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INHIBITORY ACTIVITY OF INTERFERONS AND INTERLEUKIN 1 ON THE DEVELOPMENT OF *Plasmodium falciparum* IN HUMAN HEPATOCYTE CULTURES[1]

SYLVIE MELLOUK,* RADHA K. MAHESHWARI,[2†] ALICE RHODES-FEUILLETTE,[‡] RICHARD L. BEAUDOIN,[§] NATHALIE BERBIGUIER,* HUGHES MATILE,[‖] FRANCOIS MILTGEN,[#] IRENE LANDAU,[#] SYLVIANNE PIED,* JEAN PAUL CHIGOT,** ROBERT M. FRIEDMAN,[†] AND DOMINIQUE MAZIER*

*From the *Departement de Parasitologie et Medecine Tropicale/INSERM U 313 (Pr M Gentilini) and **Service de Chirurgie, Groupe Hospitalier Pitie-Salpetriere, Paris, France; the †Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; the ‡Laboratoire de Recherches sur l'Immunomodulation, Hôpital Saint-Louis, Paris, France; the §Malaria Division, Naval Medical Research Institute, Bethesda, MD 20814; ‖ Hoffman-LaRoche, Basel, Switzerland; and the #Museum National d'Histoire Naturelle, Paris, France*

We have studied the effect of natural and recombinant human interferons (HuIFN-α, -β, and -γ), and interleukin 1 (IL-1) on development of sporozoites of *Plasmodium falciparum* in cultures of functional hepatocytes. HuIFN-γ inhibits hepatic schizogony of *P. falciparum* at very low concentrations (0.1 to 10 international units/ml), the target being the hepatocyte. Application after sporozoite inoculation is effective, suggesting an intracellular mechanism. There is also an 84% inhibition after application from 4 to 6 days following inoculation so that by day 6, there was a disappearance of a significant number of schizonts previously present at day 4, indicating more than a parasitostatic effect, and probably a postassembly action. HuIFN-α and -β were effective, but only at 1000-fold higher concentrations than HuIFN-γ. IL-1 (5 U/ml) also inhibited hepatic development of *P. falciparum* sporozoites; however, IL-1 treatment was effective only when applied before sporozoite inoculation.

Immunization of man with irradiated sporozoites of *Plasmodium falciparum* stimulates a strong protective response (1, 2), but the mechanism of this protection is not yet clearly understood. It has been suggested that antibodies directed against the repeat region of the circumsporozoite antigen were the responsible effector of the protective immunity. The inhibitory effect of these antibodies has been unequivocally demonstrated in vitro (3–5); however, in primary cultures of human hepatocytes, the inhibition of sporozoite penetration by antibody was rarely complete, regardless of the concentration of antibody used or its origin (4, 6). T cells are also required for protection against sporozoite-induced infections as demonstrated in the mouse model (7). A T cell effector mechanism may operate through the release of interferon (IFN)[3]-γ. Indeed, there was a delay in the onset and level of parasitemia, when human IFN-γ (HuIFN-α) (0.25 mg/kg/day) was administered to chimpanzees before challenge with *Plasmodium vivax* (8). Furthermore, prophylactic treatment with HuIFN-γ (0.01 to 0.1 mg/kg/day) inhibited experimental infections with *Plasmodium cynomolgi* following sporozoite inoculation into *Macaca mulatta* (9). The latter study indicated that the effect of the IFN was on the pre-erythrocytic stage of parasite development (9). Since inducers of IFN-α and -β also reduced the severity of sporozoite-initiated *Plasmodium berghei* infection in mice (10), other cytokines may be involved in such protective mechanisms. These studies have led us to investigate a convenient in vitro model to better pinpoint the mechanism of cytokine effects on malaria parasites in primary cultures of human and rat hepatocytes infected by *P. falciparum* and *Plasmodium yoelii* sporozoites (11, 12). HuIFN-α, -β, or -γ and interleukin 1 (IL-1) were applied to hepatocyte cultures at various times before and/or after sporozoite inoculation. The results indicated a dose-dependent, potent inhibitory effect of HuIFN or IL-1 on sporozoite development in the cultured human hepatocytes.

MATERIALS AND METHODS

Hepatocyte cultures. Metabolically active human and rat hepatocytes were prepared using enzymatic perfusion as previously described (11, 12). Briefly, 10^5 cells were seeded in eight-chamber plastic Lab-tek slides (Miles Research, Elkhart, IN) in minimal essential medium supplemented with 10% fetal bovine serum and allowed to attach for about 15 hr in an atmosphere of 5% CO_2 and 95% air. The cells were maintained for 24 to 48 hr before inoculation of sporozoites. The hepatocyte preparations used in seeding cultures were depleted of Kupffer cells and macrophages by differential centrifugation and by cultivation in medium containing 7×10^{-5} M hydrocortisone hemisuccinate.

Cytokines. Recombinant (rHuIFN-α)HuIFNγ was kindly provided

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[2] Please address all correspondence and requests for reprints to Dr. Radha K. Maheshwari, Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814.

[3] Abbreviations used in this paper: IFN, interferon; HuIFN, human interferon; rHuIFN, recombinant human interferon.

by C. Czarniecki, Genentech, Inc., South San Francisco, CA, and had a specific activity of at least 2×10^7 U/mg of protein. Affinity-purified HuIFN-γ (specific activity 5×10^6 U/mg protein) was purchased from Interferon Sciences, Inc., New Brunswick, NJ. Semi-purified HuIFN-γ obtained by gel filtration followed by polyethylene glycol concentration, had a specific activity of 10^6 U/mg protein. The results obtained with all three HuIFN-γ preparation were similar. Ultrapure natural HuIFN-α was from Interferon Sciences. rHuIFN-β was kindly provided by Schering-Plough (Bloomfield, NJ). The specific activities of both were 5×10^6 U/mg protein. Natural affinity-purified HuIFN-β was kindly provided by Cytotech (Lausanne, Switzerland); it had a specific activity of 5×10^6 U/mg protein. Both HuIFN-β preparations gave similar results. Human IL-1, both natural and recombinant, were purchased from Cistron Technology, Pine Brook, NJ, and contained 200 and 500 half maximal units as determined in the thymocyte proliferation assay.

Controls included specific antibody neutralization of the IFN and IL-1. These used affinity-purified rabbit polyclonal antibody to HuIFN-γ (purchased from Interferon Sciences), monoclonal antibody to HuIFN-γ (kindly provided by Roussel UCLAF), antibodies directed against IFN-α and -β (kindly provided by the Pasteur Institute, Paris, and Cytotech, respectively), and polyclonal rabbit antihuman IL-1 (purchased from Cistron Technology). All neutralizations were carried out in the presence of an excess of antibody by 1 hr incubation at 37°C followed by 17 hr at 4°C.

Sporozoites. Sporozoites of the NF54 strain of *P. falciparum* were obtained from *Anopheles stephensi* after membrane feeding on cultures of blood-containing gametocytes. Sporozoites of *P. yoelii*, strain 265 BY were extracted from *A. stephensi* 16 to 21 days after the mosquitoes received an infective mouse blood meal.

Infection of hepatocytes. Human hepatocytes were infected with either *P. falciparum* or *P. yoelii* sporozoites, whereas rat hepatocytes were inoculated with *P. yoelii* sporozoites (4). Briefly, salivary glands were aseptically dissected and homogenized. After removal of medium from the culture chambers, 5×10^4 sporozoites were added in 50 μl of fresh medium. Three hours after inoculation, medium containing the suspended sporozoites was removed and replaced once more by 300 μl of fresh medium, with or without dissolved cytokines. Medium was then changed daily.

Cultures were examined using an immunofluorescence assay (4). The number of *P. falciparum* sporozoites invading liver cells was determined at 48 hr postinoculation. The number of sporozoites developing into schizonts was determined at day 6 for *P. falciparum*, and at day 2 for *P. yoelii*, as previously described (4, 12). This was done in duplicate or triplicate cultures. The percent inhibition was then calculated by comparing the number of schizonts in the experimental cultures with that in controls.

RESULTS

Effect of HuIFN preparations. Application of 10 international units/ml of rHuIFNγ (0.5 ng/ml) 18 or 4 hr before sporozoite inoculation led to a 100% inhibition of *P. falciparum* schizogony in human hepatocyte cell cultures at 6 days after challenge (Fig. 1, *bars 1* and *3*). A strong effect was likewise observed at lower concentrations of the cytokine although the inhibition was not complete (0.1 U/ml, 74 ± 8 % inhibition; 1 U/ml, 90 ± 4 % inhibition). When natural purified HuIFN-γ was substituted for the recombinant form, equivalent inhibition resulted (data not shown). If rHuIFN-γ was added after sporozoite inoculation, potent inhibition was observed (Fig. 1, *bars 4* to *6*) in contrast to the near absence of any effect when the cytokine was only present prior to sporozoite inoculation (*bar 2*). These results suggest a postpenetration cellular mechanism blocking malaria parasite development within the hepatocytes. Significant inhibition also occurred when rIFN-γ treatment was shorter or delayed: +3 hr to + 6 days; +3 hr to +3 days; or, +4 days to +6 days (*bars 4* to *6*).

Another important feature, observed at day 6, was the total disappearance of a significant number of schizonts previously present at day 4 (60 ± 6), when IFN was added (*bar 6*). It should be noted that no morphologically altered parasites were seen in IFN-treated cells and that surviving schizonts in partially inhibited cultures appeared normal in all respects. In contrast to its effect on the intracellular parasite, there was neither morphologic evidence of HuIFN-γ-induced damage to free sporozoites, nor a decrease in numbers found in treated compared with untreated controls. Moreover, the absence of morphologic evidence for direct toxicity to the free parasite is complemented by the lack of activity of 10 U/ml of HuIFN on *P. yoelii* development in rat hepatocytes; the same concentration completely inhibited development of this parasite in human liver cells. Compared to HuIFN-γ, 1000 fold-higher titers of natural and recombinant IFN-α or -β were required for schizont inhibition (data not shown). The specificity of the inhibitory activity by each species of IFN was demonstrated by neutralization experiments with the appropriate corresponding specific antibodies.

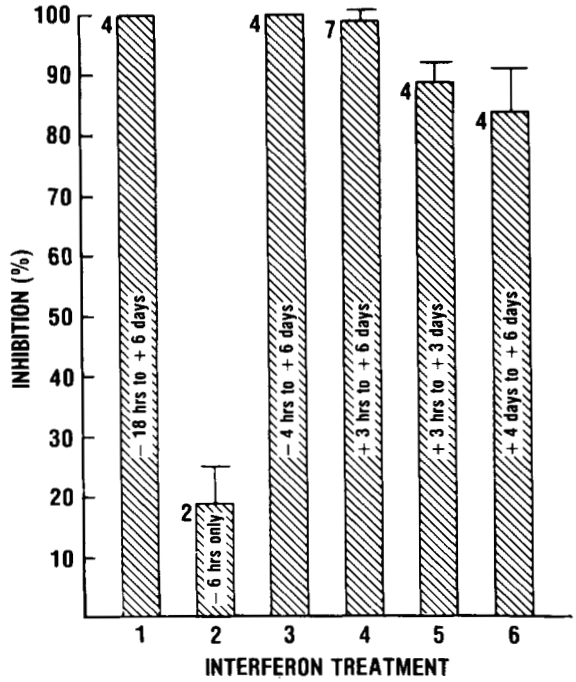

Figure 1. Time effect of rHuIFN-γ (10 U/ml) on P. falciparum schizont development in human hepatocytes. *Hatched bars* with standard errors represent the average inhibitory effect of rHuIFN (10 U/ml) compared with the untreated controls (60 schizonts ± 6/well). All cultures were evaluated at 6 days postinoculation except for those represented by *bar 2* which were evaluated at 48 hr. Time intervals shown within the bar indicate the period of time cultures were exposed to IFN with reference to the time of sporozoite inoculation. Numbers at the *top* of the bars refer to the number of experiments.

Effect of IL-1. Pretreatment of human hepatocytes with recombinant human IL-1 (5 U/ml) for 18 hr prior to introducing sporozoites led to a 97% inhibition of *P. falciparum* schizont development. When treatment was initiated 3 hr after sporozoite inoculation, or even at the time of the inoculation, no statistically significant inhibition was found (Table I). Similar results were obtained with natural, purified IL-1 (data not shown). Neutralization experiments demonstrated the specificity of the IL-1 activity. Similar results were obtained with IL-1 in rat hepatocytes inoculated with *P. yoelii* sporozoites (Table II).

DISCUSSION

Both HuIFN-γ and IL-1 strongly inhibited *P. falciparum* sporozoite development in primary cultures of functional

TABLE I

Dose response of P. falciparum in human hepatocyte cultures to recombinant human IL-1

Treatment (U/ml)	Cells Pretreated 18 hr Prior to Inoculation and throughout the Culture Period		Cells Only Treated at Time of Inoculation		Cells Treated 3 hr after Inoculation and throughout the Culture Period	
	Schizont No. (Mean ± SE)	Inhibition %	Schizont No. (Mean ± SE)	Inhibition %	Schizont No. (Mean ± SE)	Inhibition %
0	27 ± 2.5		58 ± 9		58 ± 9	
1	6 ± 5	77	ND[a]		59 ± 14	0
5	1 ± 0	96	54 ± 1	7[a]	59 ± 6.5	0
25	2 ± 0.5	93	37 ± 12.5	37[b]	53 ± 14	9[b]

[a] ND, not determined.
[b] Not statistically significant.

TABLE II

Dose-response of P. yoelii in cultures of rat hepatocytes to recombinant and natural IL-1

Treatment[a] (U/ml)	Schizont No. (Mean ± SE)	Inhibition %
0	114 ± 9	
IL-1 natural		
1	38 ± 3	66
5	7 ± 1	94
25	0	100
IL-1 recombinant		
1	33 ± 7	71
5	39 ± 2	65
25	10 ± 1	91

[a] Cultures were pretreated with IL-1 18 hr prior to sporozoite inoculation and until examined for schizonts 2 days later.

human hepatocytes, but their point of action differed. Time experiments with IFN-γ indicated a postpenetration (intrahepatocyte) cellular mechanism in contrast with IL-1 which exerted its effect on the very early phase of infection. Neither IFN nor IL-1 had a direct effect on extracellular sporozoites. The effects of HuIFN-γ on parasite development were similar whether or not exposure to this cytokine occurred while the parasite was undergoing rapid nuclear division. The late effect involved at least in part the destruction of already formed schizonts (Fig. 1, *bar 6*). The mechanism of IFN-γ activity on hepatic stages of malaria is therefore unusual, since not only was there inhibition of development, but also destruction of the parasite. This suggests a postassembly mode of action. Our hepatocyte cultures are not significantly contaminated by macrophages (Kupffer cells) or by natural killer cells. Thus, at least under our in vitro conditions IFN-γ activity appears neither to be mediated through activation of oxydative metabolism, nor to result from direct antiparasitic activity of these cells on infected hepatocytes.

These results are in basic agreement with those reported by Ferreira et al. (8) on the action of IFN-γ on *P. berghei* in cultures of the hepatoma cell line Hep-G2-A16. These authors postulated that the target of IFN activity was the hepatocyte itself. This hypothesis is also consistent with our results, since there was no activity of HuIFN-γ on *P. yoelii* infections of rodent hepatocytes in contrast to its pronounced activity on the same rodent parasite in human hepatocyte cultures. Our results likewise confirm the observation that some parasites escape the effect of the IFN-γ (13) as they have been shown to be able to evade antibodies (14, 15). In our system, however, this phenomenon appeared to be related to concentration and/or length of exposure. Nevertheless, our results do not preclude the proposed mechanism of differences in density of IFN-γ receptors on hepatocytes (15). IFN-α and -β were effective, but only at far higher titers than for IFN-γ. This result, however, does not rule out possible synergistic effects between these various species of IFN at lower concentrations.

Results of time experiments with IL-1 indicated a different point of action for this cytokine, since no activity against maturation of the parasite was observed. IL-1 activity could be related to induction of active substances by hepatocytes. Preliminary experiments with the C-reactive protein demonstrated a possible role for this acute phase reactant.

Longitudinal studies indicated that circulating IFN-γ is present several days after human infestation (16, 17), the first IFN detected being IFN-α (18). Endogenous IFN production therefore may be important in protecting against malaria reinfection during the months following an acute attack, when serum IFN is present. Moreover, other cytokines may also contribute to resistance to malaria challenge. IL-1 released by activated macrophages can not only activate T lymphocytes, resulting in a cascade effect of cytokine production, but also stimulate synthesis of C-reactive protein, a protein found in increased concentration in sera of malaria patients (19). It is possible that viable sporozoites inoculated during a challenge may trigger sensitized T cells to release protective levels of IFN-γ (8). Thus, immunogens and the means of presenting them to the host must be studied for ability to induce significant levels of lymphokines in the course of assessing them as viable vaccine candidates.

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