THE EGYPTIAN JOURNAL OF IMMUNOLOGY Vol. 17 (2), 2010
Page: 91-103

Manipulation of the Immune Evasive Properties of Circulating Cathodic Antigen Induces Protective Immunity against Schistosomiasis Mansoni in C57BL/6 Mice

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Schistosome circulating cathodic antigen (CCA) has been hypothesized to take part in immune evasion mechanisms that protect against host's immunity. To dissect its immunogenicity, lymphoproliferative responses of splenocytes were assessed in C57BL/6 mice immunized with recombinant plasmid constructs expressing full-length and truncated fragments of CCA cDNA, before and after challenge infection with *S. mansoni* cercariae. Prior to challenge, splenocytes of immunized mice showed low responses to phytohaemagglutinin (PHA) and high responses to native CCA, compared to controls. After challenge, PHA-responses increased on day 3 through day 7 and subsequently declined. On the other hand, CCA-induced responses increased on day 3 post-challenge then declined in mice immunized with CCA fragments lacking the N-terminus (-N CCA). Whereas, mice immunized with full-length or CCA fragment lacking the C-terminus (-C CCA) showed a delayed increase of CCA-induced responses that maximize on day 25. Interestingly, animals immunized with –N CCA showed a significant reduction in worm burden between 42-51%, while, mice immunized with full-length or –C CCA showed lower protection levels of about 15 and 37%, respectively. These findings suggest that CCA may contain immunosuppressive epitopes on the N-terminus. Abrogation of these epitopes could disrupt the immune evasion mechanism orchestrated by CCA, which could aid the development of an alternative vaccination approach.

The development of an effective vaccine remains the most desirable means of schistosomiasis control due to the emerging resistance to chemotherapeutic drugs and the inability of chemotherapy to protect against reinfection (Kouriba *et al*., 2010). Unfortunately, none of the tested vaccine candidates including muscle proteins, enzymes and antigens of unknown function provided a satisfactory level of protection. This has been attributed to suboptimal formulation of these antigens that resulted in inefficient antigen presentation and inappropriate immune effector responses (Bergquist *et al*., 2002).

The use of DNA vaccines may be useful in this regard. DNA vaccines offer several advantages over conventional vaccines in particular the ability to be modified to express antigens with improved immunogenicity by eliminating undesirable determinants. Otherwise, immunodominant epitopes from antigens can be expressed as minigens, or they can be buried within unrelated, but highly immunogenic core-sequences. This may be especially useful when full-length proteins are not suitable as vaccine candidates, because they are toxic to the host or contain immunosuppressive domains (Leitner *et al*., 1999; Restifo *et al*., 2000). CCA is an example of such antigens. It is a major circulating antigen secreted by cells lining the intestine of schistosomes (Nash *et al*., 1974; Deelder *et al*., 1980) and potentially involved in protecting the parasite's gut and priming hepatic egg antigen-induced granuloma formation (Jacobs *et al*., 1999). It appears as an immunogen in the urine (Deelder *et al*., 1980) and milk (Santoro *et al*., 1977) of patients, a property that may be useful for diagnosis and assessment of cure

after treatment (De Clercq *et al*., 1997; Standley *et al*., 2010).

CCA is a glycoprotein of molecular weight ranging from 50 to 300 kDa, neutral or slightly positively charged at neutral pH. It is genus specific, but not species specific (Deelder *et al*., 1976, Carlier *et al*., 1980). The carbohydrate fraction of CCA comprises a trisaccharide Galbeta1-4(Fucalpha1-3)GlcNAcbeta (Lewis-x) repeating units as a major fraction and disaccharides to hexasaccharides as a minor fraction with a Galβ(1→3)GalNac-OL core in common (Van Dam *et al*., 1994). The exact number of epitopes on CCA is unknown, but about 10 different, multiple immunogenic epitopes appear to be present (Deelder *et al*., 1996). CCA carbohydrates evoke high titers of IgM antibodies that cross-react with the repeating Lewis-x units on the surface of granulocytes (Spooncer *et al*., 1984). Consequently this may lead, in the presence of complement, to autoimmune killing of these cells (Van Dam *et al*., 1996). This lead to the hypothesis that CCA may contribute to an immune evasion mechanism that protects schistosomes from host responses by inducing an autoimmune reaction.

The current study aims to assess the lymphoproliferative responses, in spleens of mice, following immunization with plasmid DNA expressing full-length and sequentially truncated fragments of CCA. This may help to investigate the immune evasive properties of CCA and screen the molecule for domains that may play a deleterious role during infection. This information may guide the design of an alternative vaccination strategy through the disruption of the possible CCA-mediated immune evasion mechanism.

Materials and Methods

Animals and Parasitic Materials

All host and parasite materials were supplied from Biologicals Production Unit (BPU), Theodore Bilharz Research Institute (TBRI, Cairo, Egypt) including four to five weeks old female C57BL/6 (C57) mice, cercariae and adult worms of *S. mansoni*. All animal experiments were performed according to the guidelines of the National Research Council (NRC, 1997) for laboratory animal use.

Analysis of the Potentially Functional Sites of CCA

Several methods were used for protein sequence analysis based on nucleotide sequence data of CCA cDNA clone (Abdeen *et al*., 1999). These methods included; the EMBOSS package (Kolaskar and Tongaonkar, 1990) for antigenicity prediction, ProtComp 6.0 (Softberry, USA) for prediction of the putative cytotoxic T-lymphocyte (CTL) epitopes, the SCRATCH Protein Predictor (Cheng *et al*., 2005) for prediction of the probability of antigenicity and ProtScale (Gasteiger *et al*., 2005) for prediction of a wide range of properties including the optimized matching hydrophobicity (Sweet *et al*., 1983), average flexibility, % accessible residues and trans-membrane tendency.

Plasmid DNA Construction

CCA cDNA was directionally cloned between the *Bam*HI and *Xba*I sites of the eukaryotic expression plasmid pcDNAI/Amp (Full). Plasmids expressing truncated CCA fragments were constructed using combinations of restriction enzymes as follows: *Eco*RI/*Xba*I (Eco), *Xma*I/*Xba*I (Xma), *Bgl*II/*Xba*I (Bgl) and *Bam*HI/*Bgl*II (Bam). Eco, Xma and Bgl fragments were collectively referred to as -N CCA fragments as they lack the N-terminus of CCA and Bam fragment was alternatively referred to as -C CCA fragment as it lacks the C-terminus (Fig. 1). Details of plasmid construction and molecular biology work will be published elsewhere.

Immunization Protocol

Plasmid constructs were used for immunization of mice according to the protocol of Montgomery *et al*. (1993). In two independently repeated protection experiments, animals were divided into seven groups of twenty mice each. Animals were alternatively injected in the right and left quadriceps muscles with three doses of saline solution containing 50 µg plasmid DNA at one month intervals. Control groups included naive group; injected with saline and wild group; injected with wild-type plasmid. One month after the last immunization, animals of all groups were infected with 150 *S. mansoni* cercariae. Three animals from each group were sacrificed one day prior to challenge and at days 3, 7, 25 and 42 after challenge and their splenocytes collected for the lymphoproliferation assay.

At day 42, adult worms were recovered from the hepatic portal system and liver according to the method of Smithers and Terry (1965). The number of worms recovered from each group was compared with the wild control group, and protection expressed as a percentage according to the formula: % protection = (C-I)/C × 100

Where, C is the mean number of worms in the control group and I is the mean number of worms in the immunized group.

Antigen Preparation

Native CCA was used for *in vitro* stimulation of splenocytes. It was isolated from tricholoroacetic acid soluble fraction of *S. mansoni* soluble adult worm antigens (SWAP-TCA) by immunoaffinity over anti-CCA monoclonal antibody (B_3C_9) according to the method of Nash and Deelder (1985). Briefly, twenty mg of B_3C_9 was conjugated to 2 ml of packed Sepharose-4B (Pharmacia Fine Chemicals, Upsala, Sweden) as described by manufacture's manual. Following the incubation of 10 mg of SWAP-TCA with the prepared affinity column, the unbound fraction was recovered by washing with 0.01 M phosphate buffered saline, pH 7.4. The bound fraction containing native CCA was eluted with 0.05 M glycine-HCl, pH 2.5 and immediately neutralized with 1 M Tris, pH 8. The bound fraction was dialyzed against deionized distilled water at 4°C and freeze-dried.

Spleen Cell Preparation

Splenocytes were collected from each mouse and cultured as previously described (Chikunguwo *et al*., 1991) in complete RPMI 1640 (Gibco BRL, MO) medium supplemented with 2 mM L-glutamine, 4.2 mM $NaHCO_3$, 100 Units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 0.2 mM non-essential amino acids, 20 mM HEPES and 10% heat-inactivated fetal calf serum. T cell enrichment was performed with packed nylon wool (Cellular Products, Buffalo, NY). Erythrocytes were lysed with Tris ammonium chloride buffer, pH 7.2, for 15 minutes on ice. Cells that excluded trypan blue were resuspended in 2×10^6 cells per ml. The efficiency of T cell enrichment was checked by staining cells with either fluorescein isothiocyanate (FITC)-conjugated rat $IgG_{2\kappa}$ (as a negative isotype control) or FITC-CD90/Thy-1. Fluorescence was analyzed on FACSort (FACscalibur; Becton Dickinson San Jose, CA, USA). Cell acquisition and analysis of data were carried out using PC-Lysis [TM] software version 1.0 [9/92] (Becton Dickinson).

Lymphoproliferation Assay

Lymphocyte proliferation assay was performed according to the method of Mosmann (1983) using CellTiter 96TM non-radioactive cell proliferation assay kit (Promega). 100 µl of the suspended cells was incubated in the presence or absence of 5 µg/ml of PHA or CCA diluted in culture medium. Cells were incubated for three days at 37°C, 5% CO_2 in water-jacketed incubator (Jouan IG 150). Both spleen cell number and stimulus concentration were optimized from two separate titration experiments. After three days of culture, 15 µl of the dye solution were added to each well and the plates were incubated for 4 hours at 37°C, 5% CO_2. 100 µl of the solubilization/stop solution was added to each well and plates were allowed to stand overnight at room temperature to completely solubilize the formazan crystals. Absorbance values were recorded at 570 nm using ELISA reader (GDV, Roma, Italy). A reference wavelength of 630 nm was used to reduce the background contributed by cell debris and other nonspecific absorbance. The specificity of the proliferative response was controlled by the lack of response in the absence of an *in vitro* stimulus or medium alone. Accordingly, the background proliferation of unstimulated cells was subtracted from their respective stimulated cell proliferation. All assays were performed in triplicates and experiments were independently repeated twice.

Statistical Analysis

All statistical analyses were performed using SPSS 13 statistical software. A significant level of 0.05 at 95% confidence interval level was chosen. One-way ANOVA test was used to determine the statistical significance of differences between experimental and control groups. In addition, multiple regression analysis was used to determine the relationship between T cell proliferation and protection. The significance was defined as $P < 0.05$.

Results

Putative Antigenicity and Secondary Structure of CCA

Analysis of the amino acid sequence of CCA revealed 18 potentially antigenic regions, 11 putative CTL epitopes and predicted probability of antigenicity of 0.92. Changes of this configuration following restriction cutting

of CCA cDNA clone are summarized in figure (1).

In addition, CCA showed the highest hydrophobicity at the tip of the N-terminus. This short sequence has also the least average flexibility, little % accessible residues and high trans-membrane tendency. Taking this together, it could be concluded that CCA protein is probably membrane-anchored by its amino terminus. The removal of the N-terminus selectively improved the flexibility and epitope accessibility of the expressed protein. Contrarily, the removal of the C-terminus did not significantly alter the hydrophobicity, flexibility and accessibility of the full-length CCA (Fig. 2).

Lymphoproliferative Consequences of DNA Immunization

T cell enrichment increased T cell proportion from 22.1% to 71% (Fig. 3). Hence, several pilot experiments were performed on splenocytes of naive mice in order to optimize both cell number and stimulus concentration. It was found that the number of 2×10^5 cells per well and stimulus concentration of 5 µg/ml were optimal (Fig. 4).

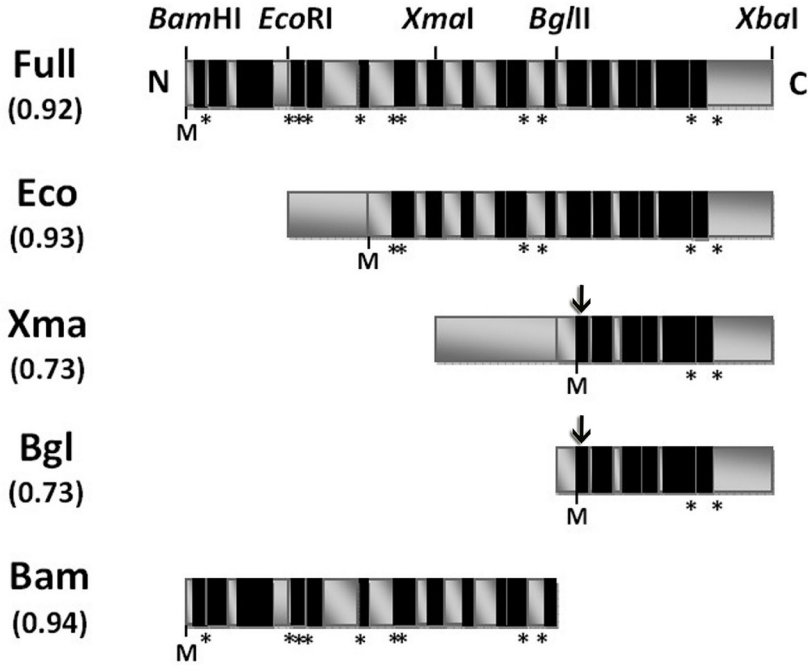

Figure 1. Schematic representation of the restriction fragments of CCA cDNA and their corresponding full-length and truncated CCA polypeptides. Antigenicity prediction criteria estimated of the antigenic regions (dark bands), putative CTL epitopes (labeled with asterikes) and probability of antigenicity (between brackets).
Note: restriction cutting of Xma and Bgl fragments may alter the first antigenic region (indicated by arrow). N, N-terminus; C, C-terminus; M, methionine.

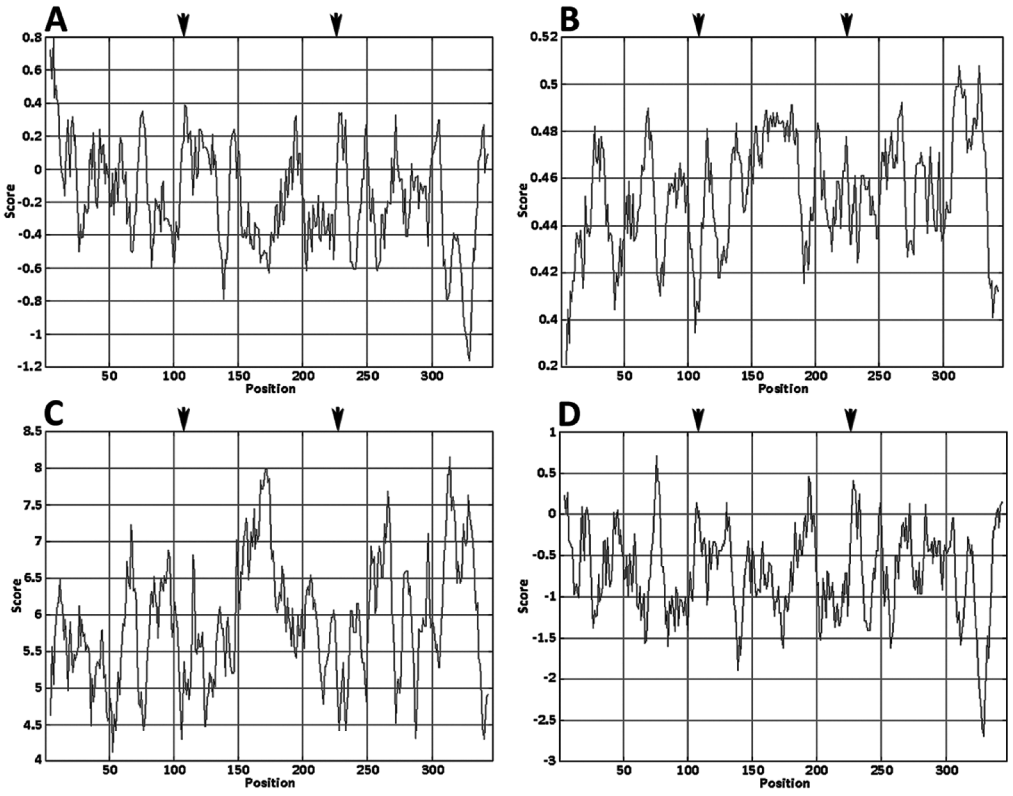

Figure 2. ProtScale sequence analysis of full-length and truncated CCA polypeptides (discriminated by arrows). Secondary structure prediction items include: the hydrophobicity (A), average flexibility (B), % accessible residues (C) and trans-membrane tendency (D).
Note: Full-length CCA is 347 residues long. CCA fragments included; Eco fragment devoid of 107 N-terminal residues, Xma and Bgl fragments, with the same open reading frame, devoid of 230 N-terminal residues and Bam fragment devoid of 117 C-terminal residues.

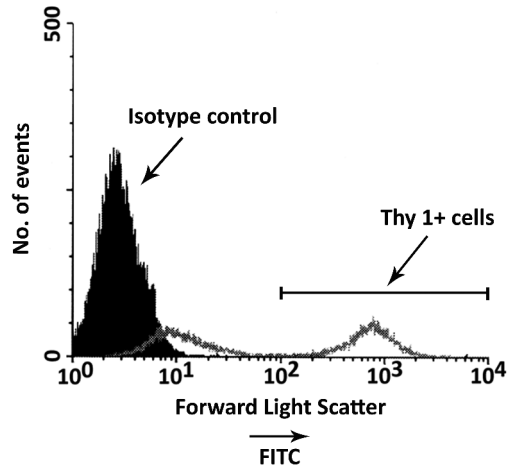

Figure 3. One-parameter histogram identifying Thy-1 expressing cells. The cells at the right grey peak are representing T cell population after nylon wool enrichment, compared to isotype control-stained cells in the left black peak.

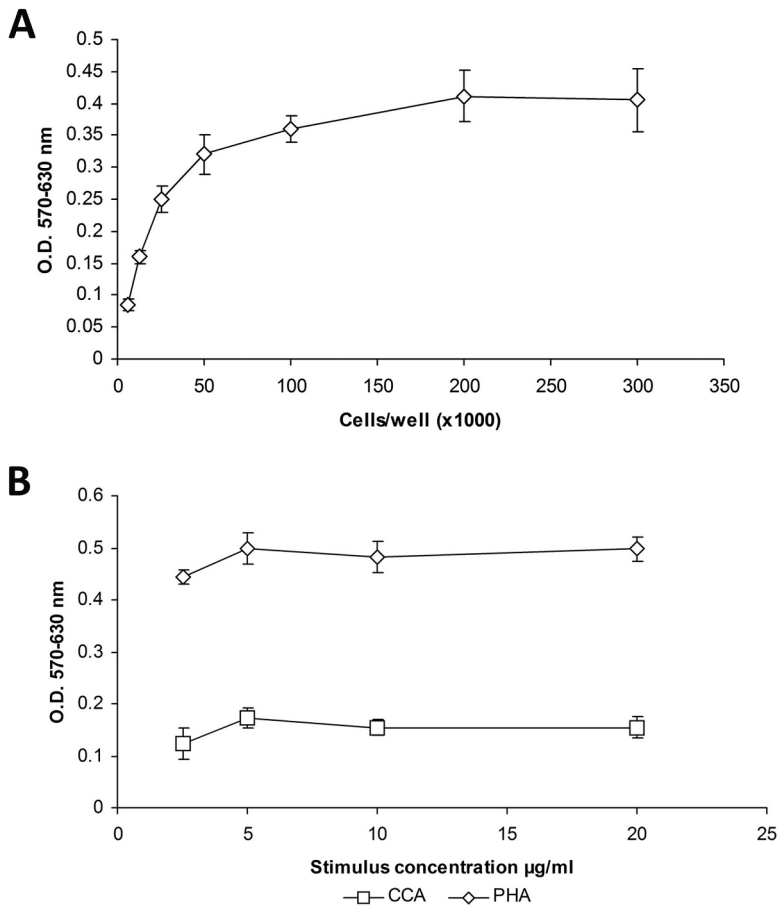

Figure 4. Optimization of spleen cell number (A) and stimulus concentration (B). The cells were proliferated optimally at a concentration of 5 µg/ml of stimulus and a number of 2×10^5 per well.

Splenocytes were obtained from all test groups one day prior to challenge and after challenge at days 3, 7, 25 and 42. Splenocytes showed declined PHA-driven proliferative responses before challenge especially in the Full and Bam groups, compared to cells of naive and wild controls. After challenge, PHA-driven responses increased differentially on day 3 through day 7 with the Eco and Xma groups showing the most pronounced responses. At day 25, PHA-responses diminished in all groups and sustained at day 42. The mitogenic responses of splenocytes from the Full and Bam mice were generally below the levels of the other groups before and after challenge (Fig. 5).

On the other hand, splenocytes from all test groups showed higher CCA-driven proliferative responses before challenge, in comparison to cells of naive and wild controls. After challenge, CCA-responses increased again on day 3 post-challenge then diminished in mice immunized with Eco, Xma and Bgl fragments (–N CCA groups). Whereas, mice immunized with full-length and Bam (-C CCA) showed a delayed increase of CCA-induced responses that maximize on day 25 (Fig. 6).

Resistance to Challenge Infection

Compared to the wild control, Eco, Xma and Bgl groups showed a significant ($P < 0.05$) reduction of worm counts that reached 41.5, 51.4 and 45.2%, respectively. However, Full and Bam groups showed significant but lower reduction of worm counts of 15.3% and 37%, respectively. Among all groups and at the different time points before and after challenge, multiple regression analysis revealed a significant ($P < 0.05$) direct relationship between T cell proliferation and resistance only at days 3 and 7 post-challenge with regression coefficient (β) equals 0.87 and 0.92, respectively (Table 1).

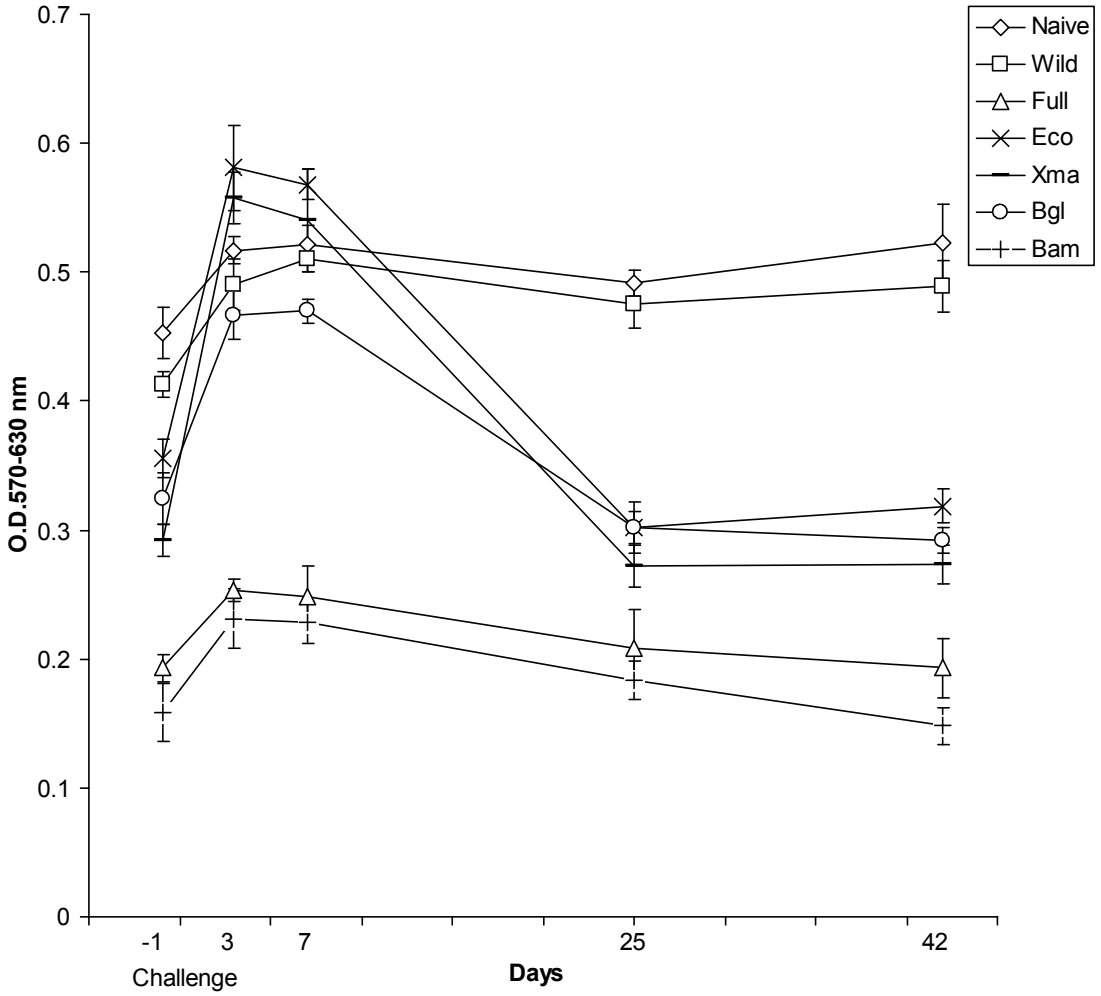

Figure 5. PHA–stimulated lymphoproliferative responses of splenocytes before and after challenge infection with *S. mansoni* cercariae.

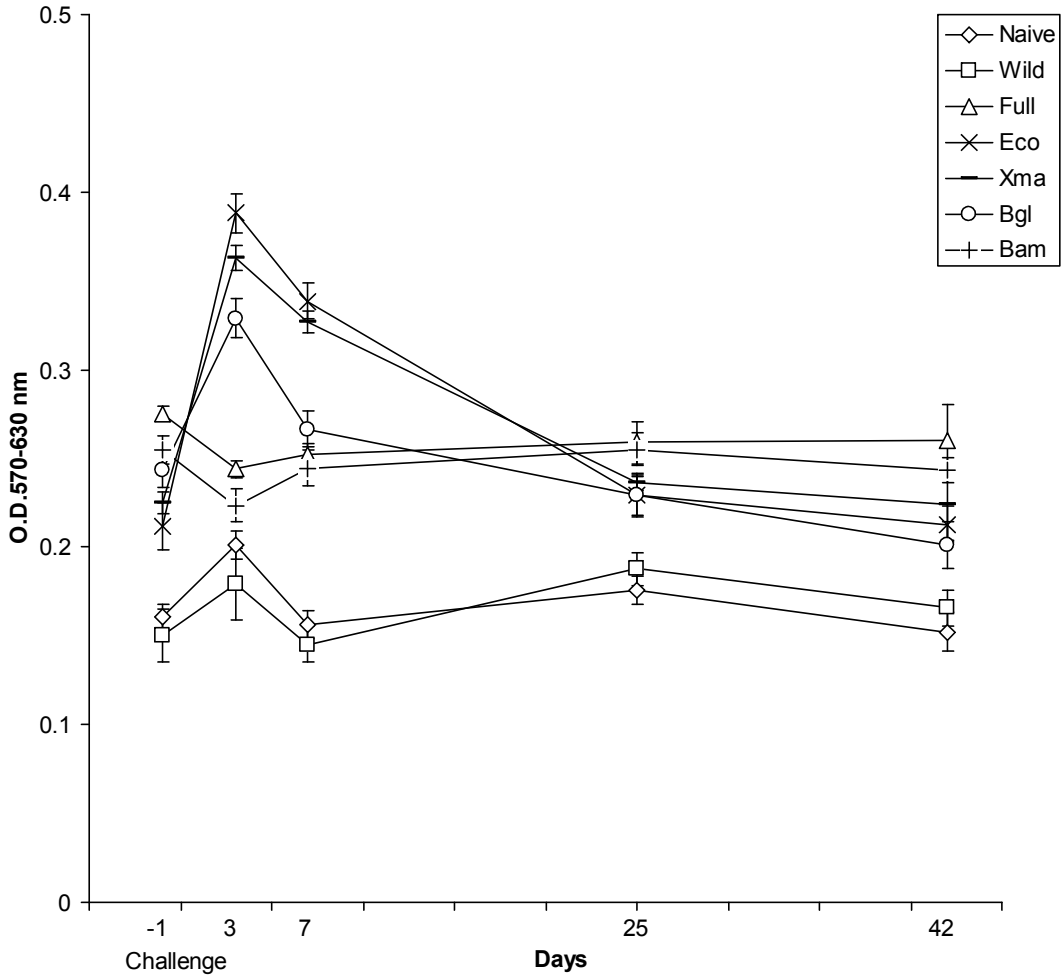

Figure 6. Native CCA–stimulated lymphoproliferative responses of splenocytes before and after challenge infection with *S. mansoni* cercariae.

Table 1. The Relationship between T cell proliferation and protection.

Mice Group	% Protection	Relationship between T cell proliferation and protection (regression coefficient, β)				
		Prior to challenge	Post challenge			
			Day 3	Day 7	Day 25	Day 42
Naive	4.2					
Wild	-					
Full	15.3*					
Eco	41.5*	0.6	0.87*	0.92*	0.62	0.51
Xma	51.4*					
Bgl	45.2*					
Bam	37*					

* Significant or $P < 0.05$.

Discussion

Most of the biochemical studies of CCA have concentrated on its carbohydrate component, since it constitutes the greater fraction of the molecule and probably contributes significantly to its immunogenicity (Bergwerff *et al*., 1994, Van Dam *et al*., 1994). The cloning of a cDNA clone encoding the polypeptide backbone and the secreted form of CCA in the urine of infected patients has allowed its characterization in the absence of the host's cross-reactive carbohydrate component (Abdeen *et al*., 1998, 1999).

The present study further investigated, at the T cell level, the immunogenicity of CCA polypeptide fragments expressed in the myocytes of mice utilizing a DNA immunization protocol. Produced in eukaryotic cells, the *in vivo* expressed CCA is likely to resemble the native molecule more than the recombinant protein, as it will contain the post-translational modifications, such as glycosylation, phosphorylation and folding to gain conformationally relevant epitopes, that would possibly induce both humoral and cellular immune responses (Manoj *et al*., 2004, Smooker *et al*., 2004).

In order to dissect CCA immunogenicity, plasmid constructs expressing full-length and sequential restriction fragments of CCA were used for immunization of mice. Lymphoproliferative responses of splenocytes were monitored after immunization (one day prior to challenge) and at different time points after challenge corresponding to the schistosomula, lung, liver and egg-depositing stages of the life cycle of the parasite. It was apparent that immunization with full-length CCA and its fragments has triggered a differential dynamics of cellular responses.

Prior to challenge, immunized mice expressed diminished PHA-induced lymphoproliferative responses compared to controls. A low PHA-stimulated response was also found in infected C57 mice shortly after the commencement of egg lying (Lewis and Wilson, 1981), as well as in the spleens of C57 and CBA mice vaccinated with irradiated cercariae (Lewis and Wilson, 1982), a consequence of the spontaneous elevation of DNA synthesis of the cells. However, after challenge infection, a regain of the PHA-stimulated proliferative responses was noted early on day three. This was found to agree with the findings of Abdeen *et al*. (2003) that showed an early rebound of the lymphoproliferative response after challenge in C57 mice immunized with anti-idiotypic antibodies mimicking adult worm antigens. The post-challenge regain of the mitogenic-induced responses is part of the overall elevated cellular responses mediated by low molecular weight antigens secreted by schistosomula during the first days of infection (Mountford and Harrop, 1998; Gobert *et al*., 2007). The genes encoding such antigens have been characterized after the identification of the parasite's genome (Berriman *et al*., 2009). Moreover, cDNA microarrays of the newly transformed parasites, during the interval from 3 hours to 5 days, confirmed the upregulation of genes involved in blood feeding and cell adhesion (Gobert *et al*., 2010).

In the present study, a drop of the PHA-responses was noted in immunized groups during the liver stage and sustained during the egg-depositing stage. This down-regulation could be attributed to the utilization of other clones of inflammatory cells such as monocytes and macrophages to mediate the immune response at these stages (Lamb *et al*., 2010). Depletion of plastic adherent suppressor and CD8+ cells has shown a possible involvement of these cells in the depressed mitogenic response during the liver

and egg-depositing stages of infection in mice (Kamal *et al*., 1991).

At the first days of infection, especially before and during the lung stage, there was an elevated CCA-induced lymphoproliferative response in the Eco, Xma and Bgl (-N CCA) mice groups and contrarily, a diminished response in Full and Bam (-C CCA) mice groups. Whereas during the liver and egg-depositing stages, the proliferation of the Eco, Xma and Bgl groups decreased to follow the level of the Full and Bam mice groups.

Interestingly, -N CCA mice groups followed a somehow similar dynamics of antigen-induced proliferation of challenged mice previously vaccinated with attenuated-cercariae whereas, those of full length and –C CCA followed the dynamics noted in challenged mice previously infected with normal cercariae (Kamal *et al*., 1991). This indicates that CCA restriction may have developed two sets of fragments; one presumed protective and the other mimicking normal re-infection. Indeed, the cellular responses during the initial stage of infection (3-5 weeks) are T helper 1 (T_H1)-like, with a shift towards a T_H2-like response after oviposition at weeks 5-6 (Hoffmann *et al*., 2002; Hewitson *et al*., 2005). Although the current investigation did not include the subsets of T cells, it represents the study of the overall cellular responses that aimed to dissect CCA.

In the present study, Eco, Xma and Bgl groups showed a significant reduction of worm burden of 41.5, 51.4 and 45.2%, respectively. Whereas, Full and Bam groups showed lower but significant reduction of 15.3% and 37%, respectively. Reduction of worm burden may indicate the induction of potentially protective immune responses mediated by DNA immunization with truncated CCA, especially the -N CCA fragments. In addition, correlation of T cell proliferation and protection in all mice groups revealed a significant positive relationship especially in days 3 and 7 post-challenge; β equals 0.87 and 0.92, respectively. This may enforce the view that the early cell-mediated immune response is the cornerstone for parasite survival in mice. Indeed, several studies have confirmed the susceptibility of the schistosomulum and lung stages to the immune response and accordingly the latter stage has been targeted for vaccine development and rational drug design (El Ridi *et al*., 2010; Gobert *et al*., 2010).

The restriction cutting of cDNA encoding CCA produced fragments of the antigen devoid of its functional sites. Of the conspicuous functional sites, the N-terminus that harbor a putative membrane anchorage-domain showing the highest hydrophobicity, the least average flexibility, low accessibility and high trans-membrane tendency. The removal of the hydrophobic N-terminus may have increased the hydrophilicity and flexibility of the expressed antigen. Such improvement of CCA formulation may trigger potentially protective immune responses. In general, most ideal antigenic epitopes are hydrophilic, surface-orientated and flexible. Since, hydrophilic regions tend to reside on the surface of proteins, while hydrophobic regions tend to be located in the interior of the protein. This may enhance the delivery to antigen-presenting cells, ensuing better processing, presentation and induction of an effector immune response. In addition, antibodies can only bind to epitopes found on the surface of proteins and tend to bind with higher affinity when these epitopes are flexible enough to move into accessible positions (Van Regenmortel, 1986). Taking into consideration, the prediction of the putative CTL epitopes that revealed low number of CTL epiopes in the Eco, Xma and Bgl clones; 6, 2, 2 compared to the 11 and 9 of the Full and Bam clones, respectively. The reduction in the number of these epitopes may

have diminished the induction of the suppressor CTLs. However, the removal of the C-terminus may have not significantly changed the characters of the full-length protein and hence engendered decreased protection. This may indicate the existence of the immune suppressive epitopes of CCA at the N-terminus.

In conclusion, these results provide evidence that may help for a better understanding of the antigenicity of CCA. Such understanding may help for the improvement of CCA diagnostic assays and worthily, the development of an alternative approach for vaccine design based on the manipulation of the evasion mechanism mediated by CCA.

Acknowledgements

This work was supported by the Faculty of Science, Mansoura University, Mansoura, Egypt. The author thanks Dr. Hesham Abdeen, Strategic Planning and Technical Support Center, Egyptian Ministry of Scientific Research, for kindly providing plasmid constructs and Prof. Dr. Ahmed El Habiby, Professor of Statistics, Mathematics Department, Faculty of Science, Mansoura University for his help with the statistical analysis.

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