Hepatic phase of malaria: a crucial role as "go-between" with other stages

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Besides potential interest in itself, the hepatic stage of malaria might play a crucial role as "go-between" with other stages. When present in the parasitophorous vacuole, antibodies induced by both sporozoite and erythrocytic stages efficiently disturb hepatic development of the parasite. Likewise previous and ensuing erythrocytic stages can modulate the "shielded" phase by cytokines, directly or as a result of a cascade of events, and by MHC-restricted or antibody-dependent cytotoxic mechanisms.

Introduction

The lack of interest in intra-hepatic malaria parasites presumed to be shielded from the immune system has now been overcome since the role played by the hepatic phase itself appears to be essential for the induction of protection against the pre-erythrocytic stage. It is clear that the hepatic stage and the previous (sporozoite) stage are interdependent, the former being in part the target for antibodies, cytokines, cytotoxic T cells and numerous other effector mechanisms induced by the sporozoite (*1*). Moreover, recent results obtained with *in vivo* and *in vitro* models tend to demonstrate that interdependence with the ensuing erythrocytic stage might also exist. Thus, besides its potential interest as a target in itself (*2*), the hepatic stage might play a crucial role as "go-between" with other stages.

Hepatic effect of antibodies induced by previous or subsequent developmental stages

The concept of functional relations between sporozoite and hepatic stages has developed from inhibition assays using anti-circumsporozoite (CS) protein antibodies and *Plasmodium falciparum* sporozoite-infected human hepatocyte primocultures (*3*). Whether antibody directed against the hepatic schizont can enter the hepatocyte is not known. There is no discernible effect when antibodies are added after sporozoite penetration is completed, whatever their isotype, origin or the epitope(s) recognized (*4*), even when the epitope is expressed at the hepatocyte surface (*5*). Nevertheless, antibodies can efficiently disturb hepatic development of the parasite: the prerequisite for antibody efficacy is that antibodies penetrate when the parasitophorous vacuole is created by invagination of the hepatocyte outer membrane during sporozoite internalization. Evidence of incorporation of antibodies directed against the CS protein has been provided by the observation that intracellular sporozoites can be stained by fluorescein isothiocyanate-labelled antimouse IgG antibodies (*4*). More surprising is the fact that identical results are obtained with antibodies unable to recognize the sporozoite stage, eliminating the hypothesis that antibodies penetrate only if bound to the parasite. It has been observed with antibodies recognizing the heatshock-like 72 kDa protein HSP70-1 from *P. falciparum* (*6–9*), an antigen expressed at the hepatic level when the parasite is binucleated (*5*). It has thus to be admitted that antibodies can passively enter and persist in the parasitophorous vacuole until appearance of the corresponding antigen in the parasite.

This post-penetration effect is one of the "positive" properties shared by the highly diverse anti-CS antibodies. In fact, although they exert an undeniably strong inhibitory effect on the entry of the sporozoite, anti-CS antibodies generally fail to totally block penetration, since some sporozoites escape the action of antibodies at whatever concentration they are used (*1*). More disquieting is the fact that, when diluted, anti-CS antibodies enhance penetration of the sporozoite. Whatever their isotype, IgG or IgM, whether they are polyclonal or monoclonal, directed against the CS protein of *P. yoelii* or *P. falciparum*, anti-CS antibodies are able to increase the number of liver parasites up to 150% *in vitro* (*4*). This phenomenon is the consequence of an antibody-induced interaction between the sporozoite and the hepatocyte membrane, not a direct effect on the sporozoite itself. The initial hypothesis was that IgG enhancement could be

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mediated either by Fc receptors (*10*) or, in the case of IgM, by complement receptors (*11*), as previously demonstrated in *in vitro* models of virus-infected cells. The fact that enhancement is also observed with monovalent Fab fragments argues against the first alternative and rather suggests that antibodies induce a change in the conformation of the CS protein, resulting in a structure capable of binding more efficiently to the putative receptor on the hepatocyte membrane. If these *in vitro* observations prove to be relevant *in vivo*, antibody-mediated enhancement might be an important phenomenon to take into account in the design of future anti-sporozoite vaccines.

The cytokines

Direct or indirect modes of action

After an infatuation with antibodies, the current trend is now to attribute an essential role to cellular mechanisms in protection against the pre-erythrocytic stage. A number of cytokines interfere with the plasmodium during its hepatic course, both directly and/or in the context of complex cytokine interactions, with the participation of various cells including the hepatocyte itself.

Interferon (γ) (IFN (γ))

Among the various cytokines released by activated T cells, the first cytokine thought possibly to interact with the hepatic stage was interferon (γ) (IFN-(γ)). *In vivo* (*12*, *13*) and *in vitro* (*12*, *14*, *15*) experiments indicate that this cytokine can significantly inhibit hepatocytic development of rodent and human malaria parasites. One of the original contributions of V. Nussenzweig's group was to hypothesize that viable sporozoites may trigger sensitized T cells to release IFN-(γ) able to inhibit the subsequent stage (*12*). As a matter of fact, administration of neutralizing MAbs against IFN-(γ) to immune hosts reverses sterile immunity to sporozoite challenge (*16*). IFN-(γ) secretion can be induced not only by the sporozoite stage but also by erythrocytic stages: longitudinal studies indicate that circulating IFN-(γ) is present several days after human infestation (*17*, *18*). Endogenous IFN-(γ) production therefore may be important in protecting against malaria reinfection during the months following an acute attack, when serum IFN-(γ) is present. Moreover, it should be underlined that IFN-(γ) has not only a parasitostatic effect, but probably also a post-assembly mode of action since addition of IFN-(γ) to developed liver stages induces a lysis of a significant number of parasites (*14*). This result might have some relevance to cytotoxicity. Indeed, even a hepatic antigen that is expressed after a delay might provide an active stimulus.

Tumour necrosis factor alpha (TNF)

While the role of T-cell released cytokines such as IFN-(γ) has been established, other cytokines, primarily produced by activated monocytes/macrophages might also be involved in the modulation of pre-erythrocytic stage development. We have therefore investigated the effect of purified *E. coli* recombinant murine TNF in *in vitro* and *in vivo* models. *In vitro*, in the presence of TNF concentrations ranging from 1 to 5 μg/ml, no detectable effect is observed on the development of *P. yoelii* in primocultures of C57BL/6, BALB/c or C3H/HeJ hepatocytes. This is also the case when TNF is added three times to the cultures, i.e., 24 hours before, at the time of and 24 hours after the infestation. These results are thus quite different from those obtained by Schofield et al. (*19*) who observed an effect of TNF on a hepatoma cell line, although it might be related to the tumorous origin of the HEP G2 A16. *In vivo*, however, and we are in agreement with Schofield et al. on this part, the intravenous administration of 1 μg of TNF interferes with the development of *P. yoelii* sporozoite infection. When injected three times (24 hours before, at the time of and 24 hours after the challenge), 60% of BALB/c mice are protected. When injected only 24 hours before the sporozoite challenge, a delay is observed. The level and the evolution of the parasitaemia are the same, however, as in the control. Obviously, under these experimental conditions, TNF might act by affecting early intra-erythrocytic development (*20*, *21*). Nevertheless recent work in our laboratory clearly showed that TNF can affect the hepatic development of parasites via IL-6 secreted by liver non-parenchymal cells (*35*).

IL-1, IL-6 and non-specific factors

Role of the CRP and other serum factors. As previously pointed out, IL-1 protects against the pre-erythrocytic stages of malaria (*14*). We have postulated that this effect of IL-1 may be mediated in part by the C-reactive protein (CRP), an acute phase protein synthetized by IL-1-stimulated hepatocytes. Evidence for this has been obtained with both *in vitro* and *in vivo* models. *In vitro* inhibition assays have demonstrated that CRP has a biological activity at an early phase of infection. This activity has been confirmed in an *in vivo* model: rats injected with turpentine oil, which causes a substantial rise in serum CRP levels, are largely protected against the inoculation of *P. yoelii* sporozoites. CRP binds to sporozoites in a calcium-dependent manner, probably via a phosphorylcholine binding site. We thus first hypothesized that CRP

binding to surface components of the sporozoite could mask recognition sites involved in the sporozoite–host cell interaction. However, the absence of inhibition of the erythrocytic stage, despite strong binding on *P. falciparum* merozoites, indicates that the mechanisms involved are more complex than foreseen (*22*).

Further experiments, using a double-staining technique permitting the steps by which the sporozoite enters and develops in the host cell to be distinguished (*23*), were thus performed to pinpoint the mode of action of purified rat CRP. In addition to an inhibitory effect on sporozoite penetration, there was also an effect at the intracellular level, which appears, not at the time of the transformation of the sporozoite into the trophozoite, but between 3 and 24 hours. CRP thus blocks the parasite at the trophozoite stage, preventing its passage to the schizont stage. This mechanism of inhibition should be considered in conjunction with the intracellular effect of anti-CS antibodies. Like the anti-CS antibodies, CRP bound to the parasite could act by blocking translocation of the CS protein to the parasitophorous vacuole membrane, leading to acidification of the vacuole and the lysosomal digestion of the parasite (*4*, *24*). This intracellular effect of the CRP is thus of potential interest for therapeutic intervention during this phase.

CRP has been found in increased concentrations in the sera of malaria patients (*25*). To what extent CRP plays a role in the resistance to plasmodial infection in individuals exposed to natural malaria infection is under investigation. It is clear, however, that CRP by itself cannot account for all the effects of IL-1, and, more generally, for the pleiomorphic manifestations of the inflammatory syndrome. When we analyse the respective roles played by the hepatocytes and/or the serum of rats in which the levels of acute-phase proteins were increased, it appears that inflammatory factors other than CRP are equally involved, their inhibitory effects being observed essentially during parasite maturation (*36*).

It has been recently demonstrated that hepatic IL-1 activity is mediated by a secretion of IL-6 in the liver (*26*). We now have evidence that the effect of IL-1 on the hepatic development of *P. falciparum* or *P. yoelii* sporozoites is in part mediated by IL-6 activity, since anti-IL-6 antibodies neutralize the effect of IL-1. However, in addition to an effect on the release of acute phase reactants, IL-6 has a direct effect on sporozoite penetration and development (*37*). Whether IL-6 secretion in liver originates within the hepatocyte itself or in the other types of cell that are always present in hepatocyte primocultures has to be investigated. Interestingly, as IL-6 has been found to be produced in large amounts during the erythrocytic phase of malaria infection (*27*), this cytokine might play a role in the resistance to further challenge with sporozoites.

Cytotoxicity: a role for parasite antigens expressed on the hepatocyte surface?

Earlier studies (*16*, *28*) suggest that suppressor/cytotoxic T cells expressing the $CD8^+$ phenotype might participate in the development of sterile immunity induced by irradiated sporozoites. Free sporozoites, in contrast to hepatocytes, able to express both intracellular parasite antigens and class I MHC molecules on the cell surface, cannot be the target of sensitized T cells (*29*). We used *in vitro* models and a panel of antibodies recognizing sporozoites and/or hepatic stages to determine whether some of the antibodies detected epitopes on the surface of infected hepatocytes. Until now, no monoclonal antibody against the CS protein of *P. yoelii* or *P. falciparum* has recognized antigens on the surface of liver cells (*1*). Nevertheless, the possibility that CS antigens might be processed in a way that prevents their recognition by MAbs directed against the "original" antigen cannot be excluded. Likewise, the CS antigens might be expressed in quantities sufficient to induce a T-cell response but undetectable in our assay. These hypotheses are in agreement with the observation that a cytotoxic response, abolished by an anti-CD8 MAb, is obtained with sensitized T cells isolated from mice immunized with a putative T-cell epitope on the CS protein of *P. yoelii*, although no antigen can be detected on the hepatocyte membrane (collaboration with D. Grillot, G. Del Giudice and P. H. Lambert, WHO, Geneva).

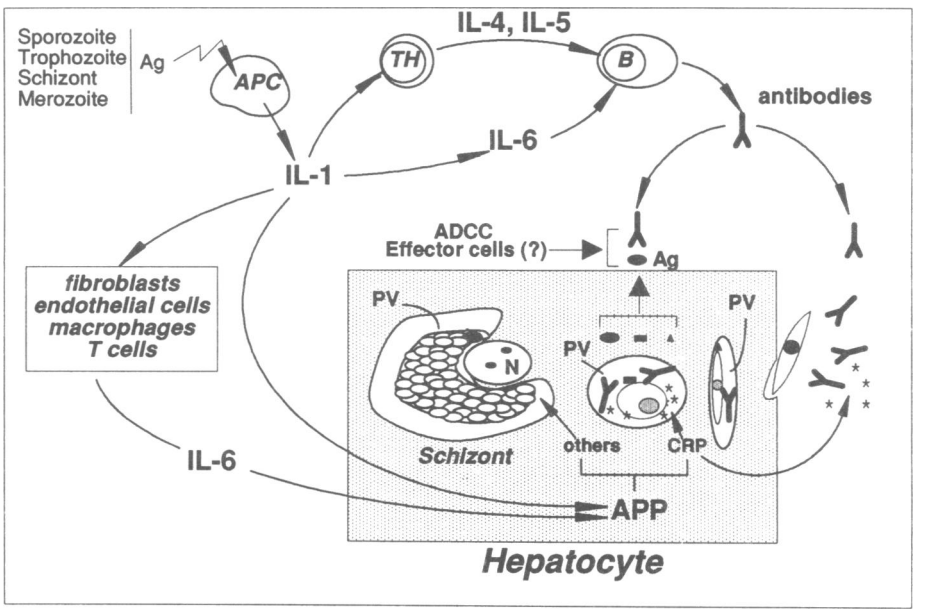
Unlike CS antigens which are not detectable on the hepatocyte surface, an epitope corresponding to the *C*-terminal fragment of the 72 kDa heat-shock-like protein HSP70-1 from *P. falciparum*, is expressed at the level of the hepatocyte membrane (*5*). Using a variety of techniques, including scanning electronic microscopy, we have observed that infected hepatocytes exhibit a staining restricted to a small area above the parasite, in the absence of staining in other areas of the infected cell, or in uninfected hepatocytes. The possibility that this epitope participates in mechanisms of MHC-restricted cytotoxicity is under investigation. Another role for this antigen, in relation to antibody-dependent cell-mediated cytotoxicity (ADCC) has already been investigated. Mononucleated cells from the spleen, the blood or the non-parenchymal population of the liver of normal mice were added, concomitantly with various dilutions of a MAb recognizing this epitope, to hepatic cultures of 24 hours, the period necessary for the appearance of the antigen on the cell surface. While no effect was obtained with peripheral blood cells, we found that 25% of the schizonts were specifically and regularly

lysed when using spleen cells at a killer : target ratio of 30:1 (*5*). More interestingly, with liver non-parenchymal cells, up to 55% of the hepatic schizonts disappear specifically with a killer : target ratio of 2:1. If the ADCC phenomenon we observed *in vitro* exists *in vivo*, an antibody response induced by an antigen expressed at the erythrocytic level might exert a feedback regulation on the previous stage, confirming the privileged status of the hepatic stage.

Molecules localized in the parasitophorous vacuole

Inhibitory effect. The additional inhibitory effect on the intrahepatic stage, occurring after penetration of the sporozoite, is interesting at several levels, and not only because it increases an effect first believed to be restricted to the sporozoite level. More noticeable is the fact that this intracellular effect is not limited to antisporozoite antibodies, nor even to antibodies. First observed with anti-CS antibodies (*3*, *4*), we have subsequently noted a similar effect with CRP. In all cases, a similar mechanism seems to be involved: incorporation of the molecules, antibodies or not, when the parasitophorous vacuole is created by invagination of the hepatocyte outer membrane during sporozoite invasion. We have previously mentioned (*30*) that a critical point in hepatic development consists in the initiation of the first nuclear division. The translocation of the CS protein to the parasitophorous vacuole membrane could be crucial to this initiation. It has been proposed that the incapacity of the

Fig. 1. **Humoral immunity.** Processing of parasite antigens (Ag) (from the sporozoite, hepatic and chiefly erythrocytic stages) by antigen-presenting cells (APC), leads to the release of IL-1 and IL-6. These two lymphokines act on the hepatic stage by two pathways.
i) In the first case, a cascade of activations result in antibody (AB, Y) synthesis. Anti-sporozoite antibodies prevent sporozoite attachment and/or penetration. When they enter the parasitophorous vacuole (PV), they not only prevent development of the trophozoites, but they also destroy them (*3*, *4*). Likewise, antibodies directed against the hepatic stage were found to partly inhibit the maturation of the schizonts (*5*). Moreover, antibodies might be effective in collaboration with cellular mechanisms: schizont antigens expressed on the hepatocyte surface are, at least in *in vitro* models, the target of ADCC mechanisms (*5*).
ii) IL-1 also induces synthesis of acute-phase proteins (APP) by hepatocytes, either directly, or through IL-6 synthesized by different cell types. Among the APP, C-reactive protein (CRP) has been demonstrated to inhibit the initial development of the sporozoite (*22*). We now have evidence that an antibody-like mechanism, inhibiting the translocation of the CS protein to the PV membrane, is also imputable to the CRP. Unlike CRP, a majority of other APP act late, inhibiting the maturation of the hepatic schizont.



CS protein of *P. falciparum* to translocate in the Hep G2 cell line would explain the inability of this hepatoma to support the development of this plasmodia (*24, 31*). It would be of major interest to delineate the mechanism(s)—either up or down regulation—present in human hepatocytes but not in the hepatoma cell line, and likewise to understand why *P. vivax* undergoes complete development in the hepatoma cell line while *P. falciparum* does not.

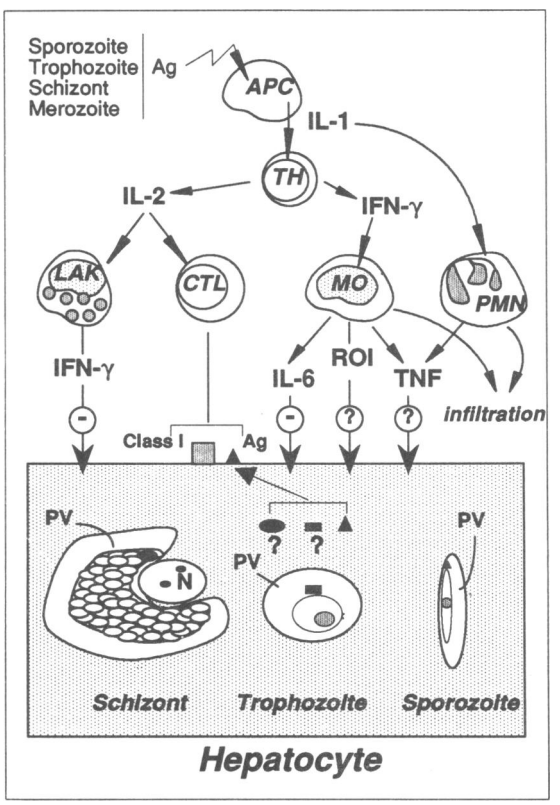
Problem of the model

Using *P. yoelii* sporozoites in different species of rodents, we have obtained evidence that the development of the hepatic stages depends on the species of rodent infected, and in a given species, on the strain. Interestingly, a good correlation exists between data obtained in culture and in infected animals. *In vivo* as well as *in vitro*, BALB/c mice are less susceptible than the C57BL/6 strain to *P. yoelii* sporozoite infection. Thus, at least in part, the hepatocytes themselves could play a role in receptivity. Among the possible mechanisms, we believe that non-specific factors may be involved. Wistar rats are *in vivo* significantly less susceptible to *P. yoelii* sporozoites than mice. This could be related to their constitutively high expression of serum CRP. Like their development, the sensitivity of hepatic stages to modulatory factors depends on the species of rodent infected. For instance, depending on the hepatocyte species used, a given MAb will cause inhibition or enhancement of the same inoculum of *P. yoelii* sporozoites (*4*). Likewise, the dose of radiation necessary to totally block passage of *P. yoelii* sporozoites from the uninuclear form differs for each species of hepatocyte (*32*). These observations underline the classical difficulties in interpreting results obtained under different experimental conditions and extrapolating from animal models to human malaria.

Conclusions

Fig. 1 and 2 attempt to summarize and integrate data recently obtained in the field of humoral and cellular immunity. Several potentially important interactions were wilfully excluded. These figures illustrate the privileged status of malaria parasite hepatic stages since they can be modulated in a number of ways, not only by the sporozoite but also by the erythrocytic stages. It should be emphasized that (1) a single molecule can exert opposite effects, depending on the amount released and its site of production; (2) cytokines can amplify uncertain phenomena, in an autocrine or paracrine manner; and (3) cytokines can also be produced in cascades, thus conferring to the system a further degree of complexity.

Fig. 2. **Cellular immunity.** IL-1 released by antigen-presenting cells (APC) activates polymorphonuclear cells (PMN) and a subset of helper T cells (TH) which will secrete IL-2 and interferon-γ (IFN-γ). Il-2 activates lymphokine-activated killer cells (LAK) and cytotoxic cells (CTL), whereas IFN-γ activates macrophages (MO) which in turn secrete IL-6. Some mechanisms have been clearly demonstrated to be interfering effectively with hepatic development of the plasmodium: phagocytosis following infiltration by polymorphonuclear cells and macrophages (*33, 34*); lysis and/or inhibition of parasite development by IFN-(γ) (*12, 14*). Unlike IL-1 (*14*), we found that IL-6 had a direct effect on parasite development. The role of reactive oxygen intermediates (ROI) is questionable and the function of tumour necrosis factor (TNF) has recently been demonstrated (*35*). Concerning the effect of CTL, we have obtained evidence of cytotoxicity directed against the hepatic stage, induced by an epitope of the non-repeated part of the CS protein of *P. yoelii*.



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