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Plasmodium falciparum Malaria: Evidence for an Isotype Imbalance Which May Be Responsible for Delayed Acquisition of Protective Immunity

HASNAA BOUHAROUN-TAYOUN AND PIERRE DRUILHE*

Laboratoire de Parasitologie Médicale, Institut Pasteur, 28 Rue du Dr Roux, 75015 Paris, France

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In view of the recent demonstration that antibodies that are protective against *Plasmodium falciparum* malaria may act in collaboration with blood monocytes, we investigated the isotype content of sera from individuals with defined clinical states of resistance or susceptibility to malaria. Profound differences in the distribution of each immunoglobulin subclass were found. Immunoglobulin G1 (IgG1) and IgG3, two cytophilic isotypes, predominated in protected subjects. In nonprotected subjects, i.e., children and adults that have sustained a primary malarial attack, four different situations were encountered: (i) an imbalance in which IgG2, a noncytophilic class, predominated (mostly seen in primary attacks), (ii) an imbalance also concerning IgG2 but only of a given antigenic specificity, (iii) an imbalance in which mostly IgM antibodies predominated (a frequent event in children), and, less frequently, (iv) an overall low level of antimalarial antibodies. Of 33 nonimmune subjects studied, all but one had one of the above defects. The function of total immunoglobulin presenting such an isotype imbalance was studied in vitro in antibody-dependent cellular inhibition assays. IgG from protected subjects cooperated efficiently with blood monocytes, whereas IgG from nonprotected groups did not. Also, the latter could inhibit the in vitro effect of the former: in competition assays whole IgG from primary-attack cases with increased IgG2 content and IgG or IgM from children from endemic areas competed with IgG from immune adults. This led us to formulate the hypothesis that nonprotected subjects have antibodies to epitopes critical for protection, but that these antibodies are nonfunctional. These results bring some clues to the very long delay required to reach protection against malaria and clearly stress the need to investigate immune responses in both quantitative and qualitative terms.

Previous studies have emphasized the critical role of antibody-cell cooperation mechanisms in the defense against the blood stages of *Plasmodium falciparum* (2, 13, 15). It was shown that the antibodies that proved protective in vivo by passive transfer of immune immunoglobulin in humans had no effect in vitro on the growth of *P. falciparum* asexual blood stages unless they were allowed to cooperate with blood monocytes. It was also shown that antibodies that were not protective in vivo were unable to exert in vitro an antimalarial effect by cooperation with monocytes (2).

In this context of a monocyte-dependent effect of antibodies, the subclasses of antibodies produced in response to infection are of particular importance, since certain isotypes such as immunoglobulin G2 (IgG2) and IgM, being noncytophilic, are unable to specifically arm cytotoxic cells.

We and others observed that the quantity of antibody produced by malaria-exposed subjects was not predictive in terms of protection (6, 16). Despite the presence of high levels of antimalarial antibodies, some individuals developed a rising parasitemia accompanied by symptoms. Such antibodies from nonprotected individuals were unable to cooperate in vitro with monocytes. In contrast, antibodies developed by individuals who had reached a state of protection were not quantitatively more abundant but could confer protection when transferred passively and furthermore, when tested in vitro, promoted an antibody-dependent cytotoxic mechanism. Finally the Western immunoblot patterns of antigens recognized by these two groups of protected and nonprotected subjects did not, at first glance, show any major qualitative differences that may be responsible for the differing in vivo and in vitro effects of these antibodies (2) (unpublished data).

In view of these observations we formulated the hypothesis that functional differences may exist among antibodies of the same antigenic specificity. We thus investigated the isotype distribution in sera from protected and nonprotected individuals. The possibility that antibodies exhibiting a different isotype pattern may be directed to the same target epitopes was investigated in vitro by means of competition assays.

MATERIALS AND METHODS

Sera, IgG, and IgM. The sera used in this study were selected from the following subjects. (i) Serum samples from 156 immune African adults living in a rural area of the Ivory Coast (used as pooled sera) proved to be effective in vivo upon passive transfer in humans (2). The state of clinical resistance to malaria was defined by the absence of clinically patent malarial attacks in those subjects. (ii) The second group comprised individual serum samples from nine immune African adults from the same area of Ivory Coast and one from Gabon. (iii) Serum samples were obtained from 12 subjects that had sustained primary *P. falciparum* attacks. Included were 10 French travelers who had received drug treatment (6 to 15 days after the start of fever) for malaria and 2 Thai children. Such individuals can be considered as susceptible to malaria, since it is well known that a single malarial attack does not protect against further infections. (iv) Serum samples from 30 individuals from the African village of Garitenga in Burkina Faso savanna were included:

* Corresponding author.

8 aged 20 to 50 years, 8 aged 11 to 15 years, 7 aged 6 to 10 years, and 7 aged 2 to 5 years. (v) Sera from 10 healthy French blood donors and a commercially available pool made from more than 1,000 healthy individuals (Biotransfusion CRTS, Lille, France) were used as controls.

In antibody-dependent cellular inhibition (ADCI) assays, only purified immunoglobulin was used. IgG from the pool of immune Africans and the pool of normal French blood donors (control IgG) was prepared at Biotransfusion CRTS by using the Cohn ethanol method as described elsewhere (2).

IgG was extracted from the remaining individual sera by ion-exchange chromatography on DEAE-Trisacryl (IBF Biotechnics, Villeneuve-La-Garenne, France) according to the manufacturer's specifications. When required, IgM was extracted by using the fast-protein liquid chromatography system of Sampson et al. (22). The IgG and IgM were dialyzed against RPMI 1640–35 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–23 mM $NaHCO_3$, adjusted by concentration to the original IgG or IgM level in the donor serum, and sterilized by filtration on 0.22-μm-pore-size Millex filters (Millipore, Continental Water Systems, Bedford, Mass.).

The presence and purity of the IgG or IgM obtained were evaluated in dot blot assays with polyclonal antibodies specific for IgG, IgM, or IgA and by serial serum dilutions in dot blots with a total extract of *P. falciparum* blood-stage proteins as the antigen and the original serum as a control.

Determination of MAb isotype specificity. Based on the results of an international meeting (11) and on their reactivity with defined human myeloma proteins, monoclonal antibodies (MAbs) were selected for their ability to recognize equally well the corresponding isotype in sera from Caucasians, Blacks, or Asians. Several MAbs had to be excluded since they were mostly directed to an allotype that was absent or very rare in one of the three races. For our experiments, we selected the following: mouse anti-IgG1 (NL16), -IgG3 (ZG4), and -IgG4 (GB7B) MAbs were purchased from Unipath (Bedford, United Kingdom), anti-IgM (MBII) was obtained from Bio yeda (Rehovot, Israël), and anti-IgG2 (HP6002) was a generous gift from R. G. Hamilton (Baltimore, Md.).

The specificity of the above MAbs, which was reported previously (11), was further studied by enzyme-linked immunosorbent dot blot assays performed with a panel of purified myeloma MAb IgG subclasses (a gift from J. L. Preud'homme and P. Aucouturier, Poitiers, France) and IgM (a gift from F. Danon, Paris, France). A range of dilutions of each MAb was used to determine the concentrations that enabled us to obtain isotype-specific results and gave about the same density of reaction, as measured with a densitometer, for each subclass. It was further determined that in such conditions each of the MAbs, at the dilution chosen, was able to provide isotype-specific results in a panel of sera from Caucasians, Blacks, and Asians.

Estimation of immunoglobulin subclass distribution with Western blots. Western blots were performed by using as the antigen mature schizonts of *P. falciparum* NF54 separated by flotation on plasmagel (20). After the parasites were washed in RPMI medium, they were extracted in Laemmli sample buffer and electrophoresed in 7.5% polyacrylamide gels containing sodium dodecyl sulfate (14). Proteins were then electroblotted onto nitrocellulose filters (BA 85; Schleicher & Schuell, Inc., Dassel, Germany) (25). Nitrocellulose strips were blocked in 5% nonfat milk (Régilait; Saint-Martin-Belle-Roche, France) and Tris buffer (50 mM Tris-HCl [pH 8], 0.15 M NaCl) and incubated with human sera diluted 1/100 in the same buffer. After washing, the strips were incubated with one of the following MAbs (dilutions given within parentheses): NL16 (1/2,000), HP6002 (1/5,000), ZG4 (1/5,000), GB7B (1/15,000), or MBII (1/15,000). After the strips were washed, they were incubated with ^{125}I-labeled (9) anti-mouse antibodies (Biosys, Compiegne, France) diluted 1/2,000, washed, and autoradiographed at −70°C. Western blots were scanned by using a scanning densitometer (GS300 Hoefer Scientific Instruments, San Francisco, Calif.). The area under each peak was calculated and expressed in arbitrary units by comparison with a known positive control reference serum used on each gel, enabling us to quantitatively compare results from different donors.

ADCI assay. An ADCI assay was performed as previously described (2) with parasites (NF54 strain) cultured in RPMI 1640 medium supplemented with 10% normal human serum (26). Normal monocytes were obtained from healthy French blood donors and separated from peripheral blood mononuclear cells by adherence on microtitration plate wells (Nunclon; Nunc, Roskilde, Denmark). *P. falciparum*-infected erythrocytes, at a 0.5% parasitemia, were added at a ratio of 200 erythrocytes per monocyte. The culture medium (RPMI 1640 plus 10% human serum) was supplemented with each of the purified IgGs to be tested at 10% of its initial concentration in the donor serum. Control wells consisted of (i) culture with control IgG, (ii) culture with test IgG without monocytes, and (iii) culture with control IgG and monocytes. The assay duration was 48 h. Parasitemia was estimated in thin smears from each well by microscopic examination of over 10,000 erythrocytes. The specific growth inhibitory index (SGI), which takes into account the possible inhibition induced by either cells or antibodies (IgG) alone, used as controls in each experiment, was calculated as follows: SGI = {1 − [(percent parasitemia with monocyte and test IgG/percent parasitemia with test IgG)/(percent parasitemia with monocyte and control IgG/percent parasitemia with control IgG)]} × 100. Competition assays were performed in exactly the same conditions as described above for direct ADCI assays, except that immunoglobulins derived from two different individuals were added in the same well. This included in each case IgGs from hyperimmune individuals known to promote an ADCI effect and a sample of either IgG or IgM from another individual that was being assayed for competitive antibodies (or IgG or IgM from control subjects). The concentrations used in these assays were uniformly derived from the concentrations of the whole IgG in the donor sera. This led us to use IgG concentrations of 2.5 mg/ml for hyperimmune subjects (10% of the serum concentration) and 1.25 mg/ml for primary-attack (PA) cases (10% of the donor IgG concentration), in both control and test wells. The results from control and test wells are expressed like those for direct ADCI, and the SGI was calculated as described above.

RESULTS

In preliminary experiments we compared the immunoglobulin distribution in the pool of African IgG previously used in passive transfer in vivo with that of three sera from nonprotected PA patients. The isotype distributions in the clinically protected African pool and in the nonprotected subjects differed markedly (Fig. 1). The most abundant antimalarial antibodies in the Africans appeared to be of the IgG1, IgG3, and, to a lesser extent, IgG4 isotypes. In contrast, IgG2, which is usually the second most abundantly

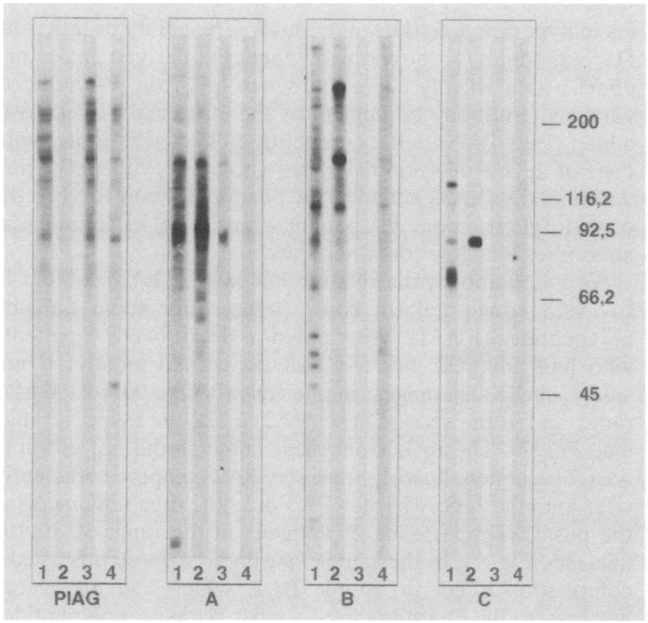

FIG. 1. Analysis of the IgG subclass distribution in a pool of immune African IgGs (PIAG) and in the sera from three PA patients (A, B, C). Western blots were performed with extracts of *P. falciparum* NF54 as the antigen and mouse MAb directed to human IgG1 (lane 1), IgG2 (lane 2), IgG3 (lane 3), or IgG4 (lane 4) as a second antibody; bands were revealed with ^{125}I-labeled anti-mouse antibody.

produced isotype after IgG1, was present in low amounts. There was thus a clear increase in IgG3 and a decrease in IgG2 as compared with the normal distribution (1). This isotype distribution differs from that observed in response to nonmalarial antigens such as *Streptococcus* antigens (18).

In contrast, IgG3 and IgG4 antibodies appeared to be consistently low in the three nonprotected (PA) subjects. The other major difference with the Africans was found in the IgG2 isotype response. IgG2 antibody levels were elevated and directed either to most antigens or to only a restricted number of parasite polypeptides (Fig. 1).

The isotype imbalance between clinically protected and nonprotected subjects indicated by the above results was further analyzed at the individual level in two groups of 10 subjects with defined histories of malaria. To enable an estimate of the relative amounts of antibodies of each subclass, the autoradiographs were scanned and the areas under the curves were measured. We are aware that such results do not express the total amount of a given isotype; however, they allow a comparative study of isotype production by individuals with different clinical statuses within a given experiment. The results obtained with six individual sera are shown in Fig. 2.

Although the relative proportions of antibodies of each isotype varied from one individual to another, the levels of IgG3 antibodies were consistently elevated in each of the African immune adults, whereas IgG2 antibody levels were relatively low. IgG3 production represented 69.7 ± 51.7% (mean ± standard deviation) and IgG2 production represented 53.9 ± 35.6% of the amount of IgG1 antibodies (Fig. 2). In the 10 Europeans recovering from a PA, more diverse situations were encountered. Some individuals simply had an overall low response in each subclass but particularly low responses in IgG3 and IgG4 (data not shown). Four individuals had high to very high levels of antimalarial antibodies but relatively low IgG3 levels compared with those of the Africans and elevated responses in the IgG2 isotype as compared with those of African adults and with their own IgG3 responses. IgG2 represented on the average 88 ± 70% of the IgG1 content, and IgG3 represented only 38 ± 39% of the IgG1 content. IgM antibodies were low in 8 of these 10 PA cases (data not shown). In the two samples where IgM antibodies were found, the IgG2 antibody levels were elevated.

In the follow-up of these cases, some antibody specificities to defined polypeptides decreased or disappeared; however, the isotype distribution remained approximately the same. The ratio of cytophilic (IgG1 and IgG3) to noncytophilic (IgG2, IgG4, and IgM) antibodies varied from 0.5 to 1.2 in

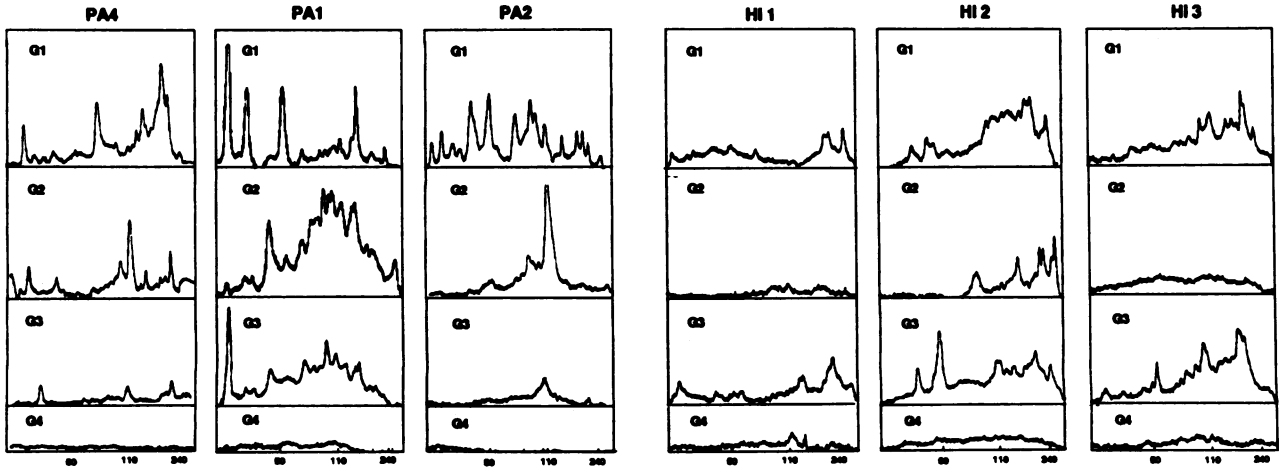

FIG. 2. Scan graphs derived from Western blots revealing IgG subclasses in HI and PA individuals. The graph shows for each isotype the density of a given band on the ordinate (optical density values; the scale is the same for all graphs shown) and the molecular mass range (in kilodaltons) on the abscissa. Western blots were performed as described in the legend to Fig. 1 with sera from three European PA patients (PA1, PA2, and PA4) and three HI African adults (HI 1, HI 2, and HI 3). The nitrocellulose strips were revealed with MAbs directed to human IgG1 (G1), IgG2 (G2), IgG3 (G3), or IgG4 (G4), autoradiographed, and scanned with a scanning densitometer.

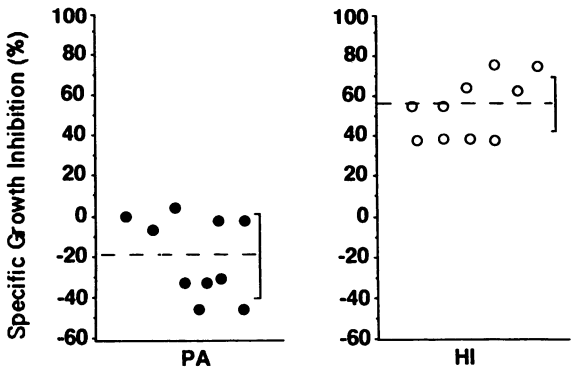

FIG. 3. SGI index recorded in ADCI assays performed with the IgG fraction from 10 PA cases and 10 HI cases. *P. falciparum* was cultured for 48 h in the presence of monocytes and the IgG to be tested. The SGI expresses the specific growth inhibitory effect of monocyte-IgG cooperation and takes into account the possible inhibitory effect of either cells or antibodies alone (see Materials and Methods). Negative values correspond to an enhancement of growth. The difference in SGI between PA and HI is highly significant (χ^2 test; $P = 0.001$).

the PA cases and from 1.4 to 3.4 in the African adults. These results led us to hypothesize that the absence of protection observed after a single infection may result not from an absence of immune response to those antigens targeted by a protective immunity but rather from an abnormal immune response (mostly noncytophilic antibodies) to those antigens. This hypothesis was investigated in vitro by using ADCI, which had been identified as one of the possible effector mechanisms of protective immunity (2).

In the presence of blood monocytes, the IgGs from all PA cases were unable to promote an inhibitory effect on *P. falciparum* monocyte multiplication, whereas IgG samples from African adults consistently yielded a significant inhibitory effect on growth (Fig. 3).

Competition assays were performed to evaluate the possibility that the higher proportion of noncytophilic antibodies in PA sera interferes with the function of cytophilic antibodies in hyperimmune (HI) individuals. The ability of IgG from HI individuals to promote a monocyte-derived inhibitory effect was markedly decreased when IgG from PA subjects with high antibody content (4 of the 10 cases studied) was added (Fig. 4). No such competitive effect was found with control IgG from nonmalarious subjects, with IgG from various HI subjects added to the positive HI control (Fig. 4), or with IgG from one PA patient whose level of antimalarial antibodies was low (data not shown).

As stated above, the relative increase of IgG2 content in PA sera concerned, in some cases, only some parasite polypeptides (Fig. 1). We studied in detail two PA patients who had an IgG2 increase that concerned mostly, if not solely, single size ranges: in one case (PA2) a 90- to 100-kDa range and in the second case (PA3) a 120- to 130-kDa range (see Fig. 7). The IgGs from these subjects did not promote ADCI (data not shown); however, PA2 competed efficiently with immune IgG, whereas PA3 did not, thus underscoring the possible importance of antigens in the range 90 to 100 kDa (see Fig. 7). In the same experiment we also observed a competition effect in sample PA1, which had an overall increase of IgG2 antibodies to most parasite polypeptides (Fig. 2).

Since African children living in areas where malaria is hyperendemic or holoendemic remain susceptible to malaria infection, with the occurrence of high parasitemias and symptoms usually up to the age of about 15 years and the development of resistance by the age of 15 to 20 years, we studied the isotype distributions in various age groups of subjects from a village of the African savanna in Burkina Faso.

The patterns obtained proved to be complex and more diverse than in the preceding group of patients studied. High levels of all classes of antibodies were found in many children. However, the levels of either the IgG2 or more frequently the IgM isotype were almost always higher in these children than in adults from the same village. Therefore the ratio of cytophilic to noncytophilic antibodies seemed to be on the average lower in the children than in adults.

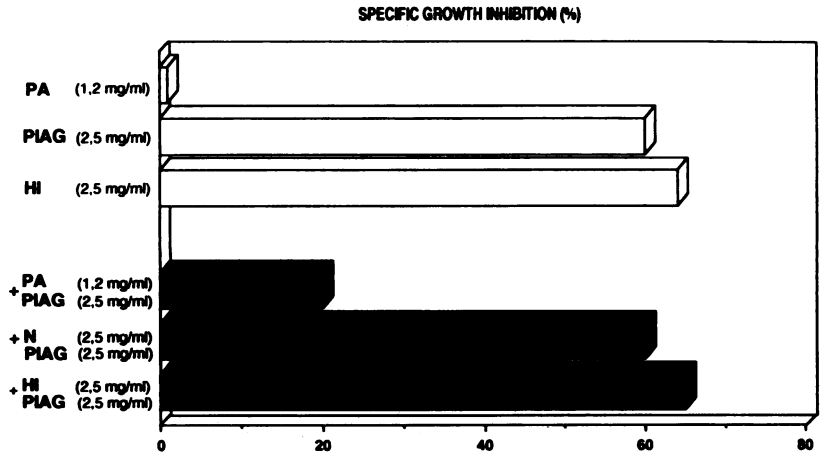

FIG. 4. Competition ADCI assays. Open bars correspond to the SGI obtained in ADCI when IgG from a single individual was added to *P. falciparum* culture in the presence of monocytes. Solid bars correspond to the SGI recorded when IgGs from two different individuals were used in the ADCI assay. PA, IgG from a PA case; PIAG, pool of immune African IgGs; HI, IgG from an individual HI African; N, pool of IgG from normal French blood donors. Shown are the final concentrations of each IgG preparation in the assay, which correspond to 1/10 of their concentration in the donor serum.

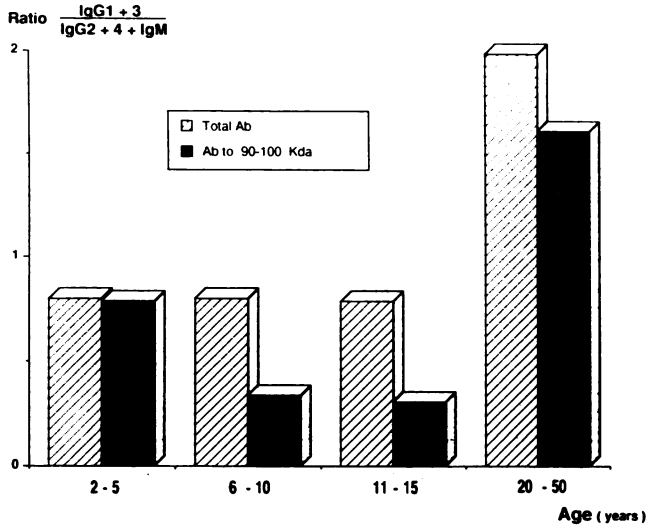

FIG. 5. Distribution of cytophilic and noncytophilic antimalarial antibodies in African subjects of different age groups. ▨, whole antimalarial antibodies; ■, antibodies specifically directed to the 90- to 100-kDa range. Autoradiographs of Western blots specific for IgG1, IgG2, IgG3, IgG4, and IgM class-specific antimalarial antibodies from each individual were scanned, the areas under the curves were measured, and the ratios of the cytophilic isotypes (IgG1 and IgG3) to the noncytophilic isotypes (IgG2, IgG4, IgM) were calculated. Shown are the geometric means of the various ratios recorded in 31 individuals (8 adults, 8 children aged 11 to 15, and 7 from each of the two remaining age groups). The differences between the results from protected subjects and those from nonprotected subjects are statistically significant both for whole antimalarial antibodies (Student-Fisher test; $P < 0.05$) and for antibodies directed to 90- to 100-kDa molecules ($P < 0.01$).

The results obtained in Western blots were scanned, the areas under the curves were measured, and the ratios of the cytophilic IgG1 and IgG3 isotypes to the remaining noncytophilic IgG2, IgG4, and IgM isotypes were calculated. There is a clear-cut difference in this ratio between children and adults when all specificities or the response to the polypeptides of 90 to 100 kDa (Fig. 5) is considered. The ratio is below 1, indicating a majority of noncytophilic antibodies, in the nonprotected children and above 1 in the protected adults, who have a majority of cytophilic antibodies. Surprisingly, the pattern of evolution does not show a progressive modification of these ratios from adolescence to adulthood but rather an abrupt switch in the immunoglobulin classes expressed above the age of 20 years. These ratios are lower in children over 5 years old than in those under 5 years of age; the low antimalarial IgG2 levels in children under the age of 5 were expected, because this subclass is known to be produced only at very low levels in younger children (17).

In the adults, IgG1 was, as before, the main isotype produced. Among the 22 children studied, the results appear to fall into three categories (Fig. 6). (i) Three children showed a relative increase of IgG2 antibodies similar to that seen in European PA patients (data not shown). (ii) More frequently (in 13 of the 22 cases), children had a high content of IgM antibodies (or sometimes of both IgG2 and IgM antibodies). This IgM imbalance was not restricted to the 90- to 100-kDa range of antigens, as was seen in the PA patients, but in all cases concerned a large range of antigens including those in the 90- to 100-kDa range (infants 4 and 6 in Fig. 6). (iii) Some children (5 of 22 cases) had an overall low total antibody content, with similar proportions of IgG1 and IgG2 and lower amounts of IgG3, IgG4, and IgM antibodies or with only IgG1 (infant 5 in Fig. 6).

A detailed study of each adult and each child proved consistent with the hypothesis that the isotype imbalance or a low antibody response may be responsible for the particular status of resistance or susceptibility to the disease. IgM was the predominant isotype in 13 of 22 children, and IgG2 was predominant in 4 of 22 children. Among the remaining five children, four had a low antibody response and only one had a pattern similar to that of adults. It is noteworthy that IgM levels reached up to 97% in one child and represented in the average of the 13 cases 39 ± 23% of the total antibodies.

Among the eight adults studied in the same village, six had antibodies predominantly of the IgG1 isotype and one had antibodies predominantly of the IgG3 isotype. In these seven cases IgG1 and IgG3 represented on the average 68 ± 12% of the total antibodies produced. Only one adult differed from the above pattern; his predominant isotype was IgG2 (38% of the total). However, in this individual, the antibodies directed to the 90- to 100-kDa antigens were mainly IgG1 and IgG3.

In view of the frequency of IgM antibodies in children, competition assays were carried out as described above with IgM antibodies purified from two children with relatively high antimalarial IgM antibody contents. Such IgM preparations proved able to compete, despite their low concentration, in the ADCI assay with IgG from HI adults (Fig. 7) but had no ADCI-promoting effect by themselves (data not shown). Finally, IgG prepared from one of the children showed an elevated IgG2 response against the 90- to 100-kDa antigens and was able to compete with clinically effective HI antibodies, as was the case with IgG from PA individuals (Fig. 7).

DISCUSSION

Several decades of clinical studies have shown the uniqueness of the immune resistance that develops in humans (5, 19, 30). No such resistance seems to take place after one or a limited number of infections. Both European adults going to malaria-prone areas and young children from the same areas remain clinically susceptible to superinfections after one, two, or three infections. Even in experimentally induced *P. falciparum* malaria, in which a single isolate or strain was used, one or two induced infections did not result in resistance to a third or fourth challenge (4).

In subjects living in areas where mosquitoes transmit malaria at least once or more per year (i.e., intertropical Africa), two types of acquired resistance can be observed. The first type, acquired by the age of 3 to 5 years, is resistance to the neurological complications induced by *P. falciparum*. The second type, which can be considered to be true antiparasite immunity, seems to occur only by the age of 15 to 20 years and corresponds to the ability to control high parasitic loads. Numerous surveys in hyperendemic or holoendemic areas have established that the pathology induced by the parasite is almost absent from adults who harbor only very low level parasitemia. Acquired antiparasite immunity has thus been coined "premunition" to reflect the fact that it is an incomplete, nonsterilizing immunity that is acquired progressively (23).

In recent years a limited number of studies have focused on the mechanisms mediating premunition in humans; to our knowledge, almost none was designed to elucidate the basis of the very long delay required to reach this state.

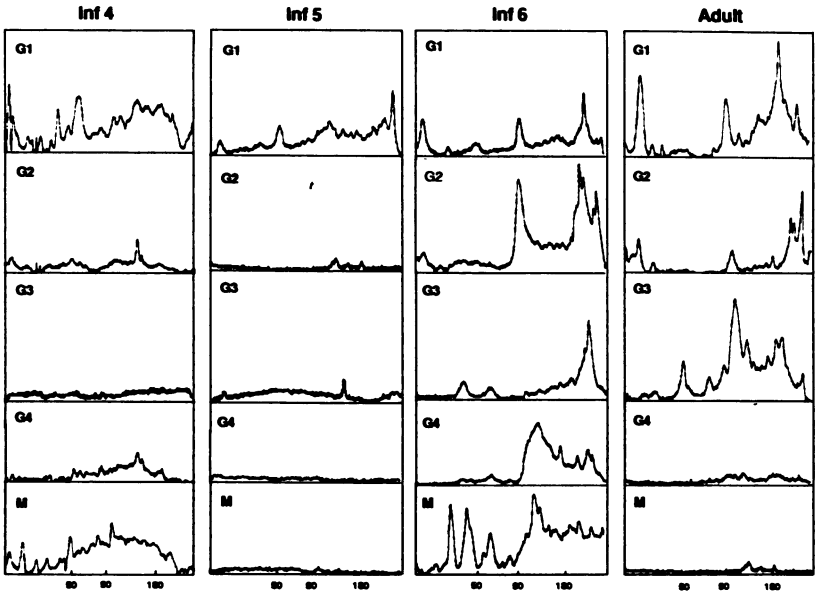

FIG. 6. Isotype distribution of antimalarial antibodies in three African children and one African adult. Shown are scans of Western blot strips from each individual in which IgG1, (G1), IgG2 (G2), IgG3 (G3), IgG4 (G4), and IgM (M) responses were measured (see legend to Fig. 2). Children were 8 years (Inf 4), 3 years (Inf 5), and 12 years (Inf 6) old.

Our previous in vitro studies led us to the idea that naturally occurring antibodies may by themselves be unable to directly limit parasite growth and caused us to suspect that such antibodies act indirectly by stimulating blood monocytes through the Fc receptors after binding to their parasite target (13, 15) (unpublished data). This hypothesis was recently strengthened by the results of a comparative in vivo-in vitro analysis (2). It was confirmed that IgG from protected African adults was able to passively transfer clinical protection to nonimmune infected Thai children. The

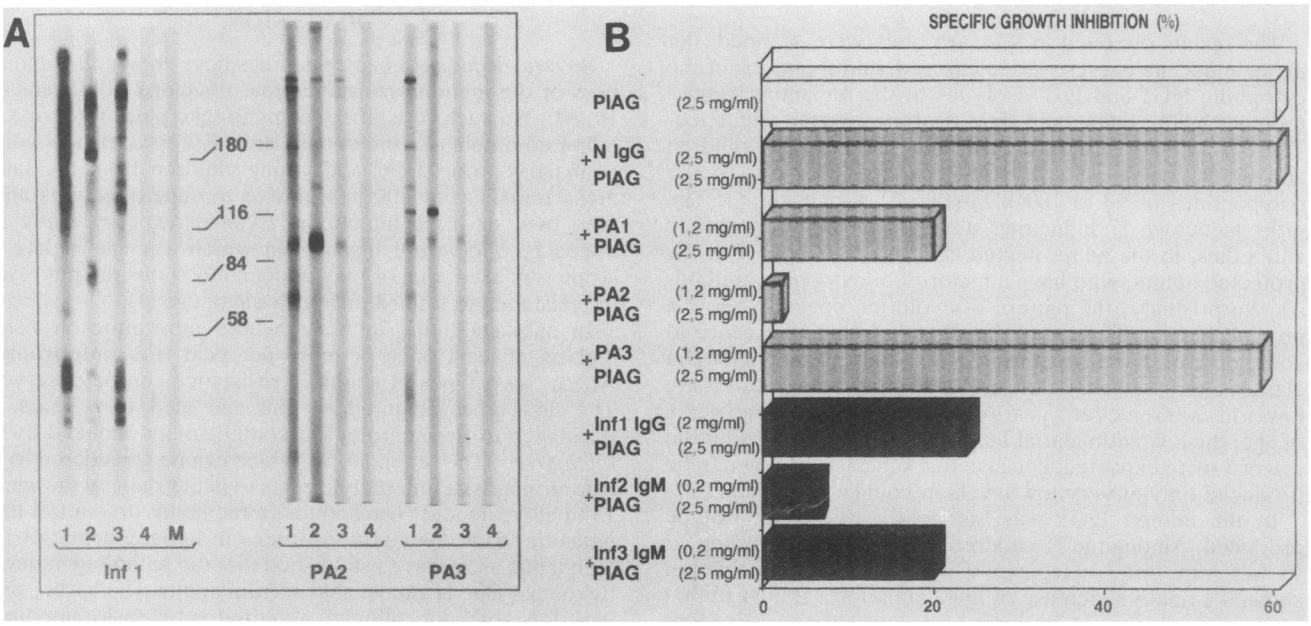

FIG. 7. Comparative analysis of isotype content (A) and the results of direct ADCI and competition ADCI assays (B). (A) Western blot showing the isotype distribution (IgG1 [lanes 1], IgG2 [lanes 2], IgG3 [lanes 3], IgG4 [lanes 4], and IgM [lane M]) of the *P. falciparum* antibody content in sera from one child (Inf 1) and two European PA adults (PA2 and PA3). (B) The SGI recorded in ADCI performed with pooled immune African IgGs (PIAG) alone (□) or pooled IgGs together with normal IgG (N IgG), two IgGs from individual PA sera (PA1, PA2, PA3), IgG from one African infant (Inf 1 IgG), or IgM from two African children (Inf 2 IgM and Inf 3 IgM). In competition ADCI assays, IgG from Inf 1 and IgG from PA2 were found, when mixed with the pooled IgGs, to reduce the ADCI effect of the latter, whereas the IgG from PA3 did not. Shown are the final concentrations of each IgG preparation in the assay, which correspond to 1/10 of their concentration in the donor serum.

strain from each receiver was also cultured and used to assess the in vitro effect of the clinically noneffective immunoglobulins from the receivers and the clinically effective IgG from the donors. The involvement of an ADCI effect induced by blood monocytes upon *P. falciparum* in vitro growth was clearly shown by results from various in vitro studies (2, 13, 15).

In studies aimed at developing a malaria vaccine, the emphasis has been put on the antigenic specificity of the antibodies studied and on the quantities of these antibodies. However, the biological function of the antibodies produced by exposed individuals or the antibodies induced in vaccinated animals has seldom been analyzed.

In view of the possibility that antibodies may act in cooperation with blood monocytes, we decided to study the relative proportions of cytophilic and noncytophilic antibodies.

The distribution of isotypes produced clearly differed between the group of immune subjects and the group of nonimmune subjects. Moreover, it correlated in almost all cases studied with the ability of their immunoglobulin to promote an ADCI effect. Seven of the eight protected adults had a majority of either IgG1 or IgG3, two cytophilic isotypes. In the two groups of nonimmune subjects studied, four different possibilities were encountered. The children and PA adults had either (i) an imbalance in which IgG2 predominated, (ii) an imbalance also concerning IgG2 but only of a given antigenic specificity, (iii) an imbalance in which mostly IgM antibodies predominated, or (iv) an overall low level of antimalarial antibodies. Among the nonimmune subjects studied, all but one had one of the above defects. Only 1 of a total of 32 individuals (22 children, 10 PA adults) had an isotype pattern similar to that of protected adults.

This pattern of distribution of antimalarial antibodies is in agreement with previous reports. Our study brings complementary information to the overall picture and enables us to discuss it in the context of a defense mechanism that relies on cytophilic isotypes. An isotype imbalance was previously found in Swedish PA cases compared with HI adults from Liberia (27). Antimalarial antibodies of various specificities were detected by using an enzyme-linked immunosorbent assay with a *P. falciparum* whole-cell extract as the antigen. Increased IgG2 levels were found in many PA cases, and increased IgG3 levels were found in many protected African subjects. Antimalarial IgM antibodies were not studied. In a further series, Wahlgren et al. (28) reported a high prevalence of IgM antibodies in African children aged 2 to 15 years; however the amounts of the antibodies were not determined, and adults from the same area were not studied simultaneously.

Similarly, in mouse malaria, distinct isotypic patterns of antibody response to *Plasmodium chabaudi* were reported to occur in protected and nonprotected animals (8). In various strains of mice the production of cytophilic classes of antibodies against *Plasmodium yoelii* was found predominantly in strains of mice that developed a mild disease but not in those that developed severe disease (24); cytophilic IgG2a was the only immunoglobulin that altered the course of parasitemia (29).

These findings along with our results can now be interpreted in the perspective of a defense mechanism that requires monocytes and antibodies that attach to them via their Fc receptors, namely, IgG1 and IgG3, and of a possible blocking effect of the noncytophilic classes, namely, IgG2, IgM, and maybe IgG4. When we attempted to correlate the isotype distribution observed with the biological function of those immunoglobulins in the monocyte-immunoglobulin ADCI cooperation assay with whole IgG preparations, very clear-cut results were obtained both in direct assays with preparations from PA cases (consistently negative) and HI cases (consistently positive) and in competition ADCI assays. When immunoglobulins from two individuals are artificially mixed, such as an IgG from a PA subject with increased IgG2 content (or an IgM from a child), the competition with HI IgG suggests that the proportion of noncytophilic antibodies was high enough in the nonimmune serum to block cytophilic antibodies in the immune serum or that the affinity of the noncytophilic antibodies to the target antigen was greater than that of the cytophilic antibodies. These results confirm indirectly that our evaluation of the isotype content was correct. They also indicate that the target epitopes of antibodies from PA or African children are similar to the epitopes against which protective antibodies from the African adults are directed. This in turn suggests that antigens that target protective mechanisms could induce a strong immune response, although of the wrong isotype, even after a single malaria attack.

Of particular interest are the cases of imbalance limited to a narrow range of molecular weights (i.e., IgG2 in PA cases); they indicate which antigens may play a major role (i.e., those in the 90- to 100-kDa range).

The above findings deserve further investigation. It is likely that the production of noncytophilic antibodies may not be the sole means of escape induced by the parasite. Our findings nevertheless modify the perspective in which immunity to malaria should be envisaged and also have implications for vaccine development.

These results lead us to raise the hypothesis that the progressive development of a state of protective immunity may, in fact, correspond to a progressive modification in the immune response regulation and not, as has been thought for a long time, to the slow development of a response to either poorly immunogenic or highly polymorphic molecules. Data from Western blot analysis and ADCI competition assays suggest that humans exposed once or a limited number of times to malaria do develop high levels of antibodies to antigens being targeted by protective mechanisms, although their antibodies are not functional. In contrast, the acquisition of a state of resistance appears to correlate with the ability to develop antibodies of the proper isotype (mostly IgG3 and also IgG1) and to reduce the proportion of noncytophilic isotypes (IgG2 and IgM) of the same specificity that block the effector mechanisms.

It is of interest to see that, depending on the immune competence of the host, various escape mechanisms can develop. Antibodies of the IgG2 isotype appear in our study to be the main blocking isotype in adult patients, whereas in endemic areas, in young children who are unable to produce high levels of IgG2, the parasite induces preferentially the synthesis of blocking IgM antibodies. In a limited number of patients, the low levels of all antibody classes produced suggest the existence of other escape mechanisms such as the induction of T-suppressor cells or the production of anti-idiotypes.

The imbalance in isotypes described herein is probably responsible for the epidemiological pattern of antibodies promoting merozoite phagocytosis described previously in subjects from various age groups in areas where malaria is endemic (6) and the differential pattern of antibodies promoting infected monkey erythrocyte phagocytosis in PA and HI individuals described recently (10). Such phagocytosis as-

says were abandoned when we observed that they could be mediated by monocytes as well as by polymorphonuclear cells and other macrophages, whereas only monocytes inhibited the asexual blood stage cycle of *P. falciparum* (15). Thus, although we no longer consider phagocytosis as a major effector mechanism, the same antibody imbalance is likely to be responsible for the results recorded in the ADCI and phagocytosis assays among subjects with various states of immunity to malaria.

These results are reminiscent of similar findings about the immune response toward another group of parasites, schistosomes, in which protective mechanisms seem also to depend on accessory cells. Several cell types were reported to have a crucial role in defense against schistosomes by antibody-dependent cellular cytotoxicity mechanisms (3). IgG2 and IgG4 competed with IgG1 and IgG3 in these assays (12), and epidemiological surveys found increased levels of IgG4 antibodies in nonprotected subjects (3, 7). The parallel between the strategies developed by the two parasites is striking, especially when one recalls that the age patterns of susceptibility or resistance to the two diseases are very similar. The mechanisms leading to the early production of IgG2, IgM, or both are far from being known. In schistosomiasis, as could be expected, such responses were found to be mainly induced by polysaccharides (7). In the study of malaria, little attention has been given to the identification of epitopes defined by polysaccharides. However, the structure of malaria parasite proteins is predominantly repetitive, and recent studies have emphasized that proper T-cell help is lacking in many instances (21). It could be proposed that malarial proteins with repetitive epitopes and defective T-helper epitopes behave immunologically as polysaccharides and would therefore induce IgG2 and IgM antibodies preferentially. It remains to be determined whether the induction of inappropriate (i.e., blocking) isotypes per se is a feature of the antigens being targeted by the ADCI mechanism or whether it is induced by some other molecule(s) presented at the same time to the immune system by the parasite.

Our results have several important implications in terms of vaccine development. Generally speaking, they stress the need to examine the quality of a given immune response and not only its quantity. This would allow better interpretation of vaccination trials aimed at identifying valuable vaccine candidates when no protection was reached in animals despite the induction of a strong immune response. The identification of antigens relevant in acquired protection has to be reevaluated with isotype-specific reagents. The cellular events leading to the preferential production of a given isotype need to be investigated, and the responsibility of the immunizing antigen or the simultaneously presented molecules in inducing an isotype imbalance has to be assessed experimentally.

Finally, these findings can be considered encouraging for the future development of a malaria vaccine. If the long delay required to reach protection is not due to an extreme polymorphism of target antigens but rather to an abnormal, nonefficient immune response to a given molecule(s), the chances of artificially producing a proper immune response with a vaccine are probably greater than if all possible variant antigens had to be identified and included in one vaccine preparation.

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