

CHARACTERIZATION OF PROTECTIVE IMMUNITY INDUCED AGAINST *SCHISTOSOMA MANSONI* VIA DNA PRIMING WITH THE LARGE SUBUNIT OF CALPAIN (SM-p80) IN THE PRESENCE OF GENETIC ADJUVANTS

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Summary:

Despite advances in control via snail eradication and large-scale chemotherapy using praziquantel, schistosomiasis continues to spread to new geographic areas particularly in sub-Saharan Africa. Presently, there is no vaccine for controlling this disease. We have concentrated on a functionally important schistosome antigen Sm-p80 as a possible vaccine candidate for schistosomiasis. Here we report the proliferation of spleen cells in response to the recombinant Sm-p80 protein and cytokine (IFN- γ and IL-4) production by the splenocytes. These spleen cells were obtained from groups of mice that were vaccinated with a DNA vaccine formulation containing Sm-p80 and one of the Th-1 (IL-2 or IL-12) or Th-2 (GM-CSF, IL-4) enhancer cytokines. The splenocytes from the groups of mice vaccinated with Sm-p80 DNA in the presence of Th-2 enhancer cytokines showed moderate but detectable proliferation. The splenocytes obtained from mice vaccinated with Sm-p80 DNA with Th-1 enhancer cytokines IL-2 and IL-12 provided the highest proliferation. The IFN- γ production by splenocytes was found to follow the similar pattern [(Sm-p80) < (Sm-p80 + IL-4) < (Sm-p80 + GM-CSF) < (Sm-p80 + IL-12) < (Sm-p80 + IL-2)], as has been observed for the proliferation and protection data. However, the elevated IL-4 production was inversely correlated to Sm-p80-induced splenocyte proliferation or the protection. These results show again that protective immune response induced by Sm-p80 is of Th-1 type.

KEY WORDS : schistosomiasis, vaccine, Sm-p80 protein.

Résumé : CARACTÉRISATION D'UNE PROTECTION IMMUNITAIRE CONTRE *SCHISTOSOMA MANSONI* INDUITE PAR L'ADN AVEC LA SOUS-UNITÉ SM-p80 ASSOCIÉE À DIVERS ADJUVANTS

Malgré les progrès dans son contrôle par les campagnes d'éradication de mollusques et de chimiothérapie par le praziquantel à grande échelle, la schistosomiase continue de toucher de nouvelles zones géographiques, notamment en Afrique sub-saharienne. Aucun vaccin n'est disponible actuellement pour contrôler la maladie. Nous nous sommes intéressés à l'importante activité antigénique de la protéine Sm-p80 en tant que candidat potentiel à une vaccination contre la schistosomiase. Nous rapportons ici la prolifération des cellules spléniques en réponse à la protéine recombinante Sm-p80, et la production de cytokines (IFN- γ et IL-4) par les splénocytes. Ces cellules spléniques ont été obtenues chez des groupes de souris vaccinées par une formulation à base d'ADN composée de Sm-p80 et de cytokines Th-1 (IL-2 ou IL-12) ou Th-2 (GM-CSF, IL-4). On observe une prolifération modérée, mais décelable, des splénocytes du groupe des souris vaccinées par Sm-p80 en présence de cytokines Th-2, et une plus importante prolifération des splénocytes des souris vaccinées par Sm-p80 en présence de cytokines Th-1 (IL-2 et IL-12). La production d'IFN- γ par les splénocytes a suivi le même modèle [(Sm-p80) < (Sm-p80 + IL-4) < (Sm-p80 + GM-CSF) < (Sm-p80 + IL-12) < (Sm-p80 + IL-2)] que ce qui a été constaté avec les données concernant la prolifération et la protection. Cependant, l'élévation de la production d'IL-4 a été inversement proportionnelle à la prolifération de splénocytes induite par Sm-p80 et à la protection. Ces résultats montrent encore que la réponse immunitaire induite par Sm-p80 est de type Th-1.

MOTS CLÉS : schistosomiase, vaccin, protéine Sm-p80.

INTRODUCTION

It is widely believed that a schistosomiasis vaccine would make an enormous contribution to current methods of control, particularly if it provides a potent, long-lasting immunity to the disease (Capron *et al.*, 2002; Pearce, 2003; Hagan & Sharaf, 2003).

However, a major problem that has hindered schistosomiasis vaccine research and development concerns the identification and selection of potential protective antigens encoded by the parasite. Recent studies have amplified these concerns (Hagan & Sharaf, 2003). Independent examination of the six "priority antigens" (paramyosin, glutathione S-transferase, fatty acid binding 14 kDa protein, IrV-5, triose phosphate isomerase and Sm23) via a standard comparative World Health Organization delineated procedure, resulted in none of them providing the stated goal of 40 % protection or better (Bergquist *et al.*, 2002). This report highlights the continued and urgent need for an examination and assessment of additional schistosomiasis vaccine candidates.

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We have identified a novel schistosome protein that was originally identified to be involved in the surface membrane biogenesis (Siddiqui *et al.*, 1993). This phenomenon has been considered to be one of the mechanisms utilized by blood-dwelling helminths to evade the protective host immune response (Podesta *et al.*, 1987; Siddiqui *et al.*, 1993). This protein designated calpain, has two subunits, the larger of which, called Sm-p80 has been demonstrated to protect up to 60 % against cercarial challenge when administered to mice in a DNA vaccine formulation (Siddiqui *et al.*, 2003a, b).

In the present study, we report the *in vitro* proliferation of splenocytes in response to the recombinant Sm-p80 protein as well as cytokine production by these cells. These splenocytes were obtained from groups of mice that were vaccinated with a DNA vaccine formulation containing Sm-p80 and one of the Th-1 (IL-2 or IL-12) or Th-2 (GM-CSF, IL-4) enhancer cytokines.

MATERIALS AND METHODS

IMMUNIZATION PROTOCOLS

As described earlier (Siddiqui *et al.*, 2003 a, b), to determine the protective effect of the large subunit of *S. mansoni* calpain, each mouse (C57BL/6 mice, 3-4 weeks old, 10-12 g) was primed with 100 µg Sm-p80-pcDNA3. First and second boosts (100 µg Sm-p80-pcDNA3 each time) were given at 4 and 8 weeks, respectively. Each mouse in the control group received 100 µg pcDNA3 on day 1, followed by two injections of 100 µg each, at week 4 and 8, as above. Similar immunization regimen was also followed to test the adjuvant effects of GM-CSF, IL-4, IL-2 and IL-12. Briefly, each mouse in the "cytokine group" received 100 µg pORF-mGM-CSF or pORF-mIL-4 or pORF-mIL-2 or pORF-mIL-12 and 100 µg Sm-p80-pcDNA3 on day 1, and two boosters of the exact same concentration and composition, 4 weeks part, as above. The control mice of the "cytokine group" received only 100 µg pcDNA3. Four weeks after, the second boost, all of the mice from the groups outlined above, were challenged with 150 cercariae. The mice were sacrificed six weeks after challenge; adult worms were recovered from each mouse and percent reduction in worm burdens in vaccinated versus control animals was calculated (Siddiqui *et al.*, 2003a, b). Each group of mice contained at least 15 animals.

SPLENOCYTE PROLIFERATION ASSAYS

The spleens from the five groups of mice, outlined above, were processed as follows: each spleen was removed aseptically then meshed through a fine screen

in 6 ml of RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA) containing 10 % fetal bovine serum (FBS). The splenocytes were collected via centrifugation at 1,000 × g for 15 min. The resultant pellet was then resuspended in 1 ml Opti-Freeze DMSO Cryopreservation Medium (Fisher Scientific, Hampton, NH), placed in - 85° C overnight and finally stored at - 135° C until used.

The day of assay, the cells were thawed, washed in 9 ml of RPMI 1640 medium containing 10 % FBS and the cells were collected by centrifugation at 1,000 × g for 15 min and the final pellet was resuspended in the medium. The viability of cells was determined via Trypan Blue, > 80 % of cells were found to be viable. In a 96-well flat-bottom plate, the splenocytes (5 × 10⁵ cells/100 µl/well) were stimulated with baculovirus generated Sm-p80 recombinant protein (1.2 µg/100 µl/well) (Siddiqui *et al.*, 2003a). ConA was used at a concentration of 0.1 µg/well. All assays were performed in triplicate wells. The plates were incubated at 37° C in 5 % CO₂-humidified incubator. After 24 h incubation, 20 µl of AlamarBlue (AB) reagent [(BioSource International, Camarillo, CA) (Ahmed *et al.*, 1994)] was added and the plates were incubated again as above for up to 72 hrs. The plates were read at 24, 48 and 72 hrs at 570 nm and 630 nm.

The reduction of AB was monitored spectrophotometrically according to the manufacturer's instructions and the reduction was expressed as a percentage (% reduced). Briefly, the calculation of % reduced is as follows when the samples are read at: λ1 = 570 nm, λ2 = 600 nm.

$$\% \text{ reduced} = \frac{(\epsilon_{\text{ox}}\lambda_2) (A\lambda_1) - (\epsilon_{\text{ox}}\lambda_2) (A\lambda_2)}{(\epsilon_{\text{red}}\lambda_1) (A\lambda_2) - (\epsilon_{\text{red}}\lambda_2) (A\lambda_1)} \times 100$$

Where: (ε_{red}λ₁) = 155,677 (Molar extinction coefficient of reduced AB at 570 nm); (ε_{red}λ₂) = 14,652 (Molar extinction coefficient of reduced AB at 600 nm); (ε_{ox}λ₁) = 80,586 (Molar extinction coefficient of oxidized AB at 570 nm); (ε_{ox}λ₂) = 117,216 (Molar extinction coefficient of oxidized AB at 600 nm); (Aλ₁) = Absorbance of test wells at 570 nm; (Aλ₂) = Absorbance of test wells at 600 nm; (A'λ₁) = Absorbance of negative control wells which contain medium plus AB but to which no cells have been added at 570 nm; (A'λ₂) = Absorbance of negative control wells which contain medium plus AB but to which no cells have been added at 600 nm.

ESTIMATION OF CYTOKINE PRODUCTION BY PROLIFERATING SPLENOCYTES

To quantify the cytokine production by the proliferating splenocytes, exact duplicate wells were run simul-

taneously, as described above. After 48 hrs of incubation, the medium was collected from each well and stored at -70°C . Productions of cytokines by proliferating splenocytes was determined via BD OptEIA ELISA kits for mouse IL-4 and mouse IFN- γ (BD Biosciences Pharmingen, San Diego, CA), according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Comparisons of cellular immune responses among groups of mice were performed by two-tailed *t* test for two groups of animals or by analysis of variance (ANOVA) for more than two groups. Bonferroni adjustments were included for multiple comparisons, to reduce the risk of reaching false conclusions based on chance. In all cases, *P* values < 0.05 were considered significant.

RESULTS AND DISCUSSION

In this study we have determined the proliferative responses and production of cytokines via Alamar Blue assays (Ahmed *et al.*, 1994) using frozen spleen cells (Wright *et al.*, 2002) from five groups of mice. The groups of mice used in this study were vaccinated with a DNA vaccine formulation as described earlier (Siddiqui *et al.*, 2003a, b) and were as follows: Group 1 (Sm-p80-pcDNA3), Group 2 (Sm-p80-pcDNA 3 + pORF-mGM-CSF), Group 3 (Sm-p80-pcDNA3 + pORF-mIL-4), Group 4 (Sm-p80-pcDNA3 + pORF-mIL-12) and Group 5 (Sm-p80-pcDNA3 + pORF-mIL-2).

The splenocytes from the vaccinated groups proliferated significantly higher than compared to their respective controls when stimulated *in vitro* with Sm-p80 protein (Figs 1-3). However, Sm-p80 driven proliferation of splenocytes was markedly lower when compared to the stimulation induced by ConA (Figs 1-3). The splenocytes from the groups of mice vaccinated with Sm-p80 DNA in the presence of Th-2 enhancer cytokines showed moderate but detectable proliferation (Fig. 2). The splenocytes obtained from mice vaccinated with Sm-p80 DNA with Th-1 enhancer cytokines IL-2 and IL-12 provided the highest proliferation (Fig. 3). Specifically, the splenocytes from the Sm-p80-pcDNA vaccinated group exhibited a 12 % higher proliferation compared to their controls ($P < 0.001$) (Fig. 1). The introduction of GM-CSF and IL-4 in the DNA vaccine formulation increased this proliferation by splenocytes to 17 % ($P < 0.001$) and 16 % ($P < 0.001$), respectively (Fig. 2). As shown in Fig. 3, the highest degree of splenocyte proliferation was observed in the IL-12 group [21 % ($P < 0.001$)] and IL-2 group [23 % ($P < 0.001$)]. The splenocyte proliferation was found to follow the similar pattern [(Sm-p80) $<$ (Sm-p80 + IL-4) $<$ (Sm-p80 + GM-CSF) $<$ (Sm-p80

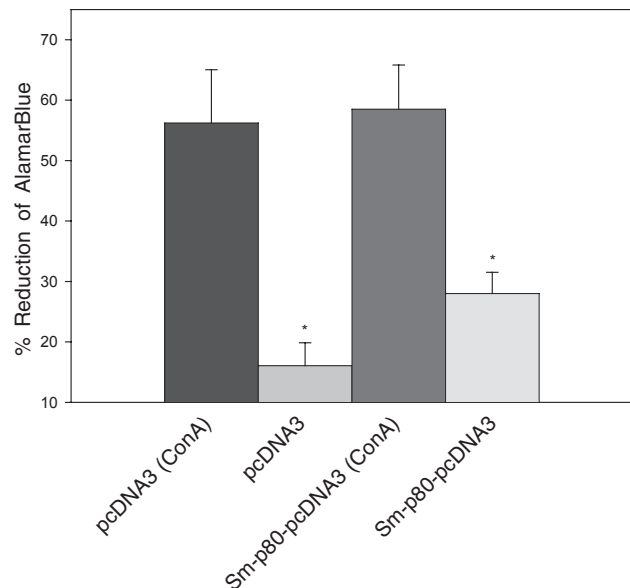


Fig. 1. – Proliferative responses by splenocytes to recombinant Sm-p80. Spleens were obtained from mice immunized with pcDNA3 or Sm-p80-pcDNA3. Results are expressed as % reduction of Alamar-Blue after 48 hrs of cultures. (* $P < 0.001$).

+ IL-12) $<$ (Sm-p80 + IL-2), as has been observed for the protection data (Siddiqui *et al.*, 2003a, b). Briefly, Sm-p80 by itself provided a 39 % protection against challenge infection in C57BL/6 mice (Siddiqui *et al.*, 2003a, b). Co-injection of plasmid DNA encoding IL-4 with Sm-p80 DNA yielded a protection level of 42 % (Siddiqui *et al.*, 2003b). The protection was found to be 44 % when plasmid encoding GM-CSF was co-administered with Sm-p80 DNA (Siddiqui *et al.*, 2003a). Co-injection of plasmid DNA encoding IL-12 with Sm-p80 DNA yielded a protection level of 45 % (Siddiqui *et al.*, 2003b). This protection was increased to 57 % when plasmid DNA encoding IL-2 was co-administered with Sm-p80 DNA (Siddiqui *et al.*, 2003b).

As shown in Table I, the degree of proliferation was also correlated by the IFN- γ production. The IFN- γ production by splenocytes was found to follow the similar trend [(Sm-p80) $<$ (Sm-p80 + IL-4) $<$ (Sm-p80 + GM-CSF) $<$ (Sm-p80 + IL-12) $<$ (Sm-p80 + IL-2)], as has been observed for the proliferation and protection data (Siddiqui *et al.*, 2003a, b). Conversely, the elevated IL-4 production (Table I) did not positively influence either the splenocyte proliferation or the protection.

Both the splenocyte proliferation profiles and the cytokine production data are in agreement with our previous findings in which immunization with Sm-p80 alone resulted in the elevation of IgG_{2A} and IgG_{2B} titers, a clear indication that a predominantly Th-1 type of antibody response was induced by this antigen (Siddiqui *et al.*, 2003b). This inclination towards a Th-1 type response following intramuscular injections of plasmid DNA is now widely accepted as an important

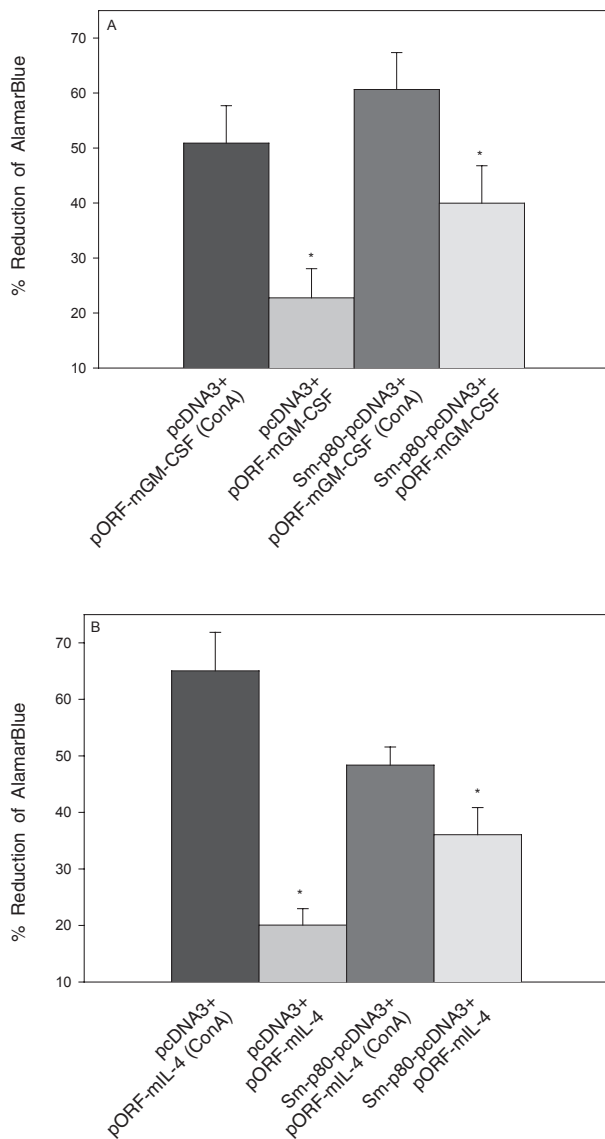


Fig. 2. – *In vitro* proliferative of spleen cells to recombinant Sm-p80 following 48 hr cultures. The spleens were obtained from mice vaccinated with (A) Sm-p80-pcDNA3 plus pORF-mGM-CSF and (B) Sm-p80-pcDNA3 with pORF-mIL-4. (**P* < 0.001).

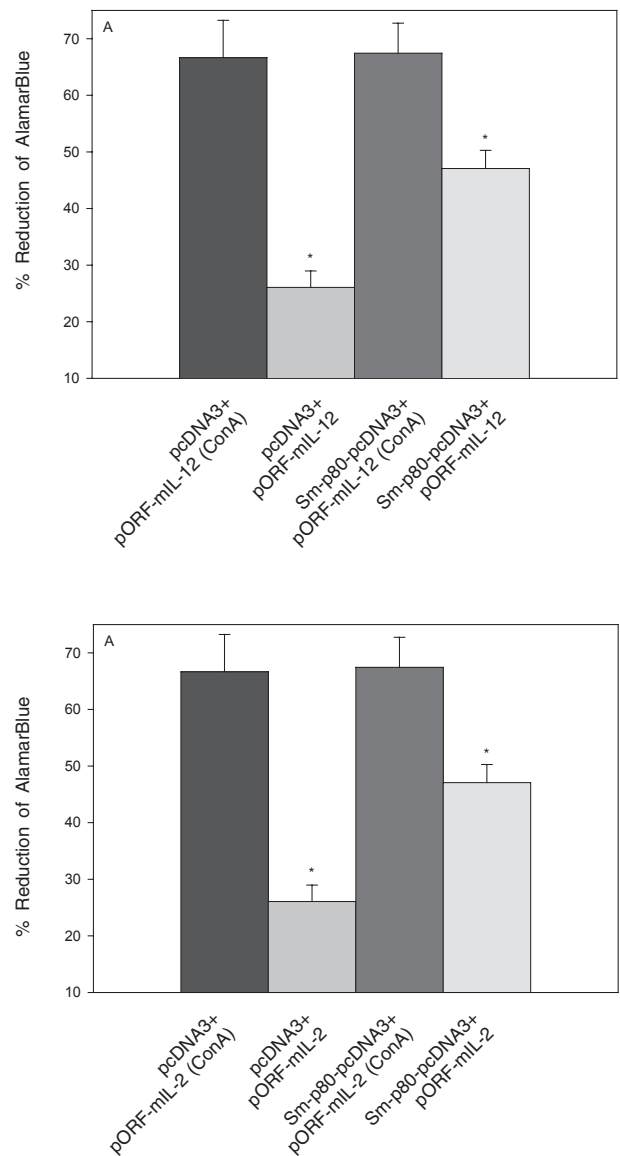


Fig. 3. – Splenocyte proliferation induced by recombinant Sm-p80 after 48 hrs of culturing *in vitro*. The spleens were obtained from mice inoculated with (A) Sm-p80-pcDNA3 plus pORF-mIL-12 and (B) Sm-p80-pcDNA3 with pORF-mIL-2. (**P* < 0.001).

Table I. – *In vitro* secretion of IFN- γ and IL-4 by lymphocytes (48 hrs) from different groups of vaccinated mice

Vaccine groups	IFN- γ (pg/ml \pm SD)	IL-4 (pg/ml \pm SD)
Sm-p80-pcDNA3	415 \pm 27.9	171 \pm 23.2
Sm-p80-pcDNA3 + pORF-mGM-CSF	488 \pm 37.9	134 \pm 12.1
Sm-p80-pcDNA3 + pORF-mIL-4	400 \pm 38.1	245 \pm 13.6
Sm-p80-pcDNA3 + pORF-mIL-12	1,071 \pm 54.3	43 \pm 2.3
Sm-p80-pcDNA3 + pORF-mIL-2	1,415 \pm 122.9	46 \pm 1.5

characteristic of naked DNA vaccinations (Min *et al.*, 2002). Furthermore, co-inoculation of Sm-p80 with GM-CSF resulted in augmentation of total IgG and IgG₁ antibody titers, indicating that Th-2 arm of the immune system was also induced. Interestingly, however, IgG_{2A} titers remained high but IgG_{2B} titers were reduced (Siddiqui *et al.*, 2003a). Elevation of IgG_{2A} titers suggested that either GM-CSF was unable to counter the already biased Th-1 type response induced by intramuscular injections of plasmid DNA containing Th-1 promoting

unmethylated CpG sequence motifs or GM-CSF was capable of inducing both Th-1 and Th-2 types of responses, as discussed previously (Scott & Hunter, 2002). Similarly, simultaneous administration of the DNA vaccine with plasmid DNA encoding GM-CSF has been shown to activate both a Th-1 and a Th-2 response (Wiess *et al.*, 1998; Kusakabe *et al.*, 2000). Intramuscular injections of Sm-p80 with IL-4 resulted in a very little enhancement of IgG₁ titers but IgG₃ titers were found to be quite elevated (Siddiqui *et al.*, 2003a). However, this combination of plasmid DNA was not able to reduce or negatively affect the levels of total IgG, IgG_{2A} and IgG_{2B}, elicited by intramuscular injections of Sm-p80 DNA (Siddiqui *et al.*, 2003b). These findings suggested that the effect of DNA vaccination is dominant over any effect of co-administered IL-4. Similar results were found with another schistosome antigen Sm23 DNA (Da'dara *et al.*, 2000 a, b). Addition of plasmid DNA encoding IL-2 with Sm-p80 resulted in marked enhancement of total IgG and its subtypes IgG_{2A} and IgG_{2B} titers when compared with antibody profiles from Sm-p80 alone group (Siddiqui *et al.*, 2003b). Furthermore, introduction of plasmid DNA encoding IL-12 with Sm-p80 augmented only total IgG and IgG_{2A} titers when compared with Sm-p80 alone group (Siddiqui *et al.*, 2003b). Taken together, data obtained in this study reinforces our previous findings (Siddiqui *et al.*, 2003a, b), on antibody titers and induction Th-1 type response by Sm-p80.

In summary, our results with Sm-p80 and of others with another schistosome antigen Sm23 (Da'Dara *et al.*, 2003), it is becoming evident that to achieve good protection against cercarial challenge, a dominant Th-1 type of response is required, at least in the murine model. The efficacy of both of these antigens is greatly reduced when using protocols that diminish Th-1 type of response (Siddiqui *et al.*, 2003; Da'Dara *et al.*, 2000a, b; 2003). As per the WHO-TDR recommendations (> 50 % consistent protection in mice) Sm-p80 is now ready to be taken into human phase I trials, but we strongly believe that this or any other schistosome antigen should not be rushed pre-maturely as has been the case with other "priority antigens". Therefore, we are presently testing the protective and therapeutic potential of Sm-p80 in non-human primates. Furthermore, studies are also underway to test the vaccine effect of Sm-p80, using different strains of mice to detect a potential "host genetic effect" on the level of protection

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