Mechanisms Underlying the Monocyte-mediated Antibody-dependent Killing of *Plasmodium falciparum* Asexual Blood Stages

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Summary

The relevance of the antibody-dependent cellular inhibition (ADCI) of *Plasmodium falciparum* to clinical protection has been previously established by in vitro studies of material obtained during passive transfer of protection by immunoglobulin G in humans. We here report further in vitro investigations aimed at elucidating the mechanisms underlying this ADCI effect. Results obtained so far suggest that (*a*) merozoite uptake by monocytes (MN) as well as by polymorphonuclear cells has little influence on the course of parasitemia; (*b*) the ADCI effect is mediated by a soluble factor released by MN; (*c*) this or these factors are able to block the division of surrounding intraerythrocytic parasites at the one nucleus stage; (*d*) the critical triggering antigen(s) targeted by effective Abs would appear to be associated with the surface of merozoites, as opposed to that of infected red blood cells; (*e*) the MN receptor for Abs effective in ADCI is apparently FcγRII, and not RI; (*f*) MN function is up- and down-regulated by interferon-γ and interleukin 4, respectively; and (*g*) of several potential mediators released by MN, only tumor necrosis factor (TNF) proved of relevance. The involvement of TNF in defense may explain the recently described increased frequency of the TNF-2 high-expression promoter in individuals living in endemic regions despite its compromising role in severe malaria.

The asexual blood stages (ABS)[1] of malaria parasites are the only stages responsible for the pathology of the disease. They are also the most accessible to investigation, because they are the only ones whose cultivation is easy. These stages have therefore been the subject of most of the studies of malaria aimed at understanding the basis of protective immunity for vaccination purposes. Based on passive transfer of Abs, there is considerable in vivo evidence that IgG constitutes an efficient arm of the immune system against erythrocytic stages in humans infected with *Plasmodium falciparum* (1–3), in South American monkeys infected with *P. falciparum* (4), and in a number of primate and rodent models (for review see reference 5). How these Abs act upon ABS, however, is far less clear. A number of hypotheses or beliefs have dominated the past decade, among which the inhibition of merozoite invasion is the most popular, together with a number of alternative hypotheses such as the inhibition of cytoadherence (thought to lead to the destruction of schizonts in the spleen), the inhibition of schizont-infected RBC (SIRBC) rosetting, the inhibition of merozoite dispersal, and opsonization of SIRBC.

Our initial observation that Abs from individuals with acquired protective immunity against ABS had no direct antiparasite effect in vitro led us to investigate other modes of action. We reported an Ab-dependent cellular cytotoxicity (ADCC)-like effect exerted by blood monocytes (MN), which was named Ab-dependent cellular inhibition (ADCI) to reflect the fact that the readout was an inhibition of parasite growth. The relevance of the MN-mediated ADCI observed in vitro was further validated by close in vivo/in vitro correlations: A strong ADCI effect could be elicited only by protective Abs, i.e., Abs whose clinical effect had been demonstrated by passive transfer in humans. Conversely, the preexisting Abs that proved clinically ineffective in the same individuals upon the same strains did not promote ADCI (6). The concept of Abs being effective by cooperation with effector cells was further supported by isotype studies showing a clear correlation between the ratio of cytophilic/noncytophilic Abs and the clinical status of protection (7).

In parallel with studies aimed at using this assay to identify protective antigen(s) (8), we have focused on the study of the ADCI mechanism itself. The present report summa-

[1] *Abbreviations used in this paper:* ABS, asexual blood stage; ADCC, antibody-dependent cellular cytotoxicity; ADCI, antibody-dependent cellular inhibition; BHA, butylated hydroxyanisole; CL, chemiluminescence; CLI, CL index; DCLI, delta CLI; FOR, free oxygen radical; HI-IgG, hyperimmune IgG; L-NMMA, N^G-monomethyl-L-arginine; MLI, maximum light intensity; MN, monocyte; N-IgG, control IgG; SIRBC, schizont-infected RBC; SGI, specific growth inhibitory index.

rizes these results and markedly advances our understanding of how cell–Ab cooperation acts in reducing *P. falciparum* intraerythrocytic replication. Results show that ADCI is dependent on IgG binding to MN via their FcγRII receptors, is triggered by a merozoite surface component, is mediated by soluble factors released by MN, and acts on young intraerythrocytic parasites.

Materials and Methods

Sera and IgG Preparations. The pool of hyperimmune (HI) African IgG, previously found to confer passive protection upon transfer (3), was purified from 180 pooled sera from protected individuals living in the Ivory Coast (6). In addition, 15 HI individual sera were collected from African adults living in a *P. falciparum*–endemic area of the Ivory Coast. These subjects are referred to as "protected" since they have reached a state of clinical immunity to malaria (9).

Control sera were obtained from healthy French adults with no history of malaria. For all individual sera, the IgG fraction was prepared using a GF05-Trisacryl column followed by a DEAE-Trisacryl column (Industrie Biologique Francaise, Villeneuve la Garenne, France). The IgG preparation was then extensively dialyzed against PBS, pH 7.4, for 48 h and finally against RPMI 1640 medium for 24 h.

Parasites and Culture. The *P. falciparum* African strains used were Uganda Palo Alto (FUP/C), NF54, and FCIP150 (8). Parasite blood stages were cultivated in RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% human serum (10). When required, parasites were synchronized by repeated sorbitol treatment (11). Schizonts were enriched by flotation on plasmagel (12).

Merozoite Preparation. Merozoites were purified according to the method described by Mrema (13). Highly synchronized schizonts were concentrated on plasmagel and resuspended at 0.5% hematocrit in RPMI 1640 10% human serum. Every 4 h the supernatant was collected after centrifugation of the culture at 300 *g* for 3 min. The pellet was cultivated again with fresh medium for another 4 h, while the supernatant was submitted to a second centrifugation at 1,200 *g* for 3 min and then passed through negatively charged nylon membranes (Versapor; Gelman Sciences, Inc., Ann Arbor, MI). Merozoites obtained after Versapor filtration were centrifuged at 3,000 *g* for 20 min and resuspended in RPMI 1640. For most experiments, this suspension was supplemented with 10% DMSO (Sigma Chemical Co., St. Louis, MO) and cryopreserved in liquid nitrogen, after it was established in parallel experiments that fresh and cryopreserved preparations had a similar effect in our assays.

Preparation of Effector Cells. Blood MN and polymorphonuclear cells (PMN) were prepared from healthy donors with no history of malaria. Mononuclear cells were separated on Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradients (14). The number of MN in the cell suspension (obtained from the upper phase of the gradient) was estimated by the nonspecific esterase stain (15). MN were then isolated by adherence to plastic in 96-well plates (Nunc, Roskilde, Denmark) as described (6). The lower phase of the gradient was used to prepare PMN. RBC were separated by sedimentation using a dextran solution: 10 ml of cell suspension in Hanks' buffer (GIBCO BRL) mixed with 3 ml of Dextran T 500 (Pharmacia LKB). After a 30-min incubation at 37°C, the supernatant was centrifuged at 200 *g* for 10 min at 4°C. The cell pellet was resuspended in 8.3% ammonium chloride solution and incubated for 10 min at 4°C, thereby lysing the remaining RBC. PMN were collected by centrifugation, washed twice, and resuspended in Hanks' buffer.

Treatment of MN with IFN-γ or IL-4. When indicated, MN were cultivated for 24 or 48 h in the presence of 100 IU/ml human rIFN-γ (supplied by Dr. Virelizier, Pasteur Institute, Paris) or for 48 h in the presence of 100 IU/ml IL-4 (Sigma Chemical Co.). IFN-γ and IL-4 were removed from the culture medium (RPMI 1640 plus 10% human serum) by several washings in RPMI 1640 before using the MN in the ADCI assay.

ADCI Assay. In the wells containing $\sim 2 \times 10^5$ adherent MN, a *P. falciparum*–synchronous culture (0.4–0.7% parasitemia) suspension in RPMI 1640 was added (100 μl/well, hematocrit 2.5%) so as to obtain a ratio of 200 RBC/1 MN. The culture medium (RPMI 1640 plus 10% human serum) was supplemented with either purified HI-IgG (at 2 mg/ml) or control IgG (N-IgG, at 1.2 mg/ml). These concentrations correspond to 10% of the initial IgG concentration in the donor's serum. The other control cultures were HI-IgG without MN and N-IgG without MN. When indicated, the culture medium was supplemented with one (or more) of the following reagents whose effect upon ADCI activity was assessed: 30 ng/ml rTNF-α (Rhône Poulenc, Paris, France); rabbit anti-TNF-α serum diluted 1:1,000 (gift from Dr. C. Rougeot, Pasteur Institute, Paris, France); rabbit anti-IL-6 serum diluted 1:100 (Seralab, Crawley Down, UK); mAb 197 directed to FcγRI or mAb 4.3 directed to FcγRII (both purchased from Medarex, Inc., West Lebanon, NH, and used at 20 μg/ml); goat anti-mouse Ig diluted 1:250 (Cappel, West Chester, UK); butylated hydroxyanisole (BHA) tested at 30 and 50 mM; butylated hydroxytoluene (30 and 50 mM); superoxide dismutase/catalase (6,000 and 12,000 IU/ml); 2.5 mM N^G-monomethyl-L-arginine (L-NMMA). In these cases control cultures were supplemented with the same reagents.

In each experiment the initial parasitemia was ~0.5% of synchronous schizonts and generally reached 5–12% after 96 h of culture. 50 μ of fresh culture medium was added to each well after 48 and 72 h.

The specific growth inhibitory index (SGI), which takes into account the possible inhibition induced by cells or Abs alone or in presence of test reagents, included as controls in each experiment, was calculated as follows: SGI = 100 × [1 − (percent parasitemia with MN and HI-IgG/percent parasitemia with HI-IgG)/(percent parasitemia with MN/percent parasitemia with N-IgG)].

It may be important to stress here that the MN function in ADCI was found to be dependent upon several subtle factors, such as the water used to prepare RPMI 1640. For example, high-purity Milli-Q water (Millipore Corp., Bedford, MA), although adequate for culturing *P. falciparum* blood stages, did not promote an ADCI effect by MN, whereas water containing traces of minerals such as double-distilled water or commercially available mineral water such as VOLVIC gave consistently good MN function. Improved and/or more consistent function of MN in ADCI could be obtained by coating the culture wells with fibronectin (i.e., coating with autologous plasma from the MN donor followed by washing with RPMI 1640). MN from subjects with a viral infection (e.g., influenza) were frequently able to induce some degree of nonspecific (non–IgG-dependent) inhibition of parasite growth, which prevented to some extent the observation of an additional IgG-dependent effect (however, this effect is taken into account by calculation of the SGI). Therefore, MN from donors suspected of having a viral infection, recovering from one, or who had a fever in the past 8 d were systematically avoided.

Two-step ADCI with Short-term Activation of MN. The two-step

ADCI was performed by (*a*) a short incubation of MN with Ig and parasites, followed by (*b*) the transfer of the supernatant to a standard *P. falciparum* erythrocytic culture without MN and without Abs. In some experiments this was done after 24 h of a standard ADCI assay performed as described above. In other experiments, adherent MN were cocultured in the presence or absence of HI-IgG with either purified *P. falciparum* merozoites at a ratio of five merozoites per MN, synchronized *P. falciparum* ring-infected RBC (at 10% parasitemia), or synchronized *P. falciparum* SIRBC (at 10% parasitemia). Supernatants were collected after 2 or 18 h of culture.

In all cases, supernatants were centrifuged at 2,000 rpm, filtered through 0.22-μm membranes (Millex-GV; Millipore, Guyancourt, France), and added immediately, at a concentration of 50% with fresh culture medium, to *P. falciparum*–asynchronous cultures (particular care was taken to reduce to a minimum the presence of leukocytes in the RBC preparation used for this second culture). Each supernatant was tested in duplicate. At 36 h of culture, 1 mCi of [^{3}H]hypoxanthine (Amersham International, Little Chalfont, UK) was added to each well. Cells were harvested after 48 h of culture, and ^{3}H uptake was estimated by counting in a liquid scintillation counter (Betaplate; LKB, Turku, Finland).

Phagocytosis Assay. This assay has been previously described in detail (16, 17). Briefly, 10^6 MN or PMN were allowed to adhere to 13-mm coverslips in multiwell culture plates for 1 h. 10^7 merozoites were then added to each well in the presence of N-IgG or HI-IgG at 2 mg/ml in RPMI 1640. After an incubation of 45 min at 37°C, the coverslips were washed twice, fixed in methanol, and Giemsa stained. The rate of phagocytosis was evaluated by counting the number of cells that had ingested one or more *P. falciparum* merozoites per 500 MN, with each test performed in duplicate.

Measurement of Free Oxygen Radical (FOR) Production. Levels of FOR production induced by merozoite uptake were estimated using a chemiluminescence (CL) assay (18, 19). 3×10^6 merozoites suspended in 10 μl of HBSS were transferred to a polystyrene counting tube containing 10 μl of either control serum (normal human AB serum) or test serum, and samples were incubated for 20 min at 20°C before the test. 3×10^5 of effector cells (MN or PMN) were transferred into the counting tubes containing either (*a*) 10 μl of the target merozoite–serum preparation; (*b*) 10 μl of HBSS alone as a CL-negative control; or (*c*) 10 μl of either latex or opsonized zymosan as positive CL controls. Tubes were incubated at 37°C with constant shaking at 120 rpm. After 10 min, 50 μl of the luminol test solution was added and the CL was measured in a photometer chamber (Biolumat LB 9500; Berthold, Widbad, Germany). For each experiment, two sera from healthy individuals and three sera from HI subjects were included as controls.

CL values were expressed as the maximum light intensity (MLI) expressed in relative light units. The CL index (CLI) was calculated as follows: CLI = [MLI (HI-IgG) − MLI (N-IgG)/MLI (HI-IgG)] × 100. The delta CLI (DCLI) is the difference between the CLI obtained in the presence of merozoites with HI serum and effector cells, and the CLI obtained in the presence of HI serum and effector cells alone.

Results

Possible Role of Phagocytosis of Merozoites in Reducing Parasite Growth. We have previously reported the existence of a close correlation at the individual level between clinical protection and the presence of Abs able to mediate the uptake of merozoites by phagocytic cells, measured either by microscopic examination (16, 17) or by CL assay (19). When examining the mechanism of ADCI, one goal was thus to estimate to what extent the phagocytosis of merozoites could play a role in the inhibition of parasite growth. Based on previous experiments suggesting that PMN were not involved in promoting ADCI but that MN were involved (20), we undertook a systematic comparative investigation of the role of MN and PMN in the ADCI mechanism, in phagocytosis of merozoites, and in FOR production. Purified IgG preparations from three HI individuals and from three controls were used in these assays.

For both cell types, antimalarial Abs promoted a significant increase of merozoite phagocytosis, as compared with N-IgG (Table 1). Because the background level of phagocytosis is consistently higher for PMN than for MN, this resulted in lower net uptake values for PMN. FOR production, which indirectly provides an estimate of macrophage phagocytic activity, was also significantly increased with HI-IgG. In this case FOR levels were usually higher for PMN than for MN.

However, in cell–Ab cooperation assays, only MN were able to influence the in vitro growth of *P. falciparum*. No significant difference was found when PMN and *P. falciparum* ABS were cultured with either N-IgG or HI-IgG. These results were surprising since parasite phagocytosis by MN as well as by PMN took place to a similar degree in the presence of HI-IgG. Merozoites, altered schizonts, and parasite debris were observed in the cytoplasm in both cases. The same clear-cut difference between MN and PMN behavior in ADCI was reproducible in independent experiments conducted using cells from six different donors. PMN were reproducibly ineffective with or without the addition of IFN-γ 24 h before the assay (results not shown). These results suggested that phagocytosis is unlikely to be the effector mechanism by which *P. falciparum* growth is reduced in ADCI assays.

ADCI Effect Is Mediated by a Soluble Factor. During the above experiments, we consistently observed the presence of numerous intraerythrocytically altered parasites in smears made at the end of operational ADCI assays (i.e., with MN and HI-IgG, but not with N-IgG or PMN). The microscopic observation of parasites revealed the presence of uninucleate, picnotic, condensed parasites that have lost their vacuole and therefore resemble the so-called "crisis forms" described by Talliafero (21). These modifications concerned only intraerythrocytic parasites, without host cell lysis. Considering the MN/RBC ratio of 1:200, effector cells were too scarce to be able to have a direct effect, i.e., by close contact with all damaged parasites. This suggested that the observed parasite alterations may be due to the release of mediators able to act at a distance from MN.

To address this hypothesis, ADCI was performed in two successive steps in two different culture vials. In the first step, parasitized RBC rich in maturing schizonts were incubated with MN and HI-IgG, and supernatants were collected at 24 h and filtered. In the second step, these supernatants were tested at dilutions of 1:2 to 1:16 in fresh complete culture medium for their effect upon *P. falciparum*–asynchronous cul-

Table 1. *Comparative Assessment of the Ability of MN and PMN to Mediate ADCI and Merozoite Phagocytosis, as Measured by Microscopy and by CL*

	Monocytes		Polymorphonuclears	
	HI-IgG	N-IgG	HI-IgG	N-IgG
Merozoite phagocytosis (%) (range)	34.3 (23.4–36.7)	5.2 (4.5–5.9)	32.7 (27.7–36.4)	10.4 (8.1–13.7)
DCLI (range)	43.5 (20–58)	1.3 (8.2–12.7)	136.3 (98–159)	19.2 (14–21)
ADCI				
Parasitemia	1.05 (±0.45)	4.2 (±0.3)	4.1 (±0.8)	3.8 (±0.5)
SGI	63% (±4)	–	3% (±2)	–

Phagocytosis of merozoites by each cell type in the presence of purified IgG from either three HI-IgG or three N-IgG individuals is expressed as the percentage of cells containing at least one merozoite in their cytoplasm (merozoite phagocytosis) or as the specific increase in CL of FOR (DCLI, expressed in arbitrary units). The range of values obtained with each IgG preparation is indicated in brackets. For the ADCI experiments, the results are expressed as the final parasitemia obtained after 96 h of culture and as the SGI, calculated as indicated in Materials and Methods. All values represent the mean ± SD obtained with three distinct IgG preparations.

tures, in the absence of MN. Parasite growth was assessed both by analysis of thin smears at 24 and 48 h of culture and by inhibition of [^{3}H]hypoxanthine uptake.

These assays showed a concentration-dependent inhibition of parasite growth and of [^{3}H]hypoxanthine uptake with ADCI assay supernatants (50–15% inhibition at 1/2 to 1/8 dilution) and resulted in the appearance of picnotic intraerythrocytic parasites. Control supernatants from ADCI performed with MN alone, with MN and N-IgG, or with HI-IgG alone were not inhibitory (results not shown). The above experiments established that direct contact between parasitized RBC and MN was not required, and they suggested that the binding of some malarial antigen(s) to antimalarial Abs could trigger MN to release a mediator able to block *P. falciparum* intraerythrocytic development.

MN Are Triggered by Free Merozoites, Not by Infected RBC. To assess which stage of the intraerythrocytic cycle carried the critical antigen(s) necessary to trigger MN via IgG, the above two-step method was used. Short-term MN stimulation assays (2 h) were performed using either infected erythrocytes from highly synchronized cultures (NF54 strain) at different times of parasite maturation or free merozoites. The effect of the resulting supernatants upon *P. falciparum* in vitro growth was then assessed by incorporation of [^{3}H]hypoxanthine.

No significant inhibitory effect was found in supernatants from MN cultures stimulated with either fully mature schizonts and HI-IgG for 2 h or ring-infected RBC and HI-IgG for 2 (not shown) or 18 h (Fig. 1). In contrast, supernatants from MN incubated for 18 h with HI-IgG and mature SIRBC contained a mediator able to markedly inhibit *P. falciparum* cultures. This time period allows schizont rupture, the release of merozoites, and their penetration into new RBC (as confirmed microscopically at the end of the experiment). These results pointed toward the merozoites, or other products released by rupturing schizonts, as the critical stage in triggering ADCI. To test this hypothesis, supernatants were recovered from 2-h cultures with HI-IgG and purified free merozoites. These proved to be strongly inhibitory (Fig. 1). Supernatants resulting from stimulation of MN with either freshly prepared or cryopreserved merozoites had similar inhibitory activity. Merozoites from two additional parasite strains (FUP/C and FCIP150) were also found to be effective. These results suggested that merozoite surface antigens (or molecules contaminating our merozoite preparations) were the critical antigens triggering the ADCI effect.

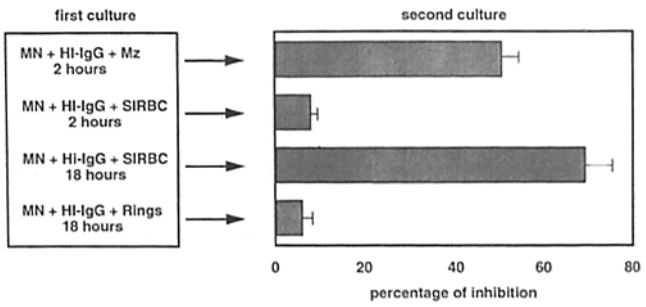

Figure 1. Results from the two-step ADCI assay. As a first step, MN were cultured for either 2 or 18 h with different developmental stages of *P. falciparum* in the presence of African IgG. In the second step, supernatants were transferred to an asynchronous *P. falciparum* culture without Abs and without MN. As shown, only the supernatants from MN cultures (*MN*) triggered by merozoites for a short time (2-h incubations) or rupturing schizonts (18-h incubations) in the presence of purified HI-IgGs are able to inhibit *P. falciparum* growth. In contrast, supernatants of MN cultured in the presence of rings or nonrupturing schizonts (SIRBC 2-h incubations) have no effect on parasite growth. Values represent the mean ± SD from two separate experiments performed in duplicate. *Mz*, merozoite.

ADCI Affects the Uninucleate Stage of the Erythrocytic Cycle. To analyze the effect of the soluble mediator released by the merozoite- and IgG-stimulated MN, synchronized cultures were started at ring stage with MN and either N-IgG or HI-IgG, and the relative proportion of each stage and of picnotic forms was determined every 24 h for 72 h. Purified merozoites (unable to reinvade a RBC) were added to these cultures at a ratio of 10 merozoites per MN at day 0 to induce the ADCI effect rapidly, before schizont rupture.

The low or nonrelevance of merozoite phagocytosis to parasite growth reduction in ADCI was supported both by the differential behavior of PMN versus MN in phagocytosis and ADCI assays and by the effect of stimulated MN supernatants, which obviously rules out merozoite removal as an important effector. This may sound surprising in view of the relatively high rates of merozoite uptake observed with both cells in the presence of Abs; however, phagocytosis was performed using purified merozoite suspensions without RBC and was expressed as the number of cells having phagocy-

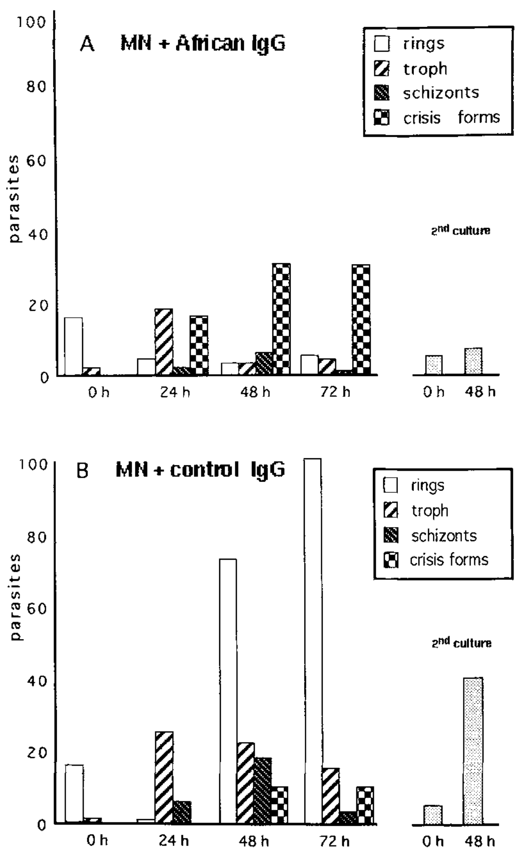

Figure 2. Progressive accumulation of uninucleate picnotic parasites during the course of an ADCI assay. (*A*) HI-IgG; (*B*) N-IgG. Differential counts of rings, trophozoites, schizonts, and crisis forms are shown (expressed as percent relative to the number of ring forms) in one ADCI assay performed with MN and either N-IgG or HI-IgG, and in which the ADCI effect was initially triggered by the early addition of purified merozoites (at the start of the culture). The smaller graph on the right-hand side represents the growth over 48 h of parasites collected at the end of the ADCI assay, washed, and then allowed to grow in normal culture medium (without MN or IgG).

the maturation of rings up to trophozoite stage was not markedly inhibited. However, there was a blocking effect at the late trophozoite stage with the accumulation of damaged uninucleate parasites. Of the few remaining schizonts seen at 48 h, some completed their maturation and released reinvading merozoites (see the proportion of schizonts at 48 h and of new rings at 72 h). Thus, over 72 h of culture there was a progressive accumulation of parasites at the uninucleate stage, i.e., altered trophozoites, and, in contrast, a seemingly more limited effect upon parasites that had reached the multinucleate stage. The accumulation of picnotic parasites was reproducibly observed at the completion of all ADCI assays.

In these experiments, the majority of, though not all, parasites submitted to the IgG-dependent MN effect were picnotic. We thus addressed the question of whether the ADCI effect could be reversed, i.e., if the remaining parasites were still viable, or capable of growing when the mediator was removed. Infected RBC were collected in 72-h ADCI culture wells by gently resuspending them and were subcultured in fresh medium with fresh RBC, without MN. Although the growth rate was higher using control parasites collected in the N-IgG ADCI assay, Fig. 2 shows that at least some parasites remaining in the HI-IgG ADCI assay were still viable and could keep growing, albeit at a slower rate. This suggested that the mediator(s) effect is reversible, or, alternatively, that all parasites or all stages (see above) are not equally susceptible to its effect.

FcγRII Is the MN Receptor Involved in ADCI. ADCC mechanisms imply that the Ig Fc fragment should bind to the MN. The ability of a given Fc receptor to be bound by IgG depends on the isotype of Ig involved and on the type of Fcγ receptors expressed by the effector cell. Previous studies have shown a correlation between the acquisition of antimalarial immunity, the results from ADCI, and the occurrence of increased levels of IgG3 and, to a lesser extent, IgG1, two isotypes known to be cytophilic (7). Among the three known Fcγ receptors, MN express only FcγRI and FcγRII. However, FcγRI has been more frequently implicated in ADCC mechanisms against nonplasmodial targets (22).

We used two mAbs, designated 197 and 4.3, which were able to bind to FcγRI and FcγRII, respectively, and which had a known ability to block, or compete with, the attachment of the Fc fragment to the corresponding receptor (23, 24). These mAbs were not toxic for the culture. The use of these mAbs in ADCI clearly implicated FcγRII as the main receptor involved (Fig. 3). ADCI was fully reversed by the addition of anti-FcγRII, whereas FcγRI does not seem to play any role. The evidence for this is twofold: (*a*) in ADCI assays the addition of anti-FcγRI was ineffective; and (*b*) no inhibition of parasite growth was obtained in direct stimulation assays of FcγRI using the mAb cross-linked by a second anti-mouse Ab, a procedure known to trigger the MN (Ravetch, J., personal communication).

Modulation of MN Function by IL-4 and IFN-γ. Human MN were first cultured alone for 12, 24, or 48 h in medium containing either IFN-γ or IL-4, two cytokines known to have up- and down-regulating effects, respectively, upon the

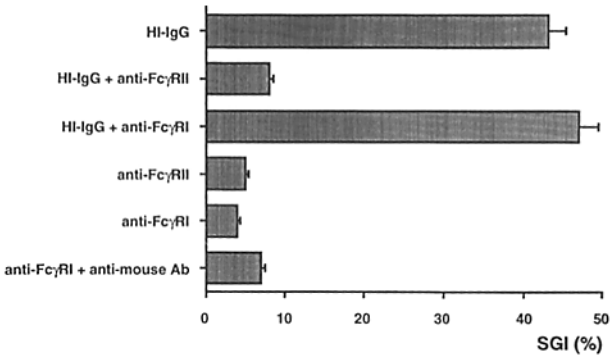

Figure 3. Reversal of the positive ADCI effect obtained with African IgG by an mAb directed against FcγRII (mAb 4.3) and having a blocking effect against this receptor. In contrast, an mAb against the FcγRI receptor (mAb 197) proved to be unable to reverse the ADCI effect. Neither mAb had any direct effect upon parasite growth. When the mAb 19.7 was further incubated with polyclonal anti-mouse Abs, a procedure known to activate the FcγRI transduction pathway, no ADCI effect was recorded. Values shown represent the mean ± SD from three separate experiments.

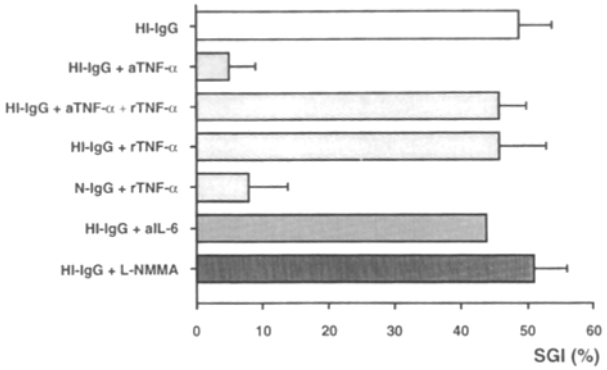

Figure 4. Identification of TNF-α as one of the soluble mediators of ADCI. Polyclonal rabbit Abs against TNF-α (known to block the biological activity of this cytokine) were able to suppress the ADCI obtained with African IgG (in the presence of MN) when added at a 1:1,000 final dilution. This suppression could be reversed by adding human rTNF-α at a 30-ng/ml concentration. However, the same concentration of rTNF-α had no direct effect upon *P. falciparum* growth either with HI-IgG (*HI-IgG + rTNF-α*) or without (*N-IgG + rTNF-α*). Values represent the mean ± SD from three separate experiments. Polyclonal rabbit Abs specific for IL-6 and able to block its biological effect did not suppress ADCI. Similarly, the arginine analogue L-NMMA was also ineffective. Values represent the mean ± SD from two experiments, except in the case of IL-6, where only one experiment was quantified.

expression of Fcγ receptors on MN (25). These MN were thereafter used in standard 72-h ADCI assays. In several independent assays, IFN-γ–treated MN were found to exert an increased ADCI effect as compared with nontreated cells. The increase, which was in the order of 15–30%, was observed in 24- and 48-h samples, though not in 12-h samples, with no clear-cut difference between 24 and 48 h. In contrast, IL-4–treated MN were no longer able to mediate ADCI, whereas control MN from the same donor, incubated in control medium for the same duration, were able to mediate ADCI (results not shown). These results were reproduced in two separate experiments.

Evidence that TNF Is a Required Mediator in ADCI. To identify the mediator(s) that may affect the intraerythrocytic growth of *P. falciparum*, we investigated the potential involvement of active radicals and selected cytokines known to be produced by MN. Of these, only TNF-α proved relevant.

The differing effects of MN and PMN in ADCI did not support the potential involvement of oxygen radicals, since FOR were found to be produced in equivalent amounts by these two cell types (see Table 1). Nevertheless, this was further investigated by ADCI assays performed in the presence of the FOR scavengers BHA, butylated hydroxytoluene, and superoxide dismutase/catalase. These proved to be unable to block ADCI. In some of these experiments there was a rather slight increase of the ADCI-specific growth inhibition, possibly because BHA neutralizes any deleterious effect of FOR upon the effector cells.

We next examined a possible role of nitric oxide radicals. Under the conditions of the ADCI experiments performed, in which the arginine analogue, L-NMMA, was added at 1- to 5-nM concentrations, no indication supporting the possible role of NO^- in mediating ADCI was obtained (Fig. 4).

The potential involvement of IFN-α, IFN-γ, IL-1, IL-6, and TNF-α was subsequently examined in parasite growth assays and in ADCI inhibition assays. The direct role of IFN-α, IFN-γ, or IL-1 was not supported by results from a series of ADCI inhibition assays performed in the presence of the corresponding anticytokine Abs (results not shown). Similarly, these cytokines were reproducibly ineffective in direct inhibition of *P. falciparum* growth, in the absence of MN, at concentrations up to 10,000 IU/ml. In contrast, Abs known to block the biological activity of human TNF-α reversed the ADCI effect (Fig. 4), whereas another Ab specific for TNF-α, although unable to act upon its biological function, did not. Finally, the specificity of the effect of anti-TNF-α Abs was demonstrated by reversing the effect of anti-TNF-α by addition of human rTNF-α (Fig. 4). These results were reproducible in two separate experiments. In contrast, anti-IL-6 Abs were ineffective.

These experiments thus identified TNF-α as a major component of ADCI. However, further experiments indicated that TNF-α alone was unable to mimic ADCI, since rTNF-α (0.1–100 μg/ml) as well as combinations of rTNF-α (100 μg/ml) plus IFN-γ (100–30,000 IU) were unable to affect *P. falciparum* growth in direct assays (results not shown). An indirect effect of TNF-α upon MN was also excluded since, in the absence of antimalarial Abs, no growth inhibition could be observed in cocultures of *P. falciparum* with MN and rTNF. These results, together with the above evidence that TNF is essential to achieve a positive ADCI effect, suggested that TNF may act in conjunction with another as yet unidentified factor.

Discussion

The relevance of the blood MN–mediated ADCI to clinical protection against *P. falciparum* malaria has been previ-

ously established by close in vivo/in vitro correlations (6). The present study offers several new findings on how ADCI operates and which are the triggering and the target stages. On the basis of our results, the following succession of events seems the most likely: At the time of schizont rupture, the contact between some merozoite surface component and cytophilic Abs bound to MN via their Fc fragment triggers the release of soluble mediators, which diffuse in the serum (or the culture medium) and which are able to block the division of surrounding parasites at the uninucleated stage.

Previous ADCI assays were performed in conditions in which the triggering stage, the target stages, and the immune effectors, MN and Abs, were all mixed, thus impeding any analysis of the succession of events. In the present study, this impediment was overcome by performing ADCI in two successive steps in two different culture vials, one used for MN stimulation and the other for the assessment of the resulting effect on the parasites.

The low or nonrelevance of merozoite phagocytosis to parasite growth reduction in ADCI was supported both by the differential behavior of PMN versus MN in phagocytosis and ADCI assays and by the effect of stimulated MN supernatants, which obviously rules out merozoite removal as an important effector. This may sound surprising in view of the relatively high rates of merozoite uptake observed with both cells in the presence of Abs; however, phagocytosis was performed using purified merozoite suspensions without RBC and was expressed as the number of cells having phagocytosed at least one merozoite, not in numbers or proportion, of merozoites removed in a normal culture (or in vivo). Therefore, in the ADCI assay conditions, the relative proportion of merozoites taken up is probably insufficient to influence the final parasitemia. Nevertheless, merozoites, more or less intact schizonts, and parasite debris are observed in MN at the end of ADCI assays. These may represent only the subset of dead merozoites, i.e., those that have been unsuccessful in reinvading an RBC. Straightforward calculations also lead us to reject merozoite phagocytosis as a critical or major mechanism in vivo. In this ADCI system, in which Abs apparently have no direct effect upon merozoite invasion (6) and in which the life span of merozoites outside RBC is short, phagocytes are simply not numerous enough compared with infected RBC to have any significant clearance effect. Most merozoites emerging from a rupturing schizont would probably reinvade one of the numerous nearest RBC before coming in contact with a phagocyte. Based on these results, our view is that the ingestion of merozoites is an essential step to triggering ADCI, although it does not account for the observed reduction of the parasitemia.

By using synchronized cultures for short periods of time (in which the percentage of each stage was assessed at the beginning and at the end of the experiment), it was possible to rule out the idea that the triggering antigens could be expressed on the host cell surface. Only those experiments in which the rupture of schizonts occurred led to efficient MN stimulation; the use of highly purified and thoroughly washed merozoites strongly suggested that the critical stage is the free merozoite. This finding is in agreement with in vivo observations performed three decades ago in the follow-up of IgG transfer experiments. I. A. McGregor noticed (26, and personal communication), as we did in our study (3), a variable delay from one individual to the other in the parasitological effect of the transferred IgG. He later related this delay to the expected time of schizont burst: "it would appear that, during serum therapy, the critical time for plasmodial survival is around schizogony. If the parasite survives this period normal trophozoite growth seems to be assured until the next schizogony is approached. This suggests that antibody-antigen interaction is only effective as segmentation advances or when the asexual merozoite emerges from the protective cover of the red blood cell" (26). Considering that only clinical and parasitological data were available at this time, his premonitory view is remarkable. The fact that merozoites would bear the critical antigen(s) responsible for triggering effector defense mechanisms against ABS may give a clue to paradoxical in vivo findings. In our previous study (3), we found that among 11 IgG treatments made in *P. falciparum*–infected receivers, the percentage of parasite reduction obtained after transfer of IgG was directly related to the initial parasitemia (correlation coefficient $r \simeq 0.85$). In other words, the higher the initial parasitemia, the stronger the effect of a given dose of IgG. In the case of a direct effect of IgG upon *P. falciparum* invasion, one could expect the opposite: the higher the parasitemia, the larger the IgG consumption and the lower the reduction of parasite load. In contrast, if merozoites are needed to trigger IgG-armed MN, the larger the number of merozoites released, the stronger the triggering of MN would be (for similar Ab concentrations).

The implication of the merozoite as the critical stage provides also an understanding of previous contradictory results. Rzepczyk et al. (27) were unable to demonstrate ADCI in 24–36-h assays in which synchronized cultures, starting at trophozoite stage, were used. In light of our findings, this absence of inhibition is understandable. Even if merozoite release occurred, it was too late at the time the experiment was stopped to enable one to observe a consecutive effect mediated by MN upon reinvaded parasites. Indeed, a full-cycle parasite development, starting from the time the mediator(s) are released, is needed to observe their effect upon parasite replication.

As the only extracellular stage in the erythrocytic cycle, merozoites have always been considered to bear valuable vaccine candidate molecules, particularly at their membrane level, and immunization attempts with whole merozoites were among the most convincing (28). Our finding that purified preparations of free merozoites are able to trigger ADCI indeed suggests that the critical antigens are constituents that would be accessible to Abs either on the merozoite membrane or at the apex (although the potential role of contaminants such as RBC membranes, residual bodies, and antigens passively adsorbed onto merozoites cannot be excluded). Such indications in favor of the role of merozoites have obvious implications for vaccine research, as they would significantly restrict the range of malarial molecules to be

investigated. It might be recalled here that we have recently used ADCI as a screen to select antigens, irrespective of their stage or their location, that are potentially responsible for the induction of Abs involved in this mechanism. One molecule identified and partially sequenced turned out to be a novel constituent of merozoite membranes, termed MSP3 (8), the full sequence of which has been recently elucidated (29). This does not rule out the possibility that other merozoite surface molecules could be targets for Abs effective in ADCI, although it points to the merozoite stage as a critical one.

In addition to being specific to merozoite antigen, the active Abs were also expected to be cytophilic for the effector cells. The involvement of Fc receptors in this Ab-dependent, MN-mediated effect was indirectly suggested by previous isotype studies. We had previously observed that the balance between cytophilic and noncytophilic classes of Abs was critical both in the establishment of clinical protection and in the in vitro triggering of ADCI. Positive ADCI effects were only obtained using total IgG, for which cytophilic classes were significantly more abundant than noncytophilic classes (7). This indirect evidence gained from isotype and ADCI studies is clearly supported by the present results, which provide direct evidence for the involvement of Fcγ receptors. This aside, the involvement of FcγRII, instead of RI, was somewhat unexpected. Indeed, FcγRI has been frequently implicated as a critical receptor involved in ADCC mechanisms against a number of targets (22). The FcγRII receptor, however, is almost as abundant on MN as is FcγRI, although it has been found to have a lower affinity to Ig, so the triggering of MN would require the involvement of at least dimers of IgG. This condition is clearly satisfied by a particulate antigen such as a merozoite. The marginal increase by IFN-γ of the ADCI intensity thus appears not to be related to its well-known induction of FcγRI (30), but rather to be the result of the IFN-γ–dependent increased ability of MN to release TNF, which is triggered by FcγRII cross-linking (31–33). This view is also consistent with our finding that a 24–48-h delay after IFN-γ stimulation was required to observe the increased ADCI effect, whereas FcγRI increased expression would occur within 8 h (Ravetch, J., personal communication).

The fact that supernatants from ADCI assays strongly inhibit parasite growth indicates that MN (triggered by merozoite antigens and the corresponding Abs, as discussed above) release soluble inhibitory mediator(s). Based on purely morphological studies, it remains difficult to identify the precise mode of action of these soluble mediators. The main observation was the accumulation of uninucleate altered parasites. At the ultrastructural level, several degrees of parasite damage, leading from ribosomal aggregation up to trophozoite fissuration, were seen (34). However, it is remarkable that this effect seemed to target nuclear division. The accumulation of uninucleate picnotic parasites has been frequently reported during both in vivo and in vitro experiments, such as in primates during the recovery phase after an acute attack (21), and in a variety of circumstances in vitro, including in the presence of activated macrophages (20, 35). The uninucleate stage may, therefore, be a more susceptible and fragile one in the malaria life cycle.

The essential requirement of TNF-α in ADCI was established by the differential effect of two distinct anti–TNF-α Abs with differing biological functions as well as by the reversibility of the anti–TNF-α effect in the presence of rTNF-α. The in vitro observation that rTNF-α alone is unable to mimic ADCI is in agreement with the in vivo observations that very high TNF-α levels during acute attacks are unable to control parasitemia (36,37) and that treatment of humans with anti-TNF does not lead to an increase in parasitemia (38). Our observations are reminiscent of other reports of TNF-dependent intraerythrocytic inactivation of the sexual stages of malaria parasites (39). In these studies, as in ADCI, TNF-mediated inactivation of the sexual stages was dependent on the presence of MN. The soluble mediators involved comprised both TNFα and "complementary factors" as found here. However, in contrast to ADCI, the inactivation of sexual stage parasites was mediated, at least in part, by nitric oxide intermediates (40). TNF-α has also been implicated in the generation of crisis forms induced in mice by bacillo Calmette-Guerin and LPS (41, 42), although this effect was most likely mediated by FOR as shown by the effect of BHA. At present, little is known of the mode of action of TNF upon parasitized erythrocytes or of the identity of the second mediator, both here and in the study of sexual stages (39). In our two-step assay, the second culture was essentially devoid of leukocytes (none could be detected on control smears), a finding which tends to exclude the possibility of TNF-dependent stimulation of MN. Receptors to TNF have not so far been identified on the surface of uninfected RBC; however, the parasites cause profound modifications in the host erythrocyte membrane, and it has been shown that large serum constituents, such as IgG, can attain the intraerythrocytic parasite (43). It has been suggested that, under certain conditions, TNF could interact and be integrated into artificial membranes independently of TNF receptors (44). Intracellular injections of TNF also indicated that binding to its receptor is not essential to its tumoricidal activity (45). Indeed, the type of alterations seen in the parasite are compatible with those induced by TNF in tumor cells. One possible explanation is that the unidentified second component is needed for TNF access to the intraerythrocytic parasite.

Interestingly, we have observed that the effect of soluble mediators was incomplete or partially reversible after a 72-h incubation. This is consistent with the identification of the major role played by TNF, given the known lability of this cytokine. An incomplete or reversible effect is also consistent with in vivo findings that malaria is a chronic disease in which some parasites persist regardless of the levels of antimalarial Abs. The implication of TNF in the ADCI defense mechanism is supported by the recent observation of an abnormally high frequency of individuals from endemic areas harboring the TNF-2 promoter. Although this may result in homozygous individuals having an increased frequency of severe malaria (46), the gene may be selected by the evolutionary advantage

of heterozygous individuals with an improved defense against malaria because of this higher TNF production phenotype.

Our study points to the implication of MN and TNF-α as essential components of defense against intraerythrocytic parasites. The question of whether this reflects a common general mechanism to both asexual and sexual stages of the human malaria life cycle or not will require complementary investigations.

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