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Role of antibody dependent cell mediated cytotoxicity (ADCC) in Sm-p80-mediated protection against *Schistosoma mansoni*

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Abstract

Schistosomiasis is a major health problem in the developing world and for international travelers to the endemic countries. Existing strategies to control schistosomiasis have had limited successes so far. The addition of an effective vaccine in existing control measures would be greatly beneficial in reducing the impact of the disease. In this regard, Sm-p80 mediated protection against intestinal schistosomiasis caused by *Schistosoma mansoni* has been observed to be promising in two animal models of infection and disease. In this study, the role of antibody dependent cell mediated cytotoxicity (ADCC) was deciphered in Sm-p80-mediated protection especially in the elimination of lung stage schistosomula. This was achieved using lung lavage cells and lung cells that were isolated from mice immunized with and without Sm-p80 formulated in a recombinant vaccine formulation. Significant differences were observed in cytotoxicity assays using immune sera with the lung lavage cells which showed 51% more killing of schistosomula and elevated levels of nitric oxide in the supernatants were detected compared to controls.

Keywords

Schistosoma; schistosomiasis; vaccine; lung lavage cells; lung cells; antibody dependent cell mediated cytotoxicity; nitric oxide

1. Introduction

Control programs implemented in schistosome-endemic areas to reduce mortality and morbidity associated with schistosome infections have so far produced suboptimal results;

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the disease has not been contained and continues to spread to new geographic areas [1–5]. An effective anti-schistosome vaccine would immensely reduce the morbidity associated with schistosomiasis through induced immune responses leading to decrease in parasite load and reduced egg production [6–11]. With the eventual aim of developing an efficacious anti-schistosome vaccine, we have targeted a functionally important antigen, Sm-p80 which is the only schistosome vaccine candidate that has shown consistent outcomes with regards to its prophylactic and antifecundity therapeutic efficacy in different vaccine formulations and approaches (e.g., naked DNA alone, recombinant protein and prime with DNA-boost with protein) in two experimental animal models (mouse and baboon) of infection and disease [12–25].

The effector mechanisms identified so far in Sm-p80-based vaccine effects from animal studies point to a mixed response involving Th1-skewed correlates and antibodies [12–25]. Additionally, transforming schistosomula are believed to be killed in lungs of resistant/vaccinated hosts via cellular and antibody-dependent effectors [26; 27]. This immune killing is mediated by different effector cells like eosinophils and macrophages [28]. The immune response in lungs is one of the important elements during the transition before the establishment of schistosome infection [26; 29]. To better understand this mechanism in Sm-80-mediated protection, we used lung lavage cells and lung cells from intact lungs obtained from mice vaccinated with rSm-p80 vaccine formulation and studied *in vitro* cytotoxicity effect of the cells in the presence of antibodies using schistosomula as targets [30–33].

2. Materials and methods

2.1. Parasites

For these experiments, *Biomphalaria glabrata* snails infected with *Schistosoma mansoni* (NMRI strain) were obtained from the Schistosomiasis Resource Center, Biomedical Research Institute, Rockville, MD, USA.

2.2. Animals and immunization groups

C57BL/6 mice were obtained from Charles River Laboratory (Wilmington, USA). For the immunization experiment, ten C57BL/6 mice were used, five controls were vaccinated with CpG oligodeoxynucleotides (ODN) plus saline and the other five were vaccinated with Sm-p80 + ODN. Each control animal received 50µg of synthetic control CpG-motif ODN (Coley Pharmaceutical Group, Wellesley, MA); whereas the experimental animals received 25µg rSm-p80 plus 50µg CpG-motif ODN #10104 [12]. Both groups were boosted twice with the same combination, one month apart, as described previously [12]. The animals were sacrificed after the last booster to isolate lung lavage cells and lung cells; the experiments were repeated twice.

2.3. Schistosomula preparation and incubation protocol

Following centrifugation schistosomula were separated from tails of mechanically transformed cercariae [34; 35]. The schistosomula were prepared for culturing in 96 well microplates in complete RPMI 1640 (Sigma-Aldrich), supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin at 37°C in a 5% CO₂ and moist atmosphere in the presence of antibodies and lung lavage cells or lung cells for 24 hrs.

2.4. Source of sera, lung lavage cells and lung cells

For ADCC assay, both sera and the cells were obtained from ODN vaccinated control C57BL/6 mice and Sm-p80 + ODN vaccinated experimental C57BL/6 mice. The blood from each animal was collected before sacrifice for sera isolation. The lung lavage cells and lung

cells were isolated from the animals as previously described by others [36; 37]. Briefly, mice were sacrificed to expose lungs and trachea; the trachea of each animal was cannulated with polyethylene tubing (PE-90; Clay Adams, Parsippany, NJ) and attached to a syringe. Then, the lung of each animal was lavaged by slowly instilling and withdrawing 1 ml of Hanks Balanced Salt Saline (HBSS) for six times. To isolate lung cells, lungs were removed from mice and minced; then incubated in collagenase digestion solution for 1 h at 37°C. The digested lungs were sieved and washed to count total lung cells. The cells were attached to Cocktail antibody labeled MicroBeads (MACS® Miltenyi Biotec, USA) and incubated for 15 min at 4°C to collect CD3⁺ T cell fraction by washing the column in the magnetic field [37]. Finally, cells were quantified and seeded in 96 well plates as: 1×10^5 cells/well (lung cells) and 5.8×10^4 cells/well (lung lavage cells), both in a 100 µl cell suspension.

2.5. Antibody dependent cell mediated cytotoxicity assays (ADCC) to *S. mansoni* schistosomula

Cytotoxicity assay was performed using effector cells: lung lavage cells and lung cells from control (ODN only) and experimental (Sm-p80 + ODN) mice, and targets as mechanically transformed schistosomula [35]. Schistosome larvae were maintained overnight in 10% FCS-RPMI at 37°C in 5% CO₂ atmosphere with pooled sera (50 µl; 1:4 dilution) obtained either from the ODN or Sm-p80 + ODN vaccinated group of mice. IgG end point titer for Sm-p80 + ODN group was 102,400 [12]. Seventy schistosomula were added to each well in triplicates [36]. After 24 hours, the supernatant of each well was saved for nitric oxide (NO) quantification. The percentage of dead larvae was determined by microscopic observation of movement, granularity, and uptake of dye by the parasites.

2.6. Measurement of nitric oxide (NO) production

The larvae were cultured with either the lung lavage cells (5.8×10^4 cells/well) or lung cells (1×10^5 cells/well) in 96 well microplates in the presence of pooled sera from ODN vaccinated or Sm-p80 + ODN vaccinated mice. Using published protocols [38], the accumulation of nitrite in the culture was quantified with Griess nitrite detection system (Promega Corporation, Madison, WI).

2.7. Flow cytometric analysis

In order to detect T cell subset of the lung cells, the T cells were stained with CD3-FITC and CD4-PerCP-CY5.5 antibodies. The gate is depicted around the acquired CD3⁺ T lymphocytes. Subsequently, data was analyzed using histogram and contour plates; the percentage of CD3⁺CD4⁺ T lymphocytes and CD3⁺CD4⁻ T lymphocytes were calculated. Flow cytometry analysis was performed on a Flowjo7.6.2 (Tree Star, Inc. USA).

2.8. Statistical analysis

SigmaPlot version 11.0 (One-way ANOVA followed by Dunnetts procedure) was used to compare differences in the experimental and control groups. For comparison of control and experimental groups involving non-normal distributions, Kruskal-Wallis Test was applied; minimum type I error of $\alpha = 0.05$ was considered for statistically significant differences.

3. Results

3.1. Maintenance of schistosomula

Mechanically transformed schistosomula were maintained in 10% complete RPMI before the cytotoxicity assays. Figure 1 shows the qualitative appearance and major changes of the parasites for different time points at 37°C in 5% CO₂. At least up to 48 hours, no or minimal morphological changes were seen in larvae as ascertained by Trypan Blue intake. After the

initial incubation of the schistosomula in 200µl media, almost all of the parasites were found dead after 72 hours (Figure 1).

3.2. Schistosomula killing in combination of sera and lung lavage cells or lung cells

The percentage killing of mechanically transformed schistosomula have been evaluated in different sets of conditions involving mock (M), sera (S), lung lavage cells or lung cells, and the cells with immune sera or pre bleed sera. The effectors-target ratio was 1.4×10^3 (cells) to 1 (larva), to evaluate the percentage killing after 24 h of incubation at 37°C in moist 5% CO₂ environment. As shown in Figure 2, higher levels of larval killing were observed in sera or the combination of the cells and the sera of Sm-p80 + ODN samples. Attachments of large cells to the schistosomula were found to be from lung lavage cells (Figure 3) and appreciable killing was recorded in the immune sera (IS) and cells or combination of lung lavage cells and the lung cells of the Sm-p80 + ODN group. However, the highest larvicidal effect was observed in the IS and the lung lavage cells.

3.3. Lung lavage cells and lung cells

The cytological classification of the lung lavage cells and lung cells of the saline group, ODN or Sm-p80 + ODN vaccinated C57BL/6 mice were performed under microscope after Giemsa staining. Cells obtained via lung lavages and through homogenization of lungs consisted of lymphocytes, macrophages, neutrophils, eosinophils and basophils. However, the percentages of major lymphocytes isolated from the intact lungs of ODN and Sm-p80 + ODN vaccinated animals are predominantly CD3⁺ CD4⁺ T cells (Table 1). The FACS analysis of total lung cells in ODN immunized C57BL/6 mice (Figure 4A) showed lesser population of CD4⁺ T lymphocytes. However, in the Sm-p80 + ODN immunized C57BL/6 group (Figure 4B), the majority of individual animals had larger populations of CD4⁺ T lymphocytes than the control animals.

3.4. Nitric oxide (NO) production by cultured cells in the presence of immune serum (IS) and schistosomula

The concentration of nitrite was measured in the supernatant of the ODN vaccinated or Sm-p80 + ODN vaccinated groups using the Griess nitrite detection system. The concentration of NO was maximum in the supernatant of the lung lavage cells and the IS from Sm-p80 + ODN samples (Figure 5); that was followed by the concentration of the NO in the lung cells and the IS of the group.

4. Discussion

The protective roles of antibodies have been implicated in different Sm-p80 vaccine approaches [12–25]. We demonstrated the transfer of protection to naïve mice with serum or purified IgG from Sm-p80 vaccinated mice and baboons [39]. This significance of antibodies was further confirmed using antibody knockout (μ Mt^{-/-}; B10.129S2 (B6)-Igh-6^{tm1Cgn/J}) mice [39]. The mechanism of protection that involves the antibodies has not clearly been understood, however, ADCC has been suggested as one of the important means of Sm-p80 mediated protection [39]. Several studies have suggested a role for macrophages and eosinophils in ADCC killing of schistosomula in rats, mice and humans; they have been shown to damage schistosomula *in vitro* [40–47]. Thus, it is logical to explore the involvement of ADCC with regards to Sm-p80 mediated protective immunity.

In the present study, we attempted to demonstrate the significance of ADCC using immune serum and fresh lung lavage cells and lung cells from Sm-p80 + ODN vaccinated C57BL/6 mice. The loss of motility and increased dye uptake showed appreciable killing of schistosomula in immune sera plus lung lavage cells or lung cells. The levels of larval

killing were directly related to amounts of NO production. The significant toxic nitrogen oxide metabolites verify the key mechanism of microbicidal activity of IFN- γ dependent effectors. Our recent studies showed that most of Sm-p80 vaccinated and protected animals produced predominantly IL-12 and IFN- γ [13; 21; 24]. These are the key activators of macrophages and endothelial cells which are known to be involved in the killing of lung stage schistosomula through nitric oxide production [48; 49]. Characteristic lung lavage cells and lung cells were observed being attached to the schistosomula. The adherence of macrophages to schistosomula is a known phenomenon in the development or the expression of microbicidal activities; the killing of the larva appeared to be mediated by activated macrophages from *S. mansoni* infected mice [33]. In the present study, the macrophages from the lung lavage cells were activated by Sm-p80 + ODN immunization; consequently the cells are able to kill the larval schistosomes *in vitro*. This suggests that both the lung lavage cells and the lung cells play important roles in the Sm-p80 vaccine mediated immunity to schistosomiasis. The higher percentage of the CD3⁺CD4⁺ T cells from the lungs of the Sm-p80 + ODN vaccinated C57BL/6 mice may indicate the involvement of the lymphocytes in the microbicidal activities. The T-cell dependent lymphokines mediated macrophage activation in response to *S. mansoni* antigen has already been implicated [33]. T cell may play a role in stimulating cytokine secretion and antibody production. Th1 cell can produce IFN- γ , which is a principal macrophage-activating cytokine. In summary, it appears from the data presented here that ADCC and NO production by adherent cells is an important component of Sm-p80-mediated protection.

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Highlights

- Sm-p80 has been exhaustively tested for its prophylactic and antifecundity efficacy
- Levels of protection obtained are comparable to those recorded using irradiated cercarial vaccine
- The role of ADCC was deciphered especially in the elimination of lung stage larvae

Figure 1. Survival of schistosomula at different time points; 0 h (A), 24 h (B), 48 h (C), and > 72 h (D), in complete RPMI media. The live parasites were not stained with the dye, but the dead larvae are stained darker as shown in (D) and in (C).

Key: *M* = Mock (media)
S = Sera
LL = Lung lavage cells
PS = Pre-bleed sera
LC = Lung cells

Figure 2.

Percentage killing of schistosomula in combination with sera and lung lavage cells or lung cells. The dark bars to the left represent sera or cells of saline group; and the middle grey bars represent sera or cells of the ODN vaccinated C57BL/6 mice, while the darker grey bars to the right represent the sera or cells of Sm-p80+ODN vaccinated C57BL/6 mice. The percentage killing was determined after 24hrs incubation, (* $P < 0.01$). The experiment was repeated twice.

Figure 3. Micrographs of the schistosomula in serum and lung lavage cells of ODN vaccinated C57BL/6 mice (left panel) and Sm-p80 + ODN vaccinated C57BL/6 mice (right panel). Panels 'A' and 'B' show larvae in media; panels 'C' and 'D' show larvae in sera; panels 'E' and 'F' show schistosomula in lung lavage cells; panels 'G' and 'H' show larvae in combination of pre-bleed sera and lung lavage cells; panels 'I' and 'J' show schistosomula in lung lavage cells and sera after the second ODN or Sm-p80 + ODN booster respectively. The arrowheads show characteristic attachment of cells (mostly macrophages) to the surface of the schistosomula.

Figure 4.

Flow cytometric analysis of the total lung cells in ODN immunized (panel A) and Sm-p80 + ODN immunized C57BL/6 mice (panel B). The gates are depicted around the acquired CD3⁺ T cells and then CD4⁺ T lymphocytes (upper panels) of interest. Subsequently, data was analyzed by quadrant separation (lower panels) to analyze single positive (lower right) CD3⁺ T lymphocytes and double positive (upper right) CD3⁺CD4⁺ T lymphocyte populations of each animal.

Key: *M* = Mock (media)
S = Sera
LL = Lung lavage cells
PS = Pre-bleed sera
LC = Lung cells

Figure 5.

Production of nitric oxide (NO) after 24hrs in *in-vitro* culture of schistosomula in media, in cells, in serum or in the combination of both cells and serum of ODN or Sm-p80 + ODN immunized C57BL/6 mice. The dark triangle represents Sm-p80 + ODN samples. The open circles represent ODN samples; but, the darker circles represent the corresponding control saline samples. All the samples were tested in triplicate wells, (* $P < 0.01$).

Table 1

The percentages of major lymphocytes isolated from intact lungs of the Sm-p80 + ODN or ODN vaccinated C57BL/6 mice.

Vaccine group	CD3 ⁺ CD4 ⁺ (%)	CD3 ⁺ CD4 ⁻ (%)
ODN	38.9±0.9	60.8±1.2
Sm-p80+ODN	49.3±4.1 *	50.5±3.7

*
p<0.01