C-Reactive Protein Protects against Preerythrocytic Stages of Malaria

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We previously reported that low doses of interleukin-1 strongly inhibited in vitro development of the hepatic stages of Plasmodium falciparum and P. yoelii. Among several hypotheses, we considered the role of C-reactive protein (CRP), a major acute-phase reactant whose concentration increases markedly in infectious disorders. We demonstrated that human hepatocytes cultured in the presence of interleukin-1 released, as early as 30 min after stimulation, an increased amount of CRP. We then established that CRP bound to the P. falciparum and P. yoelii sporozoite surface membranes, probably via a phosphorylcholine binding site. Experiments in which CRP was added to rat hepatocyte monolayers during or after inoculation confirmed that the target of the CRP-mediated inhibition was at the very early phase of infection. These in vitro functional activities were confirmed in an in vivo model; rats with increased levels of CRP in serum following an injection of turpentine oil were found to be largely protected against an inoculation of P. yoelii sporozoites. The same results were observed in animals inoculated with sporozoites previously incubated in purified CRP or in sera of rats pretreated with turpentine oil. The latter effect was inhibited after incubation of serum from turpentine-injected rats with anti-CRP serum.

The establishment of protection against preerythrocytic stages of malaria parasites depends on both humoral and cell-mediated immune responses (20a, 22, 30, 33). Particular attention has recently been given to the possible role of cytokines in protective mechanisms at the preerythrocytic stage of the infection (8, 18, 21, 29).

Gamma interferon and interleukin-1 (IL-1) were shown to strongly inhibit Plasmodium falciparum sporozoite development in primary cultures of functional hepatocytes. Neither of these two cytokines directly affects free sporozoites, but their points of action appear to differ. Time experiments with interferon indicate a postpenetration (intrahepatocyte) cellular mechanism, in contrast to IL-1, which exerts its effect at the very early phase of infection (21).

One of the hypotheses that could explain IL-1 activity is related to the secretion of inflammatory proteins by IL-1-stimulated hepatocytes, in particular C-reactive protein (CRP) (1, 6, 13). CRP, a major acute-phase reactant found in trace amounts in normal human serum, shows markedly increased levels in sera of individuals with many conditions, including infectious disorders (17, 26). CRP participation in host defense systems against infection is in part related to its capacity to bind to the phosphorylcholine-containing substances in microorganism membranes (10, 32).

We investigated the release of CRP in primary cultures of human hepatocytes after stimulation with human recombinant IL-1. We then analyzed the possible deleterious effects of CRP on sporozoites and on hepatic stages of Plasmodium spp. Finally, using inflammatory substances (e.g., turpentine oil), we investigated the possible influence of the non-specific induction of CRP in vivo on the infection of rats by P. yoelii sporozoites.

MATERIALS AND METHODS

CRP. CRP was purified from human ascitic fluid or from normal rat serum by a three-step chromatography procedure involving passage through columns of phosphoethanolamine-Sepharose 4B, protein A-Sepharose, and Sephacryl S-300 (27). The purity of CRP isolated by this procedure has been documented elsewhere (7). Preparations were checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4 to 30% acrylamide), which indicated a single band with a molecular weight of 24,000. CRP was radiolabeled by the iodogen method with carrier-free Na\(^{125}\)I (CEA, Saclay, France) and Iodobeads (Pierce Chemical Co., Rockford, Ill.) (9). Iodine-labeled protein was separated from free iodine on a Sephadex G-25 column. The specific activity of various preparations was 1 ± 0.25 μCi/μg. \(^{125}\)I-CRP retained its binding capacity, since more than 95% of the iodinated protein did bind to a gel substituted by phosphorylcholine or phosphoethanolamine. This binding was specific, since inhibition occurred in the presence of a calcium chelator agent and/or phosphorylcholine.

Antisera. Rabbit polyclonal anti-human CRP serum was purchased from Biosys, Compiegne, France. Polyclonal anti-rat CRP was raised in a rabbit immunized with purified rat CRP in the presence of complete Freund adjuvant.

IL-1. Human recombinant IL-1 was purchased from Hoffmann-La Roche, Basel, Switzerland.

Sporozoites. Sporozoites were obtained from the salivary glands of Anopheles stephensi infected with P. falciparum NF54 or with P. yoelii 17X or 265BY.

Culture of hepatic stages of malaria parasites. Human and rodent hepatocytes were isolated by collagenase perfusion of liver biopsies as previously described (19). The cells were cultured in eight-chamber plastic Lab-Tek slides (Miles Laboratories, Inc., Elkhart, Ind.) at a concentration of 100,000 cells per well and were maintained for 24 h before P.

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yoelii sporozoite inoculation and/or addition of human recombinant IL-1 at a concentration of 5 IU/ml.

The parasites were obtained by dissection of the salivary glands of *A. stephensi*. The infestation procedure of the rodent hepatocyte cultures was carried out as described previously (20). Parasites were detected by immunofluorescence assay (IFA) with a murine monoclonal antibody recognizing hepatic stages of *P. yoelii*.

**Results**

**IL-1 secretion in liver cell cultures.** (i) Qualitative assay. Samples (50 μl) of supernatants of cultures incubated for different lengths of time with human recombinant IL-1 (5 IU/ml) or control medium were mixed with an equal volume of 2× Laemmlı sample buffer (15) and boiled for 5 min. The mixture was electrophoresed under reducing conditions in 12.5% acrylamide slab gels as described by Laemmlı (15). Resolved proteins were either stained with Coomassie blue or transferred to nitrocellulose paper by electroblotting (31). In the second instance, saturation with 5% nonfat milk in phosphate-buffered saline (PBS) was performed for 2 h at room temperature. CRP was revealed by incubating the nitrocellulose strips with a 1/100 dilution of rabbit anti-human CRP serum in blocking buffer for 12 h and then with 125I-labeled *Staphylococcus aureus* protein A (Abersham, France). Dots were exposed for 3 days at −70°C on X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

(ii) Quantitative assay. An enzyme immunoassay was used to measure the amount of human CRP present in liver cell culture supernatants incubated for 18 h with human recombinant IL-1 (5 IU/ml). In brief, 10 μg of rabbit anti-human CRP immunoglobulin was attached, by passive adsorption overnight at 4°C, to polystyrene microplates (Nunc, Becton-Dickinson, Grenoble France). Purified human CRP, diluted in culture medium, was used to generate the reference curve, which was developed by using alkaline phosphatase-conjugated rabbit anti-human CRP.

**In vitro inhibition of the Plasmodium hepatic stage by CRP.** Inhibition assays were performed by adding various concentrations of human or rat CRP to culture medium at the time of sporozoite inoculation. The cultures were inoculated by adding 40,000 *P. yoelii* sporozoites to culture medium containing or not containing CRP. At 3 h later, the medium was changed and cultures were maintained in the absence of CRP. In other experiments, CRP was added 3 and 24 h after inoculation. All experiments were performed in duplicate. The percentage of inhibition of parasite development was assessed by comparing the number of schizonts in CRP-treated cultures with the number in the corresponding control cultures.

**In vitro effect of CRP on the erythrocyte stages of *P. falciparum*.** The effect of CRP on the *P. falciparum* erythrocyte stages was evaluated by using an in vitro microtest: parasite growth was estimated by [%H]thymidine incorporation (3). Nine different dilutions of CRP in RPMI 1640 medium were prepared (10 to 0.039 μg/ml) and added to wells of 96-well flat-bottom plates at the beginning of the culture. The cultures were grown for 48 h.

**CRP-parasite interactions.** (i) Binding of 125I-CRP to sporozoites. Binding studies were performed at 37°C for 3 h in 1.5-ml Eppendorf microtest tubes coated with human serum albumin. Suspensions of infected or noninfected salivary glands in calcium buffer (5 mmol of CaCl2 per liter, 150 mmol of NaCl per liter, 5 mmol of NaHCO3 per liter, 20 mmol of Tris hydrochloride [pH 7.4] per liter) containing 0.05 μg of 125I-CRP were incubated with gentle agitation in a final volume of 120 μl. Incubations were terminated by the addition of 1 ml of calcium buffer, followed by centrifugation at 10,000 × g for 7 min at 4°C. The activity present in the supernatant was determined in a gamma counter (Corning Glass Works, Corning, N.Y.), and all results are means of four determinations.

(ii) IFA. The fixation of CRP was investigated by incubating *P. yoelii* or *P. falciparum* sporozoites for 1 h at 37°C with purified human or rat CRP at concentrations of 5, 25, and 50 μg/ml in calcium buffer. After three washings, the sporozoites were transferred to poly-L-lysine-treated slides as previously described (28a). CRP binding was determined by IFA with anti-human CRP reagent as the primary antibody and a fluorescein-tagged rabbit anti-human immunoglobulin as the secondary antibody. The same experiment was performed with free *P. falciparum* merozoites isolated by the method of Heidrich et al. (11).

**In vivo experiments.** Five groups of 2-month-old female Wistar rats (IFFA CREDO, Lyon, France) were used. Each rat in group 1 (total of seven rats) was injected subcutaneously with 1.0 ml of turpentine oil. At 24 h later, each rat was inoculated intravenously with 7,000 *P. yoelii* sporozoites in 0.5 ml of PBS. Each rat in group 2 (control group for group 1, also containing seven rats) received a subcutaneous injection of 1.0 ml of PBS. Subsequent treatment was the same as that for group 1 rats. Each rat in group 3 (total of five rats) was inoculated intravenously with 7,000 sporozoites which had been previously incubated for 30 min at 37°C in 0.5 ml of rat serum collected 24 h after turpentine oil injection and diluted 1/5 in PBS. The rats in group 4 (total of five rats) were inoculated under the same conditions as those in group 3, but the rat serum was previously incubated with anti-CRP for 30 min at 37°C before the sporozoites were added. Each rat in group 5 (total of five rats) was inoculated intravenously with 7,000 sporozoites which had been mixed for 30 min at 37°C with 100 μg of purified rat CRP in 0.5 ml of PBS.

Blood smears, taken daily from days 3 to 15 after sporozoite inoculation, were stained with Giemsa stain and examined for *P. yoelii* erythrocyte stages.

**RESULTS**

**CRP secretion by human hepatocytes in response to IL-1 stimulation.** Cultures of human hepatocytes incubated for 18 h with 5 IU of human recombinant IL-1 per ml secreted CRP in larger amounts than did untreated cultures. As measured by enzyme-linked immunosorbert assay, IL-1 induced a fivefold increase in the level of CRP detected in culture supernatants: 24 μg/ml versus 4.8 μg/ml for untreated cultures. Time course analysis of CRP release, as assessed by a semiquantitative method, revealed a progressive increase in the CRP level detected in culture supernatants; this increase was detected as early as 30 min after IL-1 stimulation (Fig. 1).

**Effect of CRP on hepatic-stage *Plasmodium* cultures.** When purified CRP was added to rat hepatocyte cultures at the

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**FIG. 1.** Western immunoblot analysis of secreted CRP. CRP is visualized by using rabbit anti-human CRP antiserum followed by 125I-labeled protein A. Lanes 1 to 6 show the amount of CRP in the supernatant of cells incubated for 30 min and 1, 5, 10, 15, and 20 h, respectively, with IL-1. Abbreviations: CrP, Control purified CRP; Ccm, control culture medium.
time of sporozoite inoculation, parasite schizogony was inhibited in a dose-dependent manner (Fig. 2). Comparative studies of the effect of human and rat CRP on *P. yoelii* development in rat hepatocytes demonstrated the species specificity of CRP activity, since 50% inhibition was obtained with 25 μg of rodent CRP per ml, whereas 300 μg of human CRP per ml was necessary to achieve a similar inhibition (Fig. 3). Neutralization experiments in which anti-CRP was added to culture medium at 37°C 30 min before cell treatment demonstrated the specificity of the CRP activity. When CRP was added 3 h after sporozoite inoculation, no significant inhibition was found (data not shown).

Effect of CRP on erythrocyte-stage *Plasmodium* cultures. No inhibition was observed when purified CRP was added to erythrocyte-stage *P. falciparum* cultures.

**CRP-parasite interaction.** (i) Binding of 125I-CRP to infected and noninfected salivary glands. To investigate the possible binding of 125I-CRP to sporozoites, we compared the binding of 125I-CRP to *P. yoelii*-infected salivary glands with the binding of 125I-CRP to noninfected salivary glands (Fig. 4). It appears that 125I-CRP bound to the sporozoites, since the binding to salivary glands was increased threefold when the glands were infected. The complete inhibition of the binding observed in the presence of phosphorylcholine suggests that the CRP binding is mediated by phosphorylcholine-binding sites.

(ii) **IFA analysis.** The binding of CRP to the parasite was further studied by IFA. Significant binding to unfixed live *P. yoelii* sporozoites (Fig. 5) and *P. falciparum* merozoites (data not shown) was observed. No fluorescence appeared when the experiment was performed with calcium-free medium, confirming the importance of this divalent ion in the establishment of the interaction.

**In vivo effect of CRP on *P. yoelii* sporozoite development in rats.** In vivo experiments were carried out to find the effect of CRP on protection against sporozoite challenge. In group 1 rats, which were pretreated with turpentine oil, only one rat exhibited parasitemia, which was weak and was immediately resolved. In group 2 (the PBS-treated control group), five of the seven rats were infected. In group 3 and 5 rats, which were given sporozoites that were previously incubated in either rat serum or purified rat CRP, no parasitemia was observed. In contrast, group 4 rats, which were given CRP that was neutralized before sporozoite incubation, were all found to be infected.

**DISCUSSION**

In a previous report, we showed that low doses of natural or recombinant IL-1 strongly inhibit the in vitro hepatic development of *P. falciparum* and *P. yoelii*, since 98%
inhibition was achieved by treating hepatocyte primary cultures with 5 IU of IL-1 per ml. Analysis of time course experiments indicated that IL-1 activity did not affect the parasite maturation or the sporozoite itself but was probably due to an indirect effect on the process of liver cell invasion by the sporozoite (21).

Among several hypotheses that could explain the mode of action of the IL-1, we considered the role of the acute-phase proteins which are synthesized at a higher rate when hepatoma cell lines (6) or murine (28) and rabbit (16) hepatocytes are stimulated. One of the acute-phase proteins, the CRP, has been shown to play a significant role in nonspecific host defense mechanisms against bacteria (23, 24). In this study, we demonstrated that human hepatocytes cultured in the presence of human recombinant IL-1 released, as early as 30 min after stimulation, an increased amount of CRP. Since CRP bound to the P. falciparum and P. yoelii sporozoite surface membranes in a calcium-dependent manner and probably via a phosphorylcholine-binding site, this interaction is similar to that of CRP with bacterial membranes. Experiments in which CRP was added to rat hepatocyte monolayers during or after inoculation with P. yoelii sporozoites confirmed that the CRP-mediated inhibition occurred at the very early phase of infection. Lastly, the antiplasmodial activity displayed by CRP was substantially increased in vivo. Rats injected with pertussis oil, which caused a substantial increase in the CRP level (14), were found to be largely protected against P. yoelii sporozoite inoculation. More specifically, sporozoites preincubated in acute-phase serum were no longer infectious when administered to untreated rats. This antibacterial activity was specific, since it was abolished by anti-CRP antibodies and since it could be similarly achieved by preincubation of sporozoites with purified rat CRP. In contrast, despite a strong fixation of CRP on P. falciparum merozoites, no inhibition of the erythrocytic stages was observed in vitro.

These findings indicate that CRP, by binding to sporozoite surface components, can interfere with plasmodial infection. CRP can prevent sporozoite penetration into the hepatocyte by masking recognition sites involved in the sporozoite-host cell interaction. Complexed CRP may also activate the complement system (5, 12), which, in turn, can damage or destroy the sporozoite. Bound CRP also appears to influence the early stages of parasite maturation after penetration into hepatocytes. Besides these mechanisms, additional CRP-triggered events may occur in vivo: CRP capacity to modulate neutrophil functions (4) or platelet or macrophage (34, 35) activity or to enhance parasite opsonisation (25) can also modify the host-parasite interaction in favor of the former.

Whether the CRP level in the sera of patients is increased during the acute phase of malaria infection is presently under investigation. If it is, this might be a mechanism of resistance to sporozoite-induced superinfection. Similarly, it would be of interest to determine the extent to which acute-phase reactants, other than CRP, affect the nonspecific resistance to plasmodial infection during acute inflammatory states in individuals exposed to natural malaria infection.

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LITERATURE CITED


