Journal of Cellular Biochemistry 90:294–303 (2003)

Regionalization of DNA and Protein Synthesis in Developing Stages of the Parasitic Platyhelminth *Echinococcus granulosus*

M. Galindo, R. Paredes, C. Marchant, V. Miño, and N. Galanti*

Program of Cellular and Molecular Biology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Santiago, Chile

Abstract *Echinococcus granulosus* is a parasitic platyhelminth, which causes cystid hydatid disease, a major zoonosis involving canids as definitive hosts, and both human and herbivorous domestic animals as intermediate hosts. The disease is caused in intermediate hosts by hydatid cysts, formed upon ingestion of *E. granulosus* eggs excreted by canids. Protoscoleces, the developmental forms of the parasite infective to canids, are formed in the germinal cellular layer of hydatid cysts. We have found that protoscoleces develop from patches of proliferating cells present in the germinal layer of the hydatid cyst, while most of the other cells of the germinal layer are in a resting state. Further, patches of proliferating cells form buds, which elongate and develop a separate population of cycling cells. In these elongated buds, cell differentiation leads to the main structures of the protoscolex. Protein synthesis is very active among cells of early buds and coincides with their proliferating activity. By contrast, protein synthesis presents a much lower activity in the resting cells of the germinal layer surrounding the growing protoscoleces. In elongated buds at different stages of development, protein synthesis is found mainly close to cellular territories in which cell differentiation occurs. In free infective protoscoleces, cells in DNA synthesis are concentrated in the body of the larva while protein synthesis occurs in the entire larva. This is the first description of the regionalization of DNA and protein synthesis in developing stages of *E. granulosus*. J. Cell. Biochem. 90: 294–303, 2003. © 2003 Wiley-Liss, Inc.

Key words: *Echinococcus granulosus*; DNA synthesis; protein synthesis; protoscolex

Echinococcus granulosus is the causative agent of cystic hydatid disease or hydatidosis, recognized as a major zoonosis affecting both humans and herbivorous domestic animals in various parts of the world [Gottstein and Hemphill, 1997]. The pathological effect of the disease is due to the pressure exerted on the viscera of the intermediate hosts by hydatid cysts, which are formed upon ingestion of *E. granulosus* eggs. The inner germinal layer of these cysts is cellular, and from those cells the larval form of the parasite, the protoscolex, is developed. Mature protoscoleces are liberated into the cyst lumen. When canids feed on infected viscera, protoscoleces fix in the microvilli of their intestine and develop into the adult egg-producing worm. Although this flatworm has been studied over the years, basic aspects of its cell biology have received little attention.

An early report clearly defined the ultrastructure of the germinal layer in human hydatid cysts and proposed steps in the development of brood capsules and protoscoleces [Bortoletti and Ferretti, 1973]. This study was followed by a more detailed description of the formation of these structures using experimental infections in random bred Mongolian jirds (*M. unguiculatus*) [Thompson, 1976]. In that work, a diagramatic description of the formation of brood capsules and protoscoleces in secondary hydatid cysts was reported. Buds are formed in the germinal layer and grow towards the cyst cavity. Later on, buds become stalked and vacuolated. From the inner cellular

Grant sponsor: FONDECYT-CHILE; Grant numbers: 1010817, 1970766; Grant sponsor: NRTP/SIDA-SAREC; Grant sponsor: Fundación Andes-Antorchas-Vita; Grant sponsor: DID-University of Chile; Grant number: I013-99/2.

*Correspondence to: Norbel Galanti, Program of Cellular and Molecular Biology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Casilla 70061, Correo 7, Santiago, Chile.
E-mail: ngalanti@machi.med.uchile.cl

Received 29 May 2003; Accepted 1 July 2003
DOI 10.1002/jcb.10640

© 2003 Wiley-Liss, Inc.

layer of these cavities, a new process of budding is initiated, leading to the formation of protoscoleces. Recently, using light and scanning electron microscopy, seven stages in the formation of protoscoleces from the germinal layer of hydatid cysts were reported [Galindo et al., 2002].

The study of the development stages leading to protoscolex formation from the germinal layer of hydatid cysts in natural primary infection with *E. granulosus* is important because it determines the fertility of the cyst, which has implications both in diagnosis and therapeutic practice [Eckert et al., 1995]. For example, the antiparasitic drug praziquantel affects hydatid cyst differentiation only when applied at the beginning of this process [Urrea-Paris et al., 2002]. That kind of observation emphasize the relevance of studying cell proliferation, growth, and differentiation during protoscolex formation in relation to the effect of antihelminthic drugs. Additionally, hydatid fluid, which is secreted by the germinal layer of hydatid cysts into the cyst cavity, affects negatively the incorporation of thymidine in T cells, but not the viability of these lymphocytes [Macintyre et al., 2001]. This result suggests a possible regulatory effect of hydatid fluid on the S-phase population in nascent protoscoleces during their formation in the germinal layer of hydatid cysts.

Systematic studies on the cellular basis of the formation of protoscoleces, the infective form of *E. granulosus* in canids, are important to understand the strategy of infectivity of the parasite and to find possible targets for a rational design of drugs, other than those targeted to interrupt specific metabolic pathways. An approach to address this problem is to study DNA replication and protein synthesis in the germinal layer and in different developmental forms of the growing protoscolex in fertile hydatid cysts.

MATERIALS AND METHODS

Hydatid Cysts

E. granulosus hydatid cysts were obtained from ovine and bovine livers or lungs. The animals were slaughtered at the Lo Valledor abattoir in Santiago, and in Coyhaique and Puerto Porvenir in the south of Chile. Presence of free protoscoleces in the hydatid fluid and of buds and grown protoscoleces attached to the germinal layer were monitored under a light microscope. Other parameters of the presence of developmental stages of protoscoleces, such as a whitish color and thickness of the germinal layer, were also considered [Bortoletti and Ferretti, 1978].

Germinal Layers and Protoscoleces

Germinal layers joined to laminar layers, which are the product of secretion of cells from the germinal layer and remains attached to it, were dissected from open cysts. Protoscoleces were decanted by gravity from the hydatid fluid, washed in phosphate buffered saline (PBS) pH 7.2 at 38.5°C and treated with pepsin 0.1% in Hanks' salt solution pH 2.0 at 38.5°C for 15 min to eliminate remnants of germinal layer. Pepsin was removed by four washings with Hanks' medium.

Viability of Germinal Layer, Buds, and Protoscoleces

Viability of the protoscoleces, free or attached to the germinal layer, was evaluated on the basis of body movements and cell activity as observed under a light microscope, and by incorporation of [^{3}H]-thymidine into DNA or of [^{3}H]-leucine into proteins. Germinal layers and buds were obtained from fertile hydatid cysts presenting viable protoscoleces; both tissues showed conserved morphology and active incorporation of the labeled precursors.

[^{3}H]-Thymidine Incorporation Into DNA

To measure the incorporation of the labeled precursor into DNA, a procedure previously described was used [Rojas and Galanti, 1991]. Briefly, pieces of germinal layer and protoscoleces from fertile hydatid cysts were suspended in Hanks' salt solution and incubated with 20 μCi of [^{3}H]-thymidine (DuPont, Amersham, UK 80 Ci/mmol) at 38.5°C for 6, 12, and 24 h in 5% CO_2. The samples were washed in cold PBS pH 7.2, resuspended in the same buffer and homogenized in a Potter Elvejhem with 50 strokes at 1,400 rpm. Afterwards, the homogenate was mixed with an equal volume of ice-cold 10% trichloroacetic acid (TCA) and the sediment was recovered after spinning at 10,000*g*. The sediment was washed twice in ice-cold 5% TCA, once in 80% ethanol and once in ethanol–ether (3:1, v/v). DNA was extracted from the sediment in 5% TCA at 90°C and divided in two aliquots. One was used for DNA determination [Burton, 1956]; in the other the

radioactivity was measured in a Beckman liquid scintillation counter model LS100C.

For autoradiography, the germinal layer and protoscoleces were incubated in the presence of the radioactive precursor for 24 h as indicated above. Afterwards, the samples were washed in cold PBS and fixed in Bouin alcoholic solution for 3 h at room temperature.

[^{3}H]-Leucine Incorporation Into Proteins

Pieces of fertile germinal layer and free protoscoleces were suspended in PBS pH 7.2 and incubated with 50 μCi of [^{3}H]-leucine (60 μCi/mmol) for 15, 30, 60, 120, and 180 min at 38.5°C in 5% CO_2. To measure the incorporation of the precursor into proteins, the samples were washed in ice-cold PBS pH 7.2, resuspended in the same buffer, and homogenized in a Potter Elvejhem with 50 strokes at 1,400 rpm. The homogenate was mixed with an equal volume of cold 10% TCA and the sediment was recovered after spinning at 10,000*g*. The sediment was washed twice in 5% ice-cold TCA and dissolved in 1 N NaOH. One aliquot was used to measure protein concentration by the Bradford procedure [Bradford, 1976]. The other aliquot was neutralized and the radioactivity was measured in a Beckman liquid scintillation counter.

For autoradiography, the fertile germinal layer and protoscoleces were incubated with the precursor for 2 h as indicated above. Afterwards, the samples were washed in cold PBS and fixed in Bouin alcoholic solution for 3 h at room temperature.

Autoradiography

Fixed material was washed, dehydrated, and embedded in paraffin. Slices of 5 μm were dipped in Kodak NTB-2 photographic emulsion and exposed for 1–3 weeks at −80°C. Finally, slices were developed, stained with haematoxylin–eosin, and observed under a Nikon Opti-Phot microscope.

RESULTS

Incorporation of [^{3}H]-Thymidine and [^{3}H]-Leucine in Fertile Germinal Layer and Protoscolex Developmental Stages

Figure 1A shows the incorporation of [^{3}H]-thymidine into DNA of cells from the germinal layer of fertile hydatid cysts and protoscoleces. In the germinal layer, the incorporation of the precursor into DNA increases steadily. In protoscoleces, DNA synthesis increases slowly from 0 to 6 h, and then more sharply from 6 to 24 h. [^{3}H]-thymidine incorporation into DNA is sixfold to sevenfold higher in protoscoleces than in fertile germinal layer.

Figure 1B shows the incorporation of [^{3}H]-leucine into proteins of cells from the germinal layer of fertile hydatid cysts and protoscoleces. From 0 to 60 min there is a rather low incorporation of the precursor, reaching a plateau around 15 min in the germinal layer and 30 min in protoscoleces. From 60 min on a higher rate of incorporation of the precursor is observed in both samples. Again, incorporation of [^{3}H]-leucine into proteins of protoscoleces is slightly

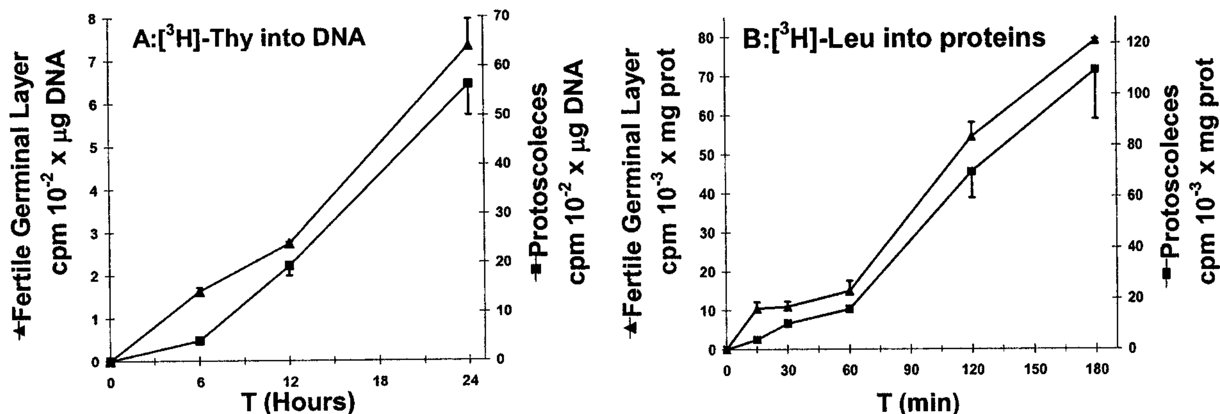

Fig. 1. DNA and protein synthesis in fertile germinal layer and protoscoleces. Samples were incubated with 20 μCi of [H^3]-thymidine (**A**) or with 50 μCi of [H^3]-leucine (**B**) at 38.5°C at the times indicated. In (A) DNA was determined following Burton [1956]. In (B) proteins were determined following Bradford [1976].

higher than in proteins of the fertile germinal layer.

DNA Synthesis in the Fertile Germinal Layer

Figure 2 shows the incorporation of [^{3}H]-thymidine in the nuclei of cells from sectors of the fertile germinal layer that are free of both buds and attached protoscoleces. Most nuclei of the germinal layer show no incorporation of [^{3}H]-thymidine; the cells are in a resting non-proliferative state (Fig. 2a). However, there are other sectors of the germinal layer showing replicative cells, with a low (Fig. 2b) or a high (Fig. 2c) number of cycling cells. Then, the germinal layer of the fertile hydatid cysts shows cells, which are mostly in G_0, with patches of cycling cells.

DNA Synthesis in the Growing Protoscolex

Figure 3 shows the incorporation of [^{3}H]-thymidine in the nuclei of cells from the germinal layer underlying buds and in the nuclei of cells from the nascent and grown buds.

In Figure 3a–e, a population of cells in DNA synthesis is observed as a continuum from the germinal layer to the buds. No signs of differentiation can be observed in the nascent buds. From Figure 3f onwards, cells replicating DNA in the buds are separated from cycling cells of the germinal layer, originating two independent dividing cell populations.

In Figures 3f–i, bud elongation is evident, apparently supported by cellular DNA replication at the base of the buds. When the buds show signs of structural organization, cells in DNA synthesis clearly diminish in number and they are mostly located in the middle-anterior region (Fig. 3j–n).

Finally, in Figures 3n,o, active cellular DNA replication is observed at the border of the forming suckers (SU, thin arrows) and around other nascent structures, thus suggesting that cell division in the area provides the cells which differentiate in the main cellular territories of the protoscolex. Most of the other cells are out of the cell cycle.

Cellular DNA Replication in the Mature Protoscolex

Figure 4A(a) shows the structure of a protoscolex obtained from the hydatid fluid of a fertile cyst. Five cellular territories following the anterior–caudal axis can be distinguished, named rostellar cone (RC), rostellar pad (RP), suckers (SU), neck-rostellum (NR), and body (BO) [Galindo, 1998]. In Figure 4A,B, synthesis of DNA in the nuclei of cells in these five territories is shown. No [^{3}H]-thymidine incorporation into DNA was found in the RC or in the RP. Cellular DNA replication was found in the NR, SU, and particularly in the body of the protoscoleces (arrows). The low number of S-phase cells in the scolex suggests that this replicative activity is mostly related to the maintenance of this cellular territory of the protoscolex rather than to an increase in cell number.

To rule out that this assymetrical distribution of labeled cells in the protoscolex was not an artifact due to the thickness of the sections, serial sections of a mature protoscolex labeled with [^{3}H]-thymidine were autoradiographed (Fig. 4B). Again, in the six sections shown (a–f) most of the labeled cells are present in the body of the protoscolex. Labeled cells could be readily distinguished from the terminally differentiated cells of the hooks (small arrows).

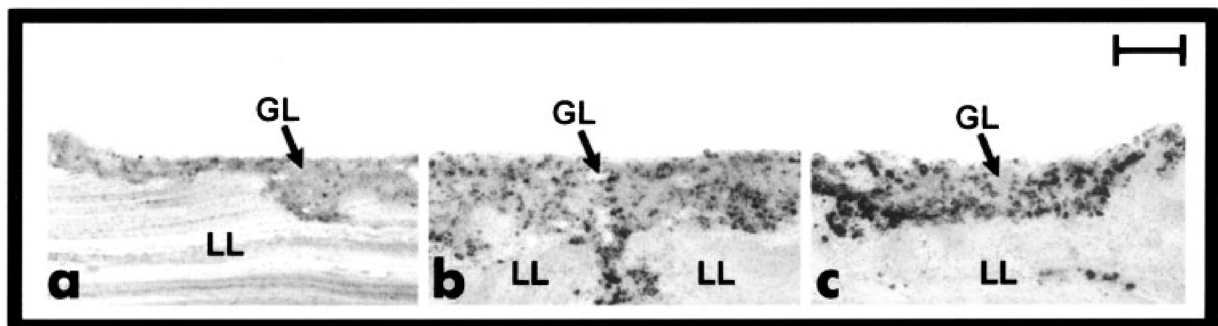

Fig. 2. [^{3}H]-thymidine DNA labeling in nuclei of cells from the germinal layer of a fertile *Echinococcus granulosus* hydatid cyst. Samples were incubated with 20 μCi of [H^{3}]-thymidine for 24 h, fixed in Bouin and processed for autoradiography. Exposition to photographic emulsion was for 1–3 weeks at −80°C. **a**: None, (**b**) low, and (**c**) high number of cells in DNA synthesis. GL, germinal layer; LL, laminar layer. Bar: 20 μm.

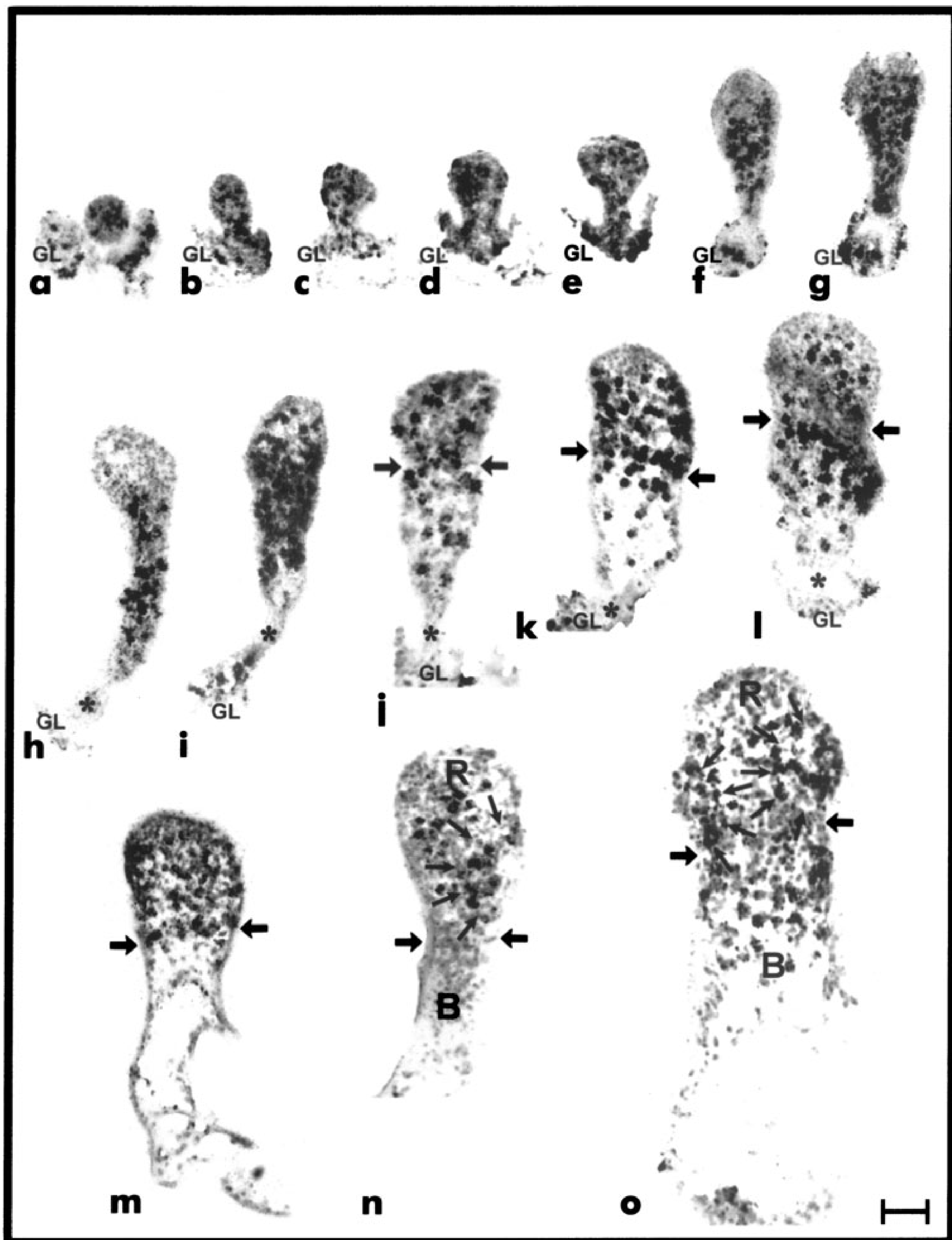

Fig. 3. [^{3}H]-thymidine DNA labeling in nuclei of cells from the germinal layer, buds, and growing protoscoleces from a fertile *E. granulosus* hydatid cyst. Samples were incubated with 20 µCi of [H^{3}]-thymidine for 24 h, fixed in Bouin, and processed for autoradiography. Exposition to photographic emulsion was for 1–3 weeks at −80°C. GL, germinal layer; *, stalk; thick arrows, scolex-body furrow; thin arrows, border of forming suckers; R, rostellum; B, body. Bar: 20 µm.

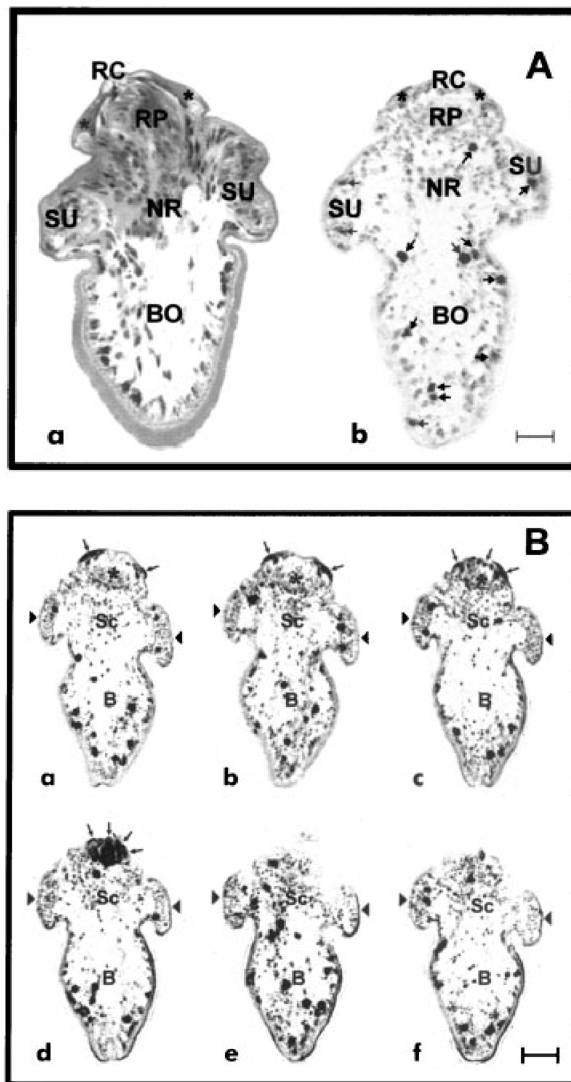

Fig. 4. **A**: Light microscopy structure of an *E. granulosus* protoscolex (**a**) and [^{3}H]-thymidine DNA labeling in nuclei of cells (**b**). RC, rostellar cone; RP, rostellar pad; SU, suckers; NR, neck-rostellum; BO, body; *, hooks; arrows, labeled cells. Bar: 20 μm. **B**: Serial sections of a protoscolex labeled with [^{3}H]-thymidine. Sc, scolex; B, body; small arrows, hooks; *, rostellar pad; arrowheads, suckers (SU). Bar: 20 μm.

These serial sections of the same organism provide further evidence for the assymetrical distribution of the replicative cells in the different cellular territories of the mature free protoscolex.

Quantitative Analysis of DNA Synthesis in Fertile Germinal Layer, Grown Buds, and Attached and Free Protoscoleces

Labeling indices were measured in the germinal layer and grown buds as well as in attached and free protoscoleces from fertile *E. granulosus* hydatid cysts. Grown buds are those in which some organization can be evidenced (anterior and caudal territories, SU outlined, hooks). Attached protoscoleces are full-differentiated larva still attached to the germinal layer while free protoscoleces are those found in the hydatid fluid.

In the fertile germinal layer free of both buds and attached protoscoleces, most of the cells are out of the cell cycle. However, there are regions of the germinal layer showing an active incorporation of [^{3}H]-thymidine into nuclear DNA. These patches of cycling cells show a variable number of nuclei in S-phase, from 19 to 59% (not shown). An important percentage of cells from the germinal layer underlying the buds and under attached protoscoleces are always in DNA synthesis (Table I). Interestingly, the number of cells in S-phase abruptly diminishes in grown buds and in protoscoleces still attached to the germinal layer (Table I). In free protoscoleces, approximately 7% of the cells present an active synthesis of DNA. However, cells in S-phase are mainly found in the body of the protoscolex, with the SU and the rostellum–neck regions showing a much lower DNA synthesis activity while cells from the RC and RP are out of the cell cycle (Table II).

Protein Synthesis in Fertile Germinal Layer, Early Buds, Grown Buds, and Attached and Free Protoscoleces

Figure 5a shows the incorporation of [^{3}H]-leucine in cells from the germinal layer and in cells from an early bud. Protein synthesis is very active in the bud (arrow) and in the underlying germinal layer, all cells showing

TABLE I. DNA Synthesis in Cells of the Fertile Germinal Layer, Grown Buds, and Attached Protoscoleces of *Echinococcus granulosus* Hydatid Cysts

Tissue	Percentage of labeled cells
Germinal layer	13.5 (±0.9)
Grown buds	1.38 (±0.1)
Attached protoscoleces	2.08 (±0.3)

Samples were incubated with 20 μCi of [H^3]-thymidine for 24 h, fixed in Bouin, and processed for autoradiography. Exposition to photographic emulsion was for 1–3 weeks at −80°C. Labeling index was obtained by counting labeled nuclei in 60 samples from ten fertile cysts. In each sample, 1,000 cells were observed. Cells from fertile germinal layer considered for counting were those below the buds and attached protoscoleces. Early buds show the same labeling index than the adjacent germinal layer.

TABLE II. DNA Synthesis in Cellular Territories of *E. granulosus* Mature Protoscoleces

Cellular territory	Percentage of labeled cells
Rostellar cone	0.0
Rostellar pad	0.0
Suckers	1.8 (±0.1)
Rostellum–neck	0.98 (±0.05)
Body	3.97 (±0.3)

Samples were incubated with 20 μCi of [H^3]-thymidine for 24 h, fixed in Bouin, and processed for autoradiography. Exposition to photographic emulsion was for 1–3 weeks at −80°C. Labeling index was obtained by counting labeled nuclei in 1,000 cells in each cellular territory, using 150 slices of protoscoleces.

high incorporation of the labeled precursor. On the other hand, cells of the fertile germinal layer that are adjacent to the bud show a much lower incorporation of [^{3}H]-leucine into proteins.

In Figure 5b–d, three developmental stages in the formation of the protoscoleces at the germinal layer are shown. At these steps, there is an evident decrease in the synthesis of proteins, as compared to the early bud. Interestingly, sites in which protein synthesis can be put in evidence seem to be spatially related to nascent structures in the forming protoscoleces. In a free, mature protoscolex (Fig. 5e), protein synthesis is scattered throughout the cellular territories of the larvae. However, the label seems to be more concentrated in cells of the NR and body regions.

DISCUSSION

Several studies have shown the participation of proliferative cell populations in the reproduction of the larvae stage and in the remodeling

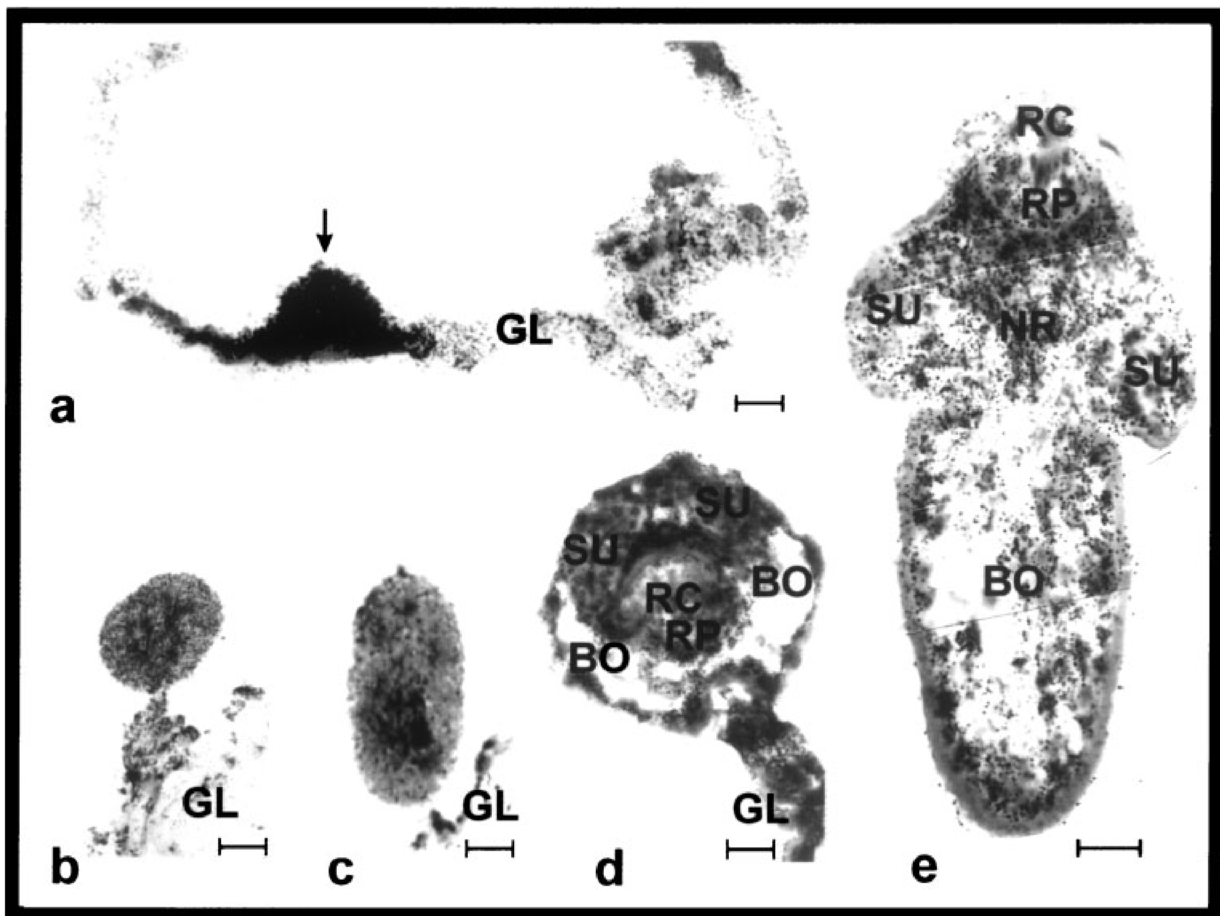

Fig. 5. [^{3}H]-leucine protein labeling of cells from the germinal layer and of cells from different stages of protoscolex development in fertile hydatid cyst. Samples were incubated with 50 μCi of [H^3]-leucine for 2 h, fixed in Bouin, and processed for autoradiography. Exposition to photographic emulsion was for 1–3 weeks at −80°C. **a**: Bud (arrow); (**b**–**d**) different stages of protoscolex development; (**e**) mature protoscolex. GL, germinal layer; RC, rostellar cone; RP, rostellar pad; SU, suckers; NR, neck-rostellum; BO, body. Bar: 20 μm.

of cellular territories during the life cycle in parasitic platyhelminths. Hess [1980] described a proliferative population of cells at the apical massif of the larva of the parasitic platyhelminth *Mesocestoides corti*, which is restricted to a small area between the SU. This apical massif represents a polynucleated cell mass which has been proposed as responsible for the anterior–posterior fission of these larvae. Coincidently, incorporation of [^{3}H]-thymidine and of bromodeoxyuridine in *M. corti* larvae has shown that proliferative cells were most numerous at the tip of the tetrathyridia [Smith and Mckerr, 2000].

Nollen [1975, 1978] has reported the presence of cycling cells in the reproductive system of *Hymenolepis diminuta* and *Philophthalmus gralli* adult forms. In *Schistosoma mansoni*, this author observed incorporation of [^{3}H]-thymidine into the mitochondria of mature oocytes [Nollen, 1981].

Similarly, adult males and females of *S. mansoni* showed [^{3}H]-thymidine labeled cells in testes and ovary, respectively. In males, there were small undifferentiated cells scattered throughout the tissues which also showed DNA replicative activity. In females, cycling cells were also found in the vitelline gland [Den Hollander and Erasmus, 1984]. When females of *S. mansoni* were exposed in vitro to males, an elevated uptake of [^{3}H]-thymidine was found, as compared to females that had not been mated [Den Hollander and Erasmus, 1985].

These studies point to the importance of studying the DNA replicative activity in these organims, in order to understand the processes of growth and development during their life cycle.

Regarding protein synthesis in the germinal layer of hydatid cysts and during protoscolex formation, we were unable to find any report in the literature, except the pioneer work of Agosin and Repetto [1967] and Agosin et al. [1971]. These detailed reports, however, were focused on metabolic aspects of RNA and protein synthesis in mature protoscoleces with no connection with the process of protoscolex formation from the germinal layer.

Our data point to a lower rate of [^{3}H]-thymidine incorporation into DNA in fertile germinal layer cells, as compared to protoscoleces. This may reflect the dilution of the label as a consequence of the patchy distribution of cycling cells in this tissue, in which most of the cells are out of the cell cycle.

Regarding protein synthesis, the rather low rate of [^{3}H]-leucine incorporation into proteins of the fertile germinal layer cells, as compared to protoscoleces, may result from the dilution of the label with proteins of the laminar layer of hydatid cysts, part of which is dissected together with the germinal layer. The laminar layer is acellular but rich in proteins. Considering the short times of [^{3}H]-leucine incorporation, labeled proteins synthesized in the cells of the germinal layer should not be secreted to the laminar layer. Indeed, we were unable to find labeled proteins in the laminar layer after autoradiography (not shown). On the other hand, the slightly higher incorporation of [^{3}H]-leucine into proteins of protoscoleces may reflect the higher proportion of cycling cells in these larvae, as compared to the fertile germinal layer, in which cycling cells are diluted among non-proliferative cells.

In the formation and growth of any tissue, three basic processes should be considered: cell proliferation (increase in cell number), cell growth (increase in cell size), and cell death (elimination of cells) [Baserga, 1985]. The formation of protoscoleces from the cells of the germinal layer of hydatid cysts may follow this general pattern. Cell proliferation may derive from the continuous activity of slowly dividing stem cells located at the germinal layer, which provides cells for another expanding population, with a much faster proliferative activity. This second population should expand rapidly originating the buds in which cell differentiation occurs, leading to functional protoscoleces.

Another strategy for protoscolex formation may be directly associated to a proliferating cell population in the germinal layer, that would produce a mass of cells (buds) that may follow two possible patterns. In one of them, the multiplying cell population of the germinal layer forms a continuum with the cycling cells of buds, which grow to almost their final dimension before starting cell differentiation. In the alternative pattern, the multiplying population of cells at the germinal layer produces buds of replicative cells and, very early, both dividing populations become spatially separated. In this case, after an elongation process of the bud involving cell multiplication, cell differentiation starts while cell division decreases. The remnant dividing cells of the buds mainly provide

specialized cells to the structural territories of the nascent protoscolex. Our results support the latter model, although the presence of stem cells dividing at a low rate in the germinal layer, close to and generating the cell multiplying population, cannot be ruled out.

From the patches of cycling cells at the germinal layer, buds seem to be formed by a flow of cells pushing towards the cyst cavity. This movement of cells may result from the pressure exerted by the growing population of dividing cells. Structural studies on the formation of buds are consistent with this model [Galindo et al., 2002].

Considering that the multiplying cell populations of the germinal layer and the buds are separated very early in the development, further elongation of the buds should be the result of cell multiplication from the dividing cells at the base of the buds. As soon as some organization appears in the growing buds, the number of dividing cells diminishes abruptly, suggesting that most of the replicative activity is directed to provide cells for the main structures of the nascent protoscolex. These cells should leave the cell cycle and become differentiated.

Protein synthesis is highly increased in cycling cells [Baserga, 1985], because of the necessary enlargement of the cell before entering mitosis. This should also be the case in the cycling cells of fertile germinal layer underlying early buds, considering the high level of protein synthesis observed in these cells. Increase in individual cell size and secretion of materials to the extracellular space should concur to the final size of the protoscolex.

Coincidently, early buds showing a high number of cells in S-phase, also show an active synthesis of proteins. The fertile germinal layer that is adjacent to the bud shows a low number of cycling cells and a much lower protein synthesis activity than cells from the bud. Indeed, protein synthesis involved in the growth of cycling cells in the bud, plays a role in the appearance and enlargement of these structures. When cell differentiation starts in the forming protoscoleces, there is a clear decay in the incorporation of labeled amino acids. Protein synthesis is mostly located close to nascent structures suggesting that is not related to cell growth but to cell differentiation. In free protoscoleces, cellular territories showing the highest incorporation of [^{3}H]-leucine into proteins (NR and body) are those in which cycling cells are more numerous. In other more specialized cellular territories which do not present cells in DNA synthesis, such as the RC and the RP, the low activity of protein synthesis may represent the housekeeping activity of these cells. We have no evidence about the participation of materials secreted by cells of the fertile germinal layer or the bud during the formation and growth of this last structure, though we cannot discard this process, particularly during protoscolex maturation.

Previous studies have shown that in *Diphyllobothrium dendriticum* adult worm, proliferating cells, also identified by [^{3}H]-thymidine incorporation into nuclear DNA, are mainly confined to the neck region [Wikgren and Gustafsson, 1967; Wikgren and Gustafsson, 1971; Gustafsson, 1976]. Similarly, *H. diminuta* [Bolla and Roberts, 1971] and *E. granulosus* [Gustafsson, 1976] show proliferating cells in the neck of adult worms. These studies point to an active participation of the neck region of cestodes in the growth of the adult form of the parasite.

Our results show that most of the S-phase cells are part of the body of mature free protoscoleces; this result suggests the participation of this replicative population in the elongation process which starts after the protoscolex fixes at the microvilli in the intestine of canids and that leads to the development of the adult worm. Indeed, an intense replicative activity has been demonstrated in the neck of the adult worm of *E. granulosus* [Gustafsson, 1976]. This observation is in agreement with our results considering that the neck region of the adult worm derives from the body cell territory of the larva. The observation that protein synthesis is also more active in the body of the protoscolex provides further evidence for the importance of this cellular territory in the induction of growth leading to worm development.

Finally, fertile cysts obtained from sheep currently show a higher number of free and attached protoscoleces than those obtained from bovines. However, the location of the population of cycling cells and its spatial distribution in the germinal layer and buds, as well as in attached and free protoscoleces, was the same for cysts from the two hosts. This result strongly suggests that the regionalization of DNA and protein synthesis in developing stages of *E. granulosus* is essentially the same in sheep and bovines infected with this parasitic platyhelminth.

REFERENCES

Agosin M, Repetto Y. 1967. Studies on the metabolism of *Echinococcus granulosus*. IX. Protein synthesis in scoleces. Exp Parasitol 21:195–208.

Agosin M, Repetto Y, Dicowsky L. 1971. Ribonucleic acid of *Echinococcus granulosus* protoscoleces. Exp Parasitol 30:233–243.

Baserga R. 1985. The biology of cell reproduction. Cambridge: Harvard University Press. pp 46–58.

Bolla R, Roberts L. 1971. Developmental physiology of cestodes. X. The effects of crowding on carbohydrate levels and on RNA, DNA, and protein synthesis in *Hymenolepis diminuta*. Comp Biochem Physiol 40:777–787.

Bortoletti G, Ferretti G. 1973. Investigation on the larval forms of *Echinococcus granulosus* with the electron microscope. Riv Parassitol 34:89–110.

Bortoletti G, Ferretti G. 1978. Ultrastructural aspects of fertile and sterile cysts of *Echinococcus granulosus* developed in hosts of different species. Int J Parasitol 8:421–431.

Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72: 248–254.

Burton K. 1956. A study on the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323.

Den Hollander JE, Erasmus DA. 1984. *Schistosoma mansoni*: DNA synthesis in males and females from mixed and single-sex populations. Parasitology 88:463–476.

Den Hollander JE, Erasmus DA. 1985. *Schistosoma mansoni*: Male stimulation and DNA synthesis by the female. Parasitology 91:449–457.

Eckert J, Pawlowski Z, Dar JK, Vuitton DA, Kern P, Savioli L. 1995. Medical aspects of *Echinococcosis*. Parasitol Today 11:273–276.

Galindo M. 1998. Cellular and molecular aspects of the formation, growth, and development of *Echinococcus granulosus* protoscoleces. PhD Thesis, Faculty of Medicine, University of Chile. pp 59–68.

Galindo M, Gonzalez MJ, Galanti N. 2002. *Echinococcus granulosus* protoscolex formation in natural infections. Biol Res 35:365–371.

Gottstein B, Hemphill A. 1997. Immunopathology of *Echinococcosis*. Chem Immunol 66:177–208.

Gustafsson MKS. 1976. Basic cell types in *Echinococcus granulosus* (Cestoda, Cyclophyllidea). Acta Zool Fenn 146:1–16.

Hess E. 1980. Ultrastructural study of the tetrathyridium of *Mesocestoides corti* Hoeppli, 1925: Tegument and parenchyma. Z Parasitenkd 61:135–159.

Macintyre AR, Dixon JB, Green JR. 2001. Mitosis and differentiation in T-cells under cytotoxic action of *Echinococcus granulosus* hydatid fluid. Vet Parasitol 96:277–289.

Nollen PM. 1975. Studies on the reproductive system of *Hymenolepis diminuta* using autoradiography and transplantation. J Parasitol 61:100–104.

Nollen PM. 1978. Studies on the reproductive system of *Philophthalmus gralli* using techniques of transplantation and autoradiography. J Parasitol 64:613–616.

Nollen PM. 1981. Localization of ^{3}H-thymidine in oocyte mitochondria from *Schistosoma mansoni*. J Parasitol 67:355–361.

Rojas MV, Galanti N. 1991. Relationship between DNA methylation and cell proliferation in *Trypanosoma cruzi*. FEBS Lett 295:31–34.

Smith AG, McKerr G. 2000. Tritiated thymidine ([^{3}H]-TdR) and immunocytochemical tracing of cellular fate within the asexually dividing cestode *Mesocestoides vogae* (syn. M. corti). Parasitology 121:105–110.

Thompson RCA. 1976. The development of brood capsules and protoscoleces in secondary hydatid cyst of *Echinococcus granulosus*. Z Parasitenkd 51:31–36.

Urrea-Paris MA, Moreno MJ, Casado N, Rodriguez-Caabeiro F. 2002. Relationship between the efficacy of praziquantel treatment and the cystic differentiation in vivo of *Echinococcus granulosus* metacestode. Parasitol Res 88: 26–31.

Wikgren BJP, Gustafsson MKS. 1967. Duration of the cell cycle of germinative cells in plerocercoids of *Diphyllobothrium dendriticum*. Z Parasitenkd 29:275–281.

Wikgren BJP, Gustafsson MKS. 1971. Cell proliferation and histogenesis in *Diphyllobothrium dendriticum*. Acta Acad Aboeusis (B) 31:1–10.