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Vaccine potential of a recombinant glutathione *S*-transferase cloned from *Schistosoma haematobium* in primates experimentally infected with an homologous challenge

Denis Boulanger[a,*], André Warter[b], Bertrand Sellin[a], Véronique Lindner[b], Raymond J. Pierce[c], Jean-Philippe Chippaux[a], André Capron[c]

[a]*Centre de Recherche sur les Méningites et les Schistosomoses (CERMES/OCCGE/ORSTOM), W.H.O. Collaborating Centre for the Control of Schistosomosis, BP 10887, Niamey, Niger*

[b]*Faculté de Médecine, Institut de Pathologie, Strasbourg, France*

[c]*INSERM U 167, "Relations Hôte-Parasite et Stratégies Vaccinales", Institut Pasteur, Lille, France*

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Abstract

Patas monkeys were twice immunized with a *Schistosoma haematobium*-derived recombinant glutathione *S*-transferase (Sh28GST) then challenged with an homologous calibrated challenge. BCG and Freund's Complete Adjuvant (FCA) were used as adjuvants in two distinct protocols. Specific IgG and IgA antibody responses were intense and homogeneous in the animals receiving Sh28GST in the presence of FCA, whereas BCG could only induce moderate and heterogeneous antibody titres. No significant effect on worm burdens was evidenced 36 weeks post-infection in either group of Sh28GST-immunized animals compared to their matched controls receiving an irrelevant protein. Although not significant, 50% reductions in the numbers of eggs located in all tissues (FCA group) and in the urogenital system (BCG group) were noted. Moreover, the total number of excreted eggs was dramatically diminished by 60% and 77% in the BCG and FCA groups, respectively. These reductions reached 75% and 80% in the urines of vaccinated monkeys. Bladder pathology was also reduced in the animals displaying the lowest urinary egg excretions. There was no clear positive or negative correlate between antibody responses and individual levels of protection. Taken as a whole, our results show that Sh28GST was capable of significantly reducing *S. haematobium* worm fecundity in experimentally infected primates. Although FCA induced higher levels of protection, the efficacy of BCG as an adjuvant appeared sufficient to justify consideration of the future application of this new formulation as a vaccine against human urogenital schistosomosis.

Keywords: *Schistosoma haematobium*; Recombinant vaccine; Glutathione *S*-transferase; Primates

1. Introduction

Reinforced by the recent emergence of strains naturally resistant to chemotherapy [1,2], need for a vaccine control of schistosomosis has become a medical priority in many endemic countries. For the last decade, several research teams have explored the protective capacities of the schistosome-derived glutathione *S*-transferases (GST) [3] family, enzymes able to neutralize the hydroperoxides resulting from the attack on the parasite tegument by the natural effector mechanisms developed by its definitive host [4]. Administered preventively, purified and recombinant GST from different schistosome species (*S. mansoni*, *S. japonicum*, *S. bovis*) have demonstrated a remarkable shared vaccine effect directed against the fecundity of adult worms [5].

For clinical and epidemiological reasons, priority for the passage to human clinical trials has been given to the severe urogenital form of human schistosomosis due to *Schistosoma haematobium*. The biological features of the interface maintained by this particular

* Corresponding author. Tel.: +227-752045; fax: +227-753180; e-mail: boulange@niamey.orstom.ne.

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species are indeed propitious: the marked focality of the urogenital lesions induced by *S. haematobium* eggs favors the choice of parasite fecundity instead of worm burden as an appropriate vaccine target. Moreover, the progressive inhibition of worm fertility evidenced in naturally *S. haematobium*-infected patients [6] supports the feasibility of such an objective.

The expected outcome of the vaccine, in terms of Public Health, together with the availability of the 28 kDa GST cloned from *S. haematobium* (Sh28GST) have led to the current launching of a Phase I clinical trial on human volunteers. The last step to be cleared was to check experimentally the potential of the *S. haematobium*-derived 28GST in a fully homologous system. We present here the results of a vaccine trial using Sh28GST in patas monkeys infected with *S. haematobium*.

2. Materials and methods

2.1. Animals

Thirty-two wild-caught female patas monkeys (*Erythrocebus patas*) were randomly distributed in four groups after weight-matching. Their average weight (±SD) was 3.9 ± 0.6 kg. During quarantine, they were confirmed as schistosome-free by repeated stool and urine examinations. Animal handling was performed according to the guidelines adopted by the Primate Vaccine Evaluation Network (PVEN).

2.2. Antigen

The recombinant Sh28GST [7] used in the experiment was produced in *Saccharomyces cerevisiae* strain TGY73.4 containing the plasmid pTG8889 (provided by TRANSGENE S.A., Strasbourg) exactly as previously described [8]. The purity of the rSh28GST (>98% pure protein of 28 kDa) was checked by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining and its concentration measured by amino acid analysis. GST activity was evaluated at 25 I.U. per mg of protein according to the method of Taylor et al. [9].

2.3. Immunizations

Animals received two injections, six weeks apart, of 100 μg of rSh28GST with either 0.1 ml of Bacillus Calmette Guérin (BCG, Institut Mérieux, Lyon) intradermally, or in the presence of Freund's Adjuvant (Complete for the first injection, Incomplete for the boost) v/v (FCA/FIA, Difco, Maurepas) subcutaneously. Control animals received an equivalent amount of bovine serum albumin (BSA) with the corresponding adjuvant. Local and general reactions were monitored for one week after each injection.

2.4. Parasite

Schistosoma haematobium miracidia, hatched from eggs excreted by children living in a highly endemic area (Liboré, Eastern Niger), were used to infect *Bulinus truncatus* snails collected from the same location then bred in the laboratory. Sixteen weeks after the second immunization, 1500 cercariae per animal (manually counted) in pond water, were used to infect paired, sedated patas monkeys by the skin pouch method [10].

2.5. Parasitological methods

Details of parasitological methods have been described previously [11]. Briefly, egg excretion was monitored fortnightly from week 8 to week 34 post-infection. At this time, the animals were humanely killed to allow measurement of worm burdens, tissue eggs and intra-uterine schistosome eggs. Only animals with a minimal number of 9 samplings throughout the experiment were included in the calculations. Total egg outputs are expressed only if urinary and faecal eggs were available on the same sampling day.

2.6. Blood samples and antibody response

Blood (5 ml) was repeatedly sampled from the femoral vein throughout the experiment and centrifuged for 30 min at 3000 rpm and at +4°C. Serum was recovered, aliquoted and stored at –20°C until use. Specific IgG antibodies were monitored following a standard ELISA procedure using 5 μg/ml of Sh28GST, a 1:50 serum dilution and a 1:1000 dilution of a horse radish peroxidase-conjugated IgG fraction anti-monkey IgG (Cappel, Turhoudt, Belgium). IgA screening used an unconjugated rabbit anti-monkey IgA (Nordic, Tilburg, Netherlands) and a horse radish peroxidase-conjugated anti-rabbit Ig (Cappel). Animals were considered as responders when their mean O.D. value was two standard deviations above the control average.

2.7. Anatomopathology

The extent of pathological changes in the bladder was assessed macroscopically on the day of sacrifice. The intensity of acute inflammatory processes was evaluated microscopically after fixation of a bladder sample in 10% formalin, embedding in paraffin and cell specific staining. Two parameters were separately scored from 0 (absence) to 5 (highly intense inflammation or pancystitis). The global degree of severity of

the bladder lesions was evaluated by the product of both indices.

2.8. Statistical analysis

Data distribution being far from normal in most cases, statistical comparisons were made by the Wilcoxon signed-rank test for paired values.

3. Results

3.1. Innocuity

Apart from indurations on the site of injection, no adverse local or systemic reactions were noted in any inoculated animal.

3.2. Antibody response

FCA induced a strong specific IgG response after the first injection which remained at a high level in most animals throughout the experiment (Fig. 1A). BCG elicited less intense and much more heterogeneous IgG titres. In this latter group, the peak value, occurring two weeks after the boost, was followed by a sharp decrease. On the day of infection, no anti-Sh28GST antibodies could be detected in any animal from the BCG group. However, the identification of the first eggs in the excreta (around week 10 post-infection) coincided with a noticeable increase in antibody titres in most animals, including the controls. Specific IgA responses were closely parallel to IgG in the BCG and control groups (Fig. 1B). In contrast, in the FCA group, the second injection provoked a detectable boost effect on IgA levels with a peak value followed by a more pronounced decrease, extending to the onset of patency. The proportion of antibody responders is shown Table 1. All the animals developed an IgG and an IgA response after immunization in presence of FCA. In contrast, most monkeys required two injections to mount an IgG response in the BCG group and half of them did not produce specific IgA antibodies even after the boost.

3.3. Egg excretion

Urinary and faecal egg excretions were dramatically and significantly reduced in both Sh28GST-immunized groups (Table 2). Quantitatively, the vaccine effect was more pronounced in the FCA group where the overall number of excreted eggs was cut by more than three-quarters. During the course of the experiment, a difference in cumulative egg output between the Sh28GST-immunized group and its corresponding control group became significant at week 21 (urines) and week 23 (faeces) post-infection in the BCG group (Fig. 2A and B respectively). This occurred earlier in the FCA group at week 19 (urines) and week 15 (faeces) post-infection. In addition, the difference continued to increase in the FCA group whereas it tended to stabilize from week 30 onwards in the BCG group.

Comparisons between matched animals indicate that most of them were protected in terms of egg excretion in both groups (Fig. 3 and Table 2). Individual levels of reductions in egg output among protected animals ranged from 56% to 95% in the urines and from 38% to 97% in the faeces. They were more consistent in the urines of the BCG group and in the faeces of the FCA group. Taken as a whole, FCA induced a more marked overall level of protection.

3.4. Perfusion data

Worm burdens, numbers of eggs in the tissues and in the female schistosome uterus were compared on the day of sacrifice (Table 3). No significant difference was evident between the Sh28GST-immunized groups and their corresponding controls. However, in the FCA group, the number of female worms was reduced by almost one-third and the number of tissue eggs by half, including the urogenital system. The latter difference was also observed in the BCG group. Large standard variations exclude conclusions on the significance of the observed reductions. The tissue egg distribution was not significantly affected by either protocol of immunization.

3.5. Anatomopathology

The type of inflammation and the extent of lesions were often heterogeneous within groups. Overall, the FCA group was the most homogeneous, showing both an eosinophilic acute inflammation centered on eggs and a chronic and sclerotic inflammation accompanied by an intense follicular lymphoid stimulation. In this group, the polarity of egg colonization frequently appeared reversed, acute phenomena predominating in the *lamina propria* and sclerotic chronic reactions in the *mucosa*. This suggests that the reaction directed against the eggs is initiated early, from the *lamina propria*. BCG and control groups displayed the same duality but with a much more heterogeneous balance between acute and chronic processes. All the stages, from pure scar to acute pancystitis, were observed. The lesser immune reactivity shown by the control animals resulted in a greater extent of lesions.

Comparison of indices between matched animals showed that immunization with Sh28GST led to an improvement over the control five times out of eight (62.5%) in the FCA group and four times out of six (66%) in the BCG group. The microscopical examin-

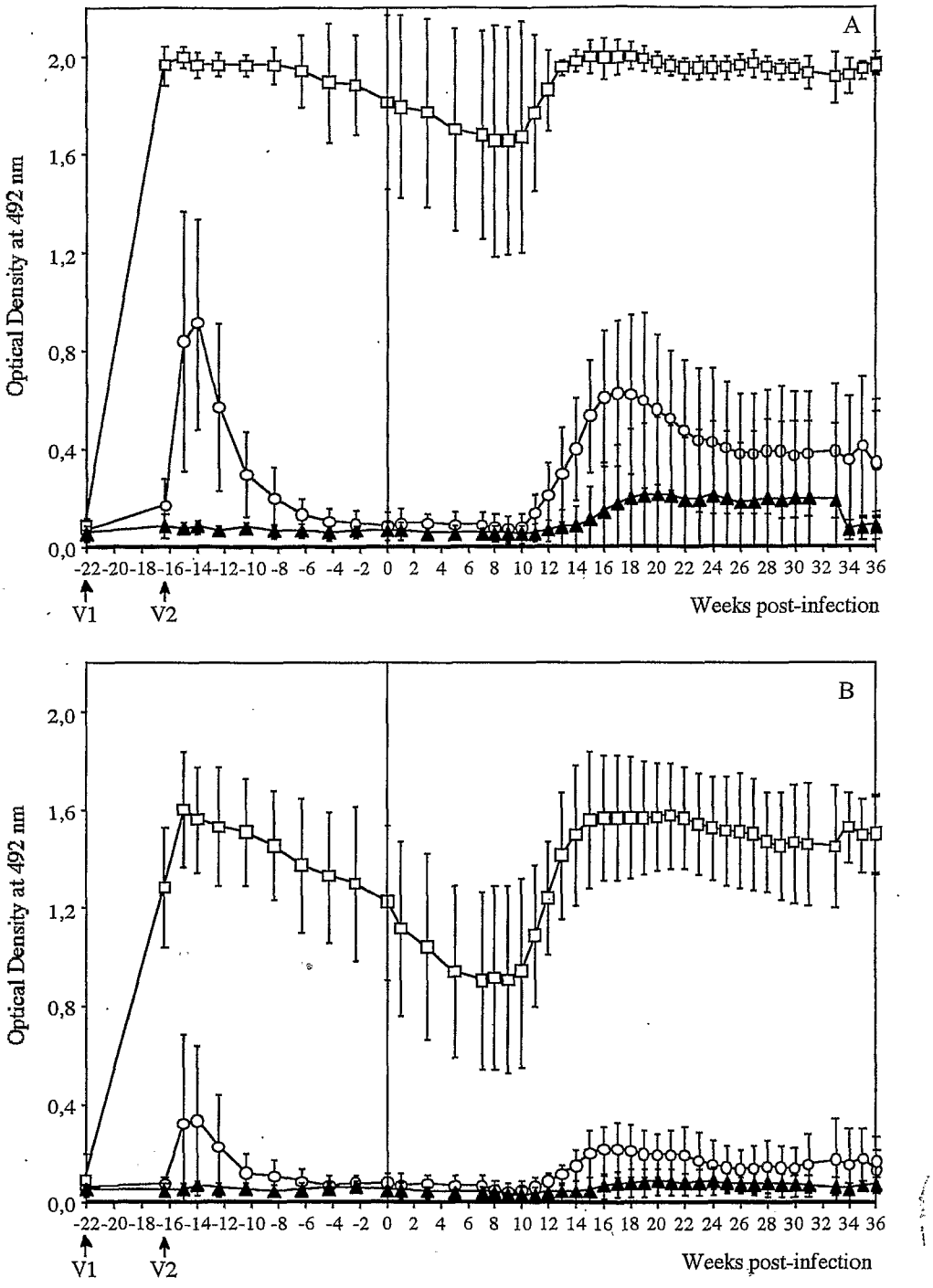

Fig. 1. Specific IgG (A) and IgA (B) antibodies in the Sh28GST-vaccinated patas monkeys (BCG group: open circles, FCA group: open squares) and in the controls (closed triangles). Results are expressed as the mean ± SD of eight animals. Data from the two control groups have been pooled. Dates of immunization (V1 and V2) are arrowed. Date of infection is indicated by a vertical bar.

Table 1
Proportion of specific IgG and IgA antibody responders among the two Sh28GST-immunized groups of patas monkeys

Group		IgG	IgA
Sh28GST + BCG	after one immunization	13% (1/8)	0% (0/8)
	after two immunizations	100% (8/8)	50% (4/8)
Sh28GST + FCA	after one immunization	100% (8/8)	100% (8/8)
	after two immunizations	100% (8/8)	100% (8/8)

Table 2
Egg excretion data from the two Sh28GST-immunized groups compared to their control groups

Egg output/24 h	n^a	Sh28GST + BCG	Control	Δ	p	No. of protected animals
Urines	77	3.8 ± 0.9	15.2 ± 3.9	−75%	< 0.005	5/6
Faeces	44	43.3 ± 16.8	113.0 ± 28.0	−62%	< 0.05	3/5
Total	43	46.4 ± 17.3	115.9 ± 29.2	−60%	< 0.05	3/5
Egg output/24 h	n^a	Sh28GST + FCA	Control	Δ	p	No. of protected animals
Urines	76	11.6 ± 2.8	58.8 ± 10.4	−80%	< 0.005	4/6
Faeces	52	50.0 ± 11.4	224.8 ± 61.4	−78%	< 0.01	4/5
Total	53	48.8 ± 10.5	209.1 ± 38.9	−77%	< 0.005	5/6

Data are expressed as mean ± S.E.M.
[a] No. of samplings.

ation clearly associated intense inflammatory reactions with a reduction in output of eggs in the urine. No sample demonstrated signs of Malpighian metaplasia.

4. Discussion

Most published vaccine trials against *S. haematobium* have involved irradiated larvae [12]. Ethical and logistical problems raised by the administration of live parasites to humans have encouraged the production of recombinant antigens [13], but none of these has been evaluated in protective assays so far. The Sh28GST used in our experiment is not only one of the first *S. haematobium* molecules of which the peptide sequence and biological activity has been elucidated, but is also the first homologous protein ever tested as a vaccine against urogenital schistosomosis in a primate model considered as relevant [14].

As the last step before Phase I clinical trials, the main goal of this experiment was to confirm the vaccine potential of Sh28GST foreseen by the heterologous Sm28GST already tested in the same model [11]. The molecule has demonstrated its innocuity and an excellent immunogenicity in patas monkeys. With FCA, Sh28GST elicited specific IgG and IgA responses in all immunized animals, auguring well for a satisfactory antibody coverage in future vaccinated human populations. However, the use of other adjuvants like BCG (in this experiment), aluminum hydroxide or liposomes (manuscript in preparation), have induced more heterogenous humoral responses

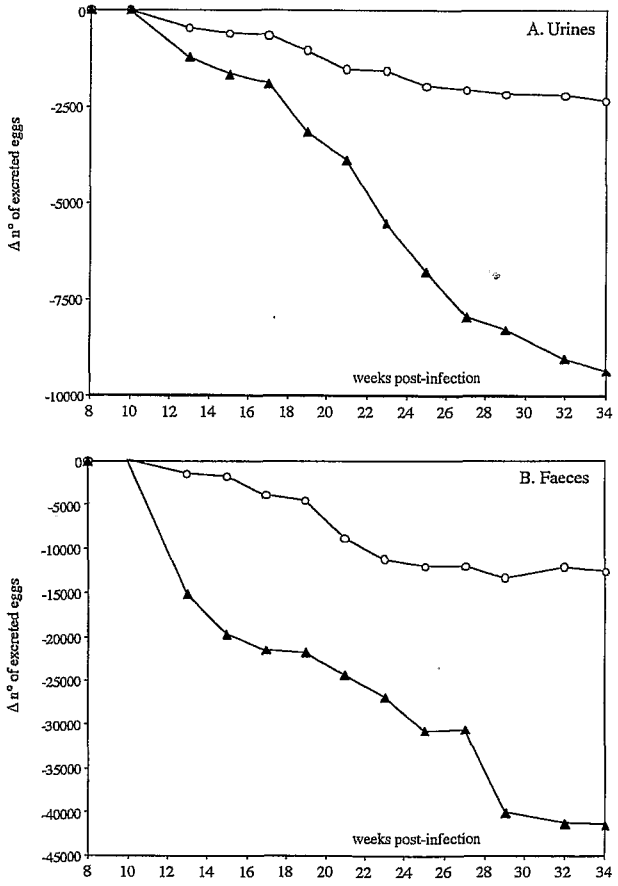

Fig. 2. Differences (Δ) between mean cumulative egg excretions in the Sh28GST-immunized groups with BCG (open circles) or FCA (closed triangles) and their corresponding control groups in the urines (A) and in the faeces (B).

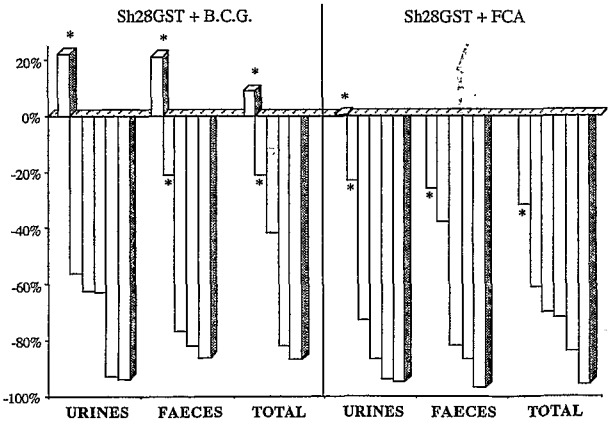

Fig. 3. Mean individual levels of reduction of urine, faecal and total excreted eggs in the two Sh28GST-immunized groups compared to their matched controls. Each bar represents a vaccinated animal. Non-significant differences are marked with an asterisk.

Table 3
Parasitological data collected on the day of perfusion from the two Sh28GST-immunized groups compared to their control groups

	Location	Sh28GST + BCG	Control	Δ (%)	p
Worm pairs	Mesenteries	9.3 ± 4.4	8.8 ± 2.1	+ 6	NS
	Urogenital	6.2 ± 2.9	5.0 ± 1.3	+ 23	NS
	Global	15.5 ± 6.9	13.8 ± 2.2	+ 12	NS
Total worms	Mesenteries	35.8 ± 12.9	42.0 ± 11.3	−15	NS
	Urogenital	16.3 ± 7.9	12.7 ± 2.8	+ 29	NS
	Global	52.2 ± 19.2	54.7 ± 10.3	−5	NS
Intra-uterine eggs		20.8 ± 1.5	18.1 ± 2.3	+ 15	NS
Tissue eggs ($\times 10^3$)	Liver	2.1 ± 0.5	2.8 ± 0.9	−27	NS
	Mesenteries	12.9 ± 10.7	14.6 ± 9.8	−12	NS
	Urogenital	24.2 ± 16.6	49.3 ± 26.1	−51	NS
	Lungs	2.3 ± 0.7	1.7 ± 0.5	+ 38	NS
	Total	41.4 ± 27.9	68.4 ± 35.1	−39	NS
	Location	Sh28GST + FCA	Control	Δ (%)	p
Worm pairs	Mesenteries	6.2 ± 1.5	8.7 ± 1.8	−28	NS
	Urogenital	4.1 ± 0.6	6.1 ± 2.0	−33	NS
	Global	10.3 ± 1.8	14.8 ± 3.5	−30	NS
Total worms	Mesenteries	41.4 ± 8.6	40.1 ± 8.0	+ 3	NS
	Urogenital	10.9 ± 1.8	15.8 ± 4.5	−31	NS
	Global	52.3 ± 8.9	55.9 ± 8.7	−3	NS
Intra-uterine eggs		19.0 ± 2.3	18.4 ± 1.4	+ 3	NS
Tissue eggs ($\times 10^3$)	Liver	1.4 ± 0.3	2.4 ± 1.0	−42	NS
	Mesenteries	4.8 ± 1.5	12.8 ± 6.7	−63	NS
	Urogenital	50.7 ± 28.7	110.4 ± 55.5	−54	NS
	Lungs	1.3 ± 0.3	2.4 ± 1.2	−44	NS
	Total	58.2 ± 28.6	128.0 ± 57.6	−55	NS

Results are expressed as the mean ± S.E.M.

than FCA, underlining the major influence of adjuvants on immune responses to highly purified antigens [15].

Unexpectedly, vaccination with the homologous Sh28GST did not influence worm burdens, reproducing the failure already encountered with the heterologous antigen [11]. Nevertheless, a comparison between the molecular sequences shows that among the 21 amino-acids which differ between Sm28GST and Sh28GST, three of them are located within the 115–131 peptide [7]. Immunization of rodents with the synthetic 115–131 peptide derived from Sm28GST induced a protective response demonstrated by a worm burden reduction after experimental infection with *S. mansoni* [16]. It might have been expected that the fully homologous anti-115–131 response elicited by Sh28GST would alter *S. haematobium* worm numbers. This was not the case, although the immunized animals from the FCA group harbored one-third fewer female worms than their matched controls. If an equilibrated cercarial sex ratio is assumed, the adult female worms still established at the 8th month of infection represent only 2% of the infecting larvae. A vaccine-dependent diminution in worm burden would be difficult to visualize when natural attrition already accounts for a 98% reduction.

On the other hand, Sh28GST was able to reproduce, with amplification, the anti-fecundity effect evidenced using the heterologous antigen [11]. This effect has been shown to be linked to inhibition of the GST enzyme activity by antibody recognizing the N- and C-terminal ends of the molecule [17], which are juxtaposed at the substrate-binding site (Mornon et al., manuscript in preparation). Moreover, this result confirms multiple observations made in different animal species immunized with GST, such as rodents infected with *S. mansoni* [17] or by *S. japonicum* [18], primates with *S. mansoni* [19], bovines with *S. bovis* [20] or pigs with *S. japonicum* [21]. The precise mechanism resulting in diminished parasite egg laying is still hypothetical in the primate models. We could not demonstrate any association between individual levels of protection and specific antibody titres, either of IgG or IgA isotypes (data not shown). However, in cattle, the passive transfer of immune serum seems to affect *S. bovis* fecundity [22]. In contrast, antibody responses are not correlated to protection in sheep vaccinated with *Fasciola hepatica* GST [23]. A more detailed analysis, including titration of antibodies directed against the different Sh28GST sequential epitopes, would be necessary to permit conclusions on the involvement of humoral mechanisms in the development of the observed anti-fecundity process. Such a titration

demonstrated a significant correlation between the anti-115–131 IgA response and resistance to reinfection in patients naturally infected with *S. mansoni* [24].

The adjuvant capacity of BCG, considered as compatible with use in humans, was another concern of this experiment. Indeed, it has already been so utilized during immunization trials against *Leishmania braziliensis* [25], against leprosy [26] and against particular cancers [27]. Despite its heterogeneous and moderate influence on the quantitative antibody response, it appeared clearly that BCG could induce levels of protection comparable to those obtained using FCA. This result reinforces the concept of a simultaneous vaccination against schistosomosis and tuberculosis, either by the physical association of both vaccines within the framework of the Expanded Program on Immunization, or through genetic manipulation. Indeed, the administration to mice of recombinant BCG expressing Sm28GST elicited the development of a specific T-cell response [28] and the production of antibodies able to neutralize the GST enzyme activity [29].

Duration of protection is an essential feature to justify the application of a vaccine in terms of medical and socio-economical impact [30]. A three month gap between vaccination and experimental infection did not prevent Sh28GST from conferring protection, suggesting the persistence of immunological memory over several months. Moreover, the reduction of egg excretion was maintained for at least 8 months post-infection and was accompanied by an alleviation of bladder pathology. This observation confirms that the use of such an anti-fecundity vaccine represents a promising strategy, able to prevent on a long-term basis the development of serious clinical forms of the disease and to significantly decrease parasite transmission [31].

Our experimental efforts focus henceforth on the follow-up of vaccinated and treated animals during ongoing infections. Special emphasis will be put on the immunopathological impact of the immunization schedule. Comprehensive analysis of the collected data should provide sufficient information to determine whether field evaluations of the molecule are justified.

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