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Research Article

Budding of *Taenia crassiceps* Cysticerci In Vitro Is Promoted by Crowding in Addition to Hormonal, Stress, and Energy-Related Signals

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Taenia crassiceps cysticerci (cysts) reproduce by budding. The cysts' production of buds was measured in vitro to explore parasite and environmental-related factors involved in the extreme individual variation in parasite loads of inbred mice. Cysts were placed in in vitro culture for 10 days at initial parasite densities of 1, 5, 10 cysts/well in 1 ml of RPMI Medium 1640 without serum. Results showed that there is considerable intrinsic initial variation among inoculated cysts in their production of buds and that increasing parasite density (crowding) stimulates the overall production of buds and recruit into budding most of the cysts. Identical cultures were then subjected to various treatments such as heating and exposure to peroxide to induce stress, or to 17ß-estradiol, insulin, glucose, or insulin+glucose to supplement putatively limiting hormonal and energy resources. All treatments increased budding but the parasites' strong budding response to crowding alone overshadows the other treatments.

1. Introduction

Taenia crassiceps is a cestode that, when adult, lives in the intestinal lumen of some carnivore species (i.e., fox) and in the subcutaneous connective tissue and pleural and peritoneal cavities of rodents (i.e., mice) in its metacestode (cysticercus) stage [1]. For experimental conditions, the infection with *T. crassiceps* is simply attained by an ip injection of a few cysticerci in laboratory mice. Once in the peritoneal cavity, the cysticerci reproduce asexually by budding [2], until reaching massive parasite loads in a matter of 3–6 months that weigh as much as the host [3]. Measuring parasite intensity in such conditions is an easy task requiring no more than a magnifying glass to count the number of parasites installed in the host at the time they are harvested by way of thoroughly washing the infected peritoneal cavity. Thus, Experimental murine intraperitoneal (ip) cysticercosis by *T. crassiceps* ORF strain (ExpMurIPTcrasCistiOrf) has been extensively used for genetical, immunological, endocrinological, and behavioral studies of host-parasite relationships [3–7].

Notwithstanding its usefulness, ExpMurIPTcrasCistiOrf is plagued by unexplained great individual mouse variability in parasite loads and in IgG antibody responses, even within the same genetic strain and sex of the murine host and time of infection [8]. A number of factors from the individual host, the parasite, and the environment have been invoked as being involved in such variability [3]. The possible role of inherent variation in the putatively identical parasites composing the infecting inoculums has received less attention. There are two major sources of possible parasite variation between inoculums, one technical and the other biological. The technical sources are the number of infecting cysticerci in the inoculums, the time of infection studied, and the degree of injury suffered by the cysticerci upon their passage through the syringes' very tight caliber needles when squirted into the peritoneal cavities of the infected mice. The biological sources of unexplained variation are also plenty

and involve both host and parasite genetic and epigenetic physiological factors at the individual level [3].

To test the hypothesis of inherent budding variability among infecting cysticerci, without the participation of the host's responses, we counted the number of buds they produced in in vitro cultures in 1, 5, or 10 mL of RPMI Medium 1640 without fetal serum or added supplements, in isolated conditions (1 cyst/culture well), and in crowded conditions (5 and 10 cysts/well) or with various supplements during 10 days in the different culture conditions.

2. Material and Methods

2.1. Parasite Collection. The cysticerci employed came from two different BalbC/AnN female mice that had been infected ip 2 months before to develop a massive parasite load [3]. Harvesting the cysticerci implies killing the donor mice by etherization (in accordance with our institute's ethical procedure in dealing with experimental animals (at http://www.biomedicas.unam.mx/CodEtico_archivos/Reglamento_Bioterio.pdf) and immediately afterwards slitting its peritoneal cavity to release hundreds of cysticerci into a Petri dish containing phosphate-buffered saline (PBS) and 100 μg/mL antibiotic (penicillin/streptomycin) at room temperature. Typically, the collected cysticerci are presented in three phases: initial (no buds and transparent vesicle), larval (filled with buds and transparent vesicle) and final (no buds and opaque vesicle), [8]. A significant fraction of the harvested cysticerci (~10%–20%) is the subpopulation of tiny (0.1–0.3 mm) nonbudded motile and transparent cysticerci, from which 10 cysticerci are selected to constitute each of the inoculums with which to infect experimental mice. Such selection of cysticerci expected would reduce variability in the resulting parasite loads between infected mice; and it does so to some extent, but significant individual variation in parasite loads usually subsists and not rarely, depending on strain and sex of infected recipient mice, some of the challenged mice are totally spared from infection [4–9]. It is from this subpopulation of tiny nonbudded cysticerci that the cysticerci employed in this in vitro study of their budding process were selected.

2.2. Parasite Culture. Microscopically nonbudded cysts were employed in two experiments. The independent variables were the initial density of cysts cultured in each well (density = 1, 5, 10 cysts/well with 1 mL of medium/well; in a dish with 6 wells), the nature of supplements to the culture medium, and the days of culture (0 to 10) at 37 or 42°C with 5% CO_2. The dependent variables were the number of buds found under light microscopy attached to each cyst (buds/cyst) in each well and the sum of all buds in each well ($\sum$ buds). The culture medium employed was RPMI Medium 1640 without serum. The cysticerci came from two different donor mice and were cultured in the three density conditions without supplements in Experiment number 1 (from donor number 1) or were subjected to various treatments in Experiment number 2 (from donor number 2), such as heating (42°C) and peroxide (30 mM) to induce stress in the cultured parasites, or supplemented with 17ß-estradiol (30 nM), insulin (1.5 U/mL), glucose (56 mM), or insulin+glucose (same concentrations as when by themselves only) to provide with energy resources and restore putatively energy limiting conditions. The culture medium in the wells was changed by fresh medium every 24 hours for the first two days and every 36 hours thereafter.

Table 1: The final budding efficiency (final $\sum$buds/$\sum$cysticerci) in Experiments 1 and 2 at each density.

Culture condition	number of buds/number of cysts		
	$d = 1$	$d = 5$	$d = 10$
Control 1	0.17	3	10.3
Control 2	3	3.7	5.9
Heat	4	2.6	3.9
ß -estradiol	6	6.1	4.5
Insulin	10	6.8	6.7
Glucose	6	5.8	5.9
Ins+Glu	6	5.4	7.9
Peroxide	5.5	5.1	5.1

2.3. Statistics. Statistical analysis was performed with SPSS a Student's t-test to study the significance of contrasts between the different densities. Statistical significance levels were set at $P < .05$.

3. Results and Discussion

Figure 1 shows that in Experiment number 1 the sum of buds produced in each well (budding) progressively increases with increasing parasite densities in a wave-like fashion more clearly visible at density = 10. It took 8 days for density = 1 to initiate budding and by only 1 of the 6 cysts, while it took 3 and 2 days for densities = 5 and 10, respectively, for most or all cysts to bud and 7 days for the higher densities to start a second wave of budding. The first line of Table 1 shows that in Experiment number 1 the final budding efficiency (final $\sum$buds/$\sum$cysticerci) at each density increased from 0.17 to 3.00 to 10.3 for densities 1, 5, and 10, respectively. From these results it is clear that there is considerable initial variation in budding among cysts and that increasing parasite density increases the production of buds and reduces the proportion of nonbudding cysts.

Thus, initial differences in the distribution of "readiness to bud" among the cysts (as defined by the time it takes a resting cysticercus to start budding plus the time taken for a bud to become a cysticercus capable of budding) may well explain the variation of parasite loads in mice infected with apparently similar inoculums. To minimize variation in parasite loads users of ExpMurIPTcrasCistiOrf may try to presynchronize in vitro the cysts meant to be inoculated at 10 cysts/mL until most (80%) are already well into budding (>1 buds/cyst) before their selection and inoculation through a procedure nondisruptive of the cysts.

But now, the questions were how is crowding controlling the budding process? Are the differences in budding related

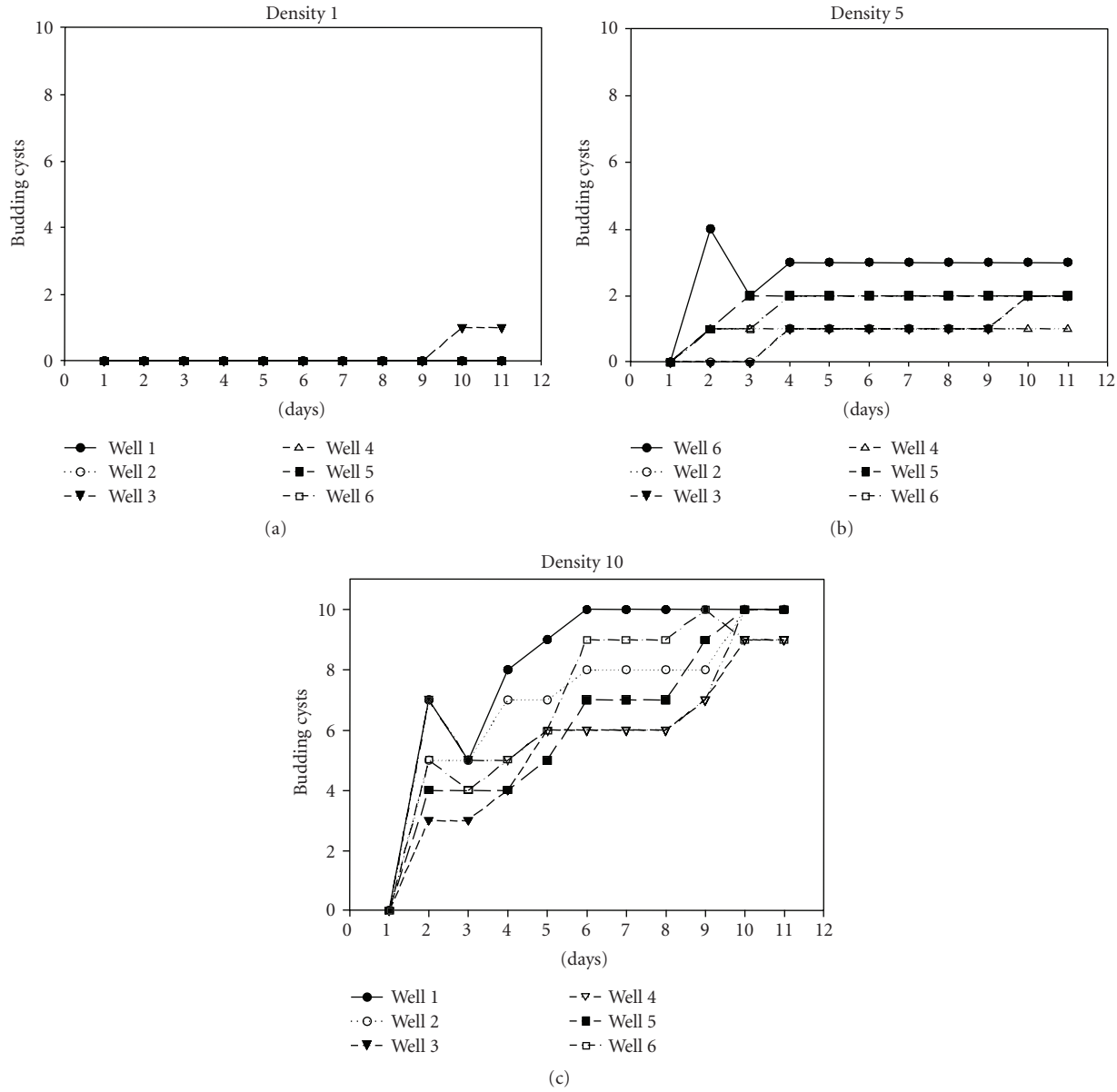

Figure 1: Sum of all buds in each well ($\sum$ buds) of *Taenia crassiceps* cysticerci at three different parasite densities. The sum of buds produced in each well progressively increases with increasing parasite densities in a wave-like fashion

to and/or result from different responses to negative or positive pressures to bud existing in the over-crowded conditions in the peritoneal cavities of the donor mice and/or in the culture tubes? [10–14].

Experiment number 2 was designed to address those questions, bearing in mind that increasing density may decrease resource availability and lead the parasites to enter into stress. Accordingly, the cultured cysticerci from Donor number 2 were submitted to standard stress (heat and peroxide) or favorable conditions (addition of 17ß-estradiol, insulin, glucose, insulin+glucose) and cultured in vitro as done in Experiment number 1. Figure 2 shows the budding process at the different conditions from day 0 to day 10. Table I most clearly shows that the total number of buds/cyst produced in vitro are increased about twofold with respect to unsupplemented control values at densities 1 and 5 but not at density 10, which is in fact reduced by the supplementations. The great difference between the control values of $\sum$buds/cyst in Experiments number 1 and number 2 (0.17 and 3.0, resp.) speaks of there being such differences between the harvested cysticerci from the two donor mice in the cysts' "readiness to bud," possibly depending on the state and terms of each host-parasite relationship established with the donor mice.

It is not surprising that the supplements stimulated budding at low parasite densities because the synthesis and the role of estradiol in stimulating cysts reproduction in vivo and in vitro have been well established [7, 15]

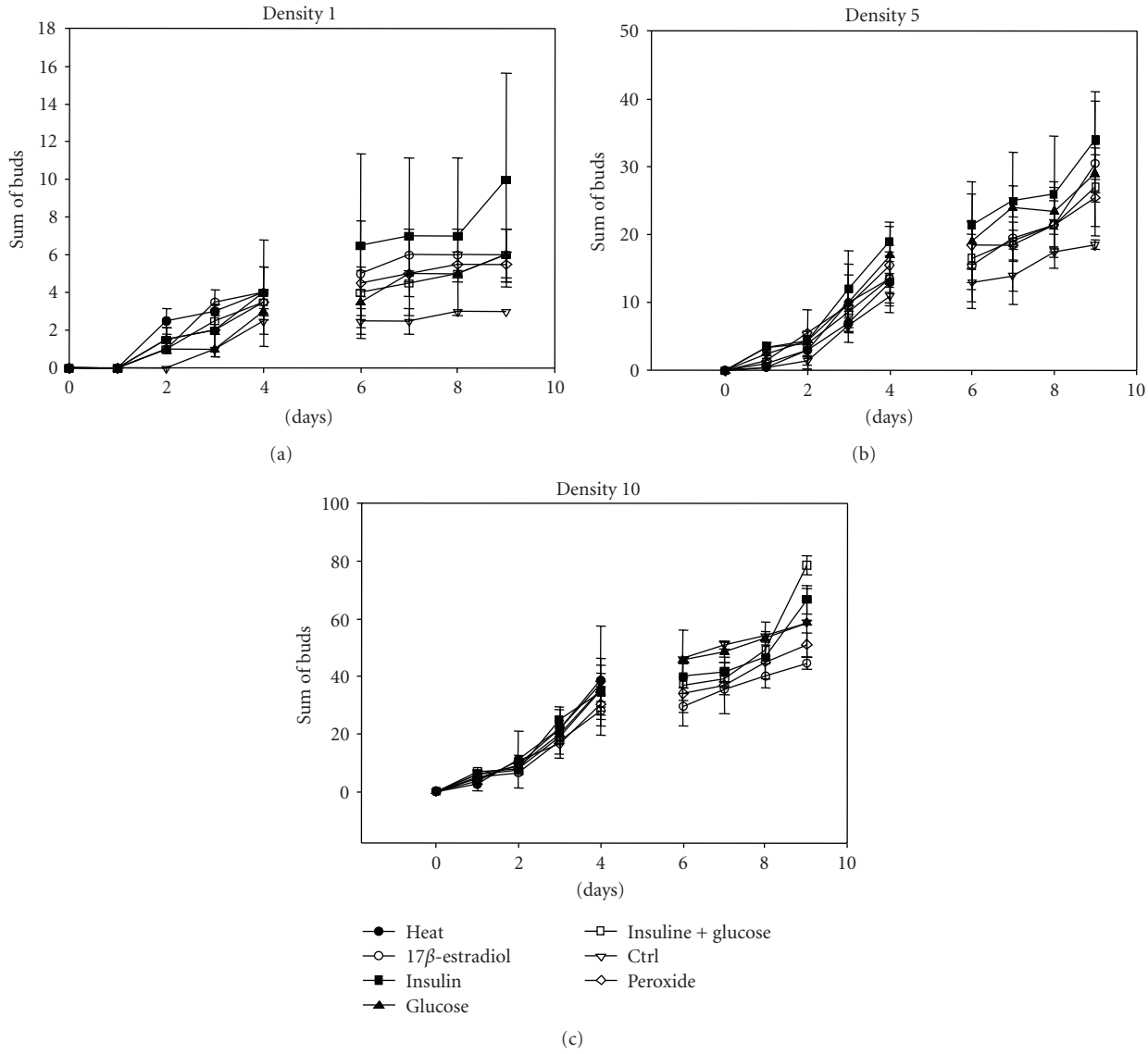

Figure 2: Budding process at the different conditions from day 0 to day 10. The cultured cysticerci from Donor number 2 were submitted to standard stressing (heat and peroxide) or favorable conditions (addition of 17ß-estradiol, insulin, glucose, insulin+glucose) and cultured in vitro as done in Experiment number 1. The results presented are from one experiment with its duplicate for each density. The graphic expresses the average value plus standard deviations. In d = 1 and d = 5, the budding in the presence of glucose and peroxide is significative. In d = 10 the budding in the presence of estradiol and insuline+glucose is significative.

and so is also the role of heat [16]. Likewise, the insulin pathway has been shown to be present in a large variety of invertebrates, including the most primitive metazoan phyla (cnidaria and sponges), and to play a central role in cell division and differentiation [17]. As other possible chemical mediators, we suspect cytokine-like substances which influence reproduction and apoptosis of heterologous cell lines, which cysticerci seem capable of producing and secreting in vivo and in vitro [18–20]. TGF-ß, EGF, and insulin pathways are conserved in helminth parasites with receptor functions probably similar to those of invertebrate and vertebrate orthologs. Indeed, host-derived signals still present in the harvested cysts could have activated parasite receptors and modulated parasite development and differentiation [16, 21].

That crowding cysts at density = 10 *per se* promotes the highest budding efficiency is a novel finding which indicates that crowding is a powerful factor controlling the population of cysts. Possibly, crowding may act by the release of growth factors [19, 20] by the cysts most differentiated and ready to begin budding when placed in vitro, which then recruit those most laggard. Additionally, crowding may be mediated by adhesive molecules or membrane sensors sensitive to contact, as it has been previously reported by Haas et al. and Loverde et al., respectively [22, 23].

That the supplements did not improve, but rather lowered, the budding efficiency at density = 10 suggests that the capacity to bud has an upper limit. Such hysteresis in the system controlling budding could also explain the wave-like form in the dynamics of budding.

Overall, the results are congruent with the hypothesis that parasite inoculums composed of 1–10 apparently identical small nonbudded cysts would likely include a variety of cysts differing in their initial "readiness to bud" and thereby induce variation in parasite loads in infected mice at early times after infection when parasite loads are relatively low, followed by a progressive tendency towards uniformity at later times [13].

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