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Immunomodulator effect of picroliv and its potential in treatment against resistant Plasmodium yoelii (MDR) infection in mice
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1. Introduction

While anti-parasitic agents used in the treatment of infectious diseases prevent rapid multiplication and hamper vital physiological activities of the parasite, it is basically the immune system of the host that plays major role in complete suppression/elimination of the pathogens from host. In fact parasites weaken immune armory of as a strategy to establish themselves in the host. It can be speculated that activation/rejuvenation of the host immune system could be an effective means to successfully combat various infections.

In spite of global efforts to develop an effective cure, malaria is still considered as one of the most prevalent and devastating disease worldwide, affecting about 300-500 million people and claiming 1.5-2.7 million human lives annually. Further, about one third of the world human population lives in area that is infested with the disease in one or other form (Butler et al, 1997). More alarmingly, the development of multiple drug resistant isolates of Plasmodium spp and the increased resistance of its vector, the Anopheles mosquito, to DDT underscore the importance of developing new chemotherapeutic means to hinder the spread of malaria. In this regard, it is always imperative to enhance anti-parasitic efficacy of already tested or existing antimicrobial compounds. For example chloroquine, the most potent antimalarial agent, has suffered a set back because of its non-effectiveness against drug resistant isolates of malaria parasite (Bjorkman and Phillips-Howard, 1990). One possible strategy to eliminate less susceptible isolates of Plasmodium spp. may rely on concomitant usage of antimalarial chloroquine in combination with some potent immunomodulators.

Picroliv, a standardized fraction isolated from the ethanol extract of root and rhizome of Picrorhiza kurroa (Scrophulariaceae; veneral name kutki) contains iridoid glycosides, and is well known for its protective action against liver damage caused by various hepatotoxins (Rastogi et al, 1996, Dwivedi et al, 1992). The compound was also found to be effective to correct liver damage induced by Plasmodium berghei infection in Mastomys natalensis (Chander et al, 1990, Puri et al, 1992). It was also reported to possess strong immunostimulant activity and showed significant
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In general, picroliv has been shown to provoke monocytes and macrophages for their natural killer activity against microbes (Puri et al 1992). In the present study, we performed elaborated studies to evaluate immunomodulatory effect of picroliv in model animals to establish its practical suitability in treatment of infectious diseases. We have also evaluated potential of combination therapy involving picroliv and chloroquine to combat less susceptible isolates of Plasmodium yoelii in model animals.

2. Materials and Methods

2.1. Chemicals

Chloroquine di phosphate was purchased from the Sigma Chemical Company St Louis Mo, USA. Picroliv was isolated according to the protocol of Dwivedi et al. (Dwivedi et al 1991). *Picrorhiza kurroa* grows abundantly in Himalayan and sub Himalayan regions at the height above the sea level 3300- 3400 m. Roots and rhizomes of the plant were collected during autumn (August-September), dried, powered and finally extracted with alcohol by cold percolation. The extract was evaporated in vacuo below 50° C. The extracts were dissolved in methanol: water (1:1) and further washed with chloroform. Chloroform phase was discarded, while aqueous methanolic phase was further extracted with ethyl acetate and butanol. Both the ethyl acetate soluble as well as butanol soluble fractions were combined and evaporated to dryness in vacuo to get picroliv. As revealed by HPLC and TLC, picroliv contained about 60 % of Kutkoside and Picroside in the ratio of 1:1.5, the remaining 40 % being a mixture of iridoid as well as cucurbitacin glycosides and some still unidentified substances.

2.2. Animals and parasites

In bred female BALB/c mice (8-10 week old), of 18± 2 gm weight, were obtained from the institute's Animal House Facility. The *Plasmodium yoelii nigeriensis* MDR (Multi drug resistant) strain was obtained from Division of Parasitolgy, Central Drug Research Institute Lucknow. The techniques used for bleeding, injection, as well as
sacrifice of animals were strictly performed following mandates approved by the Animal Ethics Committee (Committee for the purpose of control and supervision of experiments on Animals, Govt. of India).

The parasitized erythrocytes were obtained from the blood of highly infected mice (average, parasitemia 38%). The suspension was diluted in 0.9 % sodium chloride to get the stock of 5x10^7 parasitized red blood cells per ml. Animals were inoculated intraperitoneally with 1x10^7 parasitized erythrocytes in 0.2 ml of normal saline. The immunomodulatory activity of picroliv was determined by trying various dosage regimens as well as immunization schedule.

2.3. Immunization

Our pilot studies suggest that pretreatment with picroliv for 14 consecutive days before immunization (at a dose of 1mg/kg body weight) elicited effective immune response. To assess its immuno-modulatory potential, animals were pretreated with picroliv before immunization with model antigen. The picroliv pretreated animals (1mg/kg body weight) were subsequently immunized with free OVA or OVA emulsified with IFA (100 μg antigen/animal). Control animals (no picroliv treatment) received saline only. On day 7 post immunization, the T-cells were isolated from the spleen of immunized animals using published procedure as standardized in our lab (Agrewala et al 1996). The splenic tissue was macerated and erythrocytes were lysed with the hemolytic Gey’s solution (3 ml per spleen) by incubating the cell suspension for 10 minutes on ice. The macrophages were removed by panning method, while B cells were eliminated by incubating the non-adherent cells with nylon wool (1x 5cm column) as described elsewhere (Agrewala et al 1996). The cells eluted from the column, were incubated with cocktail of anti-Mac3, anti-I Ad and anti IgM Ab at 40 for 45 min. The cells were washed and then treated with baby rabbit complement for 30 min at 37 °C. Finally, the cells were again washed with RPMI-1640 and used as an enriched source of T-cell population.

2.4. Macrophages

The BALB/c mice were inoculated with 2-3 ml of thioglycollate. Peritoneal exudates cells (PEC) were isolated by peritoneal lavage four days later. The cells were washed
with cold HBSS. The macrophages were obtained, by incubating the cell suspension for 1h at 37 °C on plastic Petri dishes followed by several washing with cold HBSS.

2.5. T-cell proliferation Assay

The T-cell proliferation assay was performed following published procedure as standardized in our lab (Agrewala et al 1996). Briefly, T-cells (2x 10^4/well) obtained from spleens of various groups of mice were cultured in 96 well plate (triplicate wells). The cells were incubated with macrophages (6x10^4/well) followed by exposure with increasing doses (0.001-100 μg/ml) of ovalbumin. The macrophages were treated with Mitomycin C (50 μg/ml). The cultures were further incubated for 72 h at 37 °C / 7% CO2. The cells were pulsed with 1.0 μCi [³H] - thymidine for 16 h before harvesting by automatic cell harvester (Skatron, Tranby, Norway). The [³H]-thymidine incorporation was measured by standard liquid scintillation counting method. The results were expressed as mean cpm of triplicate cultures.

2.6. Determination of Antigen Specific IgG Isotypes by ELISA

The production of OVA specific antibodies was measured in the sera of immunized groups of mice as described elsewhere (Agrewala et al 1996). The animals were injected with two doses of free antigen, (100 μg/ animal) on days 0 and 7. The animals were subsequently bled on day 7 post last immunization, to monitor presence of antigen specific antibodies. Ninety six-well microtier plates (Costar, Boston, MA, USA) were coated overnight with 50 μl of antigen (25 μg/ml) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) at 4 °C. The antigen coated plate was blocked with 3% skimmed milk and then incubated with log 2 dilutions of test and sera of the immunized animals. The reaction was allowed to proceed at 37 °C for two hours. The micro-tier plates were washed and 50 μl biotinylated goat anti-mouse IgG1 and IgG2a ABS were added. After usual steps of washings, 50 μl of streptavidin-HRP was added in each well and the plates were incubated at 37 °C for 1 hr. The plates were washed again before adding 50 μl of orthophenylene diamine dihydrochloride (OPD 0.2M HCl) and were finally incubated at 37 °C for 20 min. The reaction was terminated by the addition of 50 μl of 7 % H₂SO₄. The absorbance of the colored complex was read at 495nm with microplate reader (Eurogenetics, Torino, Italy).
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Antibody titers were expressed as the absorbance of the colored complex determined for different serum samples at 1:4000 dilutions.

2.7. *Effect of picroliv treatment on efficacy of chloroquine against Plasmodium yoelii infection*

The efficacy of picroliv was determined by evaluating its potential to suppress drug resistant *Plasmodium yoelii* infection in mouse model. The animals were pretreated with picroliv for 14 days before challenging them with infection. The potential of combination therapy was assessed on the basis of survival rate and parasitic load in red blood cells of infected mice. In pilot study, various doses of chloroquine (i. p.) were tried to achieve best dosage regimen for treatment of infected BALB/c mice. Finally, a suboptimal dose of 8mg/kg body weight of chloroquine was selected for evaluating the efficacy of picroliv – CHQ combination.

The infected animals were divided into following four groups. Each group consisted of 10 animals.

- Group: I  
  Saline (No drug treatment)
- Group: II  
  picroliv treatment
- Group: III  
  chloroquine treatment
- Group: IV  
  treatment with chloroquine in picroliv pretreated animals

* Chloroquine (i.p.) 1mg/kg body weight was administered for three consecutive days post *P. yoelii* infection.

2.8. **Statistics**

The data were analyzed by one- way analysis of variance (ANOVA) following Dunnet's t test methods. The survival data was analyzed by Mann Whitney Test. P< 0.05 was considered statistically significant.

3. **Results:**

3.1. *Picroliv augments proliferation of OVA-specific T-cells in BALB/c mice*

The immunomodulatory potential of picroliv was assessed by immunizing picroliv pretreated animals with model antigen OVA. The picroliv pretreatment induced significantly higher T-cell proliferation in comparison to the animals that were not
pretreated with picroliv. T-cell responsiveness to antigen was observed in a dose dependent manner. The stimulation index (S.I.) value obtained in animals pretreated with picroliv prior to their exposure with OVA (Picroliv- OVA- IFA) was around 5.87± 0.6154, while control animals that were not pretreated with picroliv could induce only 1.85± 0.563 S.I. in the immunized mice (Fig 1). The PBS treated animals did not induce any T-cell proliferation.

3.2. Picroliv treatment increases the secretion of IgG2a isotype of antibodies

The level of OVA-specific total IgG present in the sera of immunized as well as control animals was assessed by ELISA. Antibody titers were estimated as the O.D. of 0.2 above the control wells. The administration of antigen in picroliv (p<0.001) pretreated animals elicited strong immunological response in terms of antibody production, while immunization of animals that were not pretreated with picroliv could induce moderate level of antibodies. We also determined isotypes of antibody generated upon administration of picroliv prior to immunization with antigen. The control animals immunized with saline or free OVA could not induce any detectable level of IgG-isotypes. Interestingly, significant increase in ratio of IgG2a/IgG1 type of antibodies was detected in the sera of animals that were primed with picroliv. While low levels of IgG1 and IgG2a isotypes were found in control animals that were not pretreated with picroliv (p<0.001). (Fig 2 & 3).
Antigen administration in picroliv pretreatment augmented the proliferation of OVA-specific T-cells. T-cells (2x10^4) were isolated from the groups of five animals immunized with various formulation of OVA and cultured with OVA pulsed macrophages (6x10^4 cells/well). After 72h, ^3[H]-thymidine was added, and its incorporation was measured 16h later by liquid scintillation spectroscopy. The stimulation index (S.I.) was calculated as mean cpm values of stimulated culture/mean cpm values of unstimulated culture. Control cultures containing cells obtained from either free OVA immunized animals or the animals immunized with PBS, gave background levels of <2000cpm of ^3[H]-thymidine incorporation. The data represents mean S.I.± SD of three determinations.
Figure 2: Increased humoral response in immunized mice that were pretreated with picroliv

Antibody levels obtained in sera of BALB/c mice immunized with ovalbumin pretreated with Picroliv. The level of OVA-specific total IgG present in the sera of all immunized as well as control animals was assessed by ELISA. Results are expressed as mean of antibody titre of five mice in each group ± SD. There was a robust increase in total IgG levels among animals immunized with picroliv- OVA- IFA than the animals immunized without treatment of picroliv.
Figure 3: Effect of picroliv pretreatment on induction of IgG isotype response against OVA in Balb/c mice

IgG isotype responses in various groups of animals immunized with ovalbumin, ovalbumin emulsified with IFA with or without treatment of picroliv. Sera obtained from normal and experimental animals were analyzed for the presence of ovalbumin-specific IgG isotype by ELISA method as described in Materials and Methods. The level of IgG isotype were expressed as absorbance ($A_{495}$) of the colored complex developed in the immunosorbant assay.
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**Figure 4: The effect of Picroliv pretreatment on survival of animals.**

The animals were challenged with \(10^6\) parasitized RBC of the lethal *Plasmodium yoelii* followed by treatment with chloroquine. Percent survival of the treated animals was determined in various experimental animal groups. Day 8, \(p<0.001\) Picroliv Vs Chloroquine Vs Pic-CHQ; Day 10, \(p<0.001\) Picroliv Vs Chloroquine Vs Pic-CHQ; Day 14, \(p<0.001\) Chloroquine Vs Pic-CHQ.
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![Graph showing parasitemia over days post-infection](image)

Figure 5: *Effect of Picroliv pretreatment of P.yoelii infection on blood parasitic load*

Groups of BALB/c mice (n = 5) were administered with 8mg/kg dose of chloroquine and picroliv (1mg/kg for 14 days prior to infection) or free chloroquine at the same dose, free picroliv. Control animals (n= 5) were given PBS only. Animals were challenged with $10^6$ P.yoelii (MDR) parasitized erythrocytes. Parasitemia was estimated by preparing thin blood smears, stained with Giemsa strain. Day 8, $P<0.001$ Picroliv Vs Chloroquine and Pic- CHQ; Day 10, $P<0.001$ Chloroquine Vs Pic-CHQ; Day 16, $P<0.001$ Chloroquine Vs Pic- CHQ.
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3.3. Effect of co-administration of Picroliv in combination with chloroquine against drug resistant isolate of P. yoelii in Balb/c mice

We also evaluated effect of picroliv pretreatment on efficacy of chloroquine against drug resistant Plasmodium yoelii infection in BALB/c mice. The treatment with 8mg/kg dose of chloroquine alone could not completely suppress resistant isolates of Plasmodium yoelii. The animals treated with chloroquine alone (same dose) died by day 12 only (Fig 4). Treatment with same dose in animals that were pretreated with picroliv (1mg/kg), ensued in 100% survival up to day 16 post infection. The parasitic burden in treated animals was monitored after three day to exposure to infection by preparing blood smears of infected animals. Interestingly there was no parasitized RBC in case of picroliv treated animals till 8th post infection, while in the group treated with free chloroquine there was significantly higher parasite load (15.8%, p<0.001) on 8th day post infection. The parasitic load shot up to 56.12% on day 12. In contrast, in the group treated with picroliv and chloroquine combination, the parasite load was 1.9% (p<0.001) on 12th day post infection (Fig 5).

4. Discussion:

In spite of tremendous advancement in the field of malaria parasitology, the exact mechanism regarding mode of action of chloroquine is not well understood. Most of the antimalarial drugs including chloroquine enter into infected red blood cell and reach to digestive vacuole of parasite by simple diffusion. As parasite resides inside erythrocyte, the effective concentration of drug reaching to cytosol of the erythrocyte play regulatory role in killing of the parasite. In order to cope up with drug on slaughter, the parasite propels drug molecules outside the cell thus preventing attainment of minimum effective drug concentration inside the cells (Krogstad et al 1987). This is a regular trend for development of drug resistant isolates of Plasmodium sps. There is at least 40 times higher drug efflux in CHQ-resistant parasites as compared to CHQ-sensitive isolates (Plowe 2005).

In general, it is pathogen specific host immune components that play active role in elimination of pathogen, while the usage of chemotherapeutic agents does not allow multiplication and growth of the parasite. The development of full blown malaria suggests non effectiveness of parasite specific antibodies and T-cells develop by the...
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host during course of establishment of infection. This in turn suggest that use of immunomodulator picroliv in combination with CHQ could be a useful choice in revoking immune system of the host ensuing in successful elimination of pathogen. The immunomodulatory role of picroliv was established by its potential to induce humoral (antibody production) as well as cell mediated immunity (T cell proliferation) in the host. As evident from data of the present study pretreatment with picroliv helps in induction of strong immune response in the animals upon their exposure to OVA. Interestingly, picroliv was found to help in induction of IgG2a isotype of antibody. This indirectly suggests that the immunomodulator helps in skewing of immune response in favor of Th1 subtype of T- helper cells. We and others have earlier shown that IgG2a subtypes of antibodies are of great importance in containing *P.yoelii* infection in model animals (Sharad *et al* 2006). Picroliv educate immune cells to help in production of IgG2a type of the antibodies against *P.yoelii* antigens that are released by lysis of parasite. Beside the antibodies also inhibit released parasite for attacking fresh erythrocytes.

The data of the present study support our hypothesis and demonstrate that combination of CHQ with picroliv is successful in treating Plasmodium infection in mice. In general, blood- induced *P. yoelii* infection in Balb/c mice led to fulminant infection that peaks up to 60 % parasitemia in 6 days. If not treated with drug the infected animals die within 5 to 8 days of the infection. This offers very convenient and effective mean for determining antimalarial efficacy of drug candidates as well as novel formulations of existing drugs.

Evidences suggest that acute malaria infection supresses immune response via autocoids (Agarwal *et al* 2002, Kubata *et al* 1998). For example, during infection with *Plasmodium sps* parasite, there is down regulation in circulating T- cells, as revealed by lymphoproliferative response and cytokine release by peripheral mononuclear cells when stimulated by malaria antigens (Riley 1988). The data of the present study advocates above fact, as picroliv mediated replenishment of type I cytokines helps in elimination of parasite. Further, picroliv seems to help in development of specific antibodies against parasite antigens. The developed antibodies play crucial role in containing the infection.
Incidentally, immunity to erythrocyte stages of the *P. yoelii* relies on activation of CD4 T cells, B-cells as well as type I cytokine and IFN- gamma as well (Burns *et al* 1997, Pamela *et al* 1999, Zhong *et al* 2003). The immune cells are claimed to be involved in both humoral as well as cell mediated protective immunity (Langhorne *et al* 1990, Hunter *et al* 1995, Melancon-Kaplan *et al* 1992). Earlier investigations have suggested that protection against blood stage malaria is complex and requires both Th2 and Th1 cellular responses (Langhorne *et al* 2002, Blomberg *et al* 1999, Mohan and Stevenson 1998, Suss and Pink 1992). Further, the protection studies suggest that by beginning of the second week after infection the surviving animals develop a very effective cellular immune response that contributes significantly to control the infection (Salmon *et al* 1990).

Chloroquine in general causes nausea, stomach upset, cramps, loss of apetite etc, while its high doses or long term usage causes irreversible retinal damage and blood dyscrasias including agranulocytosis, aplastic neutropenia or thrombocytopenia. Moreover the drug accumulates in liver and leads to many malfunctions in the body (Yam and Kwok 2006, Savarino *et al* 2003, Savarino *et al* 2006, Sotelo *et al* 2006). The effectiveness of the dosage regimen used in the present study suggests that antiparasitic drugs could be made effective at low concentration when used in combination of immunomodulator picroliv. This opens new vistas in malaria chemoprophylactic strategies and result of present study also indicate that picroliv per se has more or less no antimalarial activity; however it indirectly causes suppression of infection when used in combination with CHQ. Infact it synergistically potentiates antimalarial activity of CHQ. A suboptimal dose of chloroquine (8 mg/kg) was sufficient to inhibit of *Plasmodium yoelii* MDR strain. We have recently reported that in case of visceral leismaniasis picroliv successfully suppresses infection when used in combination with miltefosine an antileishmanial drug, in Hamesters (Mittal *et al* 1998, Gupta *et al* 2005).

The concept of using combination therapy involving potential immunomodulator and an effective antimicrobial agent is not new. The muramyl dipeptide as well as PGG glucan have been shown to help in activation of host immune system (Reeta *et al* 1985, Tziananabos and Cisnevos 1996). The former has been successfully exploited
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against fungal infection while PGG glucan was found to enhance efficacy of antibiotics against drug resistant bacteria (Reeta et al. 1985, Tziananabos and Cisnevos 1996). We also demonstrated that co administration of immunomodulator tuftsin in combination with chemotherapy could be an effective strategy in supression of fungal, bacterial and protozoa pathogens (Arif et al. 2007, Khan et al. 2006, Khan and Owais 2005).