at 27138 *g* in a Sorvall RC 2–B centrifuge with a fixed angle SS–34 rotor. Duration of infection 12 weeks. Data represent the means of 2 tubes of the same run. Density (——); cysts (· · · ·).

Table 1. *Yields of cysts of* Isospora (Toxoplasma) gondii *after centrifugation of infected mouse brain homogenate on a* 45% *Percoll gradient*

			Cysts	
Procedure	No. of tubes	Age in weeks	Initial no. of cysts added (± S.E.)	No. recovered in fraction 3 (± S.E.)
A	1	6	390 ± 100	96 ± 30
A	1	6	800 ± 249	286 ± 74
A	2	8	2050 ± 241	625 ± 56 578 ± 117
A	2	12	1450 ± 302	688 ± 116 396 ± 61
A	3	23·5	7500 ± 559	6620 ± 232 7028 ± 150 5580 ± 302
B	2	12	1450 ± 302	1320 ± 114 917 ± 200
B	2	23·5	6250 ± 467	4312 ± 258 4875 ± 486

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Table 2. *Results of the titration of purified cystozoites of* Isospora (Toxoplasma) gondii

Approximate cystozoite concentration	No. of mice inoculated	No. of mice SFT-negative
10^2	20	0
10^1	20	4
10^{0*}	20	5 or 6†
10^{-1}	19	16
10^{-2}	20	20

* Precise mean number of cystozoites/inoculum = $1{\cdot}003 \pm 0{\cdot}137$ (mean $\pm$ 2 S.E.).

† One of the mice died before the Sabin–Feldman dye test (SFT) could be performed; therefore the mean of both possibilities (5·5) was used for calculations.

0·807 and 2·256 (μ_0 for $\log \mu_0 \pm 2$ S.D. ($\log \mu_0$)). That is, the 95 % confidence interval of the titration overlapped the 95 % confidence interval of the visual count entirely; hence single, free cystozoites could be considered infective.

DISCUSSION

If we assume that the average age of cysts in mouse brains correlates with the duration of infection (Motomura & Jo, 1970; Van der Waay, 1959) young cysts appeared to be more vulnerable to high *g* forces than older cysts and fewer were recovered.

With procedure B, 100 % of the cysts were recovered with 74·2 % occurring in a single fraction with a sp.gr. of 1·056. A sp.gr. of at least 1·07 was found by Nakabayashi & Motomura (1968) with Gum Arabic and of 1·09 with sucrose. These authors, as well as Masihi and co-workers (Masihi & Jíra, 1979; Masihi, Mach, Valkoun & Jíra, 1976), considered purification and recovery to be positively correlated with the viscosity of the medium. Our results do not sustain this assumption: the viscosity of our fraction 3 (1 cP) was equal to that of the sucrose and much lower than that of the Gum Arabic solution (5 cP) with the same sp.gr. The apparent sp.gr. of cells in a gradient is not dependent on the viscosity of the gradient, but depends on the molecular weight of the gradient material: gradient material of small molecules causes the cells to band at a higher density. This phenomenon was also found in density-gradient centrifugation of oocysts of *I.*(*T.*)*gondii* in which Dubey, Miller & Frenkel (1970), using sucrose, found a main sp.gr. of 1·11 and we, using Ficoll, found a main sp.gr. of only 1·04 (Cornelissen, unpublished data). The larger the molecular weight of the gradient material, the better the resolution and hence the purity that can be obtained. Percoll and Gum Arabic both have a high molecular weight enabling high purification. However, the viscosity of the latter material most probably was responsible for the dose–dependent recovery rate of cysts found by Nakabayashi & Motomura (1968) because cysts were probably tangled in the fractions of lower sp.gr.

The recovery rate and the degree of purification obtained by our procedure B, as judged from microscopical observation, together with the fact that the infec-

tivity of cystozoites was not significantly diminished by our treatment make it the method of choice for our cloning purposes. If needed, for instance for biochemical purposes, almost absolute purification might be achieved by incubation of the cyst suspension with a rabbit antiserum against mouse brain cells (Pettersen, 1979).

The method described in this paper may also be applied to studies on other cyst-forming coccidia.

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EXPLANATION OF PLATE 1

Photo-micrographs of purified *Isospora*(*Toxoplasma*)*gondii* cyst and cystozoites.

A. Cyst purified by density–gradient centrifugation on Percoll (fraction 3).

B. Cystozoites excysted from cysts of fraction 3 by incubation in a pepsin/HCl solution for 5 min at 37 °C.

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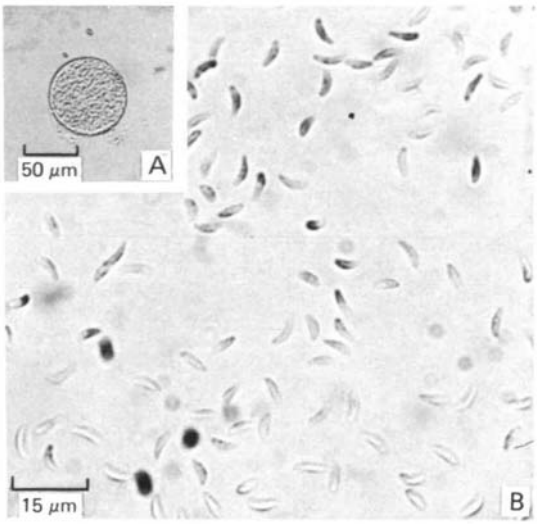

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