# A Novel Antibody-Dependent Cellular Cytotoxicity Mechanism Involved in Defense against Malaria Requires Costimulation of Monocytes Fc$\gamma$RII and Fc$\gamma$RIII[1]

**Ali Jafarshad,* Morten H. Dziegiel,† Rasmus Lundquist,† Leif K. Nielsen,† Subhash Singh,[2]* and Pierre L. Druilhe[3]***

**Clinical experiments have shown that the Ab-dependent cell-mediated inhibition of *Plasmodium falciparum* is a major mechanism controlling malaria parasitemia and thereby symptoms. In this study, we demonstrate that a single merozoite per monocyte (MN) is sufficient to trigger optimal antiparasitic activity. Using particulate Ag as pseudomerozoites, we show that only Ags, and no other parasite-derived factor, are required to trigger MN activation and that a single Ag is as potent as the complex combination of Ags constituting the merozoite surface. Moreover, we found that soluble Ags binding at least two Abs are as effective as the parasite at stimulating MN and that nonmalarial Ags are as efficient provided they are targeted by cytophilic Abs. Indeed, only cytophilic IgGs are potent and, in agreement with immunoepidemiological findings, IgG3 is superior to IgG1. Very low Ab concentrations (>700 pM), i.e., in the range of molecules having a hormonal effect, are effective, in contrast to Abs having a direct, neutralizing effect. Finally, Ab-dependent cell-mediated inhibition proved to require the synergistic activation of both Fc$\gamma$RIIa and Fc$\gamma$RIIIa which both distinguish it from other Ab-dependent cellular cytotoxicity and implies that all MN are not equally effective. These findings have both fundamental and practical implications, particularly for vaccine discovery.** ***The Journal of Immunology,*** **2007, 178: 3099–3106.**

In endemic areas, populations persistently exposed to *Plasmodium falciparum*—the parasite responsible for the lethal form of malaria in humans—develop, after ~10–20 years, a nonsterile type of immunity against the blood stages of the parasite, known as premunition (1). Having reached this state of protection, individuals are able to control their parasite densities at very low levels, thereby preventing the appearance of clinical symptoms of the disease (1).

Our approach to research on the asexual blood stages of malaria stems primarily from clinical observations (2). These observations in turn lead to ex vivo and in vitro experiments (3) aimed at elucidating the basis of the interaction of *P. falciparum* with the human immune system and the very particular pattern of infection/immunity that results (4).

Experiments of passive transfer of immune IgG have established that Abs are necessary and sufficient to reproduce in recipients the parasitological and clinical findings observed in the donors (2, 5). Abs are currently thought to act through one of three mechanisms: 1) by inhibiting merozoite invasion of RBCs, 2) by cell-Ab cooperation inhibiting intraerythrocytic parasite development, or 3) by inhibiting cytoadherence of schizont-infected RBCs (4, 6). However, detailed ex vivo studies using the material derived from one clinical experiment of passive protection (3) led us to identify monocyte (MN)[4]-IgG cooperation as one of the key components involved in defense and did not point, under our experimental conditions, to a major effect of human Abs on either merozoite invasion (3) or cytoadherence (Ref. 2 and P. Ringwald, unpublished observations).

The mechanisms underlying this Ab-dependent cellular inhibition (ADCI) have been previously partially studied (7). It was found that only blood MN, but not macrophages derived from MN, nor polymorphonuclear (PMN), lymphocytes or platelets, could mediate an ADCI effect (3, 8). The use of ADCI led to identify merozoite surface Ags as the main targets of effector Abs and showed that the activation of MN involving the Fc$\gamma$RII leads to the release by MN of soluble factors (among them TNF-$\alpha$) which block the development of surrounding intraerythrocytic parasites at trophozoite stage (7).

These findings led us to perform the first investigation into the isotype distribution of malarial Abs (9). This showed a marked predominance of cytophilic classes, mainly IgG3, among protected individuals and, conversely, of noncytophilic classes among individuals experiencing attacks. Epidemiological data thus supplied a first clue to the reason for the long delay required to achieve protection (4) and supported the view that Abs were effective through an indirect mechanism. Finally, the requirement of MN to mediate

*Bio-Medical Parasitology Unit, Pasteur Institute, Paris, France; and †H:S Blodbank KI2033, Copenhagen University Hospital, Copenhagen, Denmark

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[2] Current address: Laboratory of Malaria and Vector Research/National Institute of Allergy and Infectious Diseases/National Institutes of Health, 12735, Twinbrook Parkway, Twinbrook III, Rockville, MD 20852.

[3] Address correspondence and reprint requests to Dr. Pierre L. Druilhe, Bio-Medical Parasitology Unit, Institut Pasteur, 28, rue du Dr. Roux, 75015 Paris, France. E-mail address: druilhe@pasteur.fr

[4] Abbreviations used in this paper: MN, monocyte; ADCI, Ab-dependent cellular inhibition; PMN, polymorphonuclear; MSP, merozoite surface protein; GLURP, glutamate-rich protein; SERP, serine repeat protein; PIAG, pool of immune African globulins; N-IgG, negative control IgG; anti-RhD, anti-rhesus D; CM, culture medium; PEMS, parasitophorous vacuole membrane-enclosed merozoite structure; PMz, pseudomerozoite; SGI, specific growth inhibition; ADCC, Ab-dependent cellular cytotoxicity.

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this Ab activity, indicated by in vitro studies, was confirmed under in vivo conditions in the *P. falciparum* SCID mouse model (10).

Considering the indications for a critical role of MN-Ab cooperation in protection and the need to rationalize vaccine development, we then focused on the use of ADCI to determine merozoite surface Ags potentially involved in protection. We identified by this means merozoite surface protein-3 (MSP-3) (11) and subsequently the involvement of glutamate-rich protein (GLURP) and serine repeat protein (SERP) in this mechanism (12, 13); all three are now undergoing vaccine trials (14).

In the present study, we developed an improved, highly reproducible, two-step ADCI assay which allowed us to perform a detailed analysis of the factors responsible for the triggering of parasite killing by MN. This work uncovered several novel findings, with both fundamental and practical consequences for the understanding of human-*P. falciparum* immune interactions and vaccine development.

## Materials and Methods

### *Antigens*

Recombinant proteins and peptides derived from MSP-3, GLURP, and SERP (SERP-SERA-p126), three Ags targets of Abs active in ADCI, were used as blood-stage Ags: MSP-3Cterm$_{191-354}$, a histidine-tagged recombinant protein, and MSP-3LSP$_{181-276}$, a long synthetic peptide, both cover the highly conserved C-terminal region of MSP-3 (numbered according to *P. falciparum* 3D7 sequence, GenBank accession number: NP_700818.1) (15). Peptides MSP-3b$_{184-210}$, MSP-3c$_{203-230}$, and MSP-3d$_{211-252}$ correspond to small regions of the C-terminal part of MSP-3 previously found to define either a single (MSP-3b and c) or two epitopes (MSP-3d) (15) targets of cytophilic Abs positive in ADCI. Recombinant protein GLURP$_{94-489}$ (R0) is derived from the nonrepeat N-terminal region of GLURP (*P. falciparum* F32 strain, GenBank accession number: AAA50613) (16). Recombinant protein SE47′$_{17-382}$ (a gift from T. Horii, Osaka University, Osaka, Japan) is derived from the N-terminal domain of SERP-SERA (*P. falciparum* FCR3 strain, GenBank accession number: AAA16791) (17). Control Ags included the tetanus toxoid Ag (Sigma-Aldrich) and the recombinant protein LSA-3–729$_{223-278}$ derived from the conserved N-terminal region of liver stage Ag 3, a protein expressed in sporozoite and liver stages, though not in erythrocytic stages (*P. falciparum* K1 strain, GenBank accession number: CAB65343) (18).

### *Sera and IgG preparation*

IgG purified from a pool of hyperimmune African adults (PIAG) previously found to confer passive protection when transferred to nonimmune patients (2) were used as positive control Abs. Negative control IgG (N-IgG) was a pool of >1000 French donors with no history of malaria. IgG from test and control sera were purified using a size exclusion Trisacryl GF05M column followed by an ionic exchange DEAE Ceramic HyperD F column (both from Pall BioSepra). IgG were then dialyzed against PBS (pH 7.4) for 48 h and finally against RPMI 1640 (Invitrogen Life Technologies) overnight. Purity of IgG was assessed on 12% SDS-PAGE gels stained by Coomassie brilliant blue (Bio-Rad). Sera from LSA-3 immunized mice were selected from those with high-cytophilic IgG responses. Human sera with high-cytophilic IgG responses to tetanus toxoid were obtained from vaccinated individuals.

### *Monoclonal Abs*

The human recombinant anti-MSP-3 mAb RAM-1, specific for the MSP-3b peptide, was constructed with either IgG1 and IgG3 H chains as described previously (19). Recombinant anti-rhesus D (anti-RhD) mAb were constructed in IgG1, IgG2, IgG3, IgG4, IgA$_1$, and IgA$_2$ versions (20, 21). mAb constructs relied on those H-chain allotypes most prevalent in African populations: *G1m(a,z)*, *G2m(n$^+$)* and *G3m(b)* for IgG1, IgG2, and IgG3, respectively, and the *A2m1* for IgA$_2$ (22).

Various mouse mAbs were used to assess the phenotype of MN and THP-1 cell line: anti-CD64 (clone 10.1; DakoCytomation), anti-CD32, anti-CD16, anti-CD14 (clones AT10, 3G8, and MEM-18, respectively; Serotec) anti-CD11a, anti-CD11b, anti-CD49e, anti-CD31, and anti-CD54 (clones MEM-25 and MEM-174, NKI-SAM-1, HEC/75 and 15.2, respectively; Immunotools), anti-CD11c and anti-CD49d (clone 3.9 and 9F10, respectively; eBioscience). FITC-labeled goat anti-mouse IgG (Alexa Fluor 488; Molecular Probes) was used as a secondary Ab when necessary. Mouse mAbs of either the IgG1 (clone W3/25; Serotec) or the IgG2a class (catalog number 16-4724; eBioscience) were used as negative controls. In blockade experiments of Fc$\gamma$RI, Fc$\gamma$RII, and Fc$\gamma$RIII, F(ab′)$_2$ derived from mAbs 10.1 (1 $\mu$g/ml; Ancell), AT10 (20 $\mu$g/ml; this mAb, which targets both Fc$\gamma$RIIa and Fc$\gamma$RIIb, was a gift from Prof. C. Sautès-Friedman, Institut National de la Santé et de la Recherche Médicale (INSERM), Institut des Cordeliers, Paris, France) and 3G8 (1 $\mu$g/ml; Ancell) were used, respectively.

### *Monocytic cell line and differentiation conditions*

The human monocytic cell line THP-1 (23) was cultured in RPMI 1640 supplemented with 10% decomplemented FCS (Institut Jacques Boy, Reims, France). For differentiation studies, cells were cultured for 4 days in absence of inducers (control) or in the presence of either 1$\alpha$-25-dihydroxyvitamin 3 (100 nM; Fluka, Sigma-Aldrich), human recombinant IFN-$\gamma$ (200 U/ml; Boehringer Mannheim), recombinant human TGF-$\beta_1$ (1 ng/ml; Strathmann Biotec), or various combinations of these factors. Cells were then harvested, resuspended in RPMI 1640, and viability was assessed by trypan blue exclusion, before phenotypic analysis and ADCI functional studies.

### *Parasites*

The Uganda Palo Alto strain (FUP/C) and the 3D7 clone of *P. falciparum* were grown in a culture medium (CM) composed of RPMI 1640 supplemented with 0.5% albumax I (Invitrogen Life Technologies), as described (24). For functional assays, asynchronous blood stages parasite cultures were used. When required, parasites were synchronized by at least two successive sorbitol treatment (25) followed, after maturation over 24 h, by flotation on 1% porcine skin gelatin type A (Sigma-Aldrich) (26). Parasitemia was determined by light microscopy on Giemsa (Sigma-Aldrich) stained smears fixed by methanol by counting ≥50,000 RBCs.

### *Merozoite preparation*

Parasitophorous vacuole membrane-enclosed merozoites structures (PEMS), each containing ~20 merozoites, were obtained by using the reversible inhibitor of cysteine protease E64 (Sigma-Aldrich) as described previously (27). PEMS were then enumerated on Giemsa-stained smears and separated by centrifugation (20 min, 2000 × *g*) on a 45% (v/v) Percoll gradient (Invitrogen Life Technologies). Following washing in RPMI 1640 to remove E64, the PEMS were used in the first of a two-step ADCI assay to release, within 1–3 h, merozoites directly onto an MN monolayer.

### *Pseudomerozoites*

Defined peptides or recombinant proteins were adsorbed by hydrophobic interactions on 1-$\mu$m diameter polystyrene beads (Polysciences) mimicking merozoites. Each Ag (5 $\mu$g/ml) was added to $3 \times 10^8$ beads, incubated for 2 h at 37°C in 1.5 ml of PBS and washed three times in PBS by centrifugation at 6700 × *g* for 10 min. Free remaining nonspecific coating sites on pseudomerozoite (PMz) were further blocked by incubating beads at 37°C for 1 h in PBS supplemented with 0.1% BSA.

### *Preparation of human blood MNs*

To reduce donor to donor variability of MN preparations, blood MN were prepared from cytapheresis samples of blood donors (Lecourbe Blood Bank, Paris, France) and cryopreserved. PBMC were separated on Ficoll density gradients (J Prep; Techgen) as previously described (7) and then aliquoted at a final concentration of $15 \times 10^6$ cells/ml in heat-inactivated AB$^+$ human serum supplemented with 10% DMSO (Sigma-Aldrich). Aliquot were kept in liquid nitrogen until use. Before test, cells were rapidly thawed at 37°C in a water bath and resuspended in an excess of CM. Viability as assessed by trypan blue exclusion was ≥95%. Cells were then distributed on polystyrene 96-well flat-bottom culture plates (TPP) and adherent MN were selected by incubation for 2 h at 37°C, in a humidified 5% $CO_2$ atmosphere. More than 90% of the adherent cells obtained in this manner were MN as estimated by the $\alpha$-naphtyl acetate esterase test (Sigma-Aldrich).

### *Phenotypic analysis of monocytic cells*

The differentiation of THP-1 monocytic cells was monitored by flow cytometry. Briefly, $5 \times 10^5$ cells of each of control or differentiated cells were incubated in 100 $\mu$l of PBS supplemented with 5% FCS, for 40 min at 4°C in presence of mAbs specific for each CD marker. After three washes in PBS, at least 30,000 cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences) for each marker. Control IgGs of the same isotype as the specific mAb were used as negative controls. FITC-conjugated

anti-CD11a, -CD11b, -CD31, and -CD54; PE-conjugated anti-CD11c, -CD14, -CD16, and -CD49d; and unlabeled anti-CD49e, -CD64, and -CD32 were used. For the latter, an additional incubation for 40 min at 4°C with a secondary anti-mouse FITC-labeled goat IgG (Alexa Fluor 488; Molecular Probes) at a 1/100 dilution in PBS (pH 7.4) supplemented with 5% FCS was performed.

### *ADCI assay*

In vitro ADCI functional assays were performed in 96-well TPP plates containing $2 \times 10^5$ MN/well selected by adherence as described above. Only MN showing no direct inhibitory effect (≤15%) were used in ADCI tests. A total of 50 μl of an asynchronous parasite culture at 0.5% parasitemia and 4% hematocrit were added to each ADCI well to obtain a ratio of 1 MN:200 RBCs. Test and control Abs were then added and the total volume adjusted to 100 μl with CM. At 48 and 72 h, 50 μl of CM was added to each well and after 96 h the assay was stopped and the final parasitemia determined. Abs used included: PIAG or N-IgG at a final concentration of 1 mg/ml; anti-MSP-3 RAM1-IgG1 or IgG3 at concentrations ranging from 0.1 to 45 μg/ml; anti-RhD IgG1 or IgG3 at 45 μg/ml. Each experiment included controls to monitor nonspecific monocytic inhibition and direct inhibition by either control or test IgG. The specific growth inhibitory index (SGI) reflecting the parasite growth inhibition due to the effect of test Abs cooperating with MN was calculated as follows: SGI = 100 × (1 − (percent parasitemia with MN and PIAG/percent parasitemia PIAG)/(percent parasitemia with MN and N-IgG/percent parasitemia N-IgG)). To assess the role of each FcγR, $F(ab')_2$ of mAbs 10.1, AT10, or 3G8 were added 30 min before the addition of Ags and Abs to control and test ADCI wells.

### *Two-step ADCI assay*

The two-step ADCI protocol was adapted from the technique described previously (7). MN selected by adherence were incubated for 3 h with Abs and either PEMS or PMz at MN:merozoite ratios ranging from 1:40 to 1:0.1. The rupture of PEMS and the release of merozoites onto the MN monolayer were monitored by light microscopy on smears taken at 30-min intervals over 3 h. In the second step, supernatants from control and test wells were collected, centrifuged at 600 × *g* and 50 μl of each of them were transferred onto 50 μl of an asynchronous *P. falciparum* culture (0.5% parasitemia, 4% hematocrit) in 96-well plates. The assay was stopped after 72 h and the SGI calculated as described above. In ADCI assays assessing the role of various isotypes of Abs, PMz were directly coated with one of the six classes of anti-RhD Abs. When whole sera were used, only those sera having no substantial direct inhibitory effect on parasite growth (≤15%) were used.

### *Phagocytosis assay*

To select MN by adherence, 25 μl of a PBMC preparation containing 3800 MN suspended in HBSS were added to each well of 8-well glass chamber slides (Nalge-Nunc International) and incubated for 2 h at 37°C in a humidified 5% $CO_2$ atmosphere. The slides were then washed with RPMI 1640. MSP-3-coated polystyrene beads prepared as described above were incubated with PIAG or control N-IgG, both at 6 mg/ml, for 1 h at 37°C, washed three times in PBS and then added at a ratio of 10 beads:1 MN. Following incubation for 1 h at 37°C, cells were washed twice with PBS, fixed with methanol, stained with Giemsa and the percentage of phagocytosis determined by light microscopy of over 500 cells. An MN was considered as having phagocytosed beads when at least three beads had been ingested. To determine the role of FcγRs in ADCI, before the addition of the Ag-coated beads, MN were incubated either with mAbs 10.1, AT10, or 3G8 for 45 min at 37°C and then washed twice with RPMI 1640.

## Results

### *Low numbers of parasites are sufficient to trigger an ADCI activity by MNs*

In the two-step ADCI assay, freshly released merozoites, obtained by a recently described method allowing the preparation of PEMS, were found to be effective at triggering the activation of MN in cooperation with protective Abs (Fig. 1). The minimum number of merozoites required to trigger ADCI was studied using a range of merozoite to MN ratios. The maximal ADCI activity was reached at a ratio as low as one merozoite per MN and a substantial effect was seen at a ratio of one parasite per two MN. These figures are consistent with the very low parasitemias harbored by immune subjects.

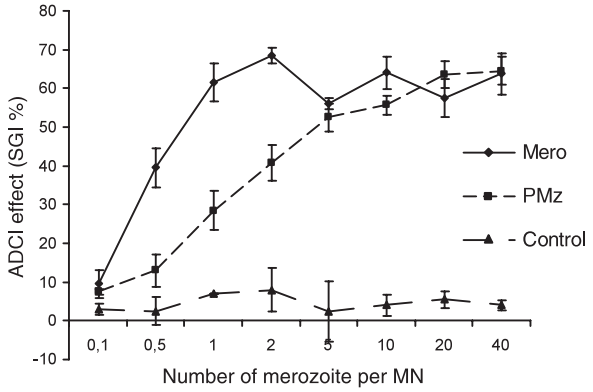


**FIGURE 1.** Determination of the minimum number of merozoites required to trigger ADCI by MN and the effect of PMz. In a two-step ADCI assay, a range of merozoites to MN was used in the first step to trigger the MN-dependant anti-*P. falciparum* inhibitory effect. Rupturing PEMS were used as the source of merozoites and PMz were obtained by coating polystyrene beads with the MSP-3-CTerm Ag or with BSA as a negative control. Ratios of merozoite to MN (plain line) or PMz to MN (dotted lines) ranging from 40:1 to 0.1:1 were tested. Merozoites or PMz were incubated for 3 h with MN and PIAG. Supernatants were transferred in the second step to an asynchronous *P. falciparum* culture (0.5% starting parasitemia) without MN for 72 h and the SGI determined. Data are the mean ± SD of two experiments.

### *Only the Ag and no other parasite molecule is required to activate the MN*

The merozoite surface expresses a very large number of Ags organized in a complex pattern (28). We thus designed PMz mimicking the merozoite but expressing only single Ags. Comparative ADCI experiments performed using either merozoites or PMz coated with the peptide MSP-3b showed that both were similarly able to trigger an ADCI effect (Fig. 1). Similar results were obtained using the GLURP-derived peptide LR70 or the SERP-SERA rAg (data not shown). These results indicate that no parasite-derived factor other than an Ag able to bind Abs is needed to achieve MN activation and, furthermore, that a single malarial Ag can trigger an ADCI effect of the same magnitude as the combination of a large number of Ags.

### *Soluble Ags are as effective as particulate Ags*

The above results also raised the question of whether soluble Ags, such as those released from schizonts, can similarly trigger ADCI. Comparative two-step ADCI assays, in which the Ags were either in particulate or soluble form, were performed. Results indicate that recombinant proteins in soluble state such as MSP-3-Cterm, and peptides such as MSP-3-LSP (Fig. 2), or GLURP-R0 (data not shown), are as effective as the merozoite or the PMz at triggering a potent ADCI effect.

### *At least two IgGs, bound to two physically linked epitopes, are required to activate ADCI*

Several MSP-3 peptides containing either single or multiple antigenic determinants which are the targets of cytophilic Abs present in the PIAG were used as Ags in two-step ADCI assays, either in particulate form, or in soluble form. Peptides known to define single antigenic determinants such as MSP-3b or MSP-3c in soluble form were not able to trigger ADCI, whereas MSP-3-LSP carrying four B cell epitopes, or the peptide MSP-3d which expresses at least two distinct epitopes, were efficient at triggering an ADCI effect (Fig. 2). However, monovalent peptides when coated on

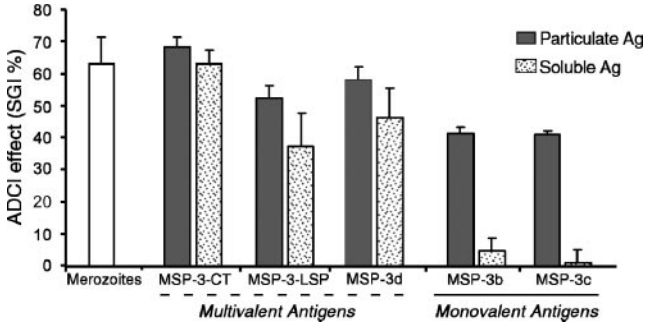


**FIGURE 2.** Induction of ADCI by Ags in soluble form. MSP-3 derived peptides defining either a single (*right panel*) or more than or equal to two (*left panel*) antigenic determinants were used either coated on microparticles as PMz or in soluble form, in a two-step ADCI assay. Following 2 h of incubation of these Ags with MN and PIAG, supernatants were transferred onto an asynchronous *P. falciparum* culture (0.5% starting parasitemia) without MN for 72 h and the SGI was determined. Data are the mean ± SD of two independent experiments each conducted in duplicate.

polystyrene beads, i.e., presenting several copies of the epitope, were also able to induce a potent ADCI activity.

In a second approach, a human mAb, the recombinant anti-MSP-3 RAM1 specific to a single epitope of MSP-3 (MSP-3b), and the polyclonal African PIAG pool were used with either the particulate or soluble form of MSP-3-CT in two-step ADCI assays. The soluble MSP-3-Cterm recombinant protein, which harbors the MSP-3b, c and d regions, was not able to activate MN in ADCI with the mAb RAM1 (Fig. 3). In contrast, the same Ag was effective when used with the polyclonal African PIAG pool, which contains Abs directed against all four B cell epitopes. Finally, the monoclonal RAM1 Ab was effective when the monomeric peptide MSP-3b was present in multiple copies adsorbed onto the PMz.

### *Nonmalarial Ags are equally effective at triggering an antimalarial effect*

The above experiments suggested that any Ag containing at least two B cell epitopes, each binding one Ab, could trigger MNs activation in ADCI. Thus, Ags from nonparasitic origins, as well as malarial Ags expressed during other stages than asexual blood ones, were tested in the two-step ADCI assay with their corresponding cytophilic Abs (Fig. 4). Both types of Ags proved as efficient as merozoite surface molecules at promoting an ADCI effect. Furthermore, microparticles coated either with the RAM1 mAb or with the polyclonal PIAG were also able to trigger parasite growth inhibition whereas albumin-coated beads, used as a control, were not efficient (Fig. 4). This finally suggests that the Ag has no other function than the cross-linking of, at least, two cytophilic IgGs to the MN.

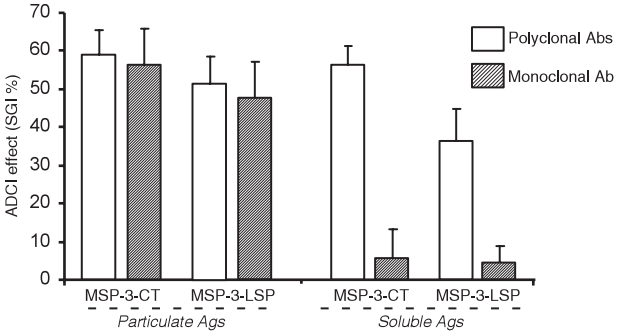


**FIGURE 3.** The binding of at least two IgG on a soluble molecule is required to trigger ADCI. MSP-3-CT and MSP-3 LSP, multiepitopic regions of MSP-3, were used as Ag either in PMz-coated or soluble form, in a two-step ADCI assay. The Abs used were either the polyclonal PIAG or the human mAb RAM1-IgG3 which is specific to a single epitope on MSP-3-CT. Following 2 h of incubation of these Ags with MN and PIAG, supernatants were transferred to an asynchronous *P. falciparum* culture (0.5% starting parasitemia) without MN for 72 h and the SGI was subsequently determined. Data are the mean ± SD of two experiments each performed in duplicate.

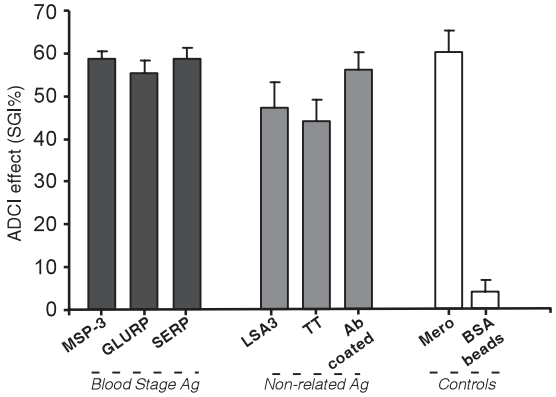


**FIGURE 4.** Induction of ADCI by various malarial and nonmalarial Ags. PMz were prepared with either asexual blood stage Ags (MSP-3CT, GLURP, and SERP) or nonrelated Ags (LSA-3-729, tetanus toxoid (TT) and PIAG-coated beads). Merozoites and BSA-coated beads were used as positive and negative controls, respectively. Abs included either PIAG (for MSP-3CT, GLURP, SERP, BSA, and merozoites) or an LSA-3 immunized mouse serum or a serum with high titer of anti-TT Abs. Following a 2-h incubation of PMz with MN and Abs, supernatants were transferred to an asynchronous *P. falciparum* culture (0.5% starting parasitemia) without MN for 72 h and the SGI determined. Data are the mean ± SD of two experiments.

### *Only cytophilic IgG1 and IgG3 are effective*

Ab-dependent cellular cytotoxicity (ADCC) type of mechanisms can operate with several cell types and several Ig classes such as IgG, IgA, or IgE (29). The purification of each class and subclass of Ab from human sera is challenging and results at best in an enrichment of certain classes, leaving some doubt that other classes present at a low concentration could be responsible for any observed effects (our unpublished material). For that reason, in the present study we used a stringent design and relied on a single human recombinant Ab, however, constructed with six distinct heavy chains, namely IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. All six versions of the mAb were tested in particulate form, in the two-step ADCI assay, as described in *Materials and Methods*. The results illustrated that only the IgG1 and IgG3 versions of the human mAb were effective (SGI of 65 ± 5 and 46 ± 11, whereas the other isotypes yielded SGI ranging from 1 ± 2 to 2 ± 1) and thereby indicated that only H chains able to bind Fc$\gamma$Rs were involved in the antiparasite effect. The absence of effect of the IgAs is noteworthy as they can act against bacteria and viruses in ADCC via Fc$\alpha$Rs (30).

### *Very low concentrations of Abs trigger a potent antiparasitic effect*

The recombinant anti-MSP-3b RAM-1, expressed as IgG1 or IgG3, both having the same specificity and affinity for the Ag, led to perform the first formal comparison between cytophilic Abs in terms of parasite killing. When studied over a wide range of concentrations in ADCI, RAM-1 IgG3 was found to have a consistently greater efficiency than RAM-1 IgG1, being ~60% more effective (Fig. 5).

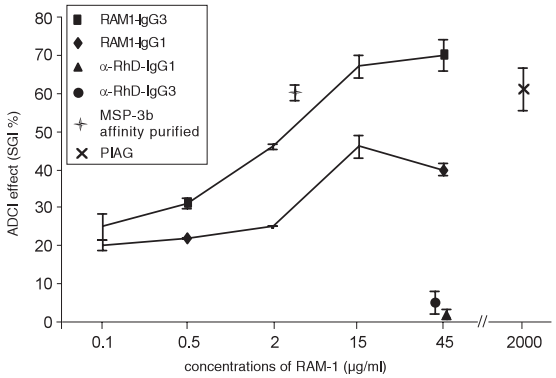


**FIGURE 5.** Minimal amount of Ab required to trigger ADCI. RAM-1 IgG1 and IgG3, two cytophilic recombinant Abs specific for MSP-3b, were tested in ADCI over a number of concentrations ranging from 100 ng/ml to 45 $\mu$g/ml. Abs and MN were incubated for 96 h with an asynchronous parasite culture (0.5% starting parasitemia). PIAG at 1 mg/ml and anti-RhD Abs at 45 $\mu$g/ml were used as positive and negative controls, respectively. Data are mean ± SD of two independent experiments.

Above all these results demonstrated that very low levels of Abs were sufficient to trigger an antiparasitic effect. Specific MN activation started to be detected at concentrations as low as 100 ng/ml, became substantial at 2 $\mu$g/ml and reached a plateau at concentrations of 15 $\mu$g/ml and above. At this concentration the ADCI effect was as high as with the pool of protected African sera (Fig. 5). This also confirms that an optimal ADCI effect does not require MN stimulation by a combination of multiple Ags with multiple Ab species.

*Phenotype of ADCI-effective MNs*

Not all leukocytes are able to exert an ADCI activity as macrophages, PMN, and lymphocytes have been shown ineffective (8). Moreover, among circulating blood MN diverse populations, characterized by distinct phenotypes and immunoregulatory functions, have been described previously (31). MN subsets were characterized by both flow cytometry and ADCI functional activity using human blood MN and THP-1 monocytic cells cultured with various maturation factors (Table I). Human MN and noninduced THP-1 were used as controls.

Noninduced THP-1 cells and those treated with IFN-$\gamma$, which leads to an increase of Fc$\gamma$RI (CD64) expression, did not show any ADCI activity (Table I). In contrast, THP-1 induced by 1$\alpha$-25-dihydroxyvitamin 3 and/or TGF-$\beta$ separately or jointly, which has been described to induce terminal monocytic differentiation of human leukemic cell lines (32), an ADCI activity was observed. The ADCI activity was always associated with the expression of Fc$\gamma$RII (CD32) and Fc$\gamma$RIII (CD16) at the cells surface. It is noteworthy that CD16 expression was observed on only 5–15% of the cells studied, yet yielded optimal ADCI activity in reproducible manner (Table I).

The expression of other monocytic maturation markers such as $\beta_2$ integrins (CD11a, CD11b, and CD11c), $\beta_1$ integrins (CD49d and CD49e), and adhesion molecules of the IgG superfamily (CD31 and CD54) did not appear, under the conditions used in our assays, to be critical for MN activity in ADCI.

*Expression of both Fc$\gamma$RII and Fc$\gamma$RIII, but not Fc$\gamma$RI, is required for ADCI activity*

Phenotypic characterization of cells active in ADCI suggested that Fc$\gamma$RIII was involved in ADCI. The roles of Fc$\gamma$RI, Fc$\gamma$RII, and Fc$\gamma$RIII were further investigated using F(ab′)$_2$ of blocking mAbs directed to each receptor (Fig. 6*A*). Blocking of Fc$\gamma$RI did not interfere with ADCI activity, whereas blocking of either Fc$\gamma$RII or Fc$\gamma$RIII totally abolished the MN mediated antiparasitic activity. These results indicate that the engagement of both Fc$\gamma$RII and Fc$\gamma$RIII is required to achieve MN-dependent parasite killing in ADCI.

Phagocytosis is a mechanism distinct from ADCI, which was used here as a control. Fc$\gamma$R-dependent phagocytosis was investigated using human MN and MSP3-CT coated PMz, opsonized with either PIAG or N-IgG (Fig. 6*B*). The phagocytic rate was found to be reduced by about half by blocking either Fc$\gamma$RI, or Fc$\gamma$RII and Fc$\gamma$RIII, by the corresponding mAb F(ab′)$_2$. Phagocytosis could be fully abolished only by blocking all three receptors simultaneously. These findings contrast with those obtained in ADCI and confirm that the pathways leading to parasite clearance by phagocytosis and ADCI are distinct.

Table I. *Phenotype of cells effective in ADCI*[a]

| | Positive Cells (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | THP-1 | | | | | | |
| | Control | VD3 | IFN-$\gamma$ | TGF-$\beta$ | VD3 + IFN-$\gamma$ | VD3 + TGF-$\beta$ | MN |
| CD64 | 70 ± 20 | 78 ± 2 | 90 ± 1 | 50 ± 3 | 92 ± 1 | 27 ± 4 | 90 ± 3 |
| CD32 | 95 ± 1 | 95 ± 2 | 93 ± 1 | 98 ± 2 | 90 ± 1 | 81 ± 3 | 93 ± 3 |
| CD16 | 0 | 4 ± 1 | 0 | 2 ± 1 | 6 ± 2 | 6 ± 3 | 19 ± 9 |
| CD11a | 52 ± 3 | 52 ± 2 | 72 ± 2 | 59 ± 7 | 78 ± 9 | 50 ± 12 | 98 ± 2 |
| CD11b | 30 ± 9 | 93 ± 4 | 29 ± 1 | 11 ± 5 | 93 ± 6 | 83 ± 11 | 75 ± 10 |
| CD11c | 15 ± 9 | 35 ± 17 | 10 ± 6 | 2 ± 1 | 28 ± 13 | 14 ± 8 | 99 ± 1 |
| CD14 | 37 ± 3 | 99 ± 1 | 59 ± 2 | 31 ± 2 | 100 | 99 | 97 ± 3 |
| CD31 | 95 ± 3 | 94 ± 3 | 96 ± 4 | 97 ± 2 | 94 ± 5 | 89 ± 6 | 93 ± 3 |
| CD49d | 99 ± 1 | 99 ± 1 | 100 | 99 ± 1 | 99 ± 1 | 98 ± 2 | 96 ± 2 |
| CD49e | 97 ± 1 | 95 ± 3 | 98 ± 2 | 98 ± 1 | 99 ± 1 | 99 ± 1 | 93 ± 3 |
| CD54 | 2 ± 1 | 3 ± 1 | 13 ± 1 | 1 | 48 ± 7 | 8 | 88 |
| SGI (%) | 7 ± 13 | 51 ± 16 | 0 | 50 ± 12 | 59 ± 8 | 33 ± 9 | 58 ± 4 |

[a] THP-1 monocytic cells were cultured for 4 days in the presence of different inducers of MN differentiation. 1$\alpha$-25-dihydroxyvitamin 3 (VD3), IFN-$\gamma$, and TGF-$\beta$ were used respectively at concentrations of 100 nM, 200 U/ml, and 1 ng/ml. Blood MN were used as controls. The percentage of cells positive for each marker, namely CD64, CD32, CD16, CD11a, CD11b, CD11c, CD14, CD31, CD49d, CD49e, and CD54, was determined by flow cytometry (with mAbs, 10.1, AT10, 3G8, MEM-25, MEM-174, 3.9, MEM-18, HEC/75, 9F10, NKI-SAM-1, and 15.2, respectively). Mouse IgG1 and IgG2a were used as negative controls. Results are expressed as mean values ± SD of three independent experiments. The functional activity of differentiated and control cells was assessed in a two-step ADCI assay. Results are the mean ± SD of three independent experiments.

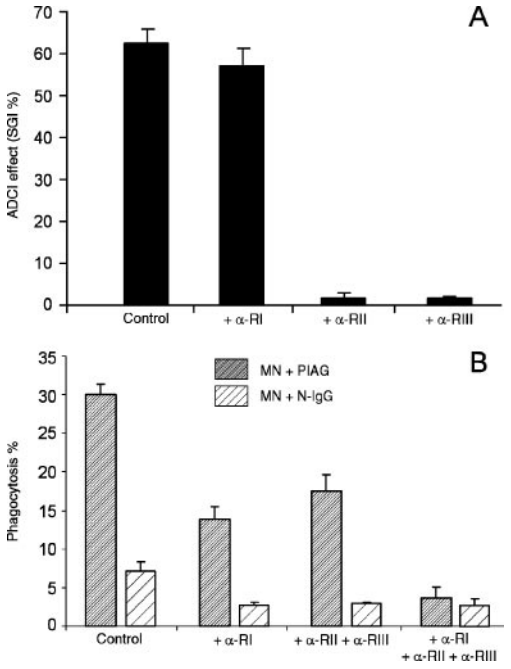


**FIGURE 6.** *A*, Identification of the FcγRs involved in the antiplasmodial ADCI effect. The role of FcγRI, FcγRII, and FcγRIII was determined in a two-step ADCI assay by preincubation of MN with $F(ab')_2$ derived from the mAbs 10.1, AT10, and 3G8 respectively, to block selectively each FcγR family expressed on MN. After a 30-min incubation, MSP-3CT-coated PMz and PIAG were added to the ADCI wells and incubated for 2 h. Supernatants were then transferred to asynchronous culture of *P. falciparum* (0.5% parasitemia) without MN for 72 h and the SGI was determined. Results are the mean ± SD of three independent experiments. *B*, Identification of FcγRs involved in phagocytosis of parasites. The role in phagocytosis of each of the three FcγR expressed on MN was determined by blocking FcγRI, FcγRII, and FcγRIII by either mAb 10.1, AT10, or 3G8, respectively. To MN were added combinations of blocking mAb 30 min before the addition of MSP-3-CT PMz opsonized by either PIAG or N-IgG. After 45 min, the percentage of phagocytosis by MN was determined by light microscopy of over 100 MN. An MN was considered as having phagocytosed particles when at least three beads were ingested. Data are mean ± SD of two distinct experiments.

## Discussion

Clinical experiments and immunoepidemiological studies, have pointed to ADCI, a mechanism relying on the cooperation between MN and IgG and inhibiting *P. falciparum* growth as playing a major role in acquired immunity to malaria (2, 3, 7, 9). In the present study, we analyzed the conditions underlying the triggering of this essential effector mechanism. This led to several novel findings that shed a new light on the circumstances of *Plasmodium* killing and that bear significant consequences.

From a practical point of view, our study addresses a major difficulty encountered when performing ADCI assays, namely that of MN variability from one donor to the other. Although several groups have confirmed the role of the ADCI mechanism in parasite killing (33–35), some have reported difficulties in obtaining reproducible results. The design of a technique to cryopreserve validated aliquots from a single cytapheresis and the identification of the conditions necessary to induce ADCI activity by a cloned cell line in a reproducible manner should permit the assay to be performed more easily by other research groups. The availability of a well-defined positive control, the human mAb RAM-1, and of the synthetic PMz, are also important components of this standardization process.

From a fundamental point of view, our results indicate that MN activation occurs when two distinct FcγRs are simultaneously engaged by a minimum of two cytophilic IgG molecules bound to an Ag containing at least two B cell determinants. Results show that MN-Ab cooperation is an ADCC type of mechanism which has similarities, but also differences, with ADCC described against other pathogens (29). Our study using, on the one hand, monoclonal human IgGs and, on the other hand, PMz presenting peptides expressing one or several antigenic determinants allowed us to determine precisely the characteristics of the Ag-Ab complex required to engage FcγRs.

Previous studies had indicated that FcγRIIa, but not FcγRI was involved in ADCI (7). However, it has been since documented that a mAb blocking a given FcγR could concomitantly trigger other FcγR via its free Fc region (36), which is the case of the anti-FcγRI IgG2a mAb197 (7, 37). Therefore, results obtained using $F(ab')_2$ of blocking mAbs exclude a nonspecific engagement of other FcγR. Our results show that FcγRI is only involved in phagocytosis whereas the role of FcγRII in ADCI is now better grounded. FcγRII exists as two isoforms: FcγRIIa which leads to the activation of the effector cell and FcγRIIb which exerts an opposite inhibitory effect. Results obtained using the mAb AT10 and previous work with mAb IV.3 (7) demonstrate that only FcγRIIa is involved in the triggering of the antiplasmodial ADCI effect. In addition, human genetics studies and epidemiological investigations lend support to this conclusion: individuals bearing a mutation at position 131 on FcγRIIa, which results in an increased affinity for IgG2, were reported to be more susceptible to malaria (38).

The requirement for FcγRIII in ADCI had not been suspected previously, possibly because it was considered not to be expressed on MN (39). Its involvement in ADCI may seem surprising as this receptor is expressed by a minor proportion of blood MN. Its absolute requirement to achieve parasite killing is supported by two convergent findings: first, anti-FcγRIII $F(ab')_2$ totally abolished ADCI and, second, using THP-1 cells cultured under various conditions of differentiation, the ADCI effect was found to directly correlate with the expression of the FcγRIII.

The total absence of an ADCI effect when either FcγRII or FcγRIII were blocked also suggests that the simultaneous activation of both is required to achieve MN activation leading to an antiparasite effect. This result distinguishes ADCI from other ADCC mechanisms described to date as the need for two distinct FcγRs has been only reported for FcγRI and RII (40), FcγRI and FcαI (41), FcγRII and FcγRIIIb (42) and not, to our knowledge, for FcγRIIa and FcγRIIIa as is the case for ADCI. This also suggests that the clinical consequences of FcγRIIIa polymorphism, and combinations of polymorphisms of FcγRIIa and/or FcγRIIIa, now deserve to be investigated as they may influence susceptibility to malarial infection, as they do for bacterial, viral, and other parasitic infections (43).

Other cells that are known to express FcγRII and FcγRIII, such as PMN or NK cells, nonetheless do not show ADCI activity. Beyond obvious physiological differences between those cells and MN, this may also rely on molecular differences. Indeed, FcγRIII expressed by PMN is a GPI-anchored protein (FcγRIIIb) while that found on MN is a transmembrane receptor (FcγRIIIa) (44). The expression of FcγRIIIa on NK cells is also remarkably heterogeneous both among individuals and from cell to cell (45).

The various classes of IgG differ notably in their H chain and only IgG1 and IgG3 show a potent affinity for FcγR (46). The design of a single mAb, constructed with either of six distinct H chains, confirmed unequivocally that neither IgG2, IgG4, IgA1 nor IgA2, but only IgG1 or IgG3 can activate ADCI. Nevertheless,

IgG2 may have an occasional role in vivo because IgG2 is cytophilic to an isoform of FcγRII (H-131) (46) which is prevalent in the African populations (47). Results also exclude the involvement of FcαR pathway in ADCI.

The use of the anti-malarial mAb, RAM-1, expressed as either IgG1 or IgG3 (19) allowed the first formal comparison of the effectiveness in ADCI of the two main cytophilic classes under identical conditions of specificity and affinity. The results confirmed that both subclasses were efficient in ADCI but that IgG3 was ~60% more active than IgG1 over a large range of concentrations. This result is supported by numerous immunoclinical findings where either total IgG3 or Ag-specific IgG3 have been consistently reported to be associated with protection against malaria (48, 49). The superiority of IgG3 over IgG1 is likely related to the length of its hinge region, leading to increased flexibility and ability to link both Ag and FcγRs (50). Moreover, IgG1 may also down-regulate MN activation by triggering the regulatory FcγRIIb (51). Though IgG3 is clearly more potent, IgG1 still plays a substantial role in ADCI as shown by our results and by the effect in ADCI of Abs induced in human volunteers by the MSP-3 vaccine, which were mostly of IgG1 subclass (14). Finally, IgG1 is also far more stable and has a longer half-life than IgG3.

Thus, in vitro results are consistent with clinical observations concerning the role of IgG3, the consequences of FcγRII polymorphism and support that Ab-MN cooperation is a main defense mechanism. Indeed, we have previously argued that an indirect mechanism such as ADCI is consistent with the phenomenon of chronicity which is characteristic of malaria in hyperendemic regions (52). The resulting low-grade chronic infection makes sense in evolutionary terms as it satisfies the need to ensure parasite survival, though at a low density to also ensure host survival. Other potentially significant findings which emerge from our study are the minute amounts of effector cells, Ab and Ag required for triggering a potent antiparasitic effect.

Indeed, only a very small number of effector cells are required. MN constitute a small proportion of the leukocyte population. In addition, our results suggest that only those expressing FcγRIII, which constitute ~5–15% of the total MN population, are able to achieve parasite killing by ADCI.

Similarly, the number of effective IgG molecules required to achieve parasite killing is remarkably low. A biological effect was detected at concentrations as low as 700 pM (100 ng/ml), i.e., at concentrations within the range at which hormones, including dopamine, cortisone, and testosterone, are effective (53). These concentrations are 1000-fold lower than those reported to achieve a 50% inhibition of merozoites invasion by Abs (54, 55). The number of Ab molecules required to neutralize all antigenic targets in an invasion inhibition mechanism is understandably far larger than that required to trigger MN activation through FcγRs. These results are in keeping with in vivo findings as anti-MSP-3 IgG3 concentrations in humans are relatively low, yet very strongly associated with protection (15). It might also be relevant that the threshold of anti-MSP-3 IgG3 concentrations distinguishing protected from nonprotected children in the African village of Dielmo was found to be ~100–150 ng/ml (C. Roussilhon, C. Oeuvray, C. Muller-Graf, A. Tall, C. Rogier, J.-F. Trape, M. Theisen, A. Balde, J.-L. Pérignon, and P. Druilhe, submitted for publication).

The Ag side of the MN activation further revealed novel and for some of them potentially provocative features as nonmalarial Ags, soluble Ags, and single Ags could be just as effective as merozoites. Along with the low numbers of MN and IgG required to promote an ADCI effect, low numbers of merozoites, i.e., a single per MN, triggered the maximal effect observed. A single malarial Ag triggered the same extent of parasite killing as the whole merozoite, expressing a large number of Ags. This suggests that the induction of maximal defenses by vaccination may not require to combine Ags, as currently thought (56).

As no other molecule than an Ag is needed, it should therefore come as no surprise that any other Ag, together with the corresponding cytophilic Abs, can also trigger MN to exert an antiparasitic effect. This finding is, at first glance, puzzling as it appears to be poorly parasite specific. However, in subjects chronically infected by *Plasmodium*, there are actually very few situations where a sufficiently large number of any other microorganisms per MN will be found to activate ADCI (although trypanosomes cannot be excluded). For instance, during an episode of septicemia, bacteria are in the range of $10^4$- to $10^5$-fold less abundant than plasmodia (57).

That soluble Ags are just as efficient as merozoite surface Ags at triggering ADCI has immediate implications for vaccine discovery and development. At a time when genome mining provides an overwhelming number of new candidates to investigate, this finding should direct future research, in addition to merozoite surface components, to the Ags that are released when an infected RBC bursts. As ADCI is an indirect mechanism of defense, we have argued that there is no direct immune pressure generating antigenic polymorphism (52). Conserved antigenic determinants offer substantial advantages for vaccine development and this is indeed the case for the MSP-3-LSP vaccine candidate recently undergoing clinical trials (14, 58). Investigations into genomic and proteomic data for molecules released by erythrocytic schizonts, presenting conserved regions, and targeted by cytophilic human Abs, shall lead to the discovery of novel vaccine candidates that have not been searched for, up to now.

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## Disclosures

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