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Mémoire.

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USE OF FLOW CYTOMETRY FOR DETERMINATION OF GROWTH RATES OF GERMINAL TISSUE FROM *ECHINOCOCCUS MULTILOCULARIS* AND *E. VOGELI*

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SUMMARY

A method to determine the cell cycle and growth rate of germinal tissue from *Echinococcus multilocularis* and *E. vogeli* using flow cytometry is described. In particular, the technique involves the use of either a mercury arc-lamp or laser-equipped system, depending on the general availability of the instruments. Of 5 DNA fluorescent stains tested, only a mixture of ethidium bromide and mithramycin (an energy-transfer method) results in adequate resolution of the cell-cycle and sufficient separation from background noise on the mercury-arc lamp system. DAPI is the stain of choice on the laser system. Enzymatic digestion with collagenase allowed the isolation of germinal cells from differentiated cells of the larval tissue, providing a more precise analysis of the percentage of cycling cells in the germinal layer. Physical separation of germinal cells from incidental host cells is unnecessary. The great disparity in DNA content between cells of *Echinococcus* spp. and mammalian cells essentially results in separate histograms in flow cytometry.

RÉSUMÉ : **Usage de la cytométrie d'écoulement pour la détermination des taux de croissance du tissu germinal d'*Echinococcus multilocularis* et d'*E. vogeli*.**

Une méthode pour déterminer le cycle cellulaire et le taux de croissance du tissu embryonnaire d'*Echinococcus multilocularis* et d'*E. vogeli* utilisant la cytométrie d'écoulement est décrite. La technique implique en particulier l'usage soit d'une lampe à arc de mercure soit d'un système laser, suivant les instruments dont on dispose. Sur les 5 colorations fluorescentes d'ADN testées, seul un mélange de bromure d'éthidium et de mithramycine (méthode de transfert d'énergie) a pour résultat une résolution adéquate du cycle cellulaire et une séparation suffisante du bruit de fond du système de la lampe à arc de mercure. Le DAPI (4,6-Diamidino-2-phénylindol) est la coloration de choix pour le système laser. La digestion enzymatique avec de la collagénase a permis la séparation des cellules germinales et des cellules différenciées du tissu larvaire, fournissant une analyse plus précise du pourcentage de cellules en division de la couche germinale. La séparation physique des cellules germinales des cellules de l'hôte n'est pas nécessaire. La grande disparité dans la teneur en ADN entre les cellules d'*Echinococcus* spp. et les cellules de Mammifères aboutit à des histogrammes séparés dans la cytométrie d'écoulement.

INTRODUCTION

Flow cytometry has been proven to be a valuable tool in the analysis of cell-cycle progression, particularly in the application of the technique for mammalian cells. This form of analysis has recently been utilized in parasitology in the study of protozoa. Most of the work has focused on blood parasites, such as *Plasmodium* spp. (Howard *et al.*, 1979; Hare and Bahler, 1986; Makler *et al.*, 1987) and *Trypanosoma* spp. (Jackson *et al.*, 1977; Midgley, 1983), but the method has also been applied in studies of *Giardia lamblia* (Kinosian *et al.*, 1988; Hoyne *et al.*, 1989). Flow cytometry for cell-cycle analysis of larval cestodes is considered here.

Investigations of the proliferation and subsequent immune responses of infections involving *Echinococcus* spp. generally determine growth rates of the larval cestode (metacestode) from increases in mass over time (Ali-Khan 1974, 1978*a*, 1978*b*; Ali-Khan and Siboo, 1982). Since larval tissue may include cells of host origin (fibroblasts and immune cells, see Ali-Khan and Siboo, 1982, and Treves and Ali-Khan, 1984), and germinal and differentiated cells (Rausch, 1954), in addition to non-cellular material, the use of the above method does not directly provide information on the proliferation of the germinal tissue. Such tissue is involved in the invasive pattern of growth in naturally infected intermediate hosts.

The purpose of this paper is to report a method utilizing flow cytometry to obtain direct data concerning the growth rate of germinal cells in the form of cell-cycle statistics. Specifically, this method provides estimates of the percentages of cells in S and G2 at particular time-points. The accumulation of time-points may result in growth curves and information pertaining to growth rates of the germinal tissues.

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This method has been developed primarily for a mercury arc-lamp system because of its general availability, although results from laser equipment will also be given here.

METHODS AND MATERIALS

Gerbils, *Meriones unguiculatus* (Milne-Edwards), were bred and maintained in the laboratory and routinely used as hosts for *Echinococcus multilocularis* and *E. vogeli.* Animals were inoculated interperitoneally with an 18 gauge needle into the right groin with approximately 0.1 g of larval tissue that had been coarsely minced by means of fine scissors and suspended in 0.5 to 1.0 ml of phosphate buffered saline (PBS) with 1 % penicillin-streptomycin. Animals were killed for examination at desired intervals postinoculation. Results are reported from 9 gerbils infected with *E. multilocularis* and from 6 with *E. vogeli.*

The inoculated tissues of the larval cestodes underwent organization and proliferation in the peritoneal cavity of the host, with quantity of tissues produced and state of development depending on interval of time postinoculation. The structure of the larval cestodes so obtained was typical for the respective species of *Echinococcus.* Tissue obtained at necropsy was processed, either immediately or after refrigeration for up to 3 days, in media lacking serum. Results obtained by the following methods indicated that refrigeration did not affect the cell-cycle statistics. A representative portion of intact larval cestode was minced in a fluorescent DNA stain and filtered through a 70 μm sieve. The sieve removed all protoscolices, which are relatively large [range for *E. multilocularis* is about 134 to 183 μm in length by 119 to 149 μm in width (x = 162.2 by 131.8 μm); for *E. vogeli,* 158 to 203 μm in length by 108 to 145 μm in width (x = 175 by 133 μm)] (Rausch *et al.,* 1981).

Five stains were tested on germinal cells of *E. multilocularis* to determine which provided the greatest resolution. With ultraviolet excitation, two stains provided emission fluorescence above 425 nm: 2′-[4-Hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2-5′-bi-1H-benzimidazole Trihydrochloride (Hoechst No. 33258) [Sigma, 1 μg/ml in 0.1 M Tris, pH 7.4, 0.9 % NaCl, 1 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.2 % bovine serum albumin (BSA), 0.1 % Nonidet-P40] or 4,6-Diamidino-2-phenylindole (DAPI) [Sigma, 10 μg in 0.1 M Tris, pH 8.0, 0.9 % NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 % BSA, 0.3 % Nonidet-P40]. The remaining 3 stains required the addition of 0.1 % RNase (Sigma, Type I, in saline) and resulted in emission fluorescence above 570 nm after excitation of 400-500 nm: Ethidium bromide (EB) [Calbiochem, 25 μg/ml in 0.1 Tris, pH 7.4, 0.6 % NaCl, 0.1 % Nonidet-P40], mithramycin (MI) [Pfizer, 50 μg/ml with 7.5 mM $MgCl_2$, 0.1 % Nonidet-P40], or with a mixture of EB and MI. The latter required the cells be stained with EB for 10 min, after which an equal volume of MI was added. All samples were RNase treated to prevent incidental staining of RNA. The detergent, Nonidet-P40, permeabilized cell membranes for access to the DNA by the fluorescent stains. Samples were syringed with a 25 gauge needle 5 times prior to analysis to remove possible clumps and to ensure a single-cell suspension. The mercury-arc lamp system used was an ICP-22 (Ortho Diagnostic Systems) equipped with the appropriate filters as mentioned above. Similar results could be achieved with a FACS (Becton Dickinson). The laser equipped Ortho Cytofluorograph 50 H with a model 2150 computer was used for comparisons between systems. DAPI and HO stains were excited by UV and emission wavelengths were measured above 400 nm. Excitation of EB stains was achieved with 488 nm wavelength with emission wavelengths of 580 nm and greater.

The remaining tissue was subjected to enzymatic digestion prior to staining. Larval tissue was coarsely minced in minimal essential medium (MEM) and washed twice with PBS. The isolation of germinal cells (and some host cells) was achieved by means of a modification of the technique of Dieckmann and Frank (1988). Type IV collagenase (Sigma, 200 IU/ml) in an isotonic buffer, as described by Dieckmann and Frank (1988), was used for enzymatic digestion of the germinal layer. Cells were isolated at 37° C with the removal and replacement of fresh collagenase solution every 5 min for a 30 min period. Collagenase activity was neutralized by addition of cold MEM with 10 % fetal calf serum (FCS). Protoscolices and tissue fragments inadvertently removed with the germinal cells could be separated from the latter by filtration. The resulting solution was centrifuged and washed twice with MEM and 10 % FCS. The final pellet of cells was resuspended in a DNA stain and syringed prior to analysis.

Cell-cycle statistics were calculated with the program MULTICYCLE (Phoenix Flow System, San Diego, California, USA) on an IBM PC compatible computer. Percentage of cells in G1, S and G2 was calculated from the area under the curves. The program is capable of subtracting background caused by debris and machine noise in calculations of the cell-cycle statistics.

RESULTS AND DISCUSSION

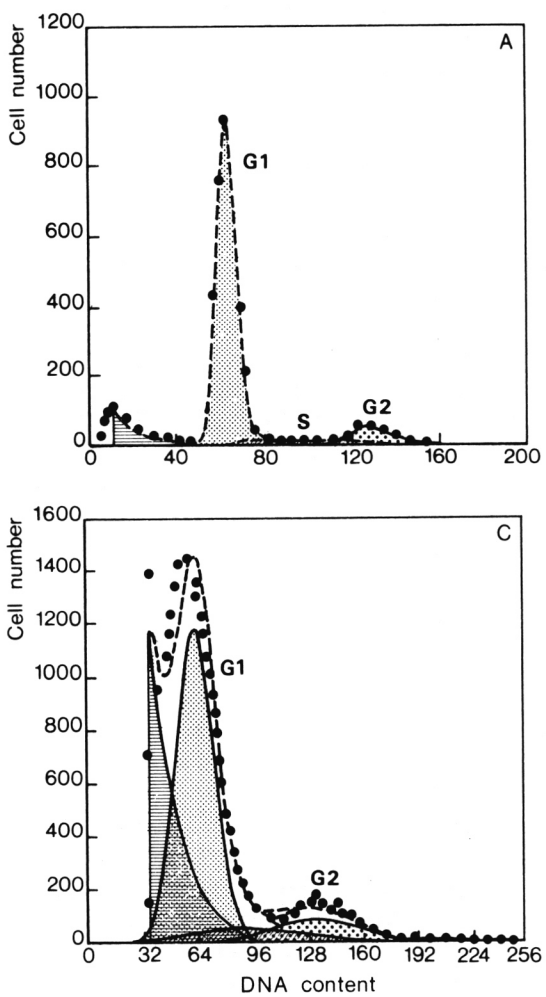
Analysis of cells from *Echinococcus* spp. for DNA content poses difficulty because of the extremely small amount of DNA (Rausch and Rausch, 1981) in relation to that in mammalian cells used for conventional cytometric analysis. Fluorescence from dyes must be sufficiently bright for adequate resolution of the cell-cycle and to provide signals sufficiently above background noise. Although G1 and G2 could be determined with the use of both Hoechst and DAPI stains, the resolution was not adequate

to provide reliable cell-cycle statistics. In particular, the peaks were so broad as to eliminate any visible S phase (Coefficient of Variability of G1 peak = 20 %).

By themselves, neither EB nor MI resulted in emission signals that could be sufficiently separated from the background noise. However, the use of both stains together resulted in the best histograms of all stains tested. The cell-cycle statistics, including S phase, were easily determined and the DNA fluorescence was appreciably above the background noise. Although either EB or MI alone did not provide enough fluorescence for adequate resolution, the use of these two stains in tandem resulted in energy transfer between MI and EB. MI is optimally excited by the deep violet wevelengths, in turn exciting the EB. The resulting fluorescence was over 7 times brighter than that from cells stained only with EB and 3 times brighter than that with MI (Shapiro, 1988). Therefore, of the five stains, only EB/MI resulted in histograms with reliable cell-cycle statistics for germinal cells on an arc-lamp system (average CV of G1 or G2 peak for *E. multilocularis* = 8.9 %; 6.6 % for *E. vogeli*).

In comparison with a mercury-arc lamp system, a laser-equipped machine provides more energy for excitation of fluorescent stains. As a result, the level of background noise can be much lower, the sensitivity of the machine is greater, and a larger number of stains is available. Of the three stains tested on this machine, DAPI was the preferred fluorescent stain for the germinal cells. It is a single-stain technique, requiring only syringing prior to analysis. Results from the laser system demonstrated low background noise, a good CV, and cell-cycle statistics comparable to those from the same cells analyzed with EB/MI on the mercury-arc lamp machine *(fig. 1)*. The same sample stained with DAPI and analyzed with the latter equipment is also shown. The high background and large CV's demostrate that this sample is at the limit of the capabilities of the mercury-arc lamp system. In this study, EB/MI was the stain of choice for germinal cells analyzed with the ICP, but systems equipped with lasers provide greater flexibility in regards to stains used.

Dieckmann and Frank (1988) indicated that, after digestion with collagenase, the final suspension consisted of individual germinal cells, erythrocytes, other host cells, and debris. Separation of the germinal cells was achieved



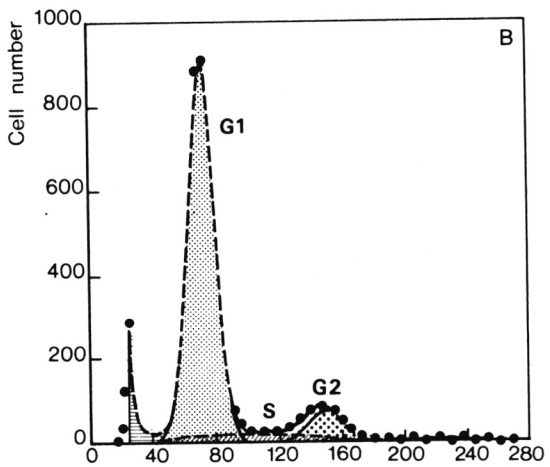


FIG. 1. — Germinal cells of *E. multilocularis* after collagenase isolation. *a*) Cells stained with DAPI and analyzed on the laser-equipped system, the Ortho Cytofluorograph 50HH. CV of 6.6 %, percentage of cells in S plus G2 estimated at 14.0 %. *b*) Same sample but stained with EB/MI and analyzed on the mercury-arc lamp system, ICP-22. CV of 9.2 %, S plus G2 estimated at 15.2 %. *c*) Same DAPI sample except analyzed on the ICP. CV of 18.1 %. Percentage of cells in S phase difficult to estimate because of the broad G1 and G2 peaks.

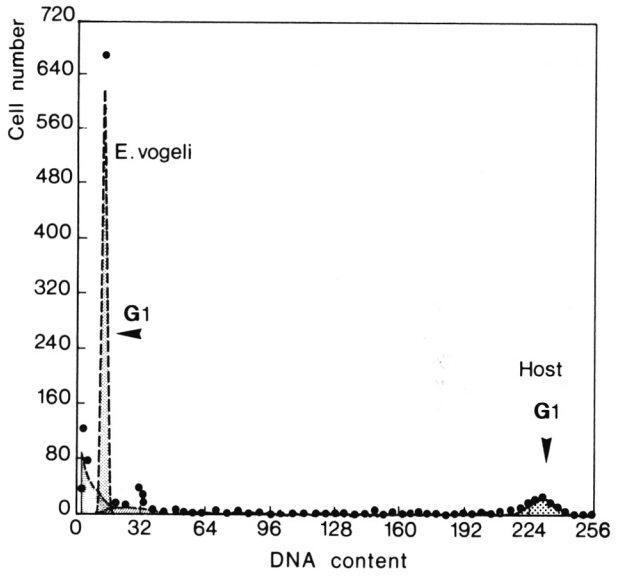


FIG. 2. — Histogram of germinal cells from *E. vogeli* and cells from the mammalian host demonstrating the complete separation of the two cell-cycles, EB/MI stain. Only the G1 phase of the host cells are shown on the far right of the histogram.

through Percoll gradient centrifugation. This last step is unnecessary in flow cytometry because of the great disparity in DNA content between cells of *Echinococcus* spp. and mammalian cells. The degree of separation between the DNA of the mammalian host and that of *E. vogeli* is shown in *figure 2*. Erythrocytes and other material lacking DNA do not form peaks.

Enzymatic digestion of larval tissue by collagenase acts as a means of enrichment. Those tissues that are more susceptible to collagenase separate into individual cells before the more resistant types do so. The resulting suspension would then have greater numbers of the former cells than the initial sample. In effect, the treated sample has been « enriched » with the more susceptible cells. In this study, germinal tissue was easily digested by collagenase, but the laminated membrane and protoscolices appeared to be impervious to that treatment. After exposure to collagenase, protoscolices were entire and active, even after incubation in MEM with 16 % FCS at 37° C for 24 hours. Dieckmann and Frank (1988) tested for viability of the germinal cells with trypan blue exclusion. They also reported that germinal cells isolated by a Percoll gradient were infective when inoculated interperitoneally into gerbils.

Comparison of cell-cycle statistics prior to collagenase isolation and afterwards indicated differences in S plus G2 ranging from 2.0 to 12.5 % and 3.1 to 10.1 % for *E. multilocularis (fig. 3)* and *E. vogeli,* respectively. The average S plus G2 for *E. multilocularis* without the enzyme treatment was 15.8 %; and 21.9 % post-isolation. For *E. vogeli,* the average S plus G2 without the treatment was 8.6 %; and

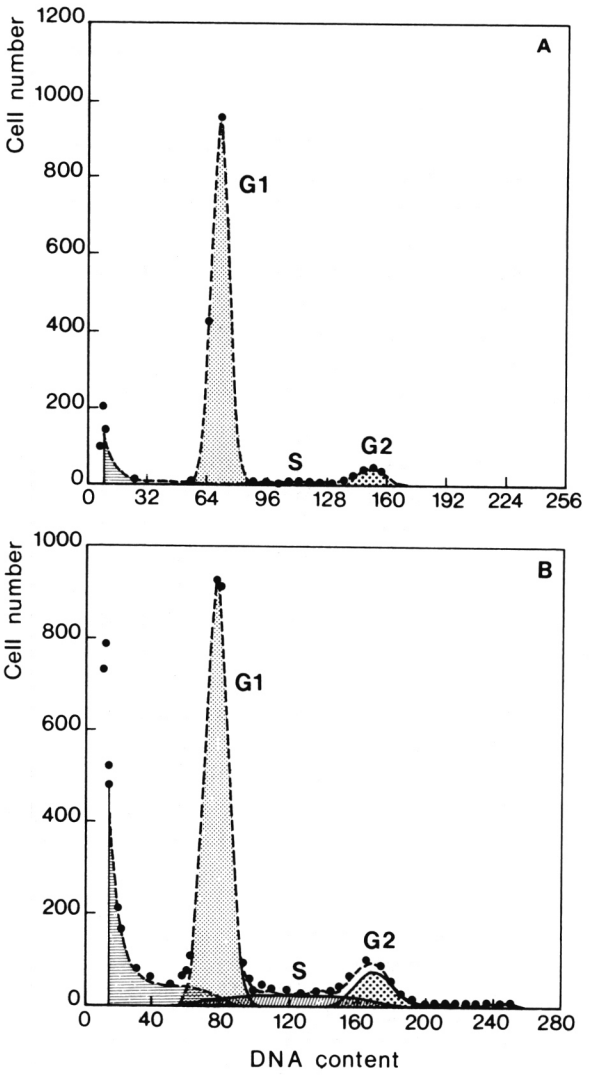


FIG. 3. — *a*) Histogram of cells from *E. multilocularis* prior to collagenase isolation. Percentage of cycling cells (S plus G2) is 10.3. *b*) Histogram of germinal cells from the same tissue after collagenase digestion. Percentage of cycling cells is 22.8. Both samples stained with EB/MI and analyzed on the mercury-arc lamp system. Background and machine noise, as seen to the left of the G1 peaks, calculated for and substracted by the program MCYCLE.

15.0 % post-isolation. In all cases, the percentage of S plus G2 was greater after collagenase isolation than before. On the average, treatment by collagenase prior to analysis resulted in cell-cycle statistics greater than a third in comparison to untreated samples.

Statistics from samples without collagenase treatment would include differentiated cells from the larval tissue. Since those cells would be growing at a considerably lower rate than those of the germinal layer, the effect on the observed cell-cycle is one of « dilution ». The differences between the treatments vary considerably and may be influenced by two factors. The degree of mincing, as illus-

trated by the number of brood capsules disrupted, would determine the exposure of protoscolices to the stains. Untreated samples with many protoscolices would have lower percentages for S and G2. Secondly, and perhaps more importantly, the age of the infection and the resulting amount of differentiation that has occurred in the brood capsules would affect the number of germinal cells in relation to other cells. Post-isolation samples from infective larvae would have considerably more germinal cells than in untreated samples. Therefore, the former would be expected to have a higher percentage of cells in S plus G2.

Results of this study indicate that flow cytometry can provide direct information concerning the cell-cycles, and therefore the growth rates, of germinal cells of *Echinococcus* spp. Of the 5 stains tested, the energy-transfer method involving EB/MI provides the best results from an arc-lamp system (ICP 22). This technique has sufficient fluorescence for reliable statistics of the cell-cycles of the larval tissue, despite the small amount of DNA present relative to that in mammalian cells. Comparable results can be achieved with access to a laser-equipped system, particularly with DAPI. Isolation of germinal cells from differentiated cells, through collagenase digestion, provides a more precise analysis of the percentage of cycling cells in the germinal (invasive) layer of the cysts. Lastly, separation of the germinal cells and incidental host cells prior to flow cytometric analysis is unnecessary because of the wide separation between the two histograms.

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