

CONCISE COMMUNICATION

***Plasmodium falciparum* Induces a Th1/Th2 Disequilibrium, Favoring the Th1-Type Pathway, in the Human Placenta**

Nadine Fievet,<sup>1,3</sup> Marlène Moussa,<sup>2</sup> Germaine Tami,<sup>3</sup> Bertrand Maubert,<sup>1,3,a</sup> Michel Cot,<sup>1</sup> Philippe Deloron,<sup>1</sup> and Gérard Chauat<sup>2</sup>

<sup>1</sup>Research Unit 10, Mother and Child Health in the Tropics, Institut de Recherche pour le Développement, Paris, and <sup>2</sup>INSERM U131, Antoine Beclere Hospital, Clamart, France; <sup>3</sup>Organisation de Coordination pour la Lutte contre les Endémies en Afrique Centrale, Yaounde, Cameroon

During pregnancy, a local and systemic Th2 bias of maternal immunity favors Th1-dependent infections such as malaria. This study measured cytokines secreted in cultures of chorionic villi, placental blood cells (PBC), and serum in term placentas from 88 malaria-infected and -noninfected Cameroon women. Interleukin (IL)-2 and -4 were consistently low; IL-1 $\beta$ , IL-6, granulocyte-macrophage colony-stimulating factor, and transforming growth factor (TGF)- $\beta$ 2 were highest in villi cultures. Tumor necrosis factor (TNF)- $\alpha$ , interferon (IFN)- $\gamma$ , and IL-10 were highest in PBC cultures. Malaria placental infection increased Th1-type cytokines, whereas Th2-type cytokines and TGF- $\beta$ 2 were unchanged. Addition of lipopolysaccharide or infected erythrocytes to cultures increased TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 secretions but not those of IFN- $\gamma$  and IL-4. Overall, *Plasmodium falciparum* induced a placental immune response involving both Th1- and Th2-type cell activation. Although the Th1 pathway was favored, IL-10 secretion was also increased, and this increase should be effective in protecting the placenta by controlling the negative effects of Th1 cytokines on pregnancy.

*Plasmodium falciparum* malaria during pregnancy is an important cause of maternal and infant morbidity and mortality [1], especially during first pregnancies. In pregnant women, *P. falciparum*-infected erythrocytes sequester in placental intervillous spaces. Histologically, infected placentas are characterized by the presence of parasites and leukocytes within intervillous spaces, pigment within macrophages, fibrin deposits, cytotrophoblastic cell proliferation, and trophoblastic basement membrane thickening [2]. Although the cytoadherence of *P. falciparum*-infected erythrocytes to chondroitin sulfate A and hyaluronic acid expressed on the syncytiotrophoblast surface [3, 4] may contribute to these phenomena, the development of a local inflammatory-like reaction [5] seems also to be involved.

Pregnancy requires a subtle tuning of systemic and local im-

mune responses [6] to enable the mother to have a foreign conceptus recognized by the production of maternal anti-paternal antibodies. The concomitant absence of an anti-paternal cytotoxic T lymphocyte response is viewed as a peripheral local split tolerance of fetal alloantigens. The mother retains an almost normal immune competence against a wide range of microorganisms, which is made possible by local immunoregulatory mechanisms exerted by a complex cytokine array at the fetomaternal interface. This may leave the mother more open to infections—mostly Th1 dependent. Conversely, excessive local production of Th1-type cytokines is detrimental to pregnancy [7, 8]. One explanation for increased malaria morbidity during pregnancy is that rejection of *Plasmodium* species is highly dependent on the Th1 response. We speculated that local deviation of maternal immune responses favors *P. falciparum* settlement in intervillous spaces and evaluated cytokine levels in placental serum samples and cytokine secretions in placental cells obtained from Cameroon women. Cytokine levels were compared by placental parasite infection and parity.

**Materials and Methods**

Women delivering at the maternity wards of Nkoldongo and Djoungolo, Yaounde, Cameroon, from June 1998 through January 1999 were enrolled in the study. After identification of an infected woman by a thick blood smear before delivery, the subsequent woman with a negative blood smear was enrolled. Term placentas were collected just after delivery. Thick blood smears from tissue sections of the maternal side of placentas were examined for the

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Informed consent was obtained from patients, and human experimentation guidelines of the Organisation de Coordination pour la Lutte contre les Endémies en Afrique Centrale were followed in the conduct of clinical research. The study was approved by the Cameroon national ethics committee.

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<sup>a</sup> Present affiliation: Hôpital de la Croix-Rousse, Service de Parasitologie et Pathologie Exotique, Lyon, France.

Reprints or correspondence: Dr. Philippe Deloron, IRD UR010, c/o INSERM U149, Hôpital Tenon, Service Gynécologie-Obstétrique, 4 rue de la Chine, 75020 Paris, France (pdeloron@club-internet.fr).

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presence of parasites, pigment within macrophages, or both, and women were categorized as infected or noninfected (no pigment and no parasite).

Placental blood was isolated after tissue incision, and serum samples were separated and were frozen at  $-80^{\circ}\text{C}$  until use. A quarter fragment of the placenta was separated and was placed in saline buffer containing 0.1% heparin and 2% penicillin-streptomycin. Placental tissues were rinsed and were flushed with 100 mL of 0.1% heparin saline buffer. The flushed placental blood cells (PBC) were isolated and were rinsed twice. Villi were isolated, minced, and rinsed. In parasitized placentas, PBC and/or placental villi contained infected erythrocytes (IEs) and/or pigment.

Villi were cultured with and without homologous PBC. A 2-g explant fragment of placental villi was cultured in 10 mL of RPMI-glutamax medium containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% sodium bicarbonate. Some flasks also had 200  $\mu\text{L}$  of homologous PBC pellet. One PBC sample was cultured separately for each villus in 10 mL of medium. Villi plus PBC samples were also cultured with IEs, noninfected erythrocytes (NIEs), or medium containing lipopolysaccharide (LPS; Sigma; final concentration, 10  $\mu\text{g}/\text{mL}$ ). IEs were obtained from an in vitro culture (5% parasite density) of the RP5 *P. falciparum* line (gift of J. Gysin; Laboratoire de Génétique et d'Immunologie, Marseille), which binds human trophoblasts via a chondroitin sulfate A-dependent mechanism [3]. Culture supernatants were harvested after 24 h, as preliminary studies demonstrated maximal cytokine expression after 24 h (data not shown). Each supernatant was centrifuged at 2000 g and was stored at  $-80^{\circ}\text{C}$  until use.

We measured the levels of Th1-type (interferon [IFN]- $\gamma$ , tumor necrosis factor [TNF]- $\alpha$ , and interleukin [IL]-2 and -1 $\beta$ ) and Th2-type cytokines (IL-4, IL-6, IL-10, and granulocyte-macrophage colony-stimulating factor [GM-CSF]) and transforming growth factor (TGF)- $\beta$ 2 in placental serum and placental cell culture supernatants. Serum cytokine concentrations represent the existing cytokine response (reflecting both their production and consumption), whereas cell cultures express the production capacity of the corresponding cells. Cytokine concentrations were measured by commercial ELISA kits (IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-1 $\beta$ , IL-4, IL-6, and IL-10, Pelikine [CLB]; GM-CSF, Immunotech [Beckman Coulter]; and TGF- $\beta$ 2, Quantikine [R&D Systems Europe]).

Differences between groups were analyzed by nonparametric methods (Mann-Whitney *U*, Wilcoxon signed rank, and Kruskal-Wallis tests). The significance limit was  $P = .05$ .

## Results

Placentas were obtained from 88 (33 primiparae and 55 multiparae) women. Of these, 49 were infected (29 with parasites and pigment, 10 with parasites alone, and 8 with pigment alone; 1 woman with *Plasmodium malariae* was excluded from analysis), and 39 were not.

A first set of cultures was done without stimulation. After 24 h, IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, GM-CSF, and TGF- $\beta$ 2 were present in all serum samples and cell culture supernatants (figure 1). Conversely, IL-4 levels were very low in all cultures but were higher in serum samples. IL-2 concentrations were measured only in villi cultures and serum, but levels were

always very low (data not shown). Of interest, the levels of IL-1 $\beta$ , IL-6, GM-CSF, and TGF- $\beta$ 2 were higher in cultures containing villi (villi and villi plus PBC) than in PBC alone and in serum (all  $P < .001$ ). In contrast, TNF- $\alpha$ , IFN- $\gamma$ , and IL-10 levels were highest in PBC cultures (all  $P < .001$ ). Th1-type cytokine levels were increased in samples from infected placentas (figure 1), and IFN- $\gamma$  levels in PBC cultures were higher in cases of placental infection ( $P = .002$ ). A similar trend was observed in villi cultures and serum (both  $P = .07$ ). Serum TNF- $\alpha$  concentrations were higher in infected placentas ( $P = .005$ ). All of these differences were particularly great when pigment was present (alone or with parasites). Conversely, levels of type 2 cytokines (IL-4, IL-6, and IL-10) and TGF- $\beta$ 2 did not differ. Among all cytokines investigated, only IL-10 levels varied with parity, being higher in villi plus PBC cultures from primiparae than in those from multiparae (10.4 vs. 5.8  $\text{pg}/\text{mL}$ ;  $P = .03$ ).

Similar cultures of villi plus PBC were made in the presence of LPS or *P. falciparum*-infected erythrocytes. After 24 h, levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 were highly increased in villi plus PBC cultures containing LPS (all  $P < .0001$ ) or IEs ( $P = .0001$  to  $.02$ ), although to a lesser extent (figure 2). Conversely, neither LPS nor IE stimulations allowed for increasing IFN- $\gamma$  or IL-4 secretions. The relative increase in cytokine production induced by IE (as the ratio in the presence of IE and its NIE control) was similar in placentas infected or not. Conversely, the relative increase in LPS-induced TNF- $\alpha$  production was lower in malaria-infected placentas (20.0 vs. 7.9;  $P = .03$ ). A similar trend was observed for IL-1 $\beta$  (5.7 vs. 3.3;  $P = .3$ ) and IL-10 (5.7 vs. 4.7;  $P = .6$ ).

## Discussion

During pregnancy, local and systemic deviation of the maternal immune response toward a Th2 bias may leave the mother more open to Th1-dependent infections, such as malaria, which offers a plausible explanation for the increased morbidity. We therefore determined cytokine secretion in cultures of chorionic villi, blood cells, and serum from term placentas from malaria-infected and -noninfected women. Malarial placental infection altered the cytokines balance, eliciting Th1-type cytokines at the placental level.

The lower concentrations of TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-10, and GM-CSF in serum than in tissues or PBC cultures suggest poor in vivo secretion or important uptake during labor. Given the involvement of proinflammatory cytokines in labor [9], the latter seems most likely. The higher TNF- $\alpha$ , IFN- $\gamma$ , and IL-10 concentrations in PBC than in villi cultures suggest that these cytokines are secreted mainly by PBC at parturition, confirming a previous report [10]. They are consumed by villi cells during culture (probably in vivo), as the addition of villi cells to PBC cultures highly decreased TNF- $\alpha$  concentrations and almost abolished those of IFN- $\gamma$  and IL-10. IL-10 is expressed at the

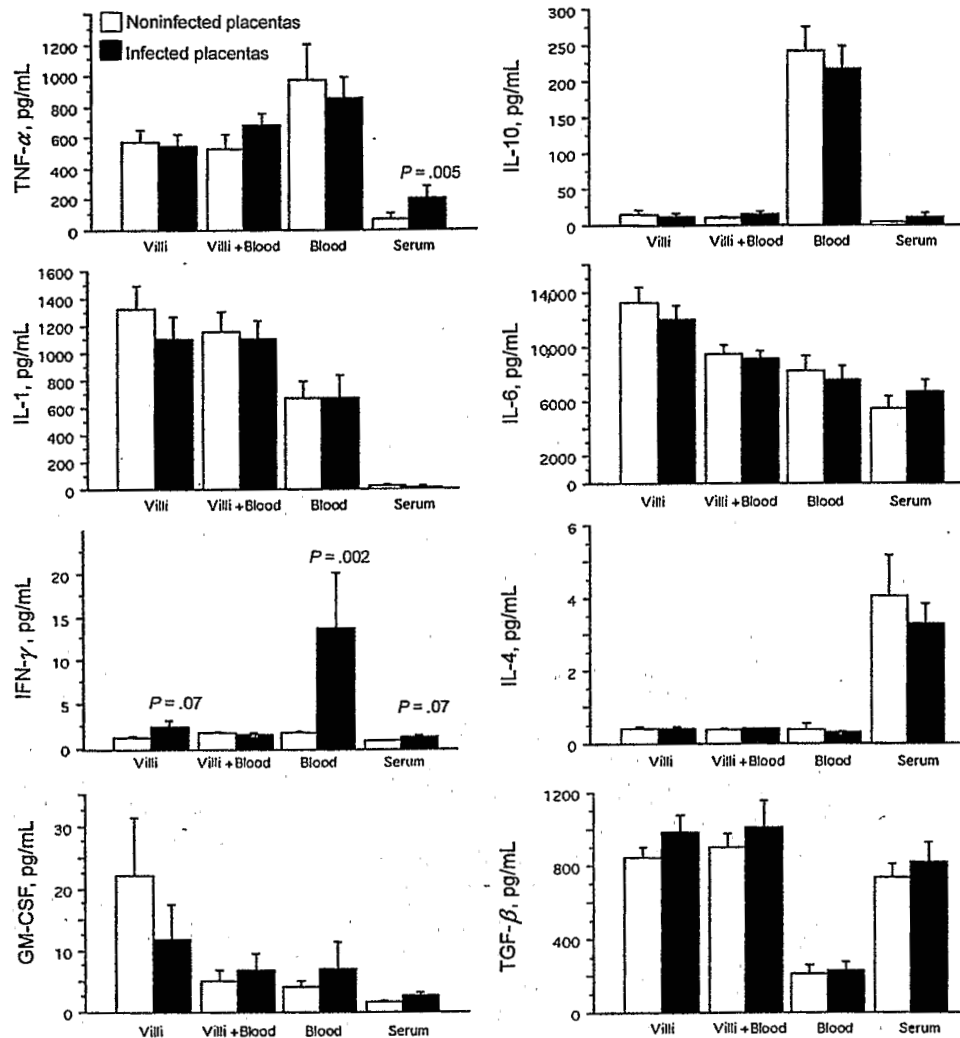
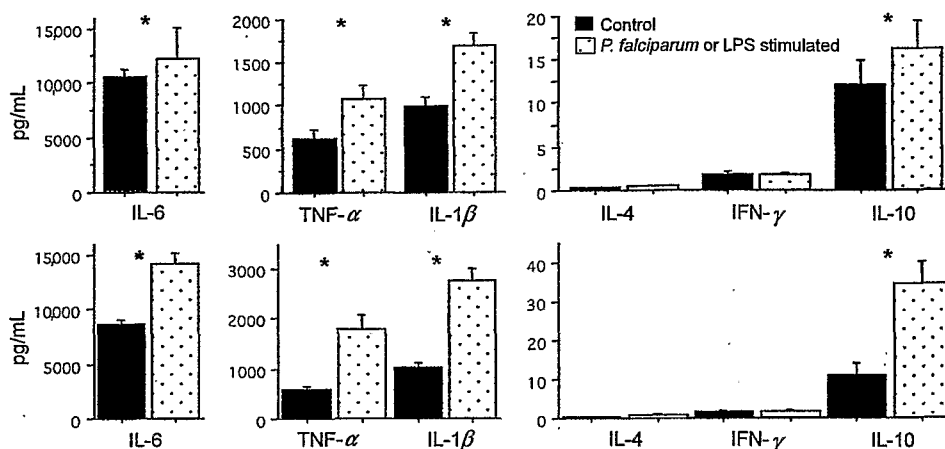


Figure 1. In vitro cytokine production by cultures of villi ( $n = 88$ ), villi and placental blood cells ( $n = 47$ ), placental blood cells ( $n = 38$ ), and placental serum ( $n = 54$ ) in placental samples from Cameroon women. Placentas were infected or not by *Plasmodium falciparum*. Data are mean + SEM. GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor.

fetomaternal interface, and its production by trophoblasts is partially regulated by GM-CSF [11], which suggests that IL-10 is produced mostly by PBC and is actively taken up by placental tissue. Conversely, IL-1 $\beta$ , IL-6, GM-CSF, and TGF- $\beta$ 2 are secreted mostly by villi cells, as their concentrations did not vary when PBC were added and were much lower in PBC cultures.

Of interest, malarial placental infection altered the balance of cytokines and elicited Th1-type cytokines at the placental level. Indeed, the concentrations of proinflammatory cytokines, such as TNF- $\alpha$  and IFN- $\gamma$ , were higher in placental serum samples from women with a *P. falciparum*-infected placenta. Villi and PBC cells from infected placentas also produced more

IFN- $\gamma$ . These levels were highest in samples from placentas containing pigment, indicating its role in triggering cells, most probably macrophages, to secrete these cytokines. This Th1-type shift is in line with a report of increased TNF- $\alpha$  mRNA expression in cells from infected placentas [12]. Another study [13] reported higher TNF- $\alpha$  serum levels in persons in malaria holoendemic areas than in nonendemic areas and in women infected with malaria. IL-10 concentrations were also decreased in placental serum samples from malaria-exposed women but did not differ with the placental infection status [13]. Although a decrease in IL-6 and TGF- $\beta$ 2 mRNA expression was observed in cells from malaria-infected placentas [12], Th2-type cytokines in placental blood were not modified in our study. Therefore,



**Figure 2.** In vitro cytokine production by villi with placental blood cell cultures ( $n = 30$ ) from Cameroon women. *Top*, Stimulation by erythrocytes infected or not with *Plasmodium falciparum*. *Bottom*, In presence or not of lipopolysaccharide (LPS). Data are mean + SEM. \*Significant differences (Wilcoxon rank sum test) are as follows: *Top*, Interleukin (IL)-6,  $P = .02$ ; tumor necrosis factor (TNF)- $\alpha$ ,  $P = .0003$ ; IL-1 $\beta$ ,  $P < .0001$ ; and IL-10,  $P = .0001$ . *Bottom*, IL-6,  $P < .0001$ ; TNF- $\alpha$ ,  $P = .0001$ ; IL-1 $\beta$ ,  $P < .0001$ ; and IL-10,  $P < .0001$ .

IL-10 may continue to play a role in modulating or promoting resolution of the inflammatory processes associated with labor and intrauterine infection-associated preterm labor [10].

Parity status did not modify cytokine concentrations in placental serum or in cell cultures, with the exception of IL-10, which was found in higher levels in villi plus PBC cultures from primiparae. This increased IL-10 production in primiparae, the group most sensitive to malaria [1], may be of importance to help buffer the increased potential Th1 stimulus of the parasite. Some differences in TNF- $\alpha$  and IFN- $\gamma$  placental serum levels have been reported by parity but with conflicting results [14].

The capacity of placental tissue to respond to stimulation was assessed in cultures of villi plus PBC containing LPS or *P. falciparum* IEs. LPS increased the production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 from 2- to 24-fold, demonstrating that culture conditions were not saturated. Conversely, LPS had no effect on IFN- $\gamma$  or IL-4 production. However, LPS induced only a very small increase in IFN- $\gamma$  secretion by chorionic villi cells [15]. Of importance, the smaller increase in TNF- $\alpha$  production after LPS stimulation in parasitized placentas suggests a parasite-induced impairment of the infected placenta's ability to produce TNF- $\alpha$ . This agrees with the lack of an LPS-induced increase in IFN- $\gamma$  and clearly confirms the Th1 pathway pregnancy-related inhibition. Stimulation of villi plus PBC cultures by *P. falciparum* elicited an increase in the production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10, but not of IFN- $\gamma$  or IL-4, even when placentas were infected by *P. falciparum*. This is similar to what was observed after stimulation with LPS, but the extent of the increase was lower. *P. falciparum* parasites induce an immune response at the placental level, and this response involves both Th1-type (mainly via TNF- $\alpha$ ) and Th2-type (mainly via IL-10) cell activation.

The timing of sampling imposes a bias, as it may not fully reflect the mother's immune status throughout the pregnancy or its evolution over time. However, in Africa, delivery is the only time for collection of placentas and for study of immune response at the fetomaternal interface. Thus, clinical events that occur during pregnancy are difficult to identify, and some women with no sign of placental infection (no parasites or pigment) may have been parasitized during pregnancy. However, such occurrences may bias our results only by decreasing the differences observed. Although *P. falciparum* clearly induces a Th1/Th2 disequilibrium favoring the Th1-type pathway, IL-10 is also increased and should remain effective in protecting the placenta by controlling the negative effects of Th1 cytokines. This is of utmost importance, as it allows the mother to keep nurturing and protecting the fetus. That numerous placentas remain infected at delivery suggests that this parasite-induced response is not effective enough to clear parasite infection from the placenta. That an additional increase in the Th1 response would be efficient in clearing infection is probable, but it would likely endanger the fetus, given the detrimental effects of TNF- $\alpha$  and IFN- $\gamma$  on pregnancy.

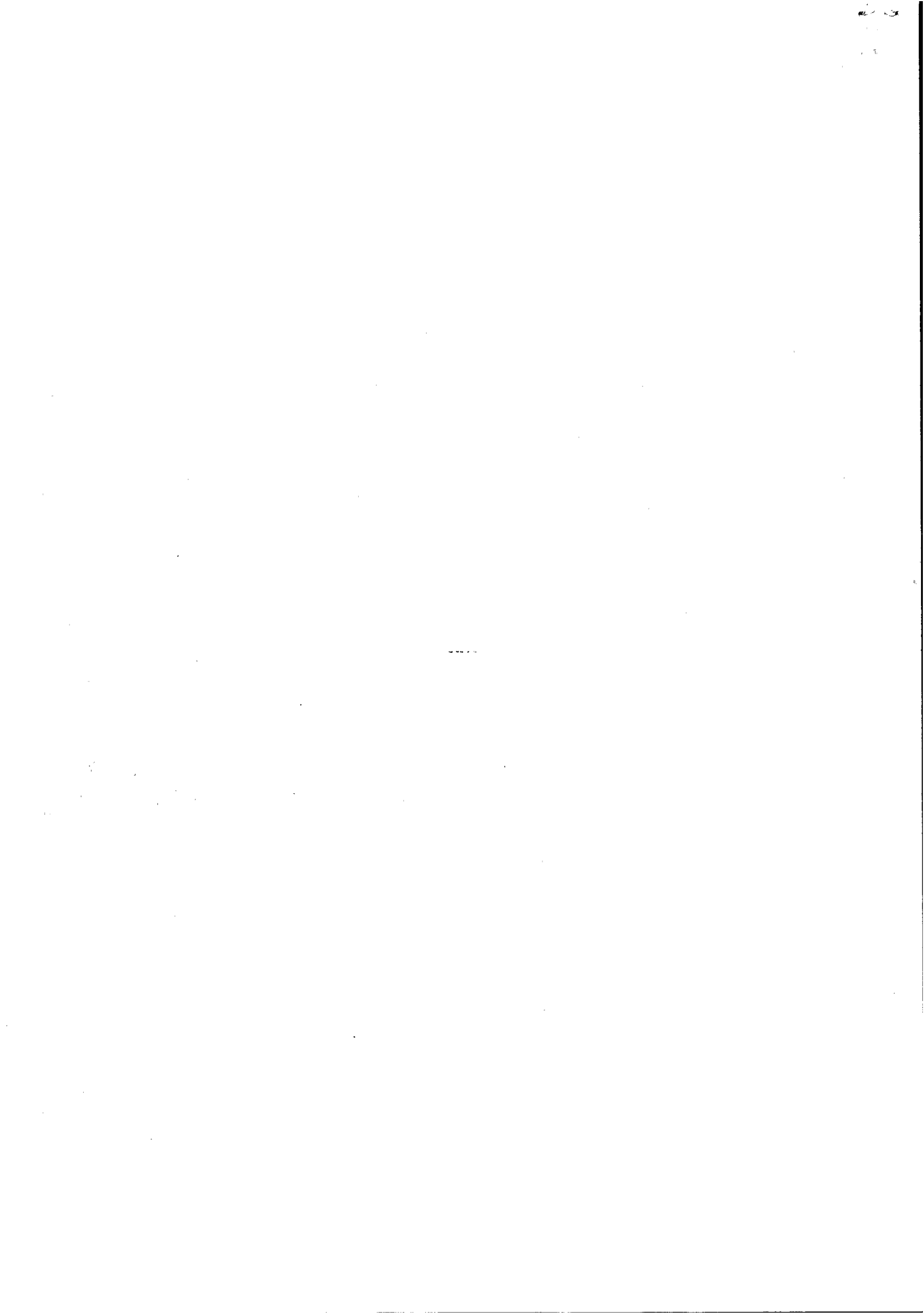
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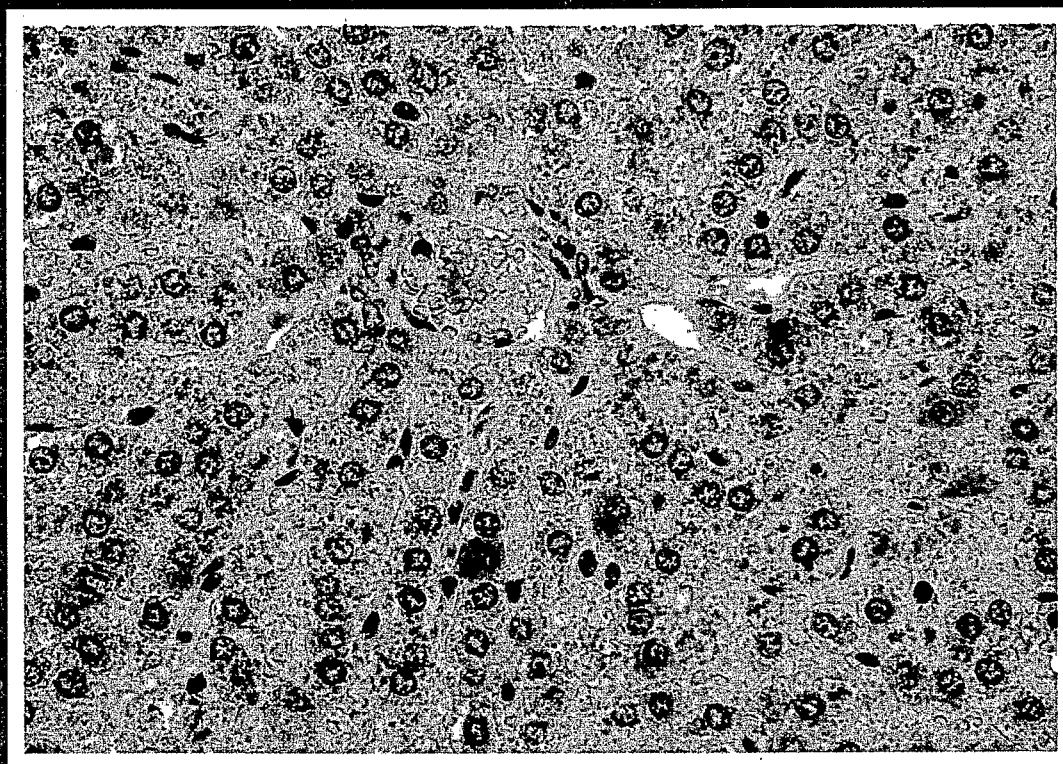
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