Effect of Ultra Violet Attenuated Cercariae on Antibody Response in Rabbits

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Abstract: Ultraviolet (UV) light is an electromagnetic radiation below the visible wavelengths. Their use in cercariae attenuation needs to be examined. In the present work, Schistosoma mansoni cercariae was exposed to UV radiation. Few studies showed previously an inflicted damage seen on the adult schistosome worms developed from irradiated cercariae. The aim of the study was to find out whether this damage was attributed to direct effect of UV-irradiation on cercariae or due to the host's immunogenicity induced by UV-irradiated cercariae. Single as well as multiple exposures of cercariae to UV Light were examined. They were exposed to UV light, for 1, 2 and 3 hrs and then, were subjected to different treatments: the first one was for the assessment of cercarial viability after one hour of the 3 different treatments. The second one was for rabbit infection and the third one was for cercarial antigen preparation. The cercarial antigens (Ag) were recognized by protective antibodies (IgG1 fractions) which then, were separated and purified from vaccinated rabbit's serum. This cercarial Ag was identified as UVISmC1 gene encoding a protein showing 100% identity at the amino acid level with previously identified S. mansoni clones: theses clones are encoding 51.7 kDa antigens elicited as a result of direct effect of UV radiation on cercariae as well as host's immunogenicity induced by UV irradiated cercariae. This was verified by the recognition of this Ag prepared from E coli clones isolated from cDNA expression library. In conclusion, the data showed a remarkable potency of the UV-radiation-attenuated cercaria in eliciting differential high effectiveness in Ab response under laboratory conditions at 1, 2 and 3 hrs of UV light output at 254 nm. An understanding of the protective immune response elicited by RA cercaria may help in designing a candidate vaccine which is still needed.

Key Words: UV light, Schistosoma mansoni, radiation-attenuated cercariae, purification of IgG and vaccine.

I. Introduction

Schistosomiasis is a parasitic disease caused by the platyhelminth worms Schistosoma mansoni, Schistosoma haematobium and Schistosoma japonicum, affecting 207 million people in the developing world, with 779 million, mostly children, at risk of the infection (Steinmann et al. 2006). It is an endemic disease in Egypt (Mohamed, 1999). Current strategies to reduce the impact of this disease on human health include the development of a defined antigen vaccine; several laboratories have selected an array of candidate vaccine antigens (Dania and Donald, 1993). However, none of the purified or recombinant antigens identified to date confers a level of protection to infection that is as high as that conferred by vaccination with radiation-attenuated cercariae (vaccine model) (Tian et al., 2010). The results of vaccination experiments in a number of animal models (murine, artiodactyl, primates, etc.) in laboratory and field trials have shown that radiation-attenuated vaccines can induce high level, stable protection against challenges with S. mansoni. Great progress has been made in the understanding of the immunological mechanisms against S. mansoni in mouse models (McManus and Loukas, 2008). In such vaccinated mice, worm burdens of challenge infections with Schistosoma mansoni are reduced by more than 90% compared with those in naive mice (Reynolds and Harn, 1992).

UV is electromagnetic radiation that is lying from about 400 nm to about 160 nm. It is usually divided into three divisions, UV-A from 400 nm to 315 nm, UV-B from 315 nm to 280 nm, and UV-C from 280 nm to about 160 nm. Antigenic preparations from Schistosoma mansoni adult worms have been tested for their efficacy in stimulating resistance to subsequent infection of experimental animals. Immunization of mice with adult worm extract resulted in 54% reduction of the parasite load while adult worm-immunized rabbits displayed 88% reduction and developed higher levels (91-100%) of cytotoxic antibodies (Tendler. et al., 1982).

Early reports on the attenuation of UV in the water column of different systems indicated that UV irradiated cercariae are rapidly attenuated. The radiation-attenuated schistosome vaccine induced a high level of protective immunity in rodents, and more than 50% protection to a challenge with normal larvae has been
achieved in primates (Yole et al., 1996). Generally, it has been found that immunization with defined antigens was generally less effective at inducing host protection against experimental infection with *S. mansoni* than vaccination with attenuated infective cercariae (Ganley-Leal et al., 2005). Li et al. (2002) made a preliminary study on *S. japonicum* cercariae antigens before and after ultraviolet irradiation; they were revealed novel antigens on UV irradiated cercariae. In addition, the protection with irradiated cercariae led to decreased pathology or reduced development of granulomas in the liver and increased the level of the inflammatory tumor necrosis factor-α (TNF-α) that could enhance inflammatory reactions due to the activation effects on macrophages, eosinophils and lymphocytes (Joseph et al., 2004 and Torben and Hailu, 2007b).

Earlier studies concluded that UV effect on aquatic organisms is minor. This view has been altered by using improved instrumentation to measure UV radiation in the water column (Kaiser and Herndl, 1997). It now appears that UV-B (315-280) although rapidly attenuating cercariae compared to UV-A (320-400 nm), it does not penetrates to considerable depth. The 10 % radiation level of the 320 nm wavelength range is at < 25 m depth while the 10 % level of the 340 and 380 nm wavelengths is at 35 and 60 m depth, respectively (Torben and Hailu, 2007a). In this context, action spectra of inhibition by UV can be constructed, but they should only be used to describe inhibition for specified time scales. Wales and Kusel (1993) concluded that vertical profiles of relative inhibition must be interpreted cautiously because inhibition by UV-radiation is likely to be a function of incubation time and the results must therefore be interpreted in the context of vertical mixing.

In schistosomiasis research, a post-irradiation expression library has been constructed that induce the UV affected transcriptions. This library was immunoscreened with protective sera from UV irradiated rabbits to isolate genes encoding immunogenic antigens which may acquire its immunogenicity after UV irradiation due to unknown mechanism (Ganley et al. 2005). At the nucleic acid level, the DNA sequence homology search revealed a striking degree of identity with *S. mansoni* antigen, encoding 51.7 kDa antigen, in the open reading frame (ORF) sequence (Francis and Bickle, 1992, Ahmed et al., 2001). UV light was reported to induce mutations (Cook et al., 2011). DNA was identified as the principle biological target for UV irradiation (Imlay and Linn, 1988). Also, (Wales et al., 1993) reported that UV irradiation caused an immediate and striking alteration in the carbohydrate antigens expressed by schistosome larvae and postulated that the structure of glycocalyx antigens released by irradiated larvae is modified in a way that alters the pattern of processing by the proteolytic enzymes of the antigen presenting cells resulting in presentation of new antigenic determinants to T helper cells, stimulating their potent protective immunity. Interleukin-2 (IL-2), a T-cell-derived lymphokine, plays an essential role in the cellular immune response by regulating the proliferation of antigen-activated T cells and in subtle regulation of complex immunological phenomenon [Cantrell, 1984 and Smith, 1980].

The aim of the present study was to find out whether infective cercaria attenuation was attributed to direct effect of UV radiation or due to the host’s immunogenicity induced by UV- irradiated cercariae. Single as well as multiple exposures of cercariae to UV Light were examined. In addition, using rabbit could be a better experimental model to study the relevant protective immune events induced by radiation-attenuated cercariae vaccine. Therefore, antigens recognized in this model may represent potent immunogens for developing future vaccine.

II. Material And Methods

Parasites and animals

An Egyptian strain of *S. mansoni* cercariae was provided from Theodore Billharz Research Institute (TBRI). Thirty male rabbits weighting 2 kg and 3 months old were provided from Egyptian Holding Company for Biological Products and Vaccines (Vacsera), Giza, Egypt and domesticated under conditions of fixed temperature 27 °C and 12 hours of light per day at the animal facility of VACSERA. They were divided into 3 groups and exposed to 1, 2 and 3 hrs of UV light exposure respectively. Rabbits were fed on pelted chow composed of 11.2% moisture, 25.4% protein; Rabbits were used in the prese...
surface, as measured by a J-255 short wave UV meter (Ultraviolet, Products, Inc.), was 110 microwatts/cm². Cercarial suspensions were exposed to UV-radiation for one and two hrs and used in different ways. The first one was for assessment of cercarial viability assessment, the second one was for rabbit infection and the third one was for cercarial antigen preparation.

**Preparation of Sera**

Two groups of rabbits were immunized by exposure to normal and UV-irradiated *S. mansoni* cercariae. In Brief, *S. mansoni* cercariae were irradiated and used within 20 min for vaccination in the range of 8,000 cercariae/dose/animal (Dean et al., 1983 and Shi et al., 1993). The animals were injected with thiopental, then *S. mansoni* irradiated cercariae were applied to the lateral shaved abdominal skin. Blood was shed from rabbits at days 0 and 25. Then, at days 51, the animals were killed, blood was collected in clean sterile tubes, and the serum was separated for further investigation. Sera were prepared according to Jwo and LoVerde, (1989).

**Isolation and purification of IgG1 fractions from whole serum**

The IgG proteins of normal and vaccinated rabbit sera (NRS and VRS) were purified by protein G agarose affinity chromatography. Readymade protein G agarose minicolumn (GIBCO BRL) was used in this study. The affinity chromatography was done as recommended by the manufacture manual for the purification of IgG 1 fraction (Mohamed, 1999). A partial purification step has been carried out by precipitation of immunoglobulins using supersaturated ammonium sulpha solution. The concentration of the IgG was determined using Lowery et al. (1951) method. The purity of IgG 1 fractions was measured using SDS-PAGE. They were stained with Coomassie blue brilliant stain. Western blot was used to detect cercarial antigcencity. The sera were kept at 4°C until used (Harlow and Lane, 1988).

**Preparation of recombinant protein lysate from host E. Coli**

Protease inhibitor was added to *E. Coli* and brought to volume equals to 45 ml with PBS or homogenization buffer. Once in aqueous solution, PMSF has a half-life of about 30 min and was freshly prepared each time. 250 µl of 100 mM EDTA (0.5 mM final conc) and 250 µl of 200 mM PMSF (1.0 mM final conc) (GIBCO BRL) were also added. Homogenization of the buffer were carried out several times on ice, and then centrifuged at 30,000g at 4°C. The supernatant was separated and used as E.Coli lysate.

**Preparation of S. mansoni cercarial antigen**

UV irradiated *S. mansoni* cercariae (5 x 10⁵ cercariae) were homogenized with 800µl tris-buffered saline (TBS) in glass-teflon homogenizer, and placed on ice. Then, they was transferred into a micro centrifuge tube and centrifuged at 10,000g for 20 minutes at 4°C. Supernatant was transferred to a new tube and protein concentration was estimated by using Lowery et al. (1951) method.

**Immunoblotting staining**

To examine the antigenicity of cercarial protein, it was loaded on mini-gel electrophoresis, then transferred to nitrocellulose membrane by electro-transfer and detected by western blotting using positive sera and compared with negative ones Immunoblotting was carried out on nitrocellulose membrane (Millipore-USA). UV-irradiated cercarial proteins were separated by electrophoresis throughout 10% polyacrylamide gel under reducing conditions according to Laemmli et al. (1970). Then, the cercarial proteins were transferred onto nitrocellulose membrane (0.45pm pore size) in a Mini Bio-Rad protein transfer unit, using transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) for 2 hr at 100 volts. The nitrocellulose membranes were blocked in 10 X TBST (tris-buffered saline Tween-20) containing 3% BSA, for 1 hr. The membranes were rinsed in TBST, and incubated with antiseraum (VRS and NRS) for 1 hr with gentle shaking. Then the blots were washed 4 times in TBST and incubated with alkaline phosphatase conjugated goat anti-human for 1 hr. After incubation, the blots were washed in TBST and soaked in Nitro-blue tetrazolium (NBT) and 5-bromo 4-chloro3-indolyil phosphate (BCIP). Once the reaction occurs (appearance of brown spots on the nitrocellulose membrane), (NBT) was discarded and the reaction was stopped by adding distilled water. Water was discarded and the membrane was allowed to dry.

**Level of serum IL-2**

The level of (IL-2), in the supernatant was measured by using the ELISA kits (Cytoscreen USTM from Bio Source, Camarillo, California, U.S.A.). Absorbance was measured with micro plates. Serum samples were analysed for OD representative to total antibody level by standard enzyme linked immunosorbant assay (ELIZA).

In brief, microtiter plates (Nunc-USA) were coated with each sabin polio virus serotypes individually as 1:100 in 1M carbonate-bicarbonate buffer pH 9.6. Plates were incubated overnight at room temperature. Well
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The contents were discarded and blocked with 4% BSA for 1 hr at room temperature (200μl/well). Wells then aspirated and dried. Serially diluted sera samples (1:50) were added to 6 wells/sample for 2 hr at room temperature (100μl/well), including positive, negative controls and blanks. Plates contents were washed 3 times with PBST. 100μl/well of anti-mouse conjugate labeled with peroxidase 1:1000 (Sigma immunochemicals) was added for 2 hr at room temperature and washed as previous step 3 times with PBST. 100μl/well of solution A (TMB peroxidase substrate) & 100μl/well of solution B (peroxidase solution B) H₂O₂ (Sigma – USA – Aldrich) was added and incubated at room temperature for 10 minutes in the dark. The reaction was stopped using 1M HCL (EGY.VAC). Optical densities were read at 492 nm using ELISA plate reader (Dynatech MRX).

Statistical analysis

Statistical analysis was performed using the analysis of variance (ANOVA) among 3 independent groups. Duncan’s multiple range tests was done to determine differences between groups (irradiated and non irradiated). Means at significance level of 0.05 were calculated. Means and standard errors of treatment were also estimated. All statistics were carried out using statistical analysis systems (SAS) program version 6.12 (SAS, 2000).

III. Results

Isolation of IgG fractions under natural conditions from rabbits sera

The IgG fractions of normal rabbits sera (NRS) and vaccinated rabbits serum (VRS) partially purified gave good results with sharp bands as shown in (Fig. 1).

![SDS-gel electrophoresis assay](image)

**Fig. (1):** SDS-gel electrophoresis assay:

(1): Protein marker.
(2): Unbound fraction of NRS.
(3): Unbound fraction of VRS.
(4): Bound fraction of VRS.
(5): Bound fraction of NRS.
(6): Normal rabbit sera (polyvalent).
(7): Vaccinated rabbit sera.

Immunoblotting assay:

The VRS and NRS IgG primary antibody were tested before and after treatment using different concentrations of E. coli phage lysate (25ng and 100ng) with one dilution of primary antibodies VRS-IgG and NRS-IgG (1:1000). The results indicated high positive reaction (as indicated from cross reaction) as was shown in lanes 2 and 3. To assess the antibody dilution for immunoscreening and to ensure the efficiency of treatment in removing E. coli antigen, Western blot was carried out after treatment using 3 different dilutions of primary antibody (1: 600; 1: 1200 and 1: 1800) VRS-IgG-1 with 2 different concentration of UV irradiated cercarial antigen 100 μg (lanes 2,4 and 6) and 25 μg (lanes 3, 5 and 7) compared with NRS-IgG 1(lanes 8 and 9) with the same dilution and 1: 600 concentration, at time one hr (Fig. 3). Western blot was also carried out with the same conditions at time 2 and 3hrs with slight increase in Ab response at 2hrs but no further changes at time 3 hrs (Fig not shown).

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Fig. (2): Immunoblotting assay: (treated and untreated VRS and NRS-IgG against E. coli XL-1-Blue phage lysate).

(1): standard protein marker
(2): Treated VRS with 150 ng of E. coli lysate
(3): Treated VRS with 25 ng of E. coli lysate
(4): Treated NRS with 150 ng of E. coli lysate
(5): Treated NRS with 25 ng of E. coli lysate
(6): Untreated VRS with 150 ng of E. coli lysate
(7): Untreated NRS with 150 ng of E. coli lysate
(8): Untreated VRS with 25 ng of E. coli lysate
(9): Untreated NRS with 25 ng of E. coli lysate

Fig. (3): Immunoblotting assay: (UV irradiated cercarial antigens recognized by VRS and NRS-IgG 1 at one hr exposure to UV).

(1): standard protein marker
(2): UV Irradiated cercarial protein (100 μg /ml) with treated VRS IgG. (1: 600)
(3): UV Irradiated cercarial protein (25 μg /ml) with treated VRS IgG. (1: 600)
(4): UV Irradiated cercarial protein (100 μg /ml) with treated VRS IgG. (1:1200)
(5): UV Irradiated cercarial protein (25 μg / ml) with treated VRS IgG. (1:1200)
(6): UV Irradiated cercarial protein (100 μg /ml) with treated VRS IgG. (1:1800)
(7): UV Irradiated cercarial protein (25 μg /ml) with treated VRS IgG. (1: 1800)
(8): UV Irradiated cercarial protein (100 μg /gm) with treated NRS IgG. (1: 600)
(9): UV Irradiated cercarial protein (25 μg /ml) with treated NRS IgG. (1: 600)

UV radiation effects of cercarial protein VRS & NRS (IgG):
As shown in Fig. 5, UV radiation significantly inhibited cercariae viability. The UV radiation effect on cercariae viability was found to be dose-dependent. Moreover, the numbers of cercaria inhibition were significantly changed in VRS compared to NRS groups.
**Level of serum IL-2**

Rabbit infected with *S. mansoni* showed a significant time-dependent increase in serum IL-2 levels (Table 1). The greatest increase in IL-2 was noted at 7 weeks post-infection. The results in Table 1 also illustrate that treatment of infected rabbit with UV radiation on cercarial exhibited a significant increase in serum IL-2 at any of the time points analyzed as compared to the levels noted in their untreated infected counterparts (p < 0.05). Also, infected rabbit with cercaria alone or in combination with UV irradiated cercarial protein with treated VRS, or UV irradiated cercarial protein with treated NRS showed a highly significant time-dependent increase in serum IL-2 relative to the control untreated infected rabbit (p < 0.001). Cercarial inhibition with UV caused significant increase in serum IL-2 after 1, 2, 3 hrs when compared to levels in untreated infected hosts. The greatest increase was found at 51 days post-infection. On the other hand, infected rabbit with cercaria alone caused no significant change in the serum IL-2 determined up to 51 days.

**Table 1. Effect of UV radiation on serum IL-2 (pg/ml) in rabbit infected with *S. mansoni***

<table>
<thead>
<tr>
<th>Groups</th>
<th>Time</th>
<th>1 hr</th>
<th>2 hrs</th>
<th>3 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Mean ± S.E.)</td>
<td></td>
<td>(Mean ± S.E.)</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>20.66 ± 0.36</td>
<td>20.66 ± 0.36</td>
<td>20.33 ± 0.35</td>
</tr>
<tr>
<td>UV irradiated cercarial with VRS</td>
<td></td>
<td><strong>40.01 ± 0.12</strong></td>
<td><strong>50.44 ± 0.02</strong></td>
<td><strong>55.33 ± 0.66</strong></td>
</tr>
<tr>
<td>UV irradiated cercarial NRS</td>
<td></td>
<td><strong>30.06 ± 0.30</strong></td>
<td><strong>44.33 ± 0.41</strong></td>
<td><strong>50.66 ± 0.13</strong></td>
</tr>
</tbody>
</table>

**P <0.001: highly significant,**  
**P <0.05: significant**

**IV. Discussion**

Schistosomiasis is the major public health problem in rural Egypt. The main agent of human schistosomiasis is *S. mansoni* (Sabatini et al., 2004). In an attempt of developing vaccine against the parasite infection, researchers have devoted a great deal of time and effort in discovering vaccine candidates, most of which have initially identified in *S. mansoni*, because this species is most easily adapted to laboratory maintenance. Unfortunately, most of these vaccine candidates including DNA vaccines and recombinant vaccines have not been proven to provide reproducibly sufficient immunity in experimental models to warrant consideration for clinical use (McManus and Loukas, 2008). The most effective and reproducible protocol to date is vaccination with radiation-attenuated (RA) cercariae. This vaccine model has been identified in *S. mansoni* and serves as a compelling model for the development of a recombinant vaccine (Coulson, 1997, Wilson and Ivens, 2006). The protective capabilities of the RA vaccine were originally established in laboratory models (Minard et al., 1978; Bickle et al., 1979 and Hsu et al., 1981) and subsequently extended to primates (Soisson et al., 1993; Yole et al., 1996 a, b, and Eberl et al., 2001).

The present study demonstrates the ability of the VRS-IgG to detect radiation-attenuated (RA) cercarial antigen bound to nitrocellulose membrane to determine the appropriate primary antibody dilution as well as to detect the presence of cross reactivity with *E. coli* phage lysate. To assess the antibody dilution for immunoscreening and to ensure the efficiency of treatment in removing *E. coli* antigen, Western blot was
carried out after treatment using 3 different dilutions of primary antibody of VRS-IgG 1 with 2 different concentration of UV irradiated cercarial antigen. Optimal stimulation by 100 ng/ml UV irradiated cercarial protein was with (1:600) treated VRS IgG 1 i.e. it was the most effective. This clearly indicates a high titre of Ab reflecting a boosting immune response. The result of this study is in agreement with Hewitson et al., 2005, Thomas et al., 2006 and Tian et al., 2010. In addition, previous studies on rabbits indicated that a single immunization with radiation-attenuated S. japonicum cercariae was able to induce 63.8% and 71.8% reductions in worm burden and hepatic eggs, respectively (Rajaram et al., 1998).

There is a slight difference in immune response in VRS exposed to 1hr UV radiation when compared to rabbits exposed to 2 hrs UV radiations. 3 hrs exposure did not show a remarkable difference when compared to rabbits exposed to 2hrs. Ritcher and Harn (1993) demonstrated that dose of radiation used to attenuate cercariae had a similar effect on Ab titer but in addition influence Ab specificity. Reynolds and Harn (1992) found that moderate dose of irradiation stimulate higher levels of resistance than do highly irradiated cercariae. On the other hand, difference in time exposure to UV radiation has a differential significant inhibitory effect on cercariae. Thus, in this study, UV radiation showed a direct inhibitory effect on exposed cercariae as well as an immunogenic effect. This effect may be due to delayed parasite migration through the skin, sdLN and lungs. This means that there is greater opportunity for interaction of parasite antigen with immune cells at these sites, which may in turn favour the priming of a protective response (Mountford et al., 1988). An alternative explanation for the immunogenicity of RA parasites is that irradiation alters the antigens present on the surface of the parasite (Mountford and Harrop, 1998). A third explanation for the immunogenicity of RA parasites is that irradiation ablates certain proteins present in the normal parasite that function to down-regulate immune responses (Hewitson et al., 2005).

In addition, in the present study, E. Coli clone was used; this clone is corresponding to gene encoding proteins (antigens) isolated from UV-irradiated S. mansoni expression library and was recognized by protective antibodies (IgG1 fraction) which was separated from vaccinated rabbits serum. Identified cDNA clone, UVISmC1 encoding an antigen that showed 100% identity at the amino acid level with previously identified S. mansoni clones, encoding 51.7 kDa antigens, isolated from S. mansoni sporocyst and 25 days schistosomula cDNA expression libraries (Francis and Bickle, 1992, Ahmed et al., 2001). However, this Ag showed a lower degree of identity with other previous studies identifying S. mansoni and S. japonicum clones, and encoding 22.6 kDa antigens, isolated from S. mansoni and S. japonicum adult worm expression library (Stein and David, 1986, Jeffs et al., 1991, Waine et al., 1994, Yang et al., 1997). Therefore, further studies to examine some genes clone encoding antigens isolated from UV irradiated S. mansoni expression library in order to engineer vaccine candidates are still needed.

The results of this study indicated that infection of mice with S. mansoni caused significant increase of IL-2 level in all time points analyzed with the greatest increase seen at 3 hrs UV exposures. These results are in agreement with Khalil et al. (1995) who revealed that IL-2 level was higher in all patients infected with schistosomiasis and the IL-2 level was higher in lightly infected group than heavily infected ones with no respect to age. On the contrary, Yamashita et al. (1987) observed an 80 to 90% reduction in the production of IL-2 produced by infected mice, which was not accounted for by a reduction in the number of IL-2 producing T-lymphocytes and suppressive macrophages. IL-2 was shown to modulate schistosome development indirectly through their effects on CD4+ T cells; however its role on schistosome development is intriguing (Rebecca et al., 2006). Also, the high level of IL-2 seen in the groups of rabbit exposed to the highest UV radiation may reflect a lower level of resistance to the parasite infection due to low immunogenicity.

In conclusion, the results of the present study showed a remarkable potency in the UV-radiation-attenuated cercariae immune response with differential high effectiveness in Ab response under laboratory conditions at 1, 2 and 3 hrs of UV light output at 254 nm.

Thus, understanding immune modulation in different animals’ model is still needed which ultimately may help to design a successful vaccine? Also, the effects of UV irradiation on the genetic material of the parasite and consequently on the released antigens and the immune response need to be correlated with human protective immune mechanisms studies.

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