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Expression and diversity of *Echinococcus multilocularis AgB* genes in secondarily infected mice: evaluating the influence of T-cell immune selection on antigenic variation

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Abstract

The T-cell-mediated immune response exhibits a crucial function in the control of the intrahepatic proliferation of *Echinococcus multilocularis* larvae in mice and humans, both being natural intermediate hosts of the parasite. Antigen B (*AgB*), a metabolized *Echinococcus* spp. lipoprotein, contributes to the modulation of the T-cell immune response, and distinct sites of the corresponding *AgB1*, *AgB3* and *AgB4* genes were shown to be under positive selection pressure. Since several *AgB* gene variants are present in a single *Echinococcus* metacestode, we used secondary *E. multilocularis* infections in BALB/c and in athymic nude mice (devoid of T-cell responses) to analyze the effect of the cellular immune response on the expression and diversity of *EmAgB1–EmAgB4* genes. We demonstrated hereby that *EmAgB* transcripts were less abundant in nude mice during the early phase of infection (at one month post-infection), and that *EmAgB2* is simultaneously down-regulated when compared to the other three genes. A negative relationship exists between the level of transcription and diversity of *EmAgB* genes. Moreover, no excess of non-synonymous substitutions was found among the distinct *EmAgB* alleles from a single host. Together, these results pointed to the effect of purifying selection, which seemed to eliminate the detrimental *AgB* variants generated during the development of the metacestode within the peritoneal cavity of its intermediate host.
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Keywords: Purifying selection; Positive selection; Evasion mechanisms; Alveolar echinococcosis

1. Introduction

Alveolar echinococcosis is a severe human life-threatening zoonosis caused by the infection with the larval stage of the fox tapeworm *Echinococcus multilocularis*. It is characterized by the growth of a tumor-like larval tissue, the so-called metacestode, which primarily infiltrates the host target organ (liver) and secondarily may form metastases in other organs or sites. Because of the growth behavior and the potential of the *E. multilocularis* metacestode to form metastases, alveolar echinococcosis is considered the most lethal helminthic infection in humans (Craig et al., 1996). The common intermediate hosts harboring

Abbreviations: AgB, antigen B; AMV, avian myeloblastosis virus; cDNA, DNA complementary to RNA; DNase, deoxyribonuclease; ds, double stranded; mpi, months post-infection; RT-PCR, reverse transcription–polymerase chain reaction; SDI, Shannon diversity index; SSCP, single strand conformation polymorphism.

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the larval stage of *E. multilocularis* are rodents, but humans can accidentally become infected as well (Thompson, 1995). Since *E. multilocularis* uses mice and humans as natural intermediate hosts, this parasite represents a suitable model to study the infective process in echinococcosis at the experimental murine laboratory level.

Host immunity can contribute to the control of the *E. multilocularis* metacestode growth. Thus, upon experimental secondary infections it became apparent that the metacestode grows faster in athymic nude mice or in severely compromised immunodeficient (SCID) mice than in fully T-cell-competent wild type control animals (Playford et al., 1992; Dai et al., 2004). Furthermore, T-cell immunity significantly contributes to the control of AE in human patients as well, as demonstrated by the rapid, fatal outcome of the infection in an immunodeficient patient co-infected with human immunodeficiency virus (HIV) (Sailer et al., 1997). Upon restoration of CD4-immunocomptence in another AIDS-patient co-infected with *E. multilocularis*, the course of disease could be positively influenced again (Zingg et al., 2004). Gottstein et al. (1994) demonstrated that mouse strains resistant to *E. multilocularis* infections show a stronger cellular immune response than susceptible mouse strains. The main role of the cellular immune response in the control of metacestode growth was confirmed later, by experiments showing that mouse strains which do not develop cellular immune response do not control metacestode growth, while mice deficient in humoral immunity control parasite growth up to a certain level (Dai et al., 2004).

Antigen B (*AgB*) is a lipoprotein secreted during the larval stage of *Echinococcus* spp. parasites. It was first isolated from *E. granulosus* hydatid fluid (Oriol et al., 1971) where it appeared as the most abundant protein (Oriol and Oriol, 1975). The native protein of *E. granulosus* is a 160 kDa polymer composed by 8 kDa monomers (González-Sapienza et al., 1996) encoded by the *AgB* gene family (Chemale et al., 2001). There are five *AgB* genes characterized in *E. multilocularis*, *EmAgB1–EmAgB5* (Frosch et al., 1994; Fernández et al., 2002; Mamuti et al., 2006), but only *EmAgB1–EmAgB4* show similarity (larger than 90%) with ortologous sequences of *E. granulosus*. Antigen B sequence variability inside a single metacestode was described for *E. granulosus* (Arend et al., 2004, Haag et al., 2004) and *E. multilocularis* (Mamuti et al., 2004). We have suggested that this variability could be adaptive, being related to the evasion of immune responses (Haag et al., 2004), but this had never been confirmed to date.

Several approaches have been used so far to examine *AgB* function. *In vitro* studies demonstrated that *AgB* is a protease inhibitor with an ability to inhibit the recruitment of neutrophils (Shepherd et al., 1991). Riganò et al. (2001) evaluated the cytokine secretion profile after *AgB* stimulation in peripheral mononuclear blood cells (PMNC) collected from patients with progressive versus stationary cystic echinococcosis (infection with *E. granulosus*). Their findings showed that *AgB* elicits a Th2 non-protective response preferentially in patients with progressive disease, and it suggested that *AgB* may contribute to the modulation of the host immune response. Chemale et al. (2005) analyzed the *AgB* lipid-binding properties and demonstrated that recombinant peptides encoded by *EgAgB1* and *EgAgB2* were able to bind but not to release fatty acids, fitting with a lipid detoxification role for the *AgB* protein.

Haag et al. (1998) verified an excess of non-synonymous over synonymous substitutions for *EgAgB1* sequences from different strains. Additional analyses of positive selection on genes encoding *AgB* identified particular codons under selection pressure in *AgB1*, *AgB3* and *AgB4*, but not in *AgB2* (Haag et al., 2006b). Since the variable sites occur in sequences from distinct strains/species of the genus *Echinococcus*, which manifestly differ in host specificity, we think that positive selection on *AgB* (among other factors, of course) is important for the adaptation to a particular host species. Interestingly, the distribution of nucleotide polymorphisms in sequences obtained from a single *E. granulosus* metacestode also departs from neutrality, but only for *EgAgB2*, and not for the other *EgAgB* genes (Haag et al., 2004). The adaptive role of nucleotide polymorphisms present inside a single *E. multilocularis* metacestode is the focus in the present study.

2. Materials and methods

2.1. Echinococcus culture and mice infection

Maintenance of *E. multilocularis* metacestodes (KF5 isolate) was done by serial passages in gerbils (*Meriones unguiculatus*). Microcysts were prepared according to Hemphill and Gottstein (1995). Fourteen 8-week-old female athymic nude mice and 14 BALB/c mice were infected by intraperitoneal inoculation of approximately 100 freshly prepared microcysts suspended in 100 µl of Hank's Balanced Salt Solution. The 14 infected mice from each strain were separated in two groups ($n=7$ each), and sacrificed by CO_2-euthanasia 1 or two 2 months post-infection (mpi). The intraperitoneal metacestode tissue was immediately recovered upon euthanasia, weighted and submitted to molecular analysis.

2.2. RNA extraction and cDNA synthesis

Total parasite RNA was isolated by the single-step guanidinium thiocyanate procedure using TRIzol Reagent

Table 1
Primers used to amplify each *EmAgB* gene (5′→3′)

	Primer F	Primer R	Amplicon size (bp)
AgB1	CGAGGAGTATGATGAAAATGC	GTAGATGGTTTATTGAGCAA	233
AgB3	GTTGATGAAGTGACTAGACGAA		255
AgB2	CAAAAGCACACAGGGGGCAAG	GTGTCCCGACGCATGACTTA	220
AgB4	CGAAAATTGAGCGAAATCCGG		191

(Gibco) according to the manufacturer's instructions. Residual genomic DNA was removed from sample RNA by performing 180 min incubation at 37 °C in the presence of RQ1 RNase-free DNase I (Promega). After heat inactivation (10 min at 80 °C) of the DNase, RNA preparations were subjected to cDNA synthesis by applying a random oligonucleotide–hexamer primer (Promega) and AMV reverse transcriptase (Promega). Finally, complementary DNA (cDNA) was purified by using a spin column from High Pure PCR product purification kit (Roche Diagnostics) and subsequently eluted with 100 µl of water before use in RT-PCR analyses.

2.3. Quantification of AgB expression

Four primer pairs were designed to specifically amplify the four *E. multilocularis AgB* genes (*EmAgB1–EmAgB4*). We used a specific forward primer annealing to the 5′ region inside the second exon and a reverse primer annealing to the 3′ flanking region of *AgB1* and *AgB3* or *AgB2* and *AgB4* (Table 1).

The amplification was performed on a LightCycler (Roche Diagnostics) using SYBR Green I as a dsDNA-specific fluorescent dye and a continuous monitoring. Amplifications of cDNA molecules derived from either different *AgB* genes or a "housekeeping" *act* gene (encoding a β-actin homolog) were carried out in triplicates. A control PCR for each sample included RNA equivalents from an aliquot that had not been treated with reverse transcriptase, to confirm that no residual genomic DNA might have resisted to DNase I digestion. PCR was initiated by a "hot start" of 15 min at 95 °C followed by 40 cycles of denaturation (94 °C, 15 s), annealing (50 °C, 30 s) and extension (72 °C, 30 s). Fluorescence was measured at each cycle at 72 °C for *AgB2*, 76 °C for *AgB1* and *AgB3* and at 79 °C for *AgB4*. The quantification of the "housekeeping" *act* gene mRNA was used in order to compensate for variations in *AgB* input RNA amounts and efficiencies of reverse transcription. Mean values from triplicate determinations were taken for the calculation of relative *AgB*-mRNA levels, using the ratio of *AgB*-mRNA to *act*-mRNA.

2.4. Cloning, SSCP screening and sequencing

The RT-PCR products of *AgB1–AgB4* obtained from *E. multilocularis* of one randomly chosen mouse for each group (nude 1 mpi, nude 2 mpi, BALB/c 1 mpi and BALB/c 2 mpi) were purified from solution using appropriate spin columns (Qiagen). One microliter of each purified RT-PCR product was cloned in a TOPO TA Vector for Sequencing (Invitrogen).

About 33–44 recombinant plasmids of each *EmAgB* cDNA were screened for sequence polymorphism by single strand conformation polymorphism (SSCP) after PCR amplification. Each recombinant colony was eluted in 50 µl water and heat denatured at 95 °C for 5 min. The PCR mix was added to 5 µl of the lysate in a final volume of 25 µl. The PCR was carried out with the same primers and conditions as used for RT-PCR. Subsequently, 3 µl of each PCR product were denatured for 3 min in a buffer containing 95% formamide, 0.0125% xylene cyanole and 0.025% bromophenol blue, and immediately chilled on ice. The electrophoresis was conducted in a GenePhor Electrophoresis Unit (GE Healthcare) using pre-casted gels (GeneGel SSCP, 12.5%), at 200 V for 2.5 h and SSCP buffer A (GE Healthcare). The electrophoresis temperature was standardized for each *EmAgB* gene: 12 °C for *AgB1*, *AgB2* and *AgB3*, and 5 °C for *AgB4*. The gels were silver stained, digitalized and analyzed with the ImageMaster package (GE Helthcare). The plasmids containing inserts from different alleles, as identified by the SSCP technique, were sequenced by cycle sequencing using primers M13 F and R (Invitrogen) and BigDye v3.0 (Applied Biosystems). The electrophoresis was performed in a capillary ABI-PRISM 3100 sequencer.

2.5. Data analyses

We used a factorial analysis of variance to search for significant differences in expression between months of infection (one or two) and mouse strains (athymic nude or BALB/c) for each gene using the SPSS (version 10.0) package. The *AgB*-mRNA quantifications relative to *act*-mRNA for each gene (*EmAgB1–EmAgB4*) and mouse were log-transformed (natural logarithm) to normalize their distribution. A one-way analysis of variance was performed to evaluate significant expression differences among genes.

Sequence quality was assessed by Phred software at www.unb.br/electropherogram/ and by visual inspection. The sequences were manually aligned and polymorphism analyses were done with DNAsp v 4.1 (Rozas et al., 2003). The allele diversity for each *AgB* gene in each mouse was estimated by the Past Software (Hammer et al., 2001) using the Shannon Diversity Index (SDI), where the total number of alleles is

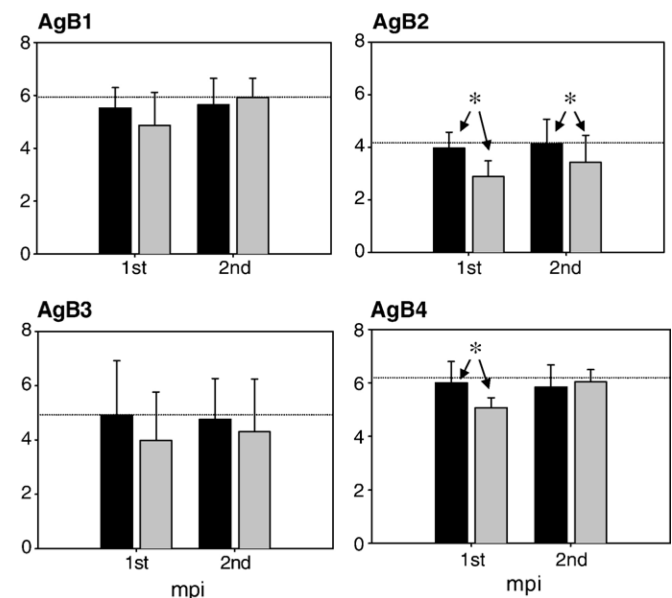

Fig. 1. Average *EmAgB1–EmAgB4* mRNA amounts in parasites collected from BALB/c (black bars) and nude (grey bars) mice at 1 and 2 mpi, quantified in relation to *act*-mRNA. Asterisks display the statistically significant differences ($p<0.05$). The horizontal dashed lines indicate the highest mean, and bars represent the standard deviation.

Table 2
Diversity of *EmAgB1–EmAgB4* alleles in the infected mice

	mpi	BALB/c				Nude				p
		n	h	sa	SDI	n	h	sa	SDI	
AgB1	1	44	5	40	0.385	44	3	42	0.193	0.346
	2	43	6	38	0.488	44	2	43	0.097	0.056
AgB2	1	42	10	33	0.883	42	5	37	0.476	0.155
	2	43	7	36	0.670	33	6	28	0.593	0.792
AgB3	1	43	3	41	0.197	44	2	43	0.097	0.503
	2	44	6	39	0.480	44	5	40	0.385	0.695
AgB4	1	44	2	43	0.097	43	4	40	0.294	0.247
	2	44	4	41	0.289	42	5	38	0.398	0.623

The statistic probabilities (p) of the Shannon Diversity Index (SDI) comparisons between BALB/c and nude hosts are indicated.
n = total number of clones; h = number of distinct alleles; sa = number of clones corresponding to standard allele.

weighted by the frequency of each allele (Poole, 1974). SDIs for each gene/mouse were compared using a t test, where means and variances were obtained by a randomization process based on 1000 permutations (Hammer et al., 2001). We calculated the nonparametric Spearman correlation between *EmAgB1–EmAgB4* expression level and the corresponding SDIs in each mouse using the SPSS (version 10.0) package.

Gene networks were obtained using the TCS software, version 1.21 (Clement et al., 2000). Both Tajima's *D* Test (Tajima, 1989) and the Fisher's exact test for excess of non-synonymous over synonymous substitutions (Nei and Kumar, 2000) were carried out with DNAsp v 4.1 (Rozas et al., 2003) using the sequences sampled from each metacestode. An additional test, based on Fitch et al. (1997), was used to calculate the probability of a given set of mutations in a coding sequence. In this binomial probability analysis we determined the probability for a synonymous and non-synonymous mutation set to be observed, considering the number of synonymous and non-synonymous sites present in the sequence. Therefore, in a sequence with m nucleotides in which s is the number of synonymous sites and n (m–s) is the number of non-synonymous sites (Nei and Gojobori, 1986) we considered that s/m was the probability of each mutation to be synonymous and n/m the probability from this mutation to be non-synonymous. Then, we used the binomial distribution properties to estimate the probability of a certain set of mutations for a given sequence.

3. Results

3.1. Parasite mass recovered from experimentally infected mice

To obtain parasites that underwent different immune challenges from the host, 14 T-cell-deficient nude mice and 14 BALB/c controls were intraperitoneally infected. At 1 mpi the mass of parasites recovered from 7 mice of each strain was relatively low and not significantly different for both nude and BALB/c mice (0.57 g±0.22 and 0.94 g±0.28, respectively). However, when the parasite was recovered 2 mpi, the nude mice contained a significantly larger amount of metacestode mass than the BALB/c (6.21 g±2.87 and 1.85 g±1.34; p<0.05). These results confirm that *E. multilocularis* metacestode growth is suppressed in a host with an intact immune system when compared to a T-cell-deficient situation.

3.2. AgB-mRNA quantification

To evaluate the relative expression of *E. multilocularis AgB* genes and to investigate if the host T-cell immune response has

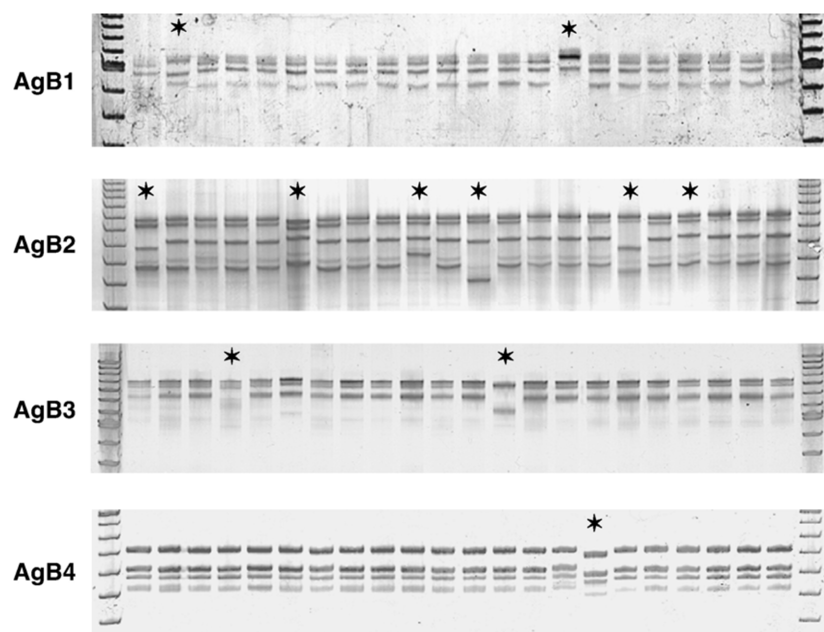

Fig. 2. Representative SSCP patterns obtained for *EmAgB1–EmAgB4* amplicons derived from distinct clones. Asterisks indicate the variant alleles, differing from the standard sequence by minor nucleotide changes, as subsequently confirmed by sequencing.

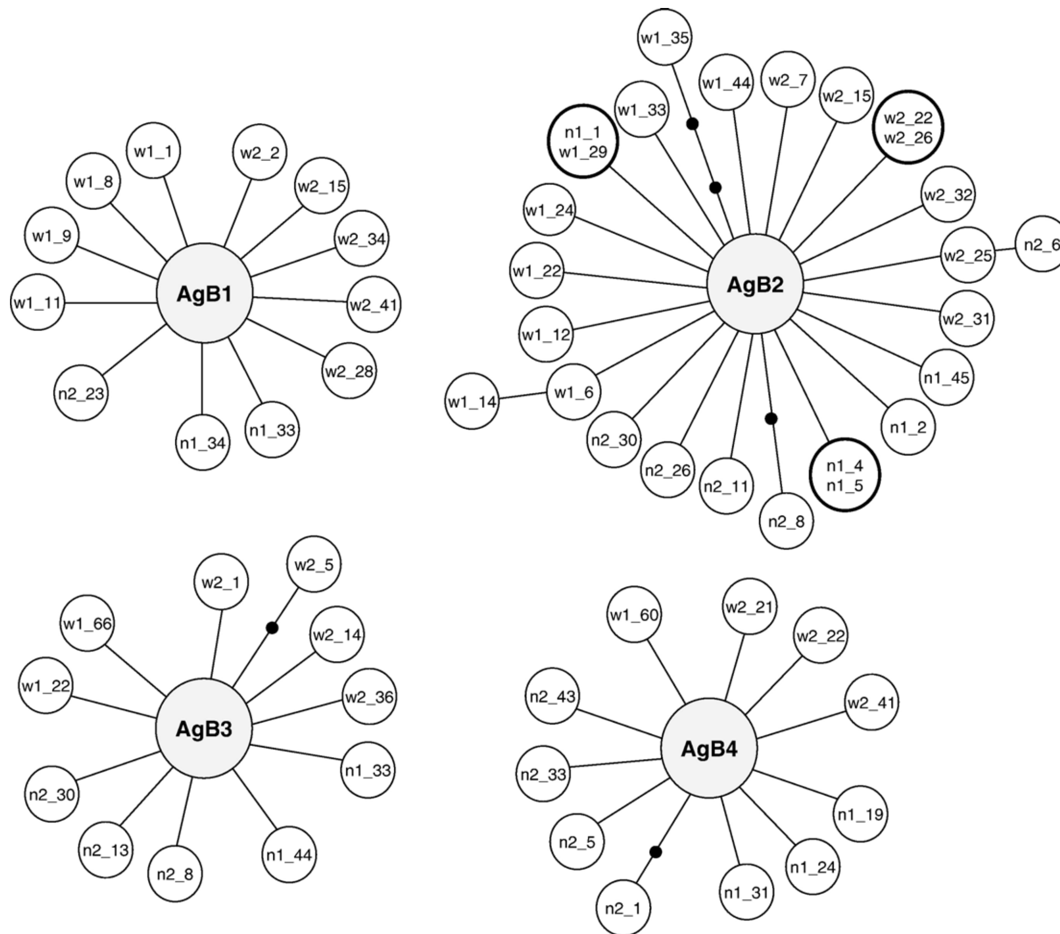

Fig. 3. *EmAgB1*–*EmAgB4* allele networks. The larger circles indicate the alleles obtained in our sample and dots represent the putative intermediate states, which were not encountered. Each line represents a single mutational step, as inferred according to a 95% confidence parsimony criterion. The standard sequences are always the most abundant, and variants appear only once, except for two variants of *EmAgB2*, which appeared twice in the same mouse or in separate mice (indicated with bold circles). Variants are denoted according to the mouse strain (w denotes the BALB/c and n the nude mice) and month of parasite collection (1 or 2 mpi). Numbers after the underline sign indicate the respective clone. GenBank accessions are DQ826464–DQ826476 (*EmAgB1*), DQ826477–DQ826503 (*EmAgB2*), DQ831832–DQ831843 (*EmAgB3*) and DQ831844–DQ831855 (*EmAgB4*).

an effect on *EmAgB* transcription, we quantified the abundance of mRNAs encoding the four known *AgB* subunits in metacestodes recovered from BALB/c and nude mice at 1 and 2 mpi. First, it became apparent that *EmAgB2* was significantly less transcribed than the other three *AgB* genes ($p<0.01$; Fig. 1). Note that the amount of mRNA is expressed in a logarithmic (ln) scale, meaning that the difference from *EmAgB2* mRNA quantities to those obtained for the other genes corresponds to about e^2. Our results also show differences in expression between the two mouse strains, most evidently visible at 1 mpi (Fig. 1). Within 1 mpi, the closely related *EmAgB2* and *EmAgB4* genes were significantly more expressed ($p<0.05$) in the BALB/c host. Although *EmAgB1* and *EmAgB3* also show more mRNA transcripts during 1 mpi in the BALB/c mice, the difference detected was statistically not significant, probably due to the large variances and small sizes in our samples. At 2 mpi, parasites from both host strains did not show differences in the amount of *EmAgB* transcripts, except for *EmAgB2*, which was consistently less transcribed in the nude mice (see Fig. 1).

3.3. AgB diversity and host T-cell immune response

The role of the T-cell dependent immune response on *AgB* gene diversification was evaluated by cloning and sequencing transcripts (cDNA) from the four *AgB* genes, sampled from parasites of four randomly selected mice from each group. The total number of alleles, and the diversity index for each gene/mouse, are shown in Table 2. The lowest allele diversity, as estimated by SDI, was obtained for *EmAgB4* from the BALB/c mouse at 1 mpi and for *EmAgB1* and *EmAgB3* from nude mice at 1 and 2 mpi, respectively, whereas the highest diversity values were from *EmAgB2*, on both months and both mouse strains. However, the differences of SDI between genes were statistically significant ($p<0.01$) only when *EmAgB2* was compared to *EmAgB3* and *EmAgB4* in the BALB/c mouse at 1 mpi. There was a negative relationship between *AgB* transcription levels and transcript diversity, but all Spearman coefficients were non-significant (data not shown). The best relationship between expression and diversity was obtained for parasites at 2 mpi ($\rho=-0.667$; $p=0.071$; $n=8$).

Table 3
Statistical tests for selection with the *EmAgB1–EmAgB4* alleles found in each infected mouse

	mpi	BALB/c				Nude			
			p				*p*		
		D	*D*	FET	BPA	*D*	*D*	FET	BPA
AgB1	1	−1.873	0.005	0.199	0.170	−1.477	0.037	0.786	0.214
	2	−1.999	0.006	0.710	0.408	−1.115	0.132	–[a]	1.000
AgB2	1	−2.176	0.000	0.618	0.384	−1.761	0.017	0.631	0.417
	2	−1.762	0.009	0.202	0.172	−1.888	0.004	0.707	0.404
AgB3	1	−1.479	0.033	–	1.000	−1.115	0.111	–	1.000
	2	−1.873	0.006	0.095	0.090	−1.873	0.006	0.477	0.377
AgB4	1	−1.117	0.125	0.785	0.782	−1.710	0.011	0.481	0.479
	2	−1.708	0.010	0.518	0.399	−1.708	0.010	0.201	0.173

Tajima's *D* value and statistical probabilities (*p*) of Tajima's *D*, Fisher exact test (FET) and binomial probability analysis (BPA) are displayed.
[a] Dashes indicate that the test could not be performed because there were no mutations inside the coding region.

The diversity values of *EmAgB1–EmAgB3* were higher in the BALB/c mice at both time points (1 and 2 mpi), while in the nude mouse the values were higher for the *EmAgB4* only. However, differences between the *EmAgB1–EmAgB4* diversities and between parasites sampled from nude and BALB/c mice were all not statistically significant (Table 2). These results do not suggest a clear relationship between *EmAgB* diversity and host T-cell immune response in the frame of secondary infections in mice. Differences in *EmAgB* diversity between 1 and 2 mpi for each gene were also not statistically significant.

3.4. *EmAgB* alleles within the host are originated by point mutations

The SSCP technique efficiently detected single nucleotide polymorphisms from the *EmAgB* amplicons obtained in the present study (Fig. 2). We sequenced ~10 clones showing the same standard SSCP pattern for each gene, and no nucleotide variation was encountered. Most of the variant SSCP patterns found in the present study, which we call alleles, differed by a point mutation.

As expected, most nucleotide substitutions were transitions (44 out of 50 substitutions). A surprising result was that six out of 26 *EmAgB2* and two out of 12 *EmAgB3* variants contained indels, which would result in non-functional proteins (data not shown). The parsimony networks generated from our set of sequences (Fig. 3) showed that each *EmAgB* variant originated independently by a single mutation from the most frequent, standard allele. There were only a few exceptions, four occurring for *EmAgB2*, one for *EmAgB3* and one for *EmAgB4*. A single *EmAgB2* variant was shared by parasites from different mice (clones b2w1_29 and b2n1_1) and another two appeared twice in the same mouse (Fig. 3). All other variant alleles occurred only once and in a single mouse.

3.5. Natural selection and murine T-cell immunity

In order to determine the evolutionary significance of *EmAgB* diversity inside a single *E. multilocularis* metacestode, we looked for evidences of natural selection on transcript variability. A Tajima's *D* Test was performed, using the total cluster of *EmAgB* alleles collected from each mouse. The values were all negative and statistically significant, except for *EmAgB1* at 2 mpi of a nude mouse and *EmAgB4* 1 mpi of a BALB/c mouse (Table 3). Negative and significant Tajima's *D* values, caused by an excess of rare alleles in the sample, could be explained either by an adaptive sweep, or by a rapid population expansion. Trying to discriminate between these two causes, we carried out additional tests, classifying each mutation from the rare variant alleles as synonymous or non-synonymous. Both the Fisher exact test and the binomial probability analysis (see Materials and methods section) would provide additional evidences about the occurrence of negative or positive (diversifying) selection, given a significant excess of synonymous or non-synonymous mutations, respectively. However, our results did not indicate departure from neutrality.

4. Discussion

Considering that, as a basic principle, the host controls *Echinococcus* metacestode growth predominantly via cellular immune responses, and that parasitic *AgB* modulates the host's T-cell response, we analyzed secondary *E. multilocularis* infections of athymic nude and T-cell-competent BALB/c mice, to assess the effect of a host T-cell response on *AgB* expression and respective diversity. For this, we quantified the transcription of *EmAgB1–EmAgB4*, hereby reporting that the *AgB* mutations originated in both mouse strains during parasite development. At variance with the former view that *AgB* variation would be adaptive even within a single host, we concluded that it is better explained by the combined effect of diversifying selection at the population level and relaxation of purifying selection at the level of individual metacestodes.

Antigen B is among the most abundant and immunogenic antigens of *Echinococcus* (Oriol et al., 1971; Lightowlers et al., 1989). There is accumulating evidence that *AgB* modulates the intermediate host immune response abrogating protective immunity and consequently maintaining a continuous status of parasite proliferation (Shepherd et al., 1991; Riganò et al., 2001, 2002). Parasite antigens which interact directly with the host immune system are usually subject to strong selective pressures and can co-evolve with host immunity (Baum et al., 2003). Primarily the cellular immune responses, but not the humoral responses, are able to contribute to the control of the *Echinococcus* metacestode growth in mice (Fotiadis et al., 1999; Dai et al., 2004); therefore BALB/c mice are anticipated to challenge the *E. multilocularis* metacestode cell proliferation more importantly than athymic nude mice.

Intriguingly, the quantity of *EmAgB* transcripts was higher in the T-cell proficient BALB/c mice during the beginning of the infection. It seemed that during the early period of metacestode proliferation, *EmAgB2* and *EmAgB4* genes were down-regulated in the athymic nude mice, which lacked of T-helper cell interaction. Two alternative explanations are possible for these findings: *AgB* genes were either 1) directly or indirectly induced by T-cell immune responses in the BALB/c mice; or 2)

down-regulated in the immune deficient nude mice. In another gene expression study, using the same infected mice analyzed here, Matsumoto et al. (2006) had found lower levels of 14-3-3 transcripts in the nude mice infected with *E. multilocularis* at 1 mpi, but not for II/3–10, a protein related to the mammalian ezrins, radixins and moezins, all involved in the cellular architecture. Both *AgB* and 14-3-3, which are known to be involved in the control of cell proliferation, must have critical roles in parasite infectivity and patogenicity.

The first step to understand the differential control of *AgB* genes during the *E. multilocularis* life cycle was made by Mamuti et al. (2006), by demonstrating that *EmAgB3* is constitutively expressed, while the other *AgB* genes are mainly transcribed in protoscoleces. The key factors linking the host immune response and the control of *Echinococcus* gene expression should be a matter for further study, since they are the basis of a host–parasite cross-talk, which determines the fate of the infection. It has already been shown for schistosome parasites that the host cellular immune responses in the definitive host have major effects on parasite early development (Davies et al., 2001). The lack of a T-cell immune response alters the normal parasite development in the early stages, leading to attenuated parasite forms which would prolong host survival.

A high sequence (allele) diversity of *E. granulosus AgB* genes inside a single metacestode was previously reported (Arend et al., 2004; Haag et al., 2004) and its origin was supposed to be related to the host-mediated immune selection pressure (Haag et al., 2004). Our results demonstrated that *E. multilocularis AgB* genes can also reach high diversities in a single metacestode. Although we found no significant difference between the SDI of *AgB* genes from parasites recovered from nude and BALB/c mice, we think that the higher values found for *EmAgB1–3* in the BALB/c mice and for *EmAgB4* in the nude model could have a biologic impact. However, due to the limited number of infected mice analyzed (one mouse per strain and mpi), we cannot draw definitive conclusions about any direct relationship between sequence diversity and T-cell immune challenge exerted by the host.

We are also aware that it is not possible to ascertain if the observed mutations had originated before the experimental infection (during gerbil passages or microcyst proliferation inside the *in vitro* culture) or after the infection (during metacestode growth inside host peritoneal cavity). Most alleles arose by a single mutation from the standard sequence, but metacestodes from distinct mice seldom shared a non-standard allele (this happened only once for *EmAgB2)*, which could be an indication that the mutations occurred after the infection. Moreover, since a similar pattern of diversity (excess of rare alleles in a single metacestode) was also described for *E. granulosus* (Haag et al., 2004), where the cyst develops from only one oncosphere, we suggest that the mutations were mostly originated inside the host, although we can not rule out that some mutations were already present in the microcyst population injected into the mice.

Another scenario which would explain both the present findings for *E. multilocularis* and the previous results obtained for *E. granulosus* would be an extremely high redundancy of *AgB* genes inside the genome. In this case, a single cell could bear a number of distinct *AgB* gene variants expressed at the metacestode stage. However, genomic Southern Blots for *E. granulosus AgB* genes did not indicate a high redundancy (Chemale et al., 2001; Haag et al., 2004), but we hypothesized that *AgB* genes can increase in number through gene rearrangements during metacestode growth (Haag et al., 2006a).

The present results failed to confirm our previous hypothesis that the *Echinococcus* within-metacestode *AgB* variability would represent some form of antigenic variation mechanism. There is no straightforward proof that *AgB* variation is directed to T-cell-mediated immune response. Moreover, our analyses showed that the substitution process in *EmAgB1–EmAgB4* alleles followed the neutral pattern, since the frequency of non-synonymous and synonymous mutations was proportional to the number of non-synonymous and synonymous sites. Two questions remain, however.

First, if particular amino acids changes do not seem to be favored by natural selection when in contact with the host immune system, how are mutations maintained? Our results of transcript quantification of *EmAgB1–EmAgB4* genes demonstrated that the lowest expressed gene is *EmAgB2.* In our mutation analysis *EmAgB2* was also the most diverse gene, and a large fraction of the variant alleles contained missense frameshift mutations (data not shown). Although not statistically significant, the inverse relationship between the amount of mRNA and genetic diversity suggests that variation in *AgB* genes within a metacestode is principally maintained through a relaxation of purifying selection.

Second, what is, then, the biological significance of the *AgB* positively selected sites previously mapped by Haag et al. (2006b)? Although the positively selected sites identified in our previous study did not seem to have a biologic significance in terms of antigenic variation within a single host, it is possible that *AgB* variation is needed at the population level. Particular *AgB* alleles might be involved in the adaptation to a particular host species, or even genetic variable individuals within a host species. Indeed, the sequences analyzed in our previous study came from different *Echinococcus* strains or species, which are specialized to distinct intermediate hosts. Being able to infect a wide range of herbivorous mammals, *Echinococcus* parasites must be able to face quite distinct immune challenges, and *AgB* diversity appears to have an important role in this process.

Our expression studies suggest that there are significant differences in the *AgB* gene transcription levels between BALB/c and nude mice, especially during the early periods of infection. However, we were not able to conclude from the present *AgB* diversity data, if the antigenic variation is directed or not to T-cell response evasion. Further studies focusing on this particular question should, therefore, rely on larger sample sizes. Furthermore, reconstituting nude mice with purified immune cell populations from normal mice will also help to address this problem in the frame of future experiments. Another aspect which might have influenced our results is that we worked with secondary infections, starting from microcysts showing a rudimental laminated layer, which might already protect the parasite from the direct attack of host immune cells. The biological role of *AgB* diversity during oncosphere establishment at the very early stage of primary infection, where innate immunity

leads into adaptive immunity, remains an open question, especially also in view of the subsequent events in the target organ where differentiation into a mature metacestode occurs.

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