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REVIEW

Why the radiation-attenuated cercarial immunization studies failed to guide the road for an effective schistosomiasis vaccine: A review

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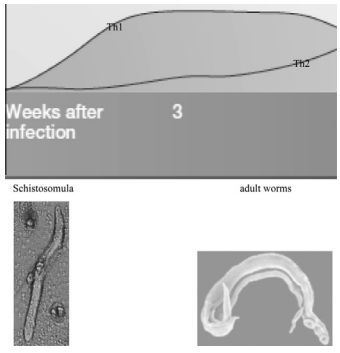
GRAPHICAL ABSTRACT



Schistosomula- and adult worms-derived antigens induce predominant Th1 immune responses. The radiation-attenuated cercariae vaccine efficacy is dependent on induction of Th1 and Th2 immune responses. Accordingly, schistosomula- and adult worms-derived antigens used for effective vaccination must be combined with Th2 immune responses-inducing cytokines or molecules as adjuvant.

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ABSTRACT

Schistosomiasis is a debilitating parasitic disease caused by platyhelminthes of the genus Schistosoma, notably *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. Pioneer researchers used radiation-attenuated (RA) schistosome larvae to immunize laboratory rodent and non-human primate hosts. Significant and reproducible reduction in challenge worm

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burden varying from 30% to 90% was achieved, providing a sound proof that vaccination against this infection is feasible. Extensive histopathological, tissue mincing and incubation, autoradiographic tracking, parasitological, and immunological studies led to defining conditions and settings for achieving optimal protection and delineating the resistance underlying mechanisms. The present review aims to summarize these findings and draw the lessons that should have guided the development of an effective schistosomiasis vaccine.

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Introduction

Schistosomiasis is a severe parasitic disease caused by members of the genus Schistosoma, notably *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*. More than 200 million persons are infected and up to 800 million, mostly children, are at risk. These statistics may well be underestimated because the stool analysis gold standard technique for diagnosis of the infection is insensitive and unreliable leading the World Health Organization to no longer provide estimates on population infected or at risk. These have been replaced by estimates of population requiring preventive chemotherapy. Egypt is among 51 countries with population requiring chemotherapy despite inaccurate and incomplete information advocating the near eradication of schistosomiasis from Egypt [1]. These hearsays have their foundation on the unreliability of diagnostic techniques and lack of sound and objective epidemiological studies. Failure to assess the prevalence of schistosomiasis leads to people unawareness of its danger. The sequelae are intense reflected in more than 70 million disability-adjusted-life-years (DALYs) and remarkably high rates of years-lived-with disability (YLD) [2]. Praziquantel is the only drug commonly used for treatment. But its efficacy is not proof, and it does not prevent reinfection necessitating its repeated use, thus increasing the threat of development of parasite resistance to the drug [1,2]. Infection and transmission can be prevented if a vaccine is in place. Vaccination studies with radiation-attenuated (RA) schistosome larvae have demonstrated that a schistosomiasis vaccine is a realistic goal [3]. These studies have provided invaluable learning and directions that should have helped developing an effective vaccine composed of purified or recombinant antigens [3]. The present review attempts to outline these lessons and clarify how and where they were disregarded or painstakingly followed.

The radiation-attenuated vaccine model

The life cycle stage used

The infective schistosome stage, the cercariae are commonly used for inducing resistance to challenge infection following radiation attenuation (RA) [4]. Mechanically transformed schistosomula (tailless cercariae) attenuated by X- or gamma irradiation and injected intramuscularly (im) successfully protected mice and cynomolgus monkeys against challenge *S. mansoni* infection [5,6]. However, percutaneously applied RA cercariae were more effective in stimulating resistance (60%) than irradiated, im-administered, schistosomula (40%) [7]. Approximately 500 RA (50 krad of gamma irradiation) 6-day-old lung *S. mansoni* schistosomula, injected im, intraperitoneally (ip), or intravenously (iv) into NIH/Nmri CV and C57BL/6J mice, were also capable of inducing significant ($P < 0.001$) levels of challenge worm reduction (36–56%) that were not very different from approximately 850 RA cercariae as immunizing agents. These findings were construed to indicate that the extravascular stages of development within the skin are not required for the induction of resistance [8]. Conversely, iv-injected RA lung-stage schistosomula derived from optimally RA cercariae failed to confer protection in C57BL/6 mice, suggesting that successful vaccination is not dependent on systemic (vascular), antigen presentation [9,10]. Additionally, irradiated day 21 (# 105) and day 28 (# 58) worms induced much less resistance (reduction in challenge worm burden of 15–27%) than RA cercariae [8].

The type and dose of radiation

Parameters of immunization of mice with ^{60}Cobalt-irradiated *Schistosoma mansoni* cercariae were first described by Minard et al. [4] and related to protection against subsequent challenge infection. Optimal protection was found to be dependent on dose of irradiation, number of immunizing cercariae, and number and time course of immunizations. Low levels of resistance were obtained with low irradiation doses. In general, resistance increased with increasing irradiation doses, up to approximately 48–56 krad. Maximal resistance (70–80% reduction in challenge worm burden) was elicited by a single exposure to 250–500 cercariae, irradiated at a dose rate of 2 krad/min to a total dose of 56 krad. In C57BL/6 mice, *S. mansoni* cercaria RA with ^{60}Co 15 krad induced higher levels of protection than 50 krad, and protection was maximal following 4× immunizations with moderately or highly RA cercariae [11]. Cobalt-60 RA cercariae and schistosomula vaccine was widely used in mice [3,4,7,12] and baboons [3,13] for protection against *S. mansoni*, in calves for protection against *Schistosoma bovis* [14], and in cattle and buffaloes for protection against homologous *Schistosoma japonicum* infection [15]. In parallel comparison studies, Cesium-137-attenuated cercariae afforded better protection than the ^{60}Co RA vaccine. The optimal total radiation with ^{137}Ce was between 45 and 50 krad [16]. Cercariae of *S. mansoni* attenuated by exposure to 30–60 krad gamma radiation from a ^{137}Ce source induced >50% protection in baboons against homologous, but not *S. haematobium*, infection challenge [17], and in the vervet monkey, where a protection ceiling of 48% was achieved following 3 vaccinations [18].

X-irradiated *S. mansoni* cercariae were also effective in protecting mice against homologous challenge infection, provided using the optimum number of immunizing cercariae (500), dose of X-irradiation (48 krad), the number of immunizations (5), the time interval between immunization and challenge (up to 1 year), and the size of the challenging dose (up to 500 cercariae) [19,20]. X-irradiated *S. japonicum* tailless cercariae were employed for protecting rhesus monkeys [21] and cattle [22] against schistosomiasis japonicum, with reduction in challenge worm burden varying between 42% and 96%.

The expenses and inconvenience of gamma and X-ray irradiation promoted studies using ultraviolet (UV) irradiated vaccine, which is cost-effective, and only requires simple devices [23]. Dean et al. demonstrated that single immunization of mice with UV-attenuated *S. mansoni* cercariae, using a small, portable S-68 Mineralight Lamp adjusted to deliver 330–440 μwatts/cm^2, conferred similar levels of resistance to infection (50–70%) as with 50 krad gamma-RA cercariae [24]. Ultra-violet-irradiated *S. mansoni* cercariae were capable of leading to reduction in challenge infection in guinea pigs (approximately 40%), but not Mongolian gerbils [25]. Of note, Mongolian gerbils were also not protected against *S. mansoni* challenge infection when vaccinated with 20 krad gamma-irradiated cercariae [26]. Likewise, UV-attenuated cercarial vaccine was highly effective with *S. japonicum* in protecting mice, water buffaloes, and pigs against homologous schistosome infection [27–31], but induced low, unstable level of protection in some inbred mice, notably C57BL/6 [32].

Fate of irradiated larvae

Studies using tissue mincing and incubation, histopathology, and autoradiographic tracking techniques revealed that similarly to normal larvae, RA cercariae are able to penetrate the epidermis of the host and henceforth to the dermis en route to the dermal blood or lymph capillaries, with only a slight difference in timing of skin exit, whereby attenuated larvae persist in the skin much longer than normal parasites [33–35]. A significant number of immunizing RA larvae were located in lymph nodes draining the skin site of exposure [34]. Migrating schistosomula derived from RA *S. mansoni* cercariae (approximately 50% of penetrants) attain the lung in 6 or 7 days, and differently from their intact counterparts linger, not to leave this site, and die therein. Indeed, schistosomula are detected in the lung for up to 3 weeks following infection with RA cercariae, and a proportion therefrom are located extravascularly within the alveoli [33–39]. Schistosomula transforming from cercariae attenuated with low doses of irradiation may make their route to the liver, but usually fail to copulate and lay eggs [35,39]. Accordingly, RA schistosome larvae confer high levels of protection without causing pathological symptoms [3].

The failure of schistosomula derived from RA cercariae to migrate beyond the lung stage was attributed to the impact of irradiation on the parasite neuromuscular function with consequent lower mobility, slow alternating body extensions and contractions, and limited maximum body elongation and extension [40]. In support, microarray examination of the gene expression in cultured schistosomula derived from normal and RA cercariae revealed down-regulation of transcripts encoding G-protein-coupled and neuro receptors, resulting into diminished parasite response to external stimuli and giving an explanation to the extended transit through skin-draining lymph nodes and the lung [41]. Radiation attenuation of *S. mansoni* larvae was reported to lead to profound inhibition of protein and glycoprotein synthesis and radiolysis of surface carbohydrates that likely enhance the immunogenicity of the larval antigens and/or stimulate exposure of cryptic epitopes [42–45]. No studies are, however, available to delineate whether the death of RA schistosomula in the lungs is a result of the radiation insult and/or to the host immune effector responses. This question might be resolved by tracking the fate of RA cercariae in thymectomized or anti-thymocyte serum-treated mice [46].

Effects on challenge worm burden and fecundity

Immunization of mice with ^{60}Co-attenuated (46–96 krad) larvae of *S. mansoni*, once or twice, resulted in a 70% reduction in challenge worm burden administered 3 and up to 15 weeks after immunization [4,7]. Treatment with immunosuppressive drugs or excision of sites of infection following immunization revealed that RA larvae need to persist in the host for between 1 and 2 weeks to stimulate optimum protection. Antigens released during protracted stay in the skin and lung likely induce the effector immune responses mediating the resistance to challenge infection [34,35,47,48]. Elucidating the challenge parasites major attrition site was a subject of controversy. Thus, in inbred CBA/Ca mice exposed to 400 *S. mansoni* cercariae attenuated

with 20 krad of ^{60}Co irradiation, challenge parasites were found to be killed within the first 4 days after challenge, i.e., at the skin stage [12,49–51]. Conversely, in mice immunized by exposure to *S. mansoni* RA cercariae (50 krad, 2 krad/min of ^{60}Co radiation), mincing and incubation [52] as well as autoradiographic studies of challenge infection with approximately 200 L-(^{75}Se) selenomethionine-labeled but otherwise normal cercariae indicated that worm elimination occurs after the skin stage, essentially in the lungs [12,33,34,39,53–56]. Challenge schistosomula were found to reach the liver in reduced numbers or are killed or cleared extravascularly in the liver in greater number in immunized mice, suggesting that the liver is a site of challenge worm attrition in mice immunized with RA larvae [53] or previously infected mice as well [57]. In guinea pigs vaccinated with ^{60}Co-RA (20 krad) *S. mansoni* cercariae, and challenged 4–5 weeks after immunization with normal cercariae, lung-stage or 2–6 week-old parasites, the liver appeared to be an important attrition site [58]. Combined microautoradiographic and histopathological studies revealed that immune elimination of challenge larvae does not result from a cytolytic hit, but is essentially due to extravascular exit during migration. Schistosomula surrounded by leukocytic foci in alveoli or in the vasculature did not show any attached leukocyte and appeared entirely free of structural damage [59].

Immune protection was found to be schistosome species-specific as mice exposed to 20 krad-irradiated *S. mansoni* cercariae showed 53–67% reduction in homologous challenge worm burden, while heterologous vaccination with *S. bovis*, *S. haematobium*, or *S. japonicum* conferred only 5–12% protection [60]. The RA vaccine cross-protection in mice was limited to species of the *S. haematobium*, but not *S. mansoni*, group [61]. In inbred mice immunized with UV-irradiated cercariae of *S. mansoni* or *S. haematobium*, homologous protection ranged from 56% to 69% for *S. mansoni* and 88% to 99% for *S. haematobium*. Significant heterologous protection was consistently induced against *S. haematobium* by immunization with *S. mansoni*, but not against *S. mansoni* by immunization with *S. haematobium* [62]. Moreover, induction of resistance with RA cercariae of *S. mansoni* varied with mouse strain, with C57BL/6 showing the highest and P/N the lowest level of reduction in challenge worm burden [63–65].

The RA schistosome vaccine induced a high level of protective immunity in experimental rodent hosts and importantly was also efficacious in baboons, whereby 9000 cercariae attenuated by exposure to 30–60 krad of gamma radiation induced $>50\%$ protection to a challenge with normal larvae [17]. Significant protection, with 64–89% reductions in worm burden and parallel reductions in egg production, was achieved in baboons immunized with gamma-irradiated *S. haematobium* cercariae [66]. Cynomolgus monkeys im-injected with ^{60}Co (50 krad at 4 krad/min)-RA *S. mansoni* tailless cercariae had 52% fewer challenge worm, and at 7 weeks post-challenge excreted 80% fewer eggs than did the control animals [6].

The data together gave strong evidence that protective immunity could be induced against schistosome infection. The RA vaccine-mediated protection was invariably partial, with surviving worms able to copulate, and daily deposit hundreds of eggs [67]. Moreover, the RA vaccine did not result in significant decrease in challenge worm fecundity in CBA and C57BL/6 mice immunized once or more with gamma-irradiated *S. mansoni* larvae [7,11]. Inbred and outbred mice receiving one exposure to UV RA *S. mansoni* cercariae, and challenged five weeks later with approximately 100 normal cercariae were assessed for worm burden and worm egg counts in liver and intestine at 5, 6, 7 and 8 weeks after infection. Reduction in worm burden varied between 27 and 65% (8 experiments). Decrease in egg counts and female fecundity was highly significant in vaccinated versus control mice at 5, 6, and 7 weeks after challenge. At 8 weeks after challenge, the egg count/mouse and per female worm was similar in immunized and control mice suggesting that the RA vaccine-mediated decrease in worm egg load is only transient [68]. In studies complete regarding egg sampling, significant reduction in fecundity of challenge worms was not observed in baboons immunized with *S. haematobium* [66], or *S. mansoni* [69] RA cryopreserved schistosomula.

RA vaccine-induced immune responses

Skin

Vaccination of CBA or C57BL mice with RA cercariae induces localized skin inflammatory foci comprising 50% macrophages and 50% eosinophils at the site of immunization that appeared to be responsible for attrition of challenge parasite within few days of entry [51,70]. In support, ip injection of a monoclonal antibody (mAb) specific to neutrophils, but apparently also effective against macrophages and eosinophils, on the day of challenge, greatly reduced (67% mean reduction) the RA-induced resistance [71]. Moreover, passive transfer of serum from RA vaccine-protected mice was able to transfer resistance against challenge infection in mice via induction of subdermal inflammatory reactions, comprising 60% mononuclear cells and 40% eosinophils [72]. Whole body irradiation of RA cercariae-immunized CBA mice 3 days prior to challenge infection revealed that eosinophils, rather than macrophages, are central to the RA vaccine-induced protection [73].

The importance of the skin-draining lymph nodes (LN) for the RA vaccine-mediated immunity was shown in mice percutaneously immunized once with 500 *S. mansoni* cercariae attenuated with 20 krad ^{60}Co radiation, LN draining the vaccination site removed five days prior, or 5, 10, 15, or 20 days after vaccination, and challenged 35 days post-immunization with 200 normal cercariae. Highly significant reduction in resistance to challenge infection was observed in the lympho-adenectomized as compared to intact mice. The results were construed to suggest that for induction of immune protection, presentation of antigens to leukocytes in the draining LN during the first days of RA larvae skin residence is more important than antigen presentation to the spleen cells (SC) during larval intravascular migration [74]. This assumption was supported by finding marked increase in T-, and to a greater extent of B-lymphocytes in skin- and lung-draining LN, but not in spleen of C57Bl/6 mice on days 2–14 post 1× vaccination with *S. mansoni* cercariae attenuated with 20 krad from a ^{60}Co source [75]. Localized hyperemia (increased blood flow) appeared to explain the accumulations of lymphocytes in draining LN [76]. This finding suggests that leukocytes in draining LN may well be stimulated by larval antigens released intravascularly and not uniquely by antigens released extravascularly, in the dermis or lung parenchyma [77]. The draining LN leukocytes of RA cercariae-vaccinated mice were shown to be essentially of the CD4+ type and responded to parasite

antigens by production of T helper (Th) dominant immune responses, notably increased production of interferon-gamma (IFN-γ) and interleukin (IL)-12 [78–82]. Yet, these LN cells released significant amounts of IL-4 and did not generate an anamnestic Th1 response to parasite antigens after challenge infection whereby IFN-γ production was profoundly down-regulated and large amounts of IL-4 were generated [83].

The results together certainly indicate that RA *S. mansoni* vaccine-induced protection of mice to challenge infection is dependent on site of vaccination-draining LN build-up of Th1 and Th2-immune responses.

Lung

Schistosomula must negotiate the thin-walled and convoluted pulmonary capillaries before attaining the liver sinusoids and then the portal vein. The migration is obligatorily intravascular, but during the strenuous journey in the lung, many larvae are detected in the alveolar spaces, destined to disintegrate and die [59,84,85]. The larval-derived antigens stimulate intense immune responses characterized by accumulation of lymphocytes and macrophages in dense foci. Similar events occur in RA cercariae-vaccinated rodents with a larger proportion of migrating schistosomula ending into the alveolar spaces and surrounded by larger leukocytic foci [85–91]. These inflammatory foci are generated in response to antigens derived from larvae destined to die, and there is no proof they are the agents responsible for parasite attrition in normal or RA cercariae-immunized mice. Indeed, in spite of the inflammation, no direct lethal cytolytic hit to the schistosomula was observed [59,85,87,92,93]. Intravascular healthy larvae release extremely minute amounts of molecules, the excretory–secretory products (ESP), the scent, and attract no or minute foci [59,92,93]. Intravascular dying or dead larvae, especially in RA vaccine-administered mice, stimulate more or less intense inflammatory foci characterized by the presence of large numbers of eosinophils [92,93]. Some histopathological studies showed the intravascular leukocytic loci destroy the blood-air barrier, thus facilitating larval exit and subsequent death, but also blood spill in the alveoli, a phenomenon rarely, if never, observed [85]. Conversely, it was reported that pulmonary intravascular foci around larvae are rather small [59,92,93]. The results together do not provide conclusive evidence that the inflammatory foci in the lung parenchyma are the agents responsible for parasite deflection in the alveoli.

The dogma stipulating that immune responses to challenge schistosome infection following RA cercariae vaccination must be Th1 polarized to achieve protection has its foundation in several studies that measured C57BL/6 mice bronchoalveolar lavage leukocytes (BAL) immune responses to parasite antigens. As stated above, BAL are situated in lung parenchyma and alveolar tissue and are stimulated by antigens released by extravasated dying larvae. Schistosome larval antigens predominantly induce Th1-related responses [78,94–98]. Accordingly, it is expected that BAL release Th1-related cytokines upon culture *in vitro* in the absence or presence of larval antigens [80,95]. Yet, there is no proof that the BAL-mediated Th1 immune responses are major players in extravasation of challenge intravascularly migrating worms.

Spleen

Schistosomes are obligatory intravascular residents. Like other blood-born antigens, ESP released by healthy parasites and molecules derived from intravascularly dying, dead and degenerated worms reach the spleen, are trapped by residents macrophages and dendritic cells (DC), and stimulate T and B lymphocytes that circulate thereafter in tissue and blood [88]. Leukocytes in blood, rather than in tissue-draining LN, are the ones that interact with developing larvae and might mediate their extravasation and potential attrition. Yet, SC immune responses in the RA vaccine model were seldom looked at. C57BL/6 mice were percutaneously vaccinated with *S. mansoni* cercariae attenuated with 20 krad of gamma irradiation from a ^{60}Co source, SC and LN cells obtained at 3 day interval for 24 days post-immunization, and tested for proliferation and cytokine release in response to soluble schistosomular (18 h-old larvae) antigens. Similarly to the axillary, inguinal and mediastinal LN, SC cultures released significant amounts of IFN-γ that reached a peak at day 18 post-vaccination; no information was shown related to SC IL-4 production [80]. Following challenge with 200 normal cercariae, SC differed from BAL in displaying vigorous proliferation but production of low levels of IFN-γ in response to *in vitro* stimulation with schistosomular antigens [95]. In our laboratory, SC obtained from C57BL/6 mice 1–6 weeks following secondary immunization with RA (25 krad of gamma irradiation from a ^{60}Co source, or 330 μW/cm^2 UV radiation) were found to consistently release IL-2, IFN-γ, and IL-4 in response to *in vitro* stimulation with electroseparated soluble schistosomular or adult worm antigens [99,100].

T cell mediated or humoral immunity?

The association between leukocytic accumulations in the lung parenchyma of RA larvae-vaccinated and challenge cercariae-infected mice and high protection levels led to the assumption that resistance in vaccinated mice may be T cell rather antibody-mediated [84,85]. In RA cercariae once vaccinated mice, results were compatible with that hypothesis and further stressed that the mechanism of immunity depends on T lymphocytes-macrophages interaction triggered by antigens released from lung larvae, leading to focal cell-mediated effector immune responses that block onward challenge larvae migration and cause their deflection in the alveoli and attrition [84–93,101]. The results together suggested that challenge larvae are predominantly eliminated through delayed-type hypersensitivity (DTH) reactions [79,90]. In support, mice of the P/N strain that are characterized as deficient in their ability to mount DTH and macrophage activity, and mice of the 129 strain with disruption of the gene encoding the tumor necrosis factor receptor consistently failed to display resistance to challenge infection following once vaccination [65,102]. In contrast, nitric oxide produced by leukocytes accumulations in the lung tissue of RA cercariae vaccinated mice was shown to be not essential for challenge parasite elimination [103]. Additionally, one-third of B cell-deficient C57BL/6 mice vaccinated once with RA cercariae failed to display resistance to challenge infection [104].

T cell and antibody reactivity to larval antigens in mouse strains differing in their level of resistance to challenge infection following once RA cercariae vaccination appeared to be of importance for the development of protection [64]. These

findings were supported in mice made deficient in T or B lymphocytes [105]. A strong evidence for the importance of antibodies came from studies of Mangold and Dean [106] who conclusively showed that passive iv transfer of serum obtained from C57BL/6 mice 3 weeks following last (of 2–3) immunization with RA (50 krad from a ^{60}Co source) *S. mansoni* cercariae into syngeneic naive mice elicited reductions in challenge worm burdens of 20–50%. The highest level of protection was achieved when immune serum was administered at a time coincident with larval migration in the pulmonary vasculature. The antibody-mediated protection levels were never as high as in the donor mice, implying that other immune effector arms, likely cell-mediated immunity, are required for optimal resistance [106] and Table 1. Highly significant protection was also achieved in C57BL/6 mice upon passive transfer of serum from RA *S. mansoni* cercariae vaccinated rabbits [107]. The serum fraction responsible for resistance transfer was conclusively shown to be antibodies of the IgG class [106,107]. Similar results were obtained in BALB/c mice passive transferred with RA *S. mansoni* vaccine immune serum from syngeneic mice or rabbits [108] and were entirely confirmed in the RA *S. japonicum* vaccine model [109]. Furthermore, protective immunity displayed by baboons vaccinated with RA *S. mansoni* cercariae was suggested to essentially be antibody-dependent [110]. In mice, the titer of antibodies following RA cercariae immunization appeared of critical importance for the development of resistance to challenge infection [111].

Th1 versus Th2?

Treatment of RA cercariae once vaccinated-mice with neutralizing mAb to mouse IL-4, IL-5, or IFN-γ, on day 14 or 7, and day 1 before and again at weekly intervals after challenge infection indicated a preponderant role for IFN-γ-dependent cell-mediated effector mechanisms in the elicited protection, while IL-4, IL-5, and eosinophils are of negligible importance [112]. Yet, mice with disrupted IFN-γ receptor gene displayed an impaired, yet not abrogated, resistance to challenge infection following vaccination with RA *S. mansoni* cercariae; of note, the reduction in worm burdens in wild type was in the range of a modest 50% [113]. The results, thus, suggest that IFN-γ-independent mechanisms are necessary for optimal protection in the RA vaccine model. Additionally, all cytokine measurements concentrated on BAL and/or total lung tissue [113,114] while it must be reiterated that *S. mansoni* strive inside the blood vasculature in lungs and elsewhere. In contrast to conclusions reported using mice treated with a mAb targeting inducible nitric oxide synthase [103], nitric oxide direct effector functions and its role in activation of macrophages and endothelial cells for killing migrating larvae were advocated as key elements in the acquisition of protection in the murine RA vaccine model [114,115]. The debate over the effector functions of nitric oxide in protection against schistosome infection is not as yet settled [116,117]. On the other hand, lung tissue or SC production of IL-4, IL-13, IL-10 and other Th2-related cytokine responses appeared to be responsible for the overall limited protection in high [115] and low [118] responder mice.

Different results were attained with 50 krad RA (from a ^{137}Cs source) *S. mansoni* cercariae once or thrice vaccination of B cell-deficient mice, whereby challenge worm burden reductions were only 33–43%, considerably less than wild type mouse. Additionally, the decrease in protection in IFN-γ knockout mice was not striking compared to wild type counterparts vaccinated in parallel with RA *S. mansoni* cercariae once (46% versus 63%) or thrice (64% versus 80%) [119]. Moreover, signaling via IL-4 receptor alpha chain was absolutely required for significant RA cercariae vaccination-mediated resistance in BALB/c mice [120]. Finally, several studies using knockout mice closed the controversy by conclusively demonstrating that optimal protection in the RA vaccine model is dependent on the induction of both type-1 and type-2-associated immune responses [121–123].

Molecules recognized by antibodies and lymphocytes of RA-immunized hosts

Antibodies of C57BL/6 mice exposed twice via tail immersion to approximately 500 *S. mansoni* RA (50 krad) cercariae selectively bound to several schistosomular molecules, notably a 38 kDa glycoprotein of *in vitro* cultured 5 day-old schistosomula, seven adult worm antigens among which a 94–97 kDa glycoprotein, as well as, an antigen of 200 kDa present in schistosomular and adult worm soluble extracts [124–127]. A cDNA encoding a 62 kDa portion of the 200 kDa molecule was cloned and sequenced and found to share homology with myosins of other species; subcutaneous or ip immunization of C57BL/6 mice with the expressed recombinant protein, designated rIrV-5, elicited 75% protection against challenge worm burden [127]. Similar studies led to identification of SmIrV1, which showed homology to calnexin and calreticulin [128,129]. Additionally, studies with SC of mice vaccinated with RA *S. mansoni* cercariae used to produce mAb against newly transformed schistosomular surface antigen resulted into selection of a larval surface membrane 18 kDa polypeptide. Polyclonal antibodies generated against the 18 kDa molecule isolated recombinant clones from an adult worm cDNA library constructed in λgt11 [130]. The target molecule was found to be of exactly 23 kDa, designated Sm23, and identified as worm

Table 1 RA cercariae vaccine efficacy varies with host species and strain immune responses.

RA vaccine	Host species and strain	Protection level	Immunity	References
S. mansoni	Mouse	C57BL/6 75–90%	Th1 and Th2	[3,11,136]
S. mansoni	Mouse	BALB/c 30–60%	High DTH	[65]
S. mansoni	Mouse	CBA 4–66%	Low Ab levels	[136]
S. mansoni	Mouse	P/N	10%-20%	Low DTH and Th1 [65,118]
S. mansoni	Baboons	30–54%	High IgM/IgG	[17]
S. japonicum	Mouse	CBA/H 50–72%	High Ab levels	[109]
S. japonicum	Mouse	C57BL/6 2–40%	Low Th1 and Th2	[32]

DTH = delayed-type hypersensitivity; Ab = antibody.

integral surface transmembrane antigen and glycosyl inositol phosphatidyl-anchored as well [131]. Furthermore, antibodies of RA *S. mansoni* cercariae-vaccinated CBA mice were found to specifically recognize schistosomulum surface antigens of >200, 38, 32, 20, and 15 kDa. The >200 and 15 kDa molecules were also recognized by CBA mice immunized with RA *S. haematobium* cercariae; conversely, the molecules of the 20–38 kDa range showed species-specificity [132,133], thus indicating that some, but, not all schistosome molecules confer cross-protection. Most importantly, when vaccinated mice of the C57BL/6 and CBA strain were compared, both strains recognized Sm23, glutathione-S-transferase (GST) and cathepsin B, thus suggesting that these molecules may be used for vaccination of different mouse strains, in contrast to Sm32 and paramyosin that were recognized only by CBA, and heat shock protein 70 exclusively by C57BL/6 mice [134].

Since T cells mediate cellular immunity and control antibody production, it was of importance to identify the schistosome antigens recognized by T cells as well as humoral antibodies of mice vaccinated with RA *S. mansoni* cercariae. Axillary LN cells of C57BL/6 and CBA mice vaccinated once with cercariae attenuated with 15 or 50 k of gamma irradiation were *in vitro* stimulated with adult worm antigens fractionated by isoelectric focusing. The LN cells proliferative and lymphokine responses and humoral antibody binding revealed that Sm23, paramyosin, heat shock protein 70, triose phosphate isomerase (TPI), and GST appeared to be the molecules that stimulate the most intense immune responses in the murine RA vaccine model [135,136]. We have used the T cell western and western blotting assays to identify the schistosomular and adult worm antigens recognized by LN and spleen T cells and serum antibody of outbred and inbred mice immunized twice with gamma or UV-radiation-attenuated *S. mansoni* cercariae [99,100,137]. The molecules most consistently recognized, and presumably of importance in inducing resistance against challenge infection in this model, were selected and identified as *S. mansoni* enolase, and *S. mansoni* calreticulin [99,100,138,139].

Some of the molecules putatively responsible for the induction of protection against challenge infection following RA cercariae vaccination, notably IrV5, Sm23, paramyosin, GST, TPI-derived peptides in a multiple antigen construct (MAP), probably emulsified in Freund's or alum were used in controlled vaccination and protection studies in C57BL/6 and BALB/c mice. None succeeded in inducing protection higher than the 40% benchmark sent by the World Health Organization for progression of schistosome vaccine antigens into pre- and clinical trials [140,141].

The outcome of the missed lessons

The majority of the murine RA vaccine model studies concentrated on the C57BL/6 strain because it proved to be the highest responder. BALB/c and CBA mice showed moderate response, A/J mice marginal resistance, while other strains, notably RF/J, and P/N appeared to display negligible protection following immunization with RA larvae [63–65]. These findings suggest that vaccination results using schistosome subunit antigens in preferred 2 or 3 inbred mouse strains may not be readily confirmed in other laboratories using different mouse strains, or extrapolated to the outbred humans. Nevertheless, the majority of studies related to development of a schistosomiasis vaccine disregarded this limitation, overrelied on the C57BL/6 strain, and neglected the use of outbred mice. Fortunately, several schistosome vaccine studies were performed in baboons, despite the challenges of the costs and experimental settings [110,142–148].

In every histopathological or mincing/incubation study regarding the RA vaccine model, no evidence was ever obtained for tight adherence of leukocytes to the lung-stage schistosomula surface, direct cytolytic hit, or structural damage presumably mediated by antibody-dependent cell-mediated cytotoxicity [39,52,56,59,67,84–87,89–93]. These results were in entire accord with the plethora of articles documenting the inaccessibility of healthy schistosome surface membrane antigens to antibody binding and the insusceptibility of developing larvae to antibody-dependent attrition mechanisms [9, reviewed in 149,150]. These well-established, confirmed, and reproducible findings imply that parasite surface membrane or tegumental antigens may not mediate access of effector immune responses to challenge infection parasites whether in the dermis or during intravascular migration and residence. Nevertheless, the great majority of articles focused on schistosome surface membrane or tegumental molecules as vaccine candidates, notwithstanding the fact that if surface membrane molecules were at any time accessible to the host effector immune responses, the parasite would not survive days, not to mention decades, in the host blood stream. The outcome of this lessons neglect is obtention of protection against challenge infection of limited significance ($P < 0.05$–< 0.01) and reduction percentages of 30–40% that are not reproduced from experiment to experiment, leading to damping of these molecules out of the vaccine candidate list [reviewed in 149,150]. An outstanding example was the *S. mansoni* glucose transporter SGTP4, a molecule at the host-parasite interface of critical importance for the parasite survival [151]. Vaccination of outbred and inbred mice with the molecule extracellular domains in recombinant or synthetic peptide constructs and emulsified in Freund's adjuvant induced considerable cellular and humoral immune responses but entirely failed to provide protection against challenge *S. mansoni* infection [152]. Fortunately, however, several antigens readily released from invading worms and potential inducers of protection in the RA vaccine model were used as vaccine candidates among which calpain [143–148], GST [142], which has now moved to phase 1 clinical trials [153], and paramyosin, whereby recombinant full-length *S. japonicum* paramyosin, rSj97 was produced and assessed for efficacy and safety in rodents and large-animal models [154].

One of the salient lessons gained from the extensive studies concerning the RA vaccine model is that protection elicited essentially depends on both Th1 and Th2-associated immune responses [3]. Since schistosome candidate vaccine molecules are documented to stimulate polarized Th1-related immune reactivity, it was of importance to look for and use an adjuvant that would skew the immunogen-induced polarized Th1 toward the Th2 immunity axis. That did not happen. On the contrary, many candidate vaccines, including calpain, were used as DNA constructs known to predominantly elicit Th1-related responses [143–145 and reviewed in 150,155]. We have used the candidate vaccine antigen and larval ESP, *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (SG3PDH) in a recombinant (*r*), linear peptide or MAP form, emulsified in Freund's or other Th1 adjuvants for immunization of outbred and inbred mice and only obtained occasional,

and barely significant ($P < 0.05$) reduction in challenge worm burden and egg load of less than 35% [156–159]. We have used other larval ESP, notably *S. mansoni* 14-3-3 and p18 protein in a recombinant form, and aldolase, calpain, and thioredoxin peroxidase (TPX) = 2 cys peroxiredoxin-derived peptides in MAP constructs emulsified in Freund's adjuvant or aluminum hydroxide for immunization of C57BL/6 and BALB/c mice. While the molecules were strongly immunogenic, eliciting biased Th1-related immune responses whether administered in conjunction with Freund's adjuvant or alum, the protection levels were suboptimal and rather erratic [160]. Not very different results were attained with the numerous trials using *S. mansoni* or *S. japonicum* tegumental and surface membrane associated molecules in conjunction with Th1-biased adjuvants for immunization of inbred mice [reviewed in 149,150,161]. The outcome is up of today, the schistosomiasis vaccine still remains an unmet clinical need [123,149].

The outcome of the well-learned lessons

We have learned our lessons and focused on the use of larval ESP, such as SG3PDH and TPX, relied on outbred mice, and most importantly performed extensive studies to find an adjuvant that would skew these molecules-mediated Th1 responses toward the Th2 axis. We found that alum [160], polyinosinic–polycytidylic acid and peptidoglycan [162] drive C57BL/6 and BALB/c to respond to *S. mansoni* larval ESP by production of IFN-γ and IL-17. Conversely, thymic stromal lymphopoietin (TSLP), the master regulator of type 2 responses, succeeded in directing the larval ESP-mediated immune responses toward a Th2-biased profile in prototypical Th1 and Th2 mice [162]. We thus understood that the type 2 cytokines, notably TSLP, IL-25, and IL-33, which stimulate the group 2 innate lymphoid cells [163–165] and type-2-cytokines-inducing molecules such as the cysteine peptidase, papain [166,167], are the immunomodulatory adjuvants needed to drive larval ESP-mediated vaccination toward generation of type 2-associated immune responses. Challenge infection larvae are, thus, met by both Th1- and Th2 cell-dependent immunity, as studies of the RA vaccine model recommended. Administration of outbred mice with rSG3PDH and TPX MAP in conjunction with papain, TSLP, IL-25, or IL-33 consistently and reproducibly elicited Th1- and Th2-associated cytokines and antibodies, and significant ($P < 0.0001$) reductions of a minimum of 50% and up to 78% in challenge worm burden and worm egg counts [168]. Since schistosome cysteine peptidases are both ESP and potential type-2 cytokines-inducers, it was reasonable to assess their protective potential in outbred mice alone or as adjuvants to the larval ESP, rSG3PDH and TPX MAP. The considerable and highly significant ($P < 0.0001$) reduction of 50–83% in worm burdens and worm egg load in each of 7 consecutive experiments, each involving 4–8 animal groups, led us to devise a formula for the schistosomiasis vaccine, notably rSG3PDH+ *S. mansoni* cathepsin B+ *S. mansoni* cathepsin L. The latter peptidase was required for its potential role in worm reproduction and impact on eliminating the Th2 cytokine-associated transient increase in challenge worm fecundity [169,170]. Benefiting from another lesson of the RA vaccine model, notably that *S. mansoni* molecules may protect hosts against *S. haematobium* infection [62], we have vaccinated outbred mice and hamsters with the *S. mansoni* antigens mentioned in the formula and obtained consistent, reproducible, and highly significant ($P < 0.0001$) reductions of 70% in challenge worm burden and worm egg counts [171].

Accordingly, we recommend retesting the various available schistosome candidate vaccine antigens, notably calpain, GST, TPI, enolase, paramyosin, and Sm14 in conjunction with cathepsin B and cathepsin L for their protective potential in laboratory outbred rodents and baboons against challenge *S. mansoni*, *S. haematobium*, and *S. japonicum* infection. Evidence regarding the longevity of the generated protection must be established in an aim of achieving the highly coveted goal of a sterilizing schistosomiasis vaccine.

Conflict of interest

The authors have declared no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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