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Evidence for Specific Genotype-Dependent Immune Priming in the Lophotrochozoan *Biomphalaria glabrata* Snail

Julien Portela[a, b] David Duval[a, b] Anne Rognon[a, b] Richard Galinier[a, b] Jérôme Boissier[a, b] Christine Coustau[c] Guillaume Mitta[a, b] André Théron[a, b] Benjamin Gourbal[a, b]

[a]CNRS, UMR 5244, Ecologie et Evolution des Interactions (2EI), and [b]Université de Perpignan Via Domitia, Perpignan, and [c]UMR 6243 Interactions Biotiques et Santé Végétale INRA, CNRS, Université de Nice Sophia-Antipolis, Nice, France

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Immune priming · Specificity · Genotype · lophotrochozoan · *Biomphalaria glabrata*

Abstract
Historically, the prevailing view in the field of invertebrate immunity was that invertebrates that do not possess acquired adaptive immunity rely on innate mechanisms with low specificity and no memory. Several recent studies have shaken this paradigm and suggested that the immune defenses of invertebrates are more complex and specific than previously thought. Mounting evidence has shown that at least some invertebrates (mainly Ecdysozoa) show high levels of specificity in their immune responses to different pathogens, and that subsequent reexposure may result in enhanced protection (recently called 'immune priming'). Here, we investigated immune priming in the Lophotrochozoan snail species *Biomphalaria glabrata,* following infection by the trematode pathogen *Schistosoma mansoni*. We confirmed that snails were protected against a secondary homologous infection whatever the host strain. We then investigated how immune priming occurs and the level of specificity of *B. glabrata* immune priming. In this report we confirmed that immune priming exists and we identified a genotype-dependent immune priming in the fresh-water snail *B. glabrata*.

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Introduction

There is currently great debate as to whether immune memory is exclusive to vertebrate animals [1–4]. The prevailing opinion had been that only vertebrates possess the mechanisms of immune memory, which occur via their adaptive immune response and allow the immune system to specifically recognize antigens through somatically generated immune receptors [5, 6], and reuse these receptors and even amplify them through the use of memory cells [7]. Until recently, no diversified molecules or memory cells had been discovered in invertebrates, which thus were thought to lack acquired adaptive immunity and instead possess innate immune mechanisms with low specificity. The immune systems of invertebrates were believed to discriminate pathogen-associated molecular patterns using a limited repertoire of invariable germ line-encoded pattern recognition receptors that engaged effector pathways capable of acting on the recognized intruders [8, 9]. However, several lines of evidence countered this viewpoint, suggesting that invertebrate immunity could possess higher levels of specificity and acquired protection. The first hints of this came from experiments on graft rejection, which revealed the presence of allorecognition processes in diverse invertebrate phyla (i.e. Porifera, Cnidaria, Annelida, Echinodermata,

KARGER

Fax +41 61 306 12 34
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Dr. Benjamin Gourbal
Université de Perpignan Via Domitia
UMR 5244 CNRS Ecologie et Evolution des Interactions (2EI)
52 Avenue Paul Alduy, FR–66860 Perpignan Cedex (France)
E-Mail benjamin.gourbal@univ-perp.fr

etc.) [1, 10–13]. These studies showed that invertebrates were able to tolerate isografts, but rejected allografts (hallmark of specificity) and possessed the ability of faster graft rejection following a secondary allograft exposure (hallmark of memory). Although it was hypothesized that this could be explained by competition occurring between colonial organisms, the benefit of such recognition events was difficult to explain for noncolonial animals, such as earth worms [12]. Consequently, it was theorized that this specific recognition could be used for the identification of aberrant self-generated cells or pathogen-derived antigens [14]. In addition, several transcriptomic approaches recently developed in different invertebrate species have revealed large and individual repertoires of putative immune receptors that could represent the molecular mechanisms underlying immune specificity. These diversified molecules have been identified in echinoderms (SRCR or Sp185/333 of sea urchin [15]), insects (DsCAM of *Drosophila melanogaster* and *Anopheles gambiae* [16, 17]), and mollusks (fibrinogen-related proteins, FREPs, of *Biomphalaria glabrata* [18]). The arguments for the involvement of these molecules in antigen recognition have recently been strengthened, especially for FREPs, which were shown to be involved in immune complexes with various antigens of the *B. glabrata*-specific trematode pathogen, *Schistosoma mansoni* [19].

Thus, invertebrates seem to be able to specifically recognize antigens/pathogens and destroy them more efficiently upon a second exposure. In this context, two secondary immune response processes could be expected. Firstly, a process of acquired resistance or sustained response could be expected. This response is characterized by the induction of an immune response following a first stimulation that confers long-lasting protection against later challenge [20]. This immune response persists at high levels even if the pathogen is neutralized [20]. Secondly, an immune response, termed 'immune memory', reminiscent of vertebrate acquired immunity could also be expected in invertebrates. It is characterized by the induction of a primary immune response following first pathogen stimulation. The primary response returns to a basal level when the infection is cleared. This first immune stimulation provides the immune system with the ability to recognize and remember specific antigens/pathogens, and to mount a faster and more powerful response against a subsequent exposure to the same antigen/pathogen [1, 21]. Both of these secondary immune responses were called 'immune priming' in invertebrates.

Several studies have found evidence for (insects, crustaceans) [2, 14, 22–24] and others have failed to detect (insects) [3, 25, 26] immune priming in invertebrates. Thus it is difficult to conclude whether priming is universal, restricted to several invertebrate groups or species, or to specific host/parasite combinations. Immune priming was described mainly for arthropods (insects [2, 24, 27–29] or crustaceans [4, 21, 30, 31]) infected by bacteria, protozoa or virus. Immune priming might also occur in insects or crustaceans via trans-generational processes, where bacterial immune-challenged or infected parents produce protected offspring via a maternal and/or paternal transfer of immune protection [30, 32–35]. Finally, to our knowledge, only one paper has investigated immune priming against a metazoan parasite and this was also identified for a crustacean [23].

Immune priming specificity also appears to be controversial. In some models immune priming could be very specific at the species or even strain level [2, 23, 27, 28], while in others immune priming appeared to be nonspecific and cross-protection occurred. For example: (i) infection with bacteria or injection of lipopolysaccharides protect against fungal [36] or protozoan pathogens [24]; (ii) fungal β-glucans protect against bacterial infections [37], and (iii) wounding was found to induce nonspecific immune responses that prevent bacterial or yeast opportunistic infections [38–40].

Most of our knowledge on immune priming comes from a few model species belonging to Ecdysozoa and much remains to be elucidated from Lophotrochozoa species. This is crucial for a better understanding of the evolutionary history of the invertebrate immune priming and is of central importance in understanding the diversity and evolution of innate memory processes from Ecdysozoa to Deuterostomia.

Here we used the *B. glabrata* snails and their natural trematode parasite *Schistosoma* spp. to investigate immune priming in a Lophotrochozoa species exposed to a metazoan parasite. Recent advances in understanding the *B. glabrata*/*Schistosoma* interaction at the phenotypic and molecular levels [18, 19, 41–46] and a previous study describing a time-dependent 'acquired resistance' in *B. glabrata* against *S. mansoni* challenges [47] make this model particularly well adapted to investigate the question of immune priming.

In this report we investigated how immune priming occurs and the level of specificity of *B. glabrata* immune priming using different approaches. First, we exposed two geographic isolates of *B. glabrata* snails to homologous challenges to test the effect of host strain on immune

priming. Second, we used a histological approach to investigate the putative role of parasite development and migration in the observed immune priming process. Third, we investigated the biological mechanisms involved in immune priming, using snails exposed to irradiated miracidia and tissue injuries. Finally, we investigated the specificity of immune priming in *B. glabrata* by comparing the infection success following homologous or heterologous challenges of four different genetic strains or species of *Schistosoma*.

Table 1. Prevalence and intensity of host/parasite combinations

Biomphalaria strain	*Schistosoma* strain	Miracidia n	Prevalence %	Intensity n
BgGUA	SmBRE	10	80	2.4
BgBRE	SmBRE	10	100	3.6
BgBRE	SmBRE-LE	10	100	5.1
BgBRE	SmVEN	10	100	3.2
BgBRE	Srod	10	75	2

Prevalence corresponds to the percentage of snails infected; intensity corresponds to the average number of SpIs for each infected snail.

Material and Methods

Ethics Statement

Our laboratory holds permit No. A66040 for experiments on animals from both the French Ministry of Agriculture and Fisheries, and the French Ministry of National Education, Research and Technology. The housing, breeding and animal care of the utilized animals followed the ethical requirements of our country. The experimenter also possesses an official certificate for animal experimentation from both French ministries (Decree No. 87-848, 19 October, 1987). Animal experimentation follows the guidelines of the French CNRS. The different protocols used in this study have been approved by the French veterinary agency from the DRAAF Languedoc-Roussillon (Direction Régionale de l'Alimentation, de l'Agriculture et de la Forêt), Montpellier, France (authorization No. 007083).

Snail and Parasite Strains

Two strains of *B. glabrata* [48] were used in this study. The Guadeloupean strain of pigmented *B. glabrata* (BgGUA) and the Brazilian strain of albino *B. glabrata* (BgBRE).

Three South American strains of *S. mansoni* originating from different geographic isolate were used, as well as two Brazilian strains (SmBRE and SmBRE-LE) and one Venezuelan strain (SmVEN). Finally, another species of *Schistosoma* was used, *Schistosoma rodhaini* (Srod), a murine species originating from Africa [49]. SmBRE and Srod had been maintained in the laboratory for thirty years and SmBRE-LE and SmVEN were recovered in 2011. All these *Schistosoma* strains or species were selected because of their similar prevalence and intensity for BgBRE snails (table 1). Here, susceptibility is estimated using snails exposed to 10 miracidia.

Each strain or species of *Schistosoma* was maintained in its homopatric strain of *B. glabrata*, and in hamsters *(Mesocricetus auratus)*, as described previously [48]. Miracidia from both strains were hatched from eggs axenically recovered from 50-day infected hamster livers according to the previously described procedures [50, 51]. Briefly, livers were collected and homogenized, and the eggs were filtered and washed to obtain miracidia.

Genotyping and Genetic Analyses of S. mansoni *Strains*

Genomic DNA was extracted from 20 adults (10 males and 10 females) of each *S. mansoni* strain according to the following protocol. Sixty microliters of TE (Tris 10 mM; EDTA 1 mM; pH 8) containing 1.67 mg/ml of proteinase K (Merck) was added to the parasite. The samples were incubated for 3 h at 55°C, with vortexes every 15 min. The samples were then heated for 10 min at 100°C for proteinase K inactivation. The genomic DNA was recovered in the supernatant and kept at –20°C until use.

S. mansoni strains were subjected to PCR-based genotyping using fourteen microsatellite markers: SmC1, SmDO11, SmDA28 [52], R95529, SmD57, SmD28, SmD25, SCMSMOXII, L46951 [53], SmBR16, SmBR10, SmBR13 [54], SmS7-1 [55] and SmBR1 [56]. PCR was performed in three multiplex reactions using a multiplex kit (Qiagen). Markers R95529, SmC1, SmDO11, SmBR16 and SmD57 were grouped in multiplex 1; SmDA28, SmBR1, SmS7-1, SmD28, SCMSMOXII were grouped in multiplex 2, and SmD25, L46951, SmBR10 and SmBR13 were grouped in multiplex 3. The multiplex reactions were carried out according to the manufacturer's standard microsatellite amplification protocol in a final volume of 10 μl and with an annealing temperature of 57°C. The PCR products were diluted in sample loading solution (Beckman Coulter) containing a red-labeled size standard (CEQ™ DNA size standard kit, 400, Beckman Coulter), and electrophoresis was performed on an automatic sequencer (CEQ™ 8000, Beckman Coulter).

Genotyping of *S. rodhaini* was not realized because microsatellite markers were not available and no microsatellite cross-amplification occurred between *S. mansoni* and *S. rodhaini*.

Deviation from Hardy-Weinberg expectancies and linkage disequilibria were analyzed using the global test in FSTAT v.2.9.3.2 [57]. The level of significance was adjusted for multiple testing using a Bonferroni correction. Furthermore, polymorphism was estimated over all loci and for each strain using the number of alleles, allelic richness, expected heterozygosity (He) and inbreeding coefficient (FIS) computed with FSTAT v.2.9.3.2. Finally, observed He and Nei's genetic distances were calculated with GENETIX software v.4.05.2 [58].

Experimental Protocol of Immune Priming

For all experiments, primary infections were performed on juvenile *B. glabrata* (5–6 mm in diameter). Snails were individually exposed for 12 h to 10 miracidia in 5 ml of pond water. Individual snails were secondarily infected at 25 days after primary infection, using 10 miracidia per snail. As controls for each experiment, 50 unprimed snails with a size equivalent to that of the primary infected snails (8–9 mm in diameter) were exposed to 10

miracidia at the same time as the experimental snails underwent secondary infection (i.e. 25 days after primary infection).

Host Effect on Immune Priming

To test whether the *B. glabrata* host strain had an influence on the observed priming, we used BgGUA or BgBRE and we performed a homologous primary/secondary infection as follow BgGUA + SmBRE + SmBRE or BgBRE + SmBRE + SmBRE.

Specific Genotype-Dependent Immune Priming

To investigate the level of immune priming specificity in *B. glabrata*, we performed homologous and heterologous primary/secondary infections using the BgBRE strain, as follows: BgBRE was primary infected with SmBRE and then challenged with SmBRE (homologous combination) or SmBRE-LE (heterologous combination, same species, same country, different strain) or SmVEN (heterologous combination, same species, different country, different strain) or Srod (heterologous combination, different species). To confirm that the different infection rates upon secondary infection are a consequence of specific priming rather than a more general effect, the same experiment was done using the SmBRE-LE strain as the primo-infection and challenged with homologous or heterologous combinations as described above.

For each experiment, all snails (unprimed or primed) were fixed 15 days after the secondary infection, and the presence and number of primary sporocysts (SpIs) were determined following the previously described method [59] to estimate the prevalence and the intensity of the infection. Briefly, snails were relaxed for 6 h in pond water containing excess crystalline menthol and each snail body was then removed from the shell and fixed in modified Raillet-Henry's solution [59, 60]. After 24 h in fixative, a dissection of the head-foot, mantle and kidney was performed, and the presence and number of SpIs in each snail was determined [59]. The SpIs could be readily observed as translucent white bodies within an opaque yellow tissue background. SpIs arising from the primary infection (40 days old at fixation time) are small and opaque white corpuscle and could be easily distinguished from those of the secondary infection (15 days old at fixation time) that appeared as big translucent white corpuscle. For all the experiments, the success of the primary infection could be determined by the presence of secondary sporocysts (SpIIs) in the hepatopancreas, and only snails harboring SpIIs were subjected to secondary infections. The results were analyzed by calculating the protection level as a ratio between prevalence in primed snails and prevalence in unprimed snails [([prevalence in unprimed – prevalence in primed snails]/prevalence in unprimed snails) × 100]. For intensity a ratio was calculated between primed and unprimed snails to estimate the effect of priming when snails were reinfected [intensity in primed snails/intensity in unprimed snails].

Histological Procedures

To investigate the intramollusk development of *S. mansoni* larvae, BgBRE snails (5–6 mm in diameter) were infected using 10 miracidia of SmBRE. Infected snails were collected at 3, 7, 10, 14, 20, 25 and 35 days postinfection (DPI; 10 snails per condition), and fixed in Halmi's fixative (mercuric chloride 4.5%, sodium chloride 0.5%, trichloroacetic acid 2%, formol 20%, acetic acid 4% and picric acid 10%). The fixed mollusks were then dehydrated and embedded in paraffin, as previously described [46, 61]. Transverse histological sections (10 μm thick) were cut and stained using azocarmine G and Heidenhain's azan (Sigma). Briefly, sections were rehydrated (in successive baths of toluene, 95% ethanol, 70% ethanol, 30% ethanol and distilled water), stained (azocarmine G, 70% ethanol + 1% aniline, 1% acetic alcohol, distilled water, 5% phosphotungstic acid, distilled water, Heidenhain's azan) and dehydrated (in 95% ethanol, 100% ethanol and toluene). The preparations were then mounted with Entellan and observed under a microscope. Pictures were taken with a Nikon MICROPHOT-FX microscope and a Nikon digital sight DS-Fi1 camera.

Experimental Infection with Irradiated Miracidia

To investigate whether immune priming depends on the development and migration of *S. mansoni* in snail tissues, we used UV-irradiated SmBRE miracidia to infect BgBRE snails. The irradiated parasites penetrated the snails normally, but then failed to develop and died. Thus, SpI growth was abolished and there was no development and migration of SpIIs.

In this experiment, juvenile BgBRE (5–6 mm in diameter) were individually exposed for 12 h to 10 irradiated SmBRE miracidia (see below for irradiation procedure) in 5 ml of pond water. Twenty-five days later, secondary infections were performed on experimental irradiated miracidia primary infected snails using 10 nonirradiated SmBRE miracidia, while 25 additional BgBRE snails of comparable size (8–9 mm in diameter) were exposed to 10 nonirradiated SmBRE miracidia and used as a positive control. Fifteen days after the secondary infection, all snails were fixed and the presence of SpI prevalence (% of snail infected) was determined following exhaustive dissection. We performed a positive control of primary infection/secondary infection using the combination BgBRE + SmBRE + SmBRE and following the procedure described in the 'Experimental Protocol of Immune Priming' section.

For irradiation, SmBRE miracidia were exposed to UV emissions from the fluorescent lamp of a BLX 254 nm crosslinker (Bio-Link; radiant exposure = 0.05 J/cm^{-2}). This intensity of UV radiation was sufficient to induce apoptosis among the pluripotent stem cells of the miracidia (germinal cells), which are involved in the development, maturation and cellular differentiation of SpIs, leading to the release of SpIIs [62]. This level of irradiation did not, however, affect the penetration of miracidia (see the following section).

PCR Diagnostics

As irradiated miracidia developed into very small SpIs not detected even under histological staining and did not produce SpIIs, it was difficult to visually assess the success of primary infection. Thus, we developed a PCR-based diagnostic procedure to confirm the penetration of irradiated miracidia and calculate their prevalence following primary infection. Genomic DNA was extracted from BgBRE snails 15 days after individuals were exposed to 10 miracidia irradiated SmBRE. Each snail was relaxed with crystalline menthol, the shell was removed, the snail body was put in DNAzol reagent (Invitrogen), and genomic DNA was recovered according to the manufacturer's instructions. Specific PCR amplification of *S. mansoni* miracidium DNA was performed using the SmAlphaFem gene (GenBank accession No. U12442.1) with the Advantage 2 PCR Enzyme System (Clontech). To ascertain SmAlphaFem gene amplification, two fragments were amplified using specific primer pairs: SmAlphaFem1 (forward, GCTT-

TATCGAGGCAATACGC; reverse, GTTTCGTTCGATTTGCCACT; 270-bp product) and SmAlphaFem2 (forward, TGCACAAGTGAGTGGCTGTGGG; reverse, TGGATGTACCTGCATCCCGTGT; 120-bp product). The PCR cycling conditions consisted of 30 s at 95°C, 30 s at 60°C and 20 s at 72°C for 40 cycles.

Tissue Injury Experiments

Snails were subjected to tissue injuries using two different procedures: (i) 15 BgBRE were pricked six times in the head-foot using a needle (26 G × 0.5″; 0.45 × 12 mm) and then infected with 10 miracidia SmBRE at 5 and 10 DPTI, and (ii) gold microparticles (0.6–1.6 μm) were used with a biolistic gene transfer apparatus (PDS-1000/He system; BioRad) to provoke numerous microinjuries on the snails' tegumental cells. Briefly, snails were relaxed in pond water containing excess crystalline menthol for 12 h so the head-foot protruded outside the shell and would not be retracted during the biolistic procedure. The gene transfer system used a burst of high-pressure helium gas (1,350 psi) to accelerate 20 μl of gold microparticles toward the snail head-foot target cells under a vacuum. Gold microparticle penetration in snail tissue was confirmed by microscopic observation. After 5 and 10 DPTI, 15 BgBRE were infected by 10 miracidia SmBRE. For each tissue injury procedure and each infection time, uninjured snails were infected under the same conditions and used as controls. For all these experimental devices, snails were assessed for their level of protection against secondary infection; the parasite prevalence was estimated following the procedure described in the 'Experimental Protocol of Immune Priming' section.

Vaccination Experiment

For vaccination a whole miracidium protein extract was prepared as follow: 1,000 miracidia from the SmBRE strain were natively extracted in 0.05% TBS-Tween 20 (TBS-T) by sonication (3 pulses of 30 s at 40% of amplitude), centrifuged and the protein amount of supernatant was quantified and conserved at –80°C until used.

Three groups of snails were anesthetized in 500 ml of fresh water with menthol for 8 h. The first group (69 individuals) was injected with 1 μg of parasite extracts in 20 μl of TBS-T. The second group (25 individuals) was injected with 20 μl of TBS-T alone and used as a control for the injection. The third group (48 individuals) constituted of naïve snails used as a control for the infection. Fifteen days after those treatments, snails of the three groups were exposed to 10 miracidia of SmBRE. Fifteen DPI, the snails were fixed in Raillet-Henry's solution and dissected to evaluate the parasite prevalence.

Statistical Analysis

All results concerning prevalence were tested using Fisher's exact test which considers two binary variables: infected/noninfected and control/experimental groups. The presence of an association between immune priming prevalence (a two category-variable) and Nei genetic distances (a variable with *k* categories) was tested using χ^2 test for trend (also called Cochran-Armitage test for trend). This test incorporates a suspected ordering in the effects of the *k* categories of the second variable. All results concerning the intensities (a continuous variable) were compared using a Mann-Whitney U test. For all the experiments, differences were considered significant at $p < 0.05$.

Table 2. Genetic information for *Schistosoma* strains

a Summary of genetic information for *Schistosoma* strains

	SmBRE	SmBRE-LE	SmVEN	Srod
Expected heterozygosity	0	10.15	5.73	ND
Observed heterozygosity	0	9.33	5.41	ND
Number of alleles	1	8.08	2.33	ND
Allelic richness	1	5.82	2.29	ND
Inbreeding coefficient	ND	0.082	0.056	ND

b Nei's genetic distances

	SmBRE-LE	SmVEN	Srod
SmBRE	0.644	0.735	ND
SmBRE-LE		0.546	ND
SmVEN			ND

ND = Not determined.

Results

Genotyping of S. mansoni *Strains*

The genetic diversities of each strain were determined using fourteen microsatellite markers (table 2). From the SmBRE strain, we obtained the following results: He = 0.0 ± 0; allelic richness = 1.0 ± 0, and FIS = not determined (table 2a). This indicates that SmBRE displays no genetic diversity based on the microsatellite markers tested herein. For the SmBRE-LE and the SmVEN stains, we observed some genetic differentiation with an expected He of 10.15 and 5.73 and an allelic richness of 5.82 and 2.29, respectively. The FIS were 0.082 and 0.056 for these two strains. Nei genetic distance between SmBRE and SmBRE-LE was equal to 0.644 and between SmBRE and SmVEN to 0.735 (table 2b). This result demonstrates that the SmBRE strain is genetically closer to SmBRE-LE than SmVEN.

Genotyping of *S. rodhaini* was not realized because microsatellite markers were not available for this species. We were not able to use the *S. mansoni* microsatellites because no cross-amplification occurred between *S. mansoni* and *S. rodhaini*. However, as *S. rodhaini* is a different species of the genus Schistosoma, we considered this strain as the more genetically distant from the SmBRE strain.

Immune Priming in Different Host/Parasite Combinations: Host Effect

To test whether the *B. glabrata* host strain had an influence on immune priming, we used two combinations

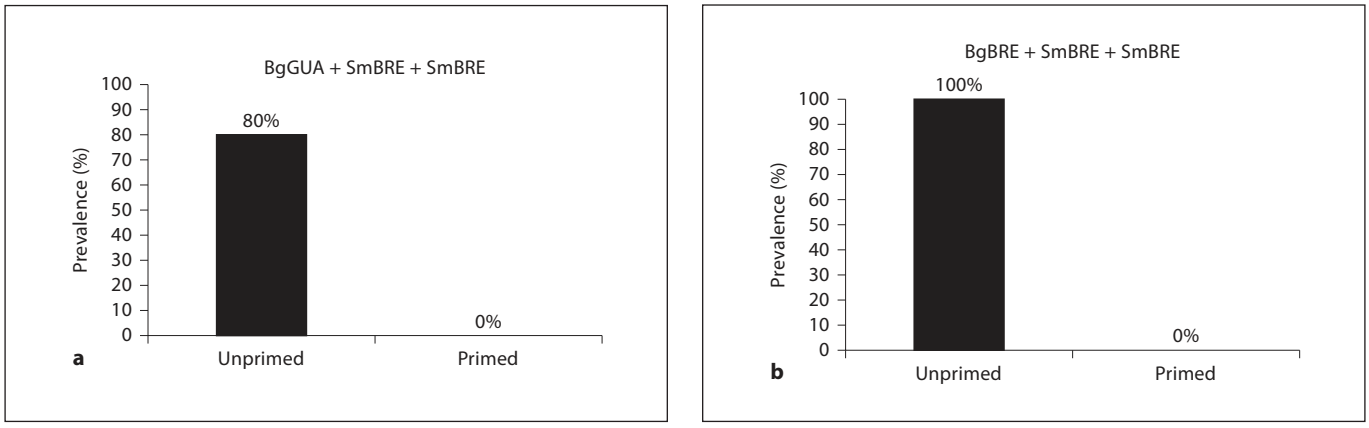


Fig. 1. Reinfection rates of BgGUA and BgBRE primary infected with 10 miracidia SmBRE (**a**) and reexposed to 10 miracidia of the homologous strain SmBRE (**b**). 'Unprimed' corresponds to snails that were not primary infected and exposed solely to the secondary infection.

involving BgGUA or BgBRE snails infected by homologous primary/secondary infection as follows: BgGUA + SmBRE + SmBRE or BgBRE + SmBRE + SmBRE (fig. 1).

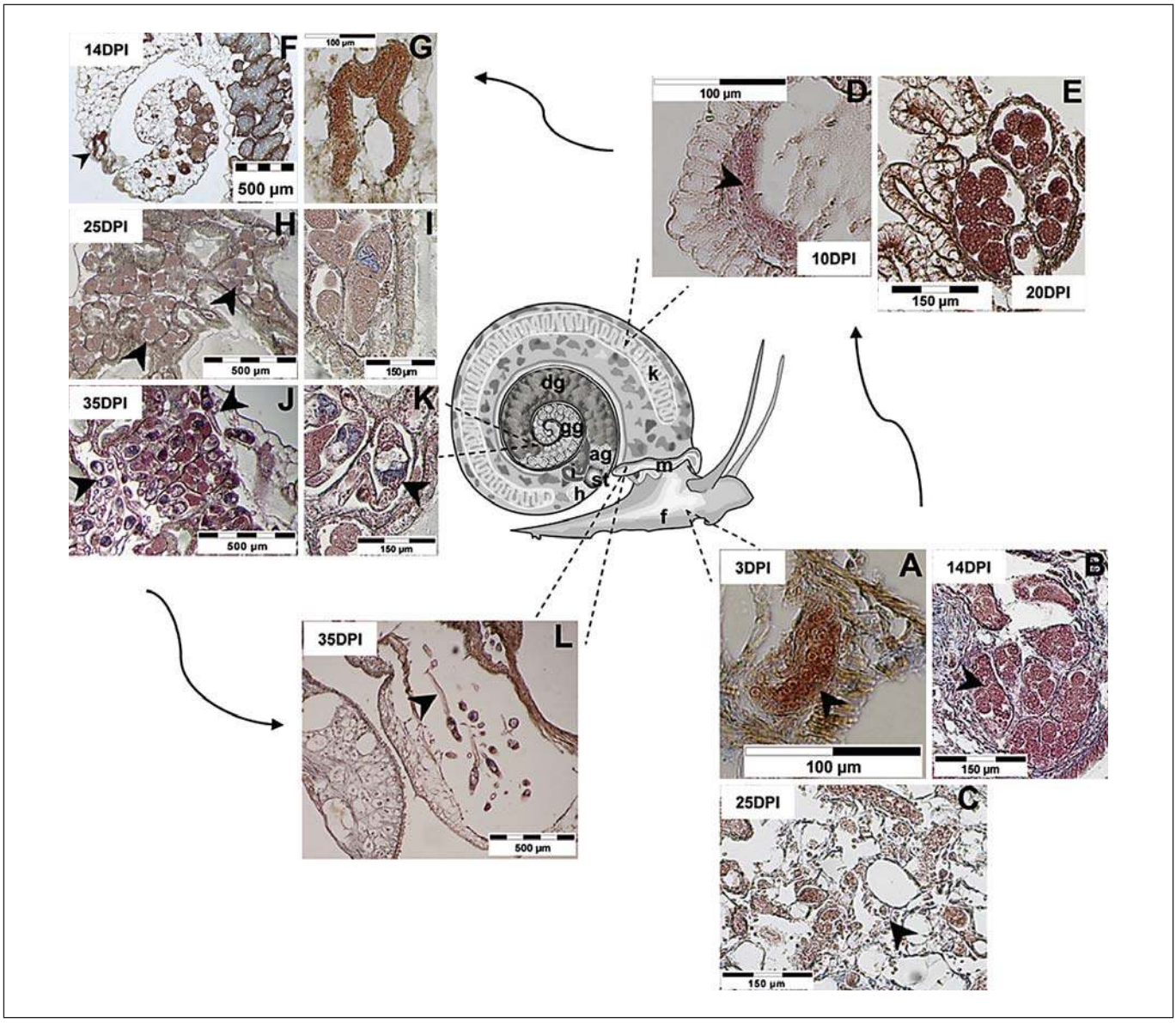
For BgGUA snails, the prevalence decreased from 80% for unprimed snails to 0% for primed snails (Fisher's exact test: $p < 0.0001$; fig. 1a). For BgBRE snails, the prevalence decreased from 100% for unprimed snails to 0% in primed snails (fig. 1b) (Fisher's exact test: $p < 0.0001$). Primary infections with SmBRE fully and equally protected against homologous secondary infection by SmBRE whatever the host strain (fig. 1a, b). These results show that the immune priming response does not depend on the snail host strain.

Intramolluskal Development of S. mansoni

In order to investigate the putative role of parasite development and migration events in the protection against secondary infection observed in *B. glabrata*, we used a histological approach to follow the infection of *B. glabrata* by *S. mansoni*. After miracidial penetration, SpIs developed in the snail head/foot. At 3 DPI, we observed growing SpIs containing dividing germinal cells that differentiated, maturated and developed into SpIIs (fig. 2A). At 14 DPI, the SpIs were full of SpIIs, some of which had left the SpIs to migrate through the host tissues toward the genital glands (fig. 2B). SpIIs began migrating at approximately 10 DPI (fig. 2D), and the first SpIIs reached the interface between the digestive and genital glands at 14 DPI (fig. 2F, G). The migration of SpIIs was very abundant at 20 DPI (fig. 2E), but it was complete by 25 DPI. At this point, the SpIs were degenerating in the snail foot tissue (fig. 2C), and the digestive/genital glands were full of SpIIs, some of which contained developing cercariae (the vertebrate infecting stage of the parasite; fig. 2H, I). At 35 DPI, the SpIIs were full of cercariae (fig. 2J, K), which were ready to escape and migrate to the snail mantle and the water environment beyond (fig. 2L).

Priming in Snails Infected with Irradiated Miracidia

To evaluate whether priming requires the development and migration of *S. mansoni* in snail tissues, *B. glabrata* were primary infected by UV-irradiated miracidia and secondarily infected at 25 DPI with nonirradiated miracidia. UV-irradiated miracidia could penetrate into the snails, but SpIs did not grow, parasitic development was interrupted and the migration of SpIIs did not occur (data not shown). When we examined protection against secondary infection among snails subjected to primary infection with irradiated miracidia, we found that no protection occurred (fig. 3a). The prevalence is similar to unprimed snails (fig. 3a). Primary infection with nonirradiated miracidia provides total protection against secondary infection (Fisher's exact test: $p < 0.0001$). The infectivity of irradiated miracidia was verified using a PCR-based diagnostic method that we developed using a specific marker of the *S. mansoni* genome (SmAlpha-Fem gene; GenBank accession No. U12442.1). This assay revealed that six of the seven individuals exposed to irradiated miracidia had been infected (fig. 3b). This represents a prevalence of 87.6%, which is similar to that observed for healthy miracidia (see controls in the present study).

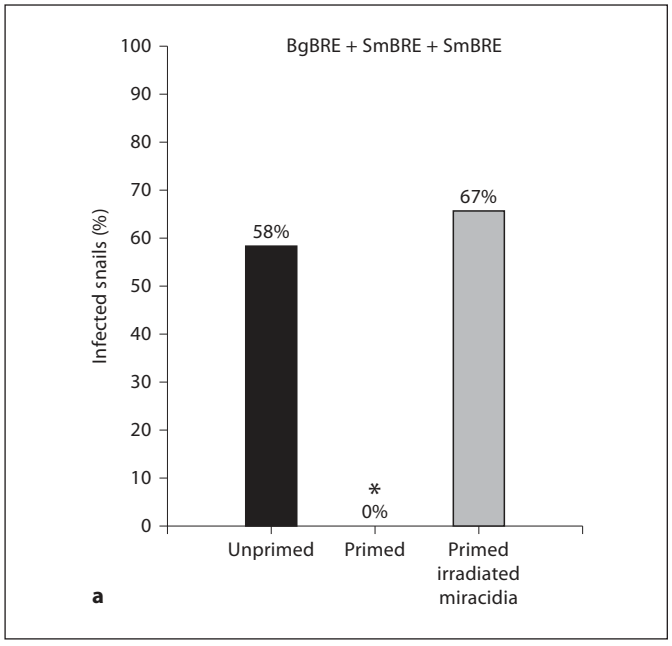


Color version available online

Fig. 2. *S. mansoni* intramolluskal-stage development in the intermediate snail host, *B. glabrata*. **A** SpIs at 3 DPI in the snail foot. **B** SpIs full of SpIIs at 14 DPI in the foot. **C** SpIs degenerating at 25 DPI in the foot. **D** One SpII migrating in the snail kidney at 10 DPI. **E** The kidney full of migrating SpIIs at 20 DPI. **F** The first SpII (black arrowhead) observed at the digestive/genital gland interface at 14 DPI. **G** Higher magnification of the adjacent image. **H** The digestive and genital gland full of SpIIs at 25 DPI, showing some developing cercariae. **I** Higher magnification of the adjacent image. **J** SpIIs full of cercariae in the digestive and genital glands at 35 DPI. **K** Higher magnification of the adjacent image. **L** Cercariae in the snail mantle at 35 DPI. All scale bars are indicated. f = Foot; m = mantle; k = kidney; h = heart; i = intestine; st = stomach; ag = albumen gland; dg = digestive gland; gg = genital gland.

Priming in Snails Subjected to Tissue Injuries

During miracidial penetration and the migration of SpIIs through the snail tissues, lesions and associated inflammatory processes may occur and could potentially be responsible for the observed priming. To test the putative involvement of tissue lesions in priming, we subjected snails to experimental tissue injuries at different times before infection. However, following needle-induced tissue injuries (fig. 4a) or biolistic particle-induced tissue injuries (fig. 4b), we failed to observe significant protection against infections by *S. mansoni* realized 5 or 10 days DPTI.



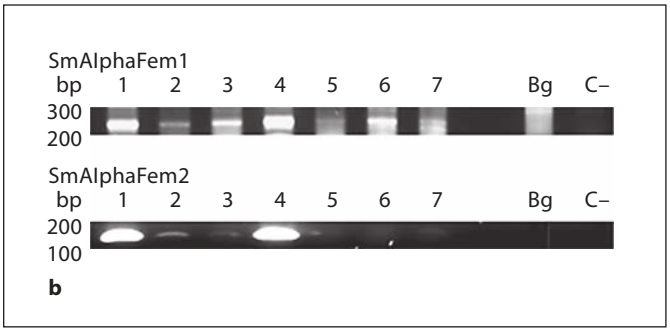
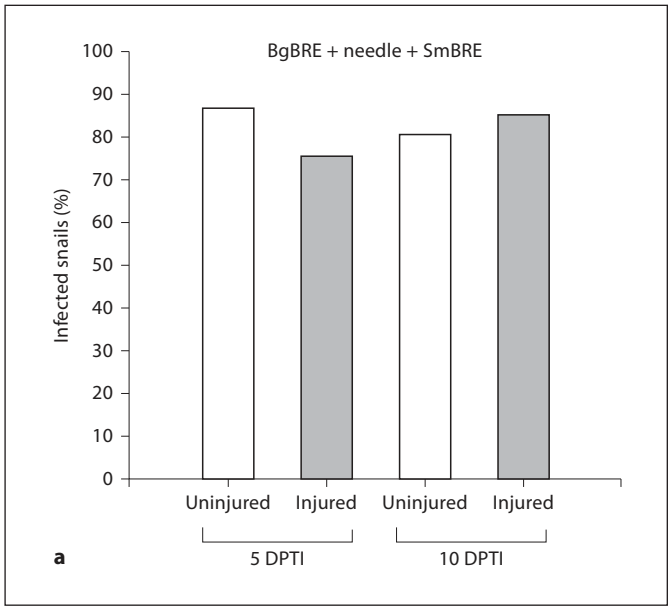


Fig. 3. a Infection rates of BgBRE snails subjected to primary infection with 10 irradiated or nonirradiated SmBRE miracidia, and then secondarily infected with 10 SmBRE miracidia. 'Unprimed' corresponds to snails that were not primarily infected and exposed solely to the secondary infection. **b** Detection of irradiate SmBRE miracidia in infected snails using diagnostic PCR amplification of the *S. mansoni*-specific SmAlphaFem gene (GenBank accession No. U12442.1) using primer pairs SmAlphaFem1 (270 bp) and SmAlphaFem2 (120 bp). * $p < 0.05$.



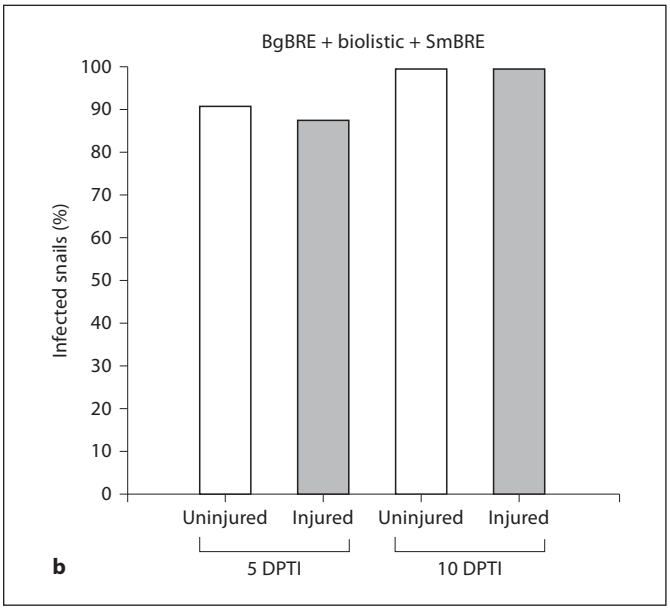


Fig. 4. Infection rates of BgBRE snails infected with 10 miracidia of SmBRE at 5 or 10 days DPTI. 'Uninjured' corresponds to healthy snails that did not receive tissue injuries. **a** Results from snails subjected to needle-induced tissue injuries. **b** Results from snails subjected to biolistic particle-induced tissue injuries.

Priming in Vaccinated Snails

Considering priming, two alternative hypotheses could be formulated to explain the observed phenomenon. Priming could be due to either an immune response of the host or an antagonistic interaction between parasites. The distinction between these alternatives would be a true challenge in this model. Thus, we developed an experimental vaccination approach as a tool to answer this question (fig. 5). One group of snails was injected with 1 μg of whole miracidium extracts from SmBRE in

Table 3. Number of snails infected or not and prevalence values

a Following SmBRE primary infection

	SmBRE	SmBRE-LE	SmVEN	Srod
Unprimed				
Infected	45	28	18	26
Uninfected	0	0	2	9
Prevalence, %	100	100	90	74.3
Primed				
Infected	0	4	12	8
Uninfected	41	32	29	11
Prevalence, %	0	11.1	29.3	42.1
p value	<0.0001	<0.0001	<0.0001	0.037

b Following SmBRE-LE primary infection

	SmBRE	SmBRE-LE	SmVEN	Srod
Unprimed				
Infected	41	21	39	35
Uninfected	3	9	4	15
Prevalence, %	93.2	70	90.7	70
Primed				
Infected	10	9	15	25
Uninfected	20	25	13	3
Prevalence, %	33.3	26.5	53.6	89.3
p value	<0.0001	0.001	0.0005	0.09

p values: Fisher's exact tests were calculated for each condition comparing unprimed and primed values.

Table 4. Mean intensity values

a Following SmBRE primary infection

	SmBRE	SmBRE-LE	SmVEN	Srod
Unprimed	3.58	4.5	1.89	2
Primed	ND	1.25	1.17	1.62
Mann-Whitney				
n1	41	28	18	26
n2	0	4	12	8
U	ND	4	62	81.5
p value	ND	0.003	0.026	0.331

b Following SmBRE-LE primary infection

	SmBRE	SmBRE-LE	SmVEN	Srod
Unprimed	2.36	2.14	2.46	1.85
Primed	1	1.3	1.25	2.08
Mann Whitney				
n1	41	21	39	35
n2	10	9	15	25
U	55	130.5	173	397.5
p value	<0.001	0.07	0.016	0.519

ND = Not determined – for primed snails in SmBRE homologous challenge condition no infected snails could be observed thus intensity and Mann-Whitney U tests could not be calculated.

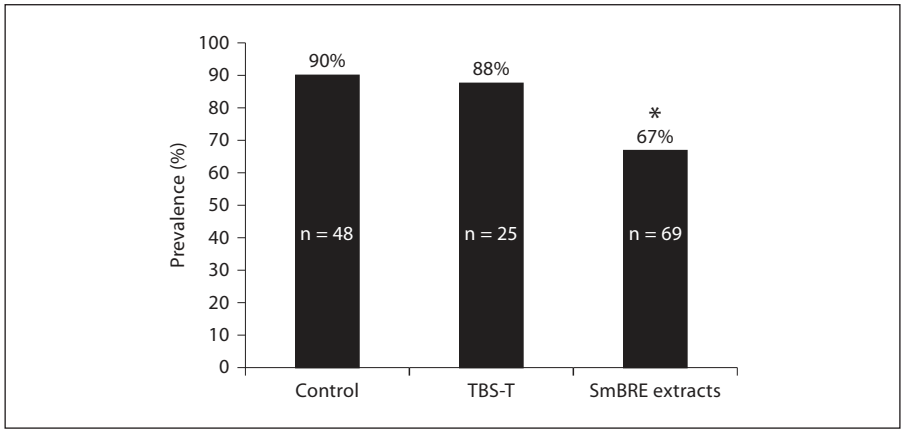


Fig. 5. Experimental vaccination of *B. glabrata* with SmBRE miracidium extracts. Prevalence of naïve snails (control), snails injected with 20 μl of TBS-T and snails vaccinated with 1 μg of SmBRE miracidium extracts in 20 μl of TBS-T. Snails were treated 15 days before the exposure to 10 miracidia of SmBRE. n = Number of snails used in each group. * $p < 0.05$.

TBS-T (SmBRE extracts). As controls, a second group received an injection of TBS-T alone (TBS-T) and a third group did not receive any treatment (control). The prevalence for the TBS-T group was 88% and did not differ significantly from the prevalence of the control group, which was 90% (fig. 5). In the SmBRE extracts group the prevalence decreased significantly to 67% compared to the control and TBS-T groups (Fisher's exact test: $p < 0.05$). This experiment invalidated the parasite antagonistic interaction hypothesis. Indeed, when infections were made

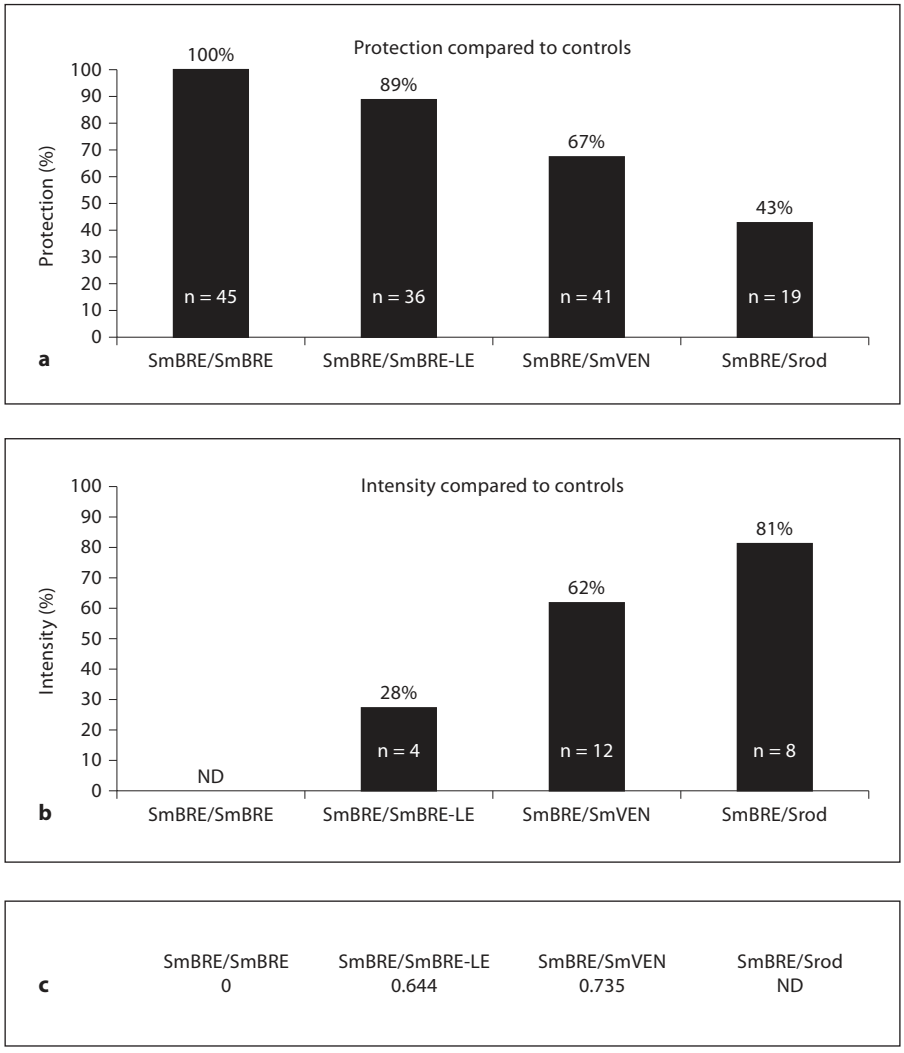


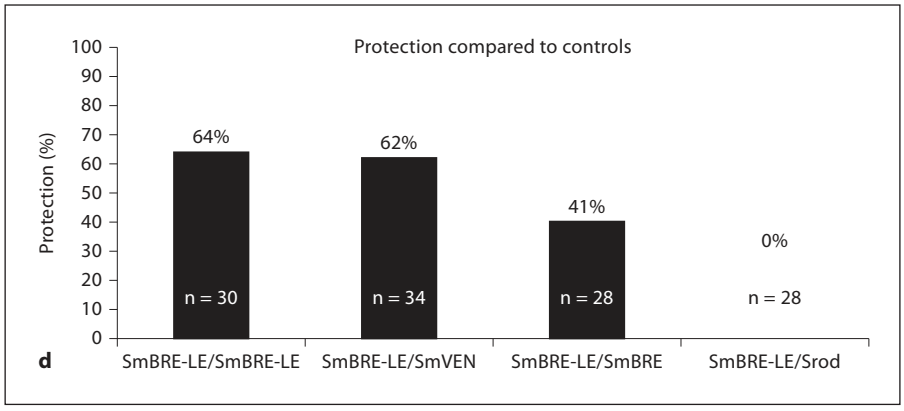
Fig. 6. Specific genotype-dependent immune priming in *B. glabrata* snails. Effect of a primary infection with SmBRE on prevalence (**a**) and intensity (**b**) after secondary infections with different *Schistosoma* strains. ND = Not determined – no intensity rate could be calculated because there was no snail infected. **c** Nei's genetic distances between the strain used for the primary infection and the strain used for the secondary infections (table 2b); Nei's distance could not be calculated because microsatellite markers for *S. rodhaini* were not available.

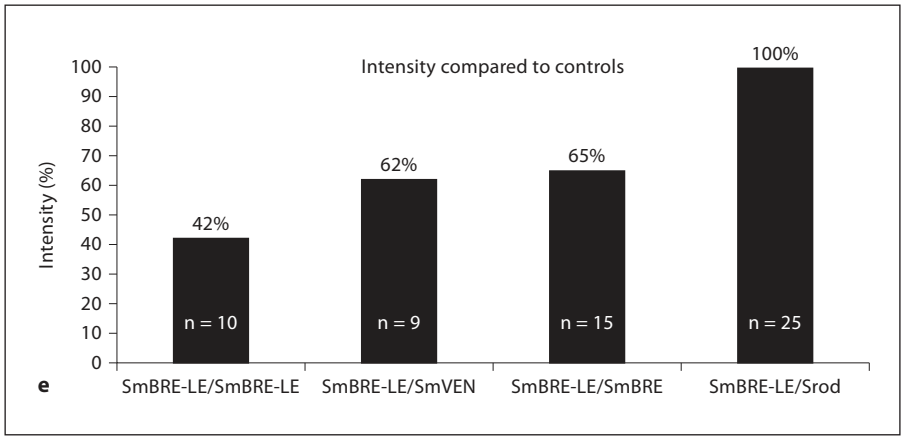
15 days after SmBre extract injection, we could suppose that all the parasite proteins injected had been ubiquitinated and addressed to the proteasome to be destroyed and recycled. Thus a direct parasite antagonism could not be considered and the host immune response hypothesis appeared to be more relevant.

Specific Genotype-Dependent Immune Priming

Primary infection of BgBRE snails was done with SmBRE (fig. 6a) or SmBRE-LE (fig. 6b) and snails were secondary infected with 4 different *Schistosomes*: SmBRE, SmBRE-LE, SmVEN and Srod.

For SmBRE primary infection (fig. 6a) the prevalence values for all the secondary infections tested decreased significantly compared to unprimed snails (table 3a). Thus, immune priming is efficient for each condition. When comparing the prevalence for all the secondary infections tested we could observe that prevalence in primed snails increased from 0% for SmBRE to 42.1% for Srod secondary infections. The link between this increase of prevalence and genetic distance was tested using a χ^2 for trends that are highly significant (χ^2 for trend = 18.384; d.f. = 1; $p < 0.0001$). Protection levels were calculated as a ratio between prevalence values of primed and controls snails (fig. 6a). In the case of homologous combinations (SmBRE/SmBRE) the protection level was 100%. In the case of heterologous combinations protection levels were 89% for SmBRE-LE (same species, same country, different strain), 67% for SmVEN (same species, different country, different strain), and 43% for Srod (different species). Concerning intensity values, for the homologous combination (SmBRE/SmBRE) no reinfection occurred





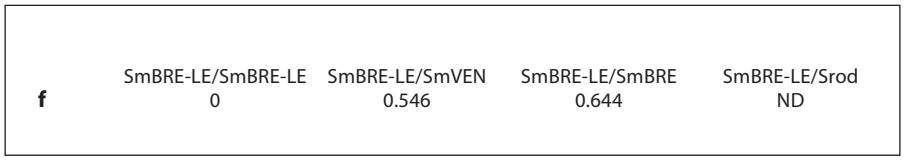


Fig. 6. Effect of a primary infection with SmBRE-LE on prevalence (**d**) and intensity (**e**) after secondary infections with different *Schistosoma* strains. **f** Nei's genetic distances between the strain used for the primary infection and the strain used for the secondary infections (table 2b); Nei's distance could not be calculated because microsatellite markers for *S. rodhaini* were not available.

(protection 100%; fig. 6a), hence intensity could not be calculated (table 4a). For heterologous combinations intensity levels were calculated as described in the Material and Methods section. We could observe that intensity deceased in primed snails compared to unprimed snails. This decrease was significant for secondary infection with SmBRE, SmBRE-LE and SmVEN (table 4a). Investigating the link between intensity and genetic distance, we observed that intensity levels increased regularly with the genetic distance (fig. 6b, c): 28% for SmBRE-LE secondary infection (same species, same country, different strain), 62% with SmVEN (same species, different country, different strain) and 81% with Srod (different species).

For SmBRE-LE primary infection (fig. 6d–f) the prevalence values for all the secondary infections tested decreased significantly compared to unprimed snails except for Srod secondary infection for which the prevalence was not affected by SmBRE-LE primary infection (table 3b). When comparing the prevalence for all the secondary infections tested we could observe that prevalence in primed snails increased from 26.5% for SmVEN to 89.3% for Srod. The link between this increase of prevalence and genetic distance was tested using a χ^2 for trends that are significant (χ^2 for trend = 8.52; d.f. = 1; p = 0.0035). Protection levels were calculated as a ratio between prevalence values of primed and controls snails (fig. 6d). In the case of homologous combinations (SmBRE-LE/SmBRE-LE) the protection level was of 64%. In the case of heterologous combinations protection levels were 62% for SmVEN (same species, different country, different strain), 41% for SmBRE (same species, same country, different strain) and 0% with Srod (different spe-

cies). The intensity decreased in primed snails compared to unprimed snails except for the secondary infection with Srod for which the intensity was not affected by SmBRE-LE primary infection (table 4b). This decrease is significant for secondary infection with SmBRE-LE and SmBRE (table 4a). Investigating the link between intensity and genetic distance, we observed that intensity levels increased regularly with genetic distance (fig. 6e, f): 42% for SmBRE-LE secondary infection (homologous combination), 62% for SmVEN (same species, different country, different strain), 65% for SmBRE (same species, same country, different strain) and 100% with Srod (different species).

These results confirmed that immune priming affects prevalence and intensity, i.e. the number of SpIs that penetrated and developed in snail tissues decreased in primed snails compared to unprimed snails. However, whatever the strain used for the primary infection (SmBRE or SmBRE-LE), protection levels decreased and intensity levels increased with the increase of the Nei genetic distance between primary and secondary infections (fig. 6a, b). Immune priming appeared to be less efficient when genetic distance increased. These results concerning prevalence and intensity indicate that priming in *B. glabrata* is highly specific, and seems to be genotype-dependent.

Discussion

A better understanding of Lophotrochozoan innate immunity, which remains poorly investigated, appears to be crucial to bridge the gap between Deuterostoma and Ecdysozoa immunity and will help achieve a better understanding of the diversity and evolution of innate immune processes. In this study, we investigated the occurrence, the origin and the specificity of immune priming among fresh-water snails *B. glabrata* exposed to trematode pathogens of the genus *Schistosoma*. The existence of efficient immune priming was confirmed using homologous primo/secondary infections in different *B. glabrata* strains. When homologous primo/secondary infections were performed no secondary infections were observed, regardless of whether it was the host strain (fig. 1). After a primary infection of BgGUA or BgBRE snails, the mollusks were totally protected from secondary infection.

We were able to confirm the occurrence of immune priming in our model; however, the characterization of immune priming in an invertebrate animal model requires consideration regarding the timing of this process (i.e. when priming first appears after infection and how long it is maintained thereafter). The first study reporting the discovery of this process in *B. glabrata* showed that BgBRE snails developed a time-dependent 'acquired resistance' starting 3 DPI [47]. The success of secondary infection decreased from 3 to 7 DPI, the snails became totally protected against secondary infections between 7 and 14 days, and they remained protected until the end of the snail's life [47].

To further investigate the cause of this partial protection against secondary infection within the first 7 days following primary infection, and the total protection against secondary infections occurring between 7 and 14 DPI, we examined the intramolluskal development of the parasite (fig. 2). The histological approach conducted in the present paper showed that SpIs grew and developed in snail tissues to produce SpIIs during the first 14 DPI (fig. 2A, B); the migration of SpIIs through host tissues started at 10 DPI (fig. 2D) and the first SpIIs reached the digestive/genital gland interface at 14 DPI (fig. 2F, G). Furthermore, the growth of SpIs and the development of SpIIs, which occurred during the first 7 DPI, were correlated with the decreased success of secondary infection, while the start of SpII migration (10 DPI) was concomitant with the acquisition of complete protection against secondary infections. These observations suggest that the dynamics of parasitic development are linked to the acquisition of protection. In this context, we can hypothesize that the tissue damage induced by the parasite during miracidial penetration and SpII migration could activate the snail inflammatory and immune processes responsible for the observed immune priming. In order to test the impact of miracidial penetration on immune priming, we used UV-irradiated miracidia that were capable of infecting the host snails, but did not show subsequent development (no growth, no SpII differentiation or migration). Under these irradiated conditions, no resistance was observed (fig. 3a). This result is in agreement with a previous study showing that immune stimulation of *B. glabrata* with irradiated miracidia did not induce protection [63]. One hypothesis could be that tissue lesions resulting from the growth and development of SpIs/SpIIs and/or the migration of SpIIs could trigger a nonspecific acquired protection; this phenomenon was previously described for different invertebrate species where wounding was found to induce nonspecific immune responses that prevented opportunistic infections [38–40]. We tested this hypothesis by submitting snails to simple needle- or biolistic particle-induced tissue injuries (fig. 4) without

any specific antigenic stimulation prior to infection, as previously examined in insects [64]. No significant protection against parasitic infection was observed, showing that lesion-induced immune stimulation was not responsible for the observed protection against *S. mansoni*.

These results supported the view that SpI/SpII growth and development in snail tissues resulted in the stimulation of snail immunity that was probably the cause of the immune priming response developed against secondary infections. However, an alternative hypothesis has to be considered. The priming effect disappeared when irradiated parasites were used for primary infection (fig. 3). Based on this observation the acquired protection against secondary infections observed could be interpreted as kin-mediated competition among parasites [2, 47] rather than as the result of the host immune priming response. Indeed, after the primary infection, the parasite *S. mansoni* remained in the host's tissues throughout the snail's life. Sire et al. [47] have thus suggested that the failure of secondary infection could well be due to intraspecific larval antagonism. However, this is a subject of controversy, as some authors expected a higher competition between more closely related parasites that use similar resources [3], while others hypothesized that kin cooperation should facilitate rather than reduce secondary infection rates [23]. Moreover, it was recently shown that coinfections could enhance certain parasite life history traits (reproduction, growth, etc.) [65] or increase parasite prevalence [66] indicating no evidence for regulatory processes or larval antagonism [48]. Axenic cultivation studies performed on several trematode species, including *S. mansoni*, did not show any evidence for a direct antagonistic effect in vitro [67]. Finally, we developed recently a global bi-dimensional proteomic approach conducted on primed snail plasma following *S. mansoni* infection (data not shown). This approach did not identify any circulating parasite molecules in these plasmas, another clue for the absence of a direct larval antagonism in this model.

However, all these assumptions constituted indirect arguments. To be fully convinced of the existence of efficient immune priming in the *B. glabrata/Schistosoma* spp. model, we developed a vaccination experiment (fig. 5). We showed that vaccination with miracidium protein extracts significantly reduce prevalence (fig. 5), resulting in a partial protection against secondary infection. This partial protection could not be related to a direct larval antagonism but supports the view that SpI/SpII growth and development were important steps toward the acquisition of a total immune priming response.

The fact that immune stimulation along the parasite development from miracidium penetration to SpII migration appeared to be essential to the acquisition of a total immune priming response (a partial protection is obtained with miracidium proteins) asks the question of the specificity of immune priming response in *B. glabrata*. Most of the models used for studying immune priming in invertebrates were conducted for arthropods (insects [2, 24, 27–29] or crustaceans [4, 21, 30, 31]) infected by bacteria, yeast or virus. For eukaryote hosts, it could be easy with a limited set of pattern recognition receptors to discriminate or recognize lipopolysaccharides, peptidoglycans or β-glucans, some very specific pathogen-associated molecular patterns of micro-organisms and respond to them efficiently. This was perfectly illustrated for Drosophila immune priming response for which response to fungal and bacterial infection occurred through two distinct signaling cascades, which are known as the Toll and immune deficiency pathways, respectively [9]. These activations resulted in separated intracellular signaling cascades inducing the synthesis of seven families of antimicrobial peptides that were directed against fungi, Gram-positive or Gram-negative bacteria and protect the fly against subsequent infections more or less specifically [9, 27].

In the *B. glabrata/Schistosoma* spp. model both partners are metazoan eukaryotes belonging to the Lophotrochozoan group and this phylogenetic proximity is particularly interesting when studying the mechanisms involved in the specificity of immune priming. Here a higher level of specificity is expected because of the potential molecular proximity between host and parasite antigens. The mechanisms involved in host immune recognition were expected to be sophisticated to discriminate between self- and nonself-eukaryote cells and avoid autoimmunity.

When studying immune priming specificity in invertebrate systems, care is needed to ensure that the specificity of the putative changes in immune reactivity is fully addressed by secondary challenge with a wide range of related and unrelated pathogens or parasites. The different geographic isolates or species of parasites maintained in our laboratory were used to show a high degree of specificity using homologous and heterologous challenges (fig. 6, table 2). The protection decreased from homologous to heterologous conditions alongside the genetic distance between parasites used for primary infections and challenges (different geographic isolates or different species of parasites were used; fig. 6). Parasite intensity also gives the same interesting results. Indeed,

figure 6 showed that intensity in primed snails increases together with the genetic distance. This indicates that prior exposure to genetically closer parasites resulted in fewer parasites that penetrated and developed in the host than occurred after exposure to genetically distant parasites.

This result is interesting because it shows that the first stimulation activated immune components that were able to discriminate more or less efficiently between the parasite strain used for the primary infection and for the challenge. In this context, it is important to note that the strains used are genetically distinct (table 2). Immune priming specificity appeared to be dependent on the genetic distance between the parasite used for the primary infection and for the challenge and illustrated that the specificity of the immune priming in *B. glabrata* is probably genotype dependent.

To date, to our knowledge, only one paper has investigated immune priming specificity in a host/parasite metazoan eukaryote interaction. In that paper, evidence for specific immune priming has been uncovered in the small crustacean copepod *Macrocyclops albidus* infected with different strains of its natural tapeworm parasite, *Schistocephalus solidus* [23]. The authors reported that prior exposure to related parasites resulted in less secondary infection than occurred after exposure to unrelated parasites. Here also the authors were able to demonstrate a genotype-dependent immune priming [23]. However, this effect was studied over only 3 days after primary infection and the specificity observed could result from the primary response and not from immune priming or memory.

Collectively, our observations demonstrate the specificity of the protection process. Better protection against a homologous (vs. heterologous) secondary infection in immune priming (fig. 1, 6) may arise via processes that involve specific immune receptors and/or effectors that are mobilized to target certain subsets of *S. mansoni* genotypes. Previous reports make the FREPs, some polymorphic and diversified putative immune receptor variants, promising candidates for involvement in the immune priming taking place in *B. glabrata* [18, 42, 68]. FREPs are diversified recognition and/or effector proteins involved in *B. glabrata* defense against parasitic infection that exhibit functional specialization with respect to the pathogen encountered [69–72]. These molecules are hemolymph lectins [73] that exhibited a remarkable degree of diversification [18]. Finally, their crucial role in the fate of infection was previously demonstrated using siRNA-mediated knockdown, which rendered approximately 30% of constitutively resistant adult snails susceptible to *Echinostoma paraensei* [68].

Interestingly, we recently showed that a specific set of these highly variable FREPs from *B. glabrata* forms immune complexes with mucin molecules from *S. mansoni* (*S. mansoni* polymorphic mucins; SmPoMucs), which are also highly polymorphic and individually variable [19]. This was the first evidence of an interaction between FREP, one of the putative diversified immune receptors and antigenic variants in an invertebrate host/pathogen model [19]. Each *S. mansoni* individual expresses a particular SmPoMuc profile [44, 45], which may be recognized by a specific set of FREPs produced by the mollusk. Consequently, the specific sets of FREPs produced in response to the parasitic strains or genotypes found in the primary infection may form the basis for the specific immune priming described herein. The snails would then be protected against secondary infection, with the degree of protection depending on the antigenic similarities between the strains used for the primary and secondary infections. Confirming the role played by FREPs in this priming process deserves further functional experiment using siRNA approaches.

Until now, priming observations in invertebrates were mainly phenomenological and based on ecological or phenotypic studies, and they lacked a clear understanding or description of the potential underlying molecular and/or cellular mechanisms. The exception to this was the enhanced phagocytosis described in two prior studies in *Porcellio scaber* (Crustacea, Isopoda) and *A. gambiae* (Insecta, Diptera) [24, 28]. The international community working on invertebrate innate immunity believes that observations cannot be used in isolation and should solely be used to construct hypotheses but such hypotheses must be exhaustively tested and backed by rigorous functional cellular, biochemical and molecular methods to eliminate all alternative explanations [3, 74].

Thus, future studies should use gene-discovery approaches (e.g. global comparative proteomic or transcriptomic studies) to identify all of the determinants involved in the specific immune priming of the Lophotrochozoan snail, *B. glabrata*, in response to *S. mansoni* infections. A better understanding of the immune priming response of the Lophotrochozoan snails could help us decipher the evolutionary history of innate immune memory or immune priming in organisms ranging from the Ecdysozoa to the Deuterostomia.

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