DENSITY GRADIENT CENTRIFUGATION FOR SEPARATION OF DIFFERENT STAGES OF TOXOPLASMA GONDII

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Abstract. Suitable procedures for separation of various stages of Toxoplasma gondii by density gradient centrifugation have been delineated using peritoneal exudate of infected mouse as a model. Separation from host cells was effected by gradient centrifugation at 450 g for 30 minutes. Using Ficoll, dextran and sucrose, average parasite recoveries by pooling up to and including the peak fraction of 73.65 %, 66.18 % and 65.68 % respectively were obtained. Toxoplasma trophozoites peaked at density of 1.040 g/ml with Ficoll, 1.060 g/ml with dextran and 1.110 g/ml with sucrose. In view of successful separation of exo-enteric stages of Toxoplasma by density gradient centrifugation, possible application of this method to isolation of various endo-enteric stages is discussed.

Until recently, only asexual exo-enteric trophozoites (tachyzoites, endozoites) and cystozoites (bradyzoites) of Toxoplasma gondii were recognized. After intensive research effort, the endo-enteric stages of the parasite were discovered independently by a number of investigators (Frenkel et al. 1970, Hutchison et al. 1970, Werner and Voos 1970). The development of both asexual and sexual stages were observed in the epithelial cells of small intestine of the domestic cat and it became apparent that T. gondii was a coccidian parasite with a complicated life cycle.

At present little is known about the immunological behaviour of the enteroepithelial types A, B, C, D, E (Dubey and Frenkel 1972) and the macro- and microgametocytes which develop into oocysts. Studies with Plasmodium have shown that different parasite stages e. g. sporozoites, merozoites and gamonts, can be used for protective immunization. An impediment to similar experiments with Toxoplasma is the necessity of obtaining the various stages in relatively pure and sufficient quantities. In a preliminary study, however, a parasite stage could be used which is easily available. It is possible to apply the selected purification method to it with a view to delineating guide-lines for future experiments involving various enteric stages.

Among many methods currently available for selective purification of cell populations, there has been an emphasis on density gradient centrifugation with use of a variety of gradient materials. This method has high resolution since differences as small as 0.022 g/cm³ density can be detected and separated (Cutts 1970). In the present paper, peritoneal exudate from infected mouse was used as a model for defining suitable procedures for future separation of other stages of Toxoplasma by density gradient centrifugation using ordinary laboratory equipment.

MATERIAL AND METHODS

Three materials were used for density gradients. Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), an inert synthetic polysucrose with average molecular weight of 400,000, Dextran 40 (Léčiva, Nat. Corp., Prague) with molecular weight of 40,000 and sucrose (Mann Research Labs., New York) of density gradient grade.

Performed gradients prepared in phosphate buffered saline, pH 7.2, were loaded in an 10 ml poly-
carbonate centrifuge tube (I E C Scientific Products, Massachusetts). The gradient composition with Ficoll and with dextran ranged from 5% (x/v) at the top to 30% (w/v) at the bottom, while with sucrose it was 25% (w/v) to 50% (w/v). If facilities are available, gradients can be easily prepared in a mixing vessel with a peristaltic pump or in a device with two vessel gravity-feed mixer. Alternatively, gradients can be individually prepared by mixing the stock solution of the highest gradient concentration to be used with appropriate amounts of buffered saline.

The sharp interfaces between adjacent steps were minimized by allowing the preparation to equilibrate for 24 hours at 4°C. When interfaces are maintained, the cells tend to accumulate momentarily as they meet the high density interface and can become agglutinated. In addition, the pressure produced by packing cells against a succession of high density interfaces leads to removal of water with a concomitant increase in the cell density.

Appropriate concentration of cells in the starting band is of utmost importance if “streaming” is to be avoided. It is desirable when separating cells in a gradient medium, to start with a small volume on top, thus allowing the cells to enter the gradient layer almost simultaneously. In the present experiments, mouse peritoneal exudate collected aseptically on the 3rd day after infection with virulent P strain of Toxoplasma was used. This strain was isolated in Prague laboratory from a fatal case of congenital toxoplasmosis. Peritoneal exudate was centrifuged, the pellet resuspended with phosphate buffered saline, pH 7.2, and two further washings performed. Exudate diluted 1:9 with buffered saline was also used. One milliliter of diluted or 0.1—0.2 ml of washed exudate was carefully overlaid on top of the gradient and centrifuged in a swing-out rotor at 450 g for 30 minutes. Centrifuge with a swing-out rotor was preferred to one with an angle-head rotor as the latter tends to produce convection currents which favour sedimentation along the outer wall of the tube. To reduce mechanical swirling and mixing, gradual acceleration and deceleration was used and the rotor allowed to come to a stop without brake.

Separated cells can be recovered in various ways. Where the preparation yields sharp bands of cells, these can be collected by aspiration by capillary pipette or using needle and syringe, puncturing just below the desired band. Tube slippers can also be employed but these require precise alignment. When the resolution between bands is not so sharp and the cells form no distinct layers, the contents should be recovered as fractions. This can be done either by collecting the tube contents dropwise from the bottom of the tube or by forcing the gradient and its contents upward through a restricted orifice. In the present experiments, a tapping cap was fitted for upward ejection of gradients by pumping heavy sucrose at the bottom of the centrifuge tube. Fractions of 1 ml or 10—15 drops were collected and the refractive index of each was measured on a Zeiss Jena refractometer. Densities at 20°C were extrapolated from curves prepared by plotting known concentrations of gradients against their densities as determined by calibrated pycnometer. Cells were counted in a Bürker chamber using phase contrast microscope.

RESULTS

Initial experiments were performed to determine the centrifugation time necessary to achieve optimal separation of cells. During centrifugation, speed and duration are of critical importance as the cells start off together, separate widely for a time and then come together again. The results given in Table 1 show that satisfactory separation was obtained following centrifugation at 450 g for 30 minutes. After 40 minutes, the

Table 1. Centrifugation time necessary to achieve optimal separation of toxoplasmas and host cells in Ficoll density gradient

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Density</th>
<th>Percent recovery</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toxoplasmas</td>
</tr>
<tr>
<td>1</td>
<td>1.030</td>
<td>41.4</td>
</tr>
<tr>
<td>2</td>
<td>1.045</td>
<td>33.6</td>
</tr>
<tr>
<td>3</td>
<td>1.060</td>
<td>14.5</td>
</tr>
<tr>
<td>4</td>
<td>1.065</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>1.080</td>
<td>1.3</td>
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beginning of pellet formation was observed. Low speed centrifugation was considered desirable as *Toxoplasma* seem to collapse when submitted to more than 1000 g (Lycke and Lund 1964).

**Fig. 1.** Separation of toxoplasmas and mouse peritoneal leucocytes in Ficoll density gradient.

**Fig. 2.** Separation of toxoplasmas and mouse peritoneal leucocytes in dextran density gradient.

When undiluted exudate was used, small clumps were formed after centrifugation and persisted even when the gradients were prepared using anti-coagulants. They could, however, be avoided by prior washing of the cells twice with phosphate buffered saline. A total of 20 experiments were performed, 10 with Ficoll and 5 each using dextran and sucrose. Typical results with each are shown in Figures 1, 2 and 3. The average parasite recoveries by pooling the banded cells, up to and including the peak fraction, in case of Ficoll was 73.65 %, with dextran 66.18 %, and with sucrose 65.68 %. The contaminations with host cells were respectively 11.16 %, 11.18 % and 10.72 %. By selectively pooling the fractions, contamination could be reduced to 2.72 % for Ficoll, 7.18 % for dextran, 4.04 % for sucrose giving parasite recoveries of 55.62 %, 57.60 % and 44.54 % for each.

**Fig. 3.** Separation of toxoplasmas and mouse peritoneal leucocytes in sucrose density gradient.
Normally the parasite peak banded at density of 1.040 g/ml with Ficoll, 1.060 g/ml with dextran and 1.110 g/ml with sucrose. The host cell peak for each was usually found respectively at densities of 1.065 g/ml, 1.080 g/ml and 1.160 g/ml. There was some variation from experiment to experiment in the position and densities of the peaks. However, their position relative to each other remained constant.

DISCUSSION

Particles that differ in their densities or sizes sediment at different rates when they are subjected to a centrifugal force. This means that by choosing a suitable system of centrifugation, it should be possible to separate individual types of cells from a mixed population. Rate-zonal centrifugation which is dependent on size, as opposed to isopycnic-zonal centrifugation dependent on density, was used in the present study. Infected peritoneal exudate contains host cells varying in type and size and parasites. Applying the sedimentation equation to non-special particles and the fact that these cell types were suspended in the same medium indicated that their sedimentation would be more dependent on size than density.

The results of this study demonstrate that high recovery of the desired cell population, in present case Toxoplasma trophozoites, can be obtained by density gradient centrifugation. Individual recoveries in excess of 80% were obtained in some experiments with Ficoll and of 93% in one experiment with dextran. The higher molecular weights and consequently low osmotic pressures of these gradients may account for their greater yields and better resolution than sucrose. The viscosity of the gradient solution may also influence the outcome of separation. At the same concentration, Ficoll has a greater viscosity than dextran (Maeh and Lacco 1968). In purification of Toxoplasma cysts for cystozoite preparations, higher recoveries were obtained using Gum Arabic solution which is more viscous than sucrose (Nakabayashi and Motomura 1968).

Important exo-enteric stages of Toxoplasma have been separated in relatively pure form by density gradient centrifugation. Recently, trophozoites have been separated into two distinct peaks in a Ficoll gradient using a zonal rotor (Masahi et al. 1976). The parasites in the smaller second peak were larger than the ones in the primary peak. Satisfactory purification of brain cysts has also been reported using Gum Arabic solution and sucrose (Nakabayashi and Motomura 1968). The enteroepithelial and extra-intestinal stages of Toxoplasma show significant difference in their sizes compared to each other and the surrounding host cells. It should be possible to carry out large-scale purification of various stages by using tubes with a greater capacity or by zonal rotors. With proper density gradient solutions and with reasonable attention to handling etc., the gradient method would seem to have much to offer where enrichment of a particular cell population and elimination, or reduction to a low level, of host elements is desired.
REFERENCES


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Dr. Konstantin Aleksandrovich Breev (1910—1978)

On 15 May 1978 Konstantin Aleksandrovich Breev, an outstanding zoologist and parasitologist, the leading scientific worker of the Zoological Institute, the USSR Academy of Sciences, passed away suddenly in Leningrad.

K. A. Breev was born on 29 November 1910 in Voronezh. After completing his secondary school education he began studying natural sciences at the Pedagogical Faculty of the Rostov University and in 1930 transferred to the University of Leningrad, from which he graduated in 1935, specializing in entomology. After graduation he worked for two years in Turkmenia in an expedition organized by the USSR Academy of Sciences, and lectured at the Pokrovsky Pedagogical Institute in Leningrad. In this early period he was already engaged in the studies on the ecology of parasitic Diptera. After the outbreak of the Second World War he joined the army in 1941 as a volunteer and fought against fascism until 1945. He was twice wounded and decorated with military orders.

After the war he completed his post-graduate studies at the Institute of Zoology, the USSR Academy of Sciences, where he received his Candidate of Sciences degree in 1949, using for his thesis “Activity of blood-sucking Diptera and