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POLYCLONAL B-LYMPHOCYTE ACTIVATION AND SENSITIZATION OF ERYTHROCYTES BY IgG IN HUMAN MALARIA: RELEVANCE TO THE DEVELOPMENT OF ANAEMIA IN A HOLOENDEMIC AREA IN NORTHWESTERN BRAZIL (ARIQUEMES – RONDÔNIA)

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In order to investigate whether the phenomena of polyclonal B-cell activation (PBA) and of red blood cell (RBC) sensitization by immunoglobulins (Ig) and complement were relevant to the development of the malaria anaemia 138 malaria infected and 49 non-infected individuals from an endemic region in the northwest of Brazil were studied for the degree of activation of Ig (G, A and M) secreting cells (IgSC) by a reverse haemolytical plaque assay, for the degree of RBC sensitization by a sensitive immunoradiometric assay and for the anaemia. The numbers of activated IgGSC and IgMSC were found to be significantly increased in MI when compared to NMI, the same was true for the amount of RBC associated IgG (but not IgM or C3d). The degree of anaemia was not related to the parasitaemia but was positively and significantly related to the degree of RBC-sensitization by IgG. This increase in the amount of IgG-RBC was not related to the increase in the numbers of IgGSC. These data suggest that although the sensitization of erythrocytes by IgG molecules can be involved in the pathogenesis of the malaria anaemia it does not seem to be a direct consequence of the malaria associated PBA phenomenom.

The human *Plasmodia* are obligate intracellular parasites that infect and destroy RBC during their cycle in the vertebrate host. Therefore malaria infection is usually accompanied by a variable degree of anaemia that however does not correlate with the level of parasitaemia and quite often becomes evident when no more parasites can be detected in the circulating blood. These facts suggested the participation on immunological factors in its pathogenesis.

In fact, besides mechanical rupture or an anti-plasmodial antibody (Ab) dependent lysis of parasitized RBC (reviewed by Seed & Kreier, 1980) and a mild degree of bone marrow depression (Woodruff, Ansdell & Pettitt, 1979) several immune mechanisms could operate in the production of the malarial anaemia (Zuckerman, 1966). Classicaly the evoked mechanisms are the adsorption of parasite antigen (Ag) or non erythrocytic auto-Ag to the surface membrane of non parasitized RBC with the consequent fixation of the corresponding Ab; the adsorption of preformed immune complexes to the RBC membrane through its complement receptor and finally the fixation of anti-erythrocyte auto-Abs. These auto-Abs could appear as a result of cross-reactions between *Plasmodium* and erythrocyte as those that exist between RBC allo-Ag and other parasites (Damian, 1964) or of a polyclonal activation of erythrocyte specific autoreactive clones of B-lymphocytes (Hammarstrom et al., 1976).

Indeed, the PBA phenomenon has been demonstrated to exist both in rodent (Freeman & Parish, 1978; Rosenberg, 1978; Daniel Ribeiro, 1983) and in human malaria (Banic, Galvão Castro & Daniel Ribeiro, 1986, manuscript in preparation) and moreover it is known that PBA can activate autoreactive cells both *in vivo* and *in vitro* leading to the formation of several auto-Abs including anti-erythrocyte Ab (Hammarstrom et al., 1976). In the same way PBA can induce the formation of immune complexes (e.g. idyotype/anti-id Ab-Lambert et al., 1983) that could adhere to the RBC membrane.

In order to study these points we investigated the relationship between the degree of PBA, that of RBC sensitization and that of anaemia in 138 malaria infected (MI) and 49 non-infected (NMI) individuals from a holoendemic area of malaria in the northwest of Brazil (Ariquemes – Rondônia).

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MATERIAL AND METHODS

Individuals – Individuals from the city of Ariquemes (State of Rondônia) 200 km far from Porto Velho by the Porto Velho–Cuiabá road were studied. Ariquemes is a holoendemic area of malaria in the State of Rondônia which concentrates a large proportion of the recorded cases of malaria in Brazil (40% in 1984 – Fundação SESP, 1985) (Fig. 1).

Blood samples were collected by venipuncture of all examined individuals between August and October 1985. All 138MI were seen at the out-patients clinic of the SUCAM station or hospitalized at the Hospital unit of the Fundação Serviços de Saúde Pública (SESP) and had their diagnosis established on the basis of the direct observation of *Plasmodium* parasites in the thick blood smear. After the blood sample had been collected all MI were treated according to the schedules precluded by SUCAM. The 49 NMI considered as normal controls of the region were asymptomatic and had no previous history of malaria.

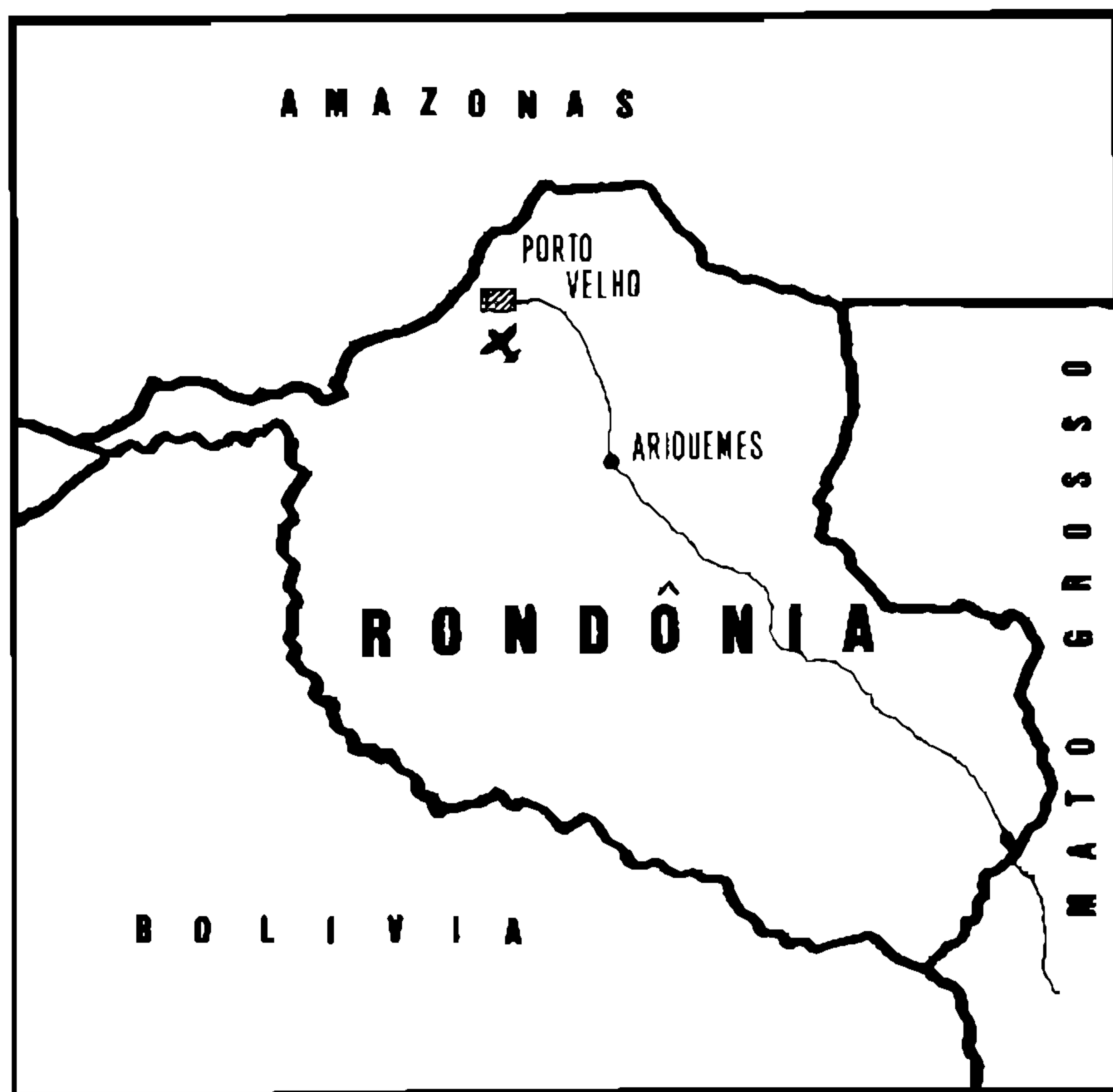

Fig. 1: Geographical situation of the Rondônia state's village of Ariquemes in the northwest of Brazil.

Peripheral blood mononucleated cells (PBM) – Blood samples were collected with heparin (10μ/ml final concentration) and diluted twice with RPMI 1640 (Sigma Chemical Company, St. Louis, Mo., USA) supplemented with 25 mM hepes buffer (Sigma Chemical Company) and 5% fetal calf serum (FCS) (heat inactivated at 56°C for 30 min.). PBM were obtained by standard methods using Ficoll-Hypaque (Histopaque-Sigma Chemical Company) density gradient centrifugation (400g, 30 min. at RT). After washing twice with RPMI 1640-hepes the cells (> 95% viable as determined by trypan blue exclusion) were incubated for 60 min. at 37°C in RPMI 1640-hepes + 5% FCS and washed twice with RPMI 1640-hepes.

Detection of Ig (G, A or M) secreting cells in the peripheral blood – In order to evaluate the number of cells spontaneously secreting Ig in the peripheral blood we used the reverse protein-A Plaque Forming Cell (PFC) assay as described by Gronowicz, Coutinho & Melchers (1976) and adapted to the Cunningham & Szenberg (1968) system by Dammaco et al. (1984). This technique employs *Staphylococcus aureus* protein A (Spa), which has a specificity to the Fc portion of the IgG molecules, and rabbit anti-human Ig, and detects Ig secretion of all specificities and classes.

Protein A coupled sheep red blood cells (SpA-SRBC) – (SpA-SRBC) was prepared by incubating two parts of SpA (Pharmacia Fine Chemicals, 575104, Uppsala-Sweden) (1 mg/ml 0.15M NaCl) and two parts of packed SRBC 6x washed in 0.9% NaCl 10 min. at RT. 0.1 part of a 1/2 dilution of a $CrCl_3$ ($6H_2O$) (13.3 mg/ml destilled water) solution and 20 parts of 0.15M NaCl were then added, all components were mixed and allowed to react for 15 min. at 37°C and

at RT for 20 min. The SpA-SRBC were washed three times with 0.15M NaCl, resuspended 25% v/v in RPMI 1640 and used within three days of preparation. The success of this coupling procedure was assessed by routine haemagglutination reactions using rabbit Ig and sheep anti-rabbit Ig.

The protein A reverse PFC assay – Each assay was done in quadruplicata and, unless otherwise specified, with all reagents diluted or suspended in RPMI 1640.

Briefly, 25μl of a 25% suspension of SpA-RBC and 25μl of an appropriate dilution of a rabbit anti-human IgG, IgA or IgM (1 in 20, 1 in 10 and 1 in 10) (Hoechst-Behring) were mixed in microtiter wells. One hundred μl of the washed PBM to be assayed containing approximately $2x10^5$ cells and 50μl of a 1 in 8 dilution of SRBC absorbed guinea pig serum as source of complement were added to the mixture. Double chambers capable of holding 100μl each were filled with this mixture, sealed with liquid paraffin and incubated at 37°C in a humidified atmospher for one hour, removed to room temperature and analyzed under a stereomicroscope by dark field illumination after another hour. Results were expressed as numbers of IgSC per 10^6 PBM or per mm^3. In all experiments negative controls without complement and/or without anti-Ig were included to determine the presence of background PFC to SpA-SRBC.

DETECTION OF RBC ASSOCIATED IgG, IgM or C3d

Erythrocytes – Erythrocytes were obtained from the pellet used for separation of PBM after the removal of the buffy coat layer and washed five times with 0.15M phosphate buffered saline (PBS) with 0.2% bovine serum albumim (BSA) and 0.1% sodium azide (PBS-BSA) at 4°C.

Iodination of SpA and immunoradiometric assay (IRMA) for detection of erythrocyte bound Ig (E-Ig) – SpA was labelled with 125_I (NaI, New England Nuclear, Boston, Mass, USA) to specific activities of 0.8-1.1 ci/g by the method of Fraker & Speck (1978), employing 1, 3, 4, 6 tetrachlore 3a, 6a, diphenylglycouril (Iodogen, Pierce Chemical, Il., USA) as oxidizing agent (Pontes de Carvalho et al., 1982).

IRMA for detection of E-IgG – Three hundred μl (15ng) of a radiolabelled SpA solution in PBS-BSA, pH8, were mixed with 300μl of packed erythrocytes in 1.8ml microfuge tubes and incubated overnight at 4°C on an inversion rotator. Tubes were then centrifuged at 4°C (500g 15 min.) the supernatant discarded and the radioactivity in the pellet counted in a gamma counter (Beckman Gamma 4000 counting System, Beckman Instruments Inc., Fullerton, CA, USA), each sample being assayed in duplicata.

IRMA for detection of E-IgM and E-C3d – Three hundred μl of packed erythrocytes were incubated with 300μl of a 1/600 dilution of a rabbit anti-human IgM or C3d (Hoechst-Behring) for one hour at 37°C and one hour at 4°C. Erythrocytes were then washed three times in PBS-BSA (500g 5 min 4°C) and 300μl of iodinated SpA (15ng) were distributed in each tube and incubated overnight at 4°C. Cells were then washed three times with PBS-BSA 0.2% and the radioactivity present in the pellet counted in the gamma counter, 300μl of the iodinated SpA gave the 100% radioactivity of the test.

Haematology – Haematocrite (Ht), Haemoglobin (Hb) and Erythrocyte sedimentation rate (ESR) were determined by classical procedures (Dacie & Lewis, 1975).

Statistical analysis – Statistical significance of the data was assessed by the student's T test for unpaired data and the r coefficient correlation as appropriate.

RESULTS

Polyclonal B-cell activation (PBA) – As shown in Table I the mean numbers of IgGSC and of IgMSC of both *P. falciparum* and *P. vivax* MI were significantly higher than those recorded for NMI of the same region. In both *P. falciparum* MI and *P. vivax* MI the relationship observed between each possible pair of groups of class specific IgSC (IgG and IgM; IgG and IgA, IgM and IgA) was always positive and statistically significant. Fig. 2 shows representative data for IgMSC and IgASC ($r = 0.9$ $p < 0.001$) in *P. falciparum* MI. Considering the mean value calculated for NMI plus two standard errors (SE) as the highest value accepted as normal the percentages of individuals presenting "high" numbers of IgSC were always significantly higher among MI than among NMI ($P < 0.01$ for IgGSC and IgMSC and $P < 0.05$ for IgASC) (Fig. 3b).

A significant positive relationship was observed between the numbers of IgGSC, IgASC and IgMSC and the parasitaemia ($r = 0.3$ – $P < 0.005$ in all cases) but no relationship was observed between the IgSC numbers and those of past attacks of malaria (results not shown).

TABLE I

IgG, IgA and IgM secreting cells per 10^6 peripheral blood mononucleated cells or per mm^3 ($m \pm 1SD$) in malarious compared to non malarious individuals in Ariquemes (Rondônia) in northwestern Brazil

individuals infected with:	IgGSC /10^6C	IgGSC /mm^3	IgASC /10^6C	IgASC /mm^3	IgMSC /10^6C	IgMSC /mm^3
P. falciparum:	**** 569±929 (73)	** 3.4±6.6 (60)	125±363 (55)	* 0.8±1.75 (49)	*** 178±460 (52)	** 1±2.3 (47)
P. vivax:	**** 538±790 (68)	*** 3.7±5.1 (58)	* 71±126 (60)	0.6±1.3 (53)	***** 86±166 (52)	** 0.6±1.15 (53)
Non Malarious: Controls	204±210 (52)	1.5±1.55 (50)	31±39 (46)	0.3±0.4 (42)	22±42 (39)	0.2±0.3 (38)

*$p < 0.05$; **$p < 0.02$; ***$p < 0.01$; ***$p < 0.002$; *****$p < 0.001$

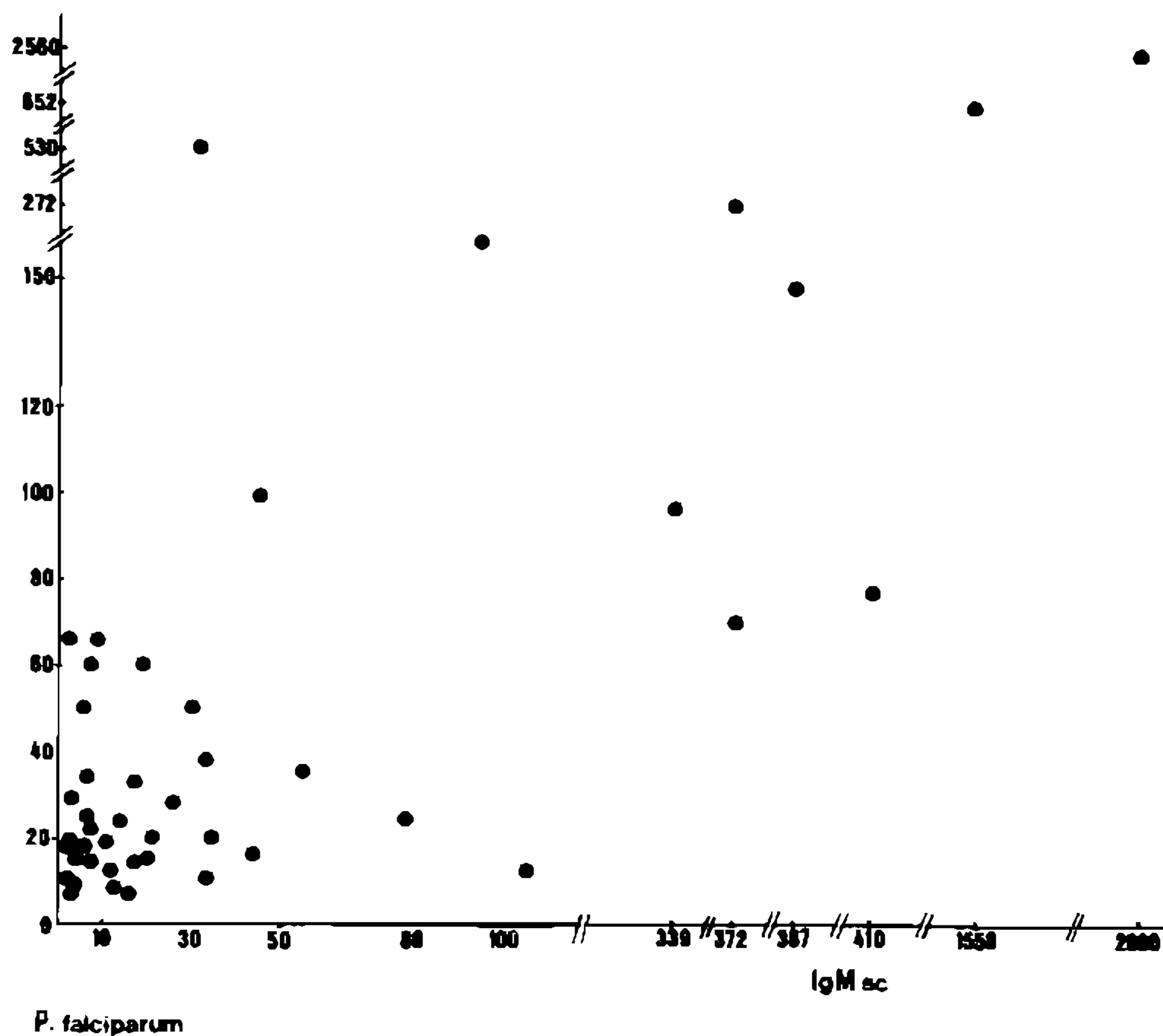

Fig. 2: Relationship between the numbers of IgA and of IgM secreting cells (sc) per 10^6 peripheral blood mononucleated cells in *P. falciparum* infected individuals.

Similarly no difference was observed in the number of Ig (G, A or M) SC between males and females ($P > 0.1$) or those above and below thirty. ($P > 0.8$ – IgGSC and IgMSC; $P > 0.3$ – IgASC).

Red blood cells sensitization by immunoglobulins and complement – As it can be seen in Table II the percentages of IgG (but not of IgM or C3d) sensitized RBC were significantly higher in both *P. falciparum* MI and *P. vivax* MI than in NMI and a significant positive relationship was observed in malarious individuals between the numbers of IgM and those of IgG sensitized RBC ($r = 0.8 P < 0.001$) and between those of IgM and those of C3d-RBC ($r = 0.32 P < 0.001$).

TABLE II

RBC sensitization by IgG, IgM and C3d and anaemia in malarious compared to non malarious individuals in Ariquemes (RO)

	IgG	IgM	C3d	ESR	Hb	Ht
P. falciparum:	13±34.7* (77)	6.5±7.7 (77)	4.5±9.1 (77)	33.3±15.4* (86)	11.8±1.7* (83)	38.2±7.4* (96)
P vivax:	11.7±14.1* (69)	6.8±9 (66)	5.6±11.2 (70)	30.2±14.7* (76)	11.9±3* (77)	41.2±0.6 (86)
Non malarious	–13.9±12.9 (41)	3.6±7.9 (38)	7±11.6 (42)	12.8±8.7 (60)	13.5±1.4 (58)	42.7±7 (62)

*P < 0.001 ESR: Erythrocyte Sedimentation Rate; Hb: Haemoglobin; Ht: Haematocrite

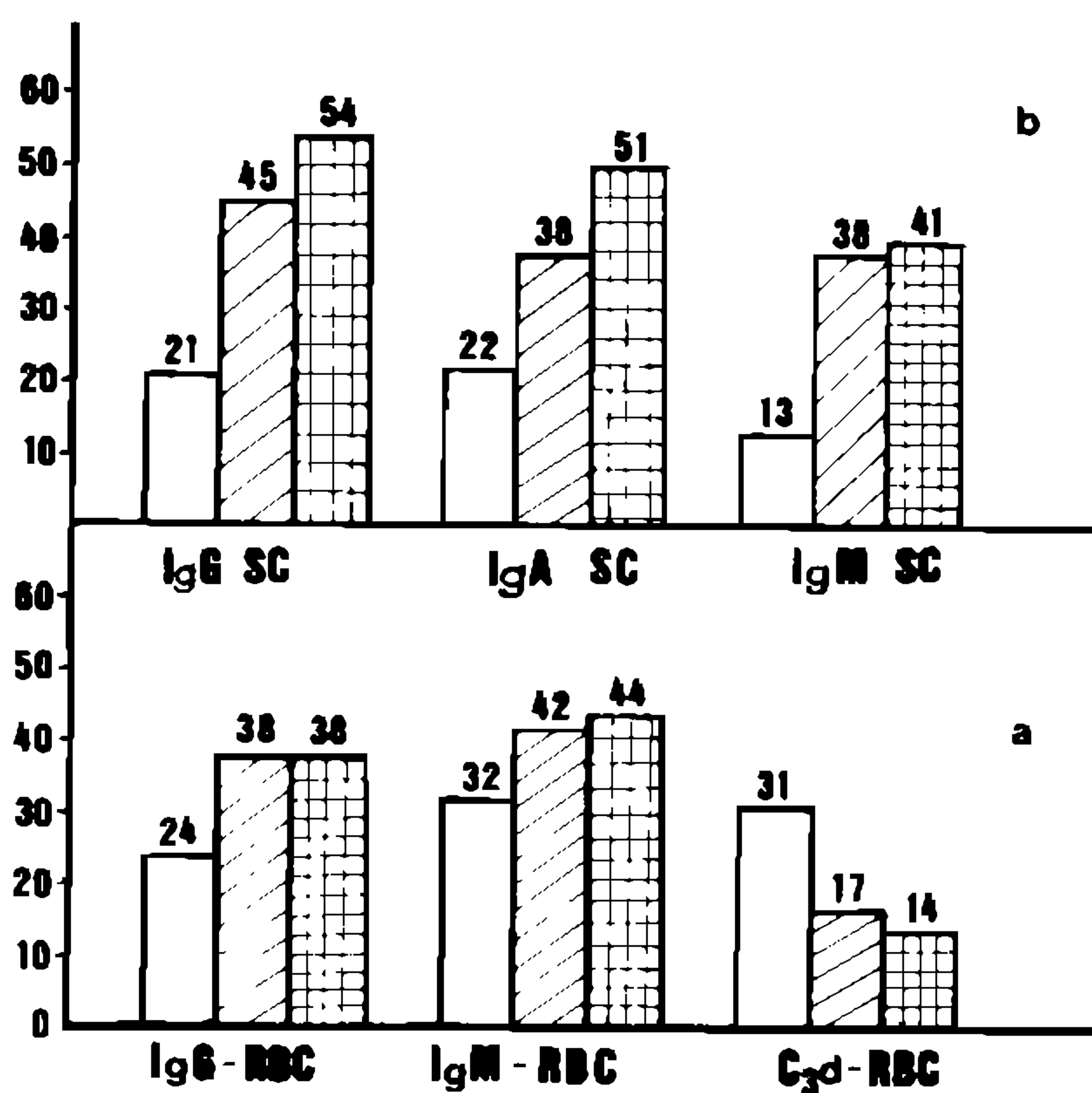

Fig. 3: Percentages of individuals presenting a) sensitized RBC in larger amounts than the highest limit of normality b) "high" numbers of IgSC. For definition of the highest limit of normality and of the "high" numbers see results. ☐ NMI, ▨ *Pf*MI, ▦ *Pv*MI.

Considering the mean value calculated for NMI + 2SE as the highest limit of normality only the percentage of IgG-RBC positive individuals was significantly higher (x^2 = 32.2 P < 0.001) in MI than in NMI. Curiously NMI were more often positive for C3d-RBC than *Pv*.MI and *Pf*.Mi but the differences were not statistically significant (Fig. 3a). No difference was observed among MI in the levels of sensitized RBC between males and females (P > 0.1) nor between those above and below thirty (P > 0.3). Similarly no relationship was observed between the amount of sensitized RBC and the parasitaemia.

Anaemia – As expected the mean Ht and Hb values of MI were significantly lower than those observed in NMI. MI also showed an increase in the ESR (Table II). Considering the mean Ht value of NMI-2SE as the lowest limit of normality 21% of NMI, 56% of *Pf*Mi and 33% of *Pv*MI were anaemic (x^2 = 22.03 P < 0.001). In the same way 40% of NMI, 85% of *Pf*ii and 88% of *Pv*ii presented an increase in the ESR (x^2 = 49.2 P < 0.001). Although a positive relationship was observed between the ESR and the parasitaemia (r = 0.21 P < 0.05) no correlation could be detected between the latter and the Ht and Hb values (P > 0.1). Again no differences were observed in the values of Ht, Hb or ESR between those above and below thirty (P > 0.1). However, as expected the mean Ht value observed in infected females (37.1 + –9) was significantly lower (p < 0.05) than that recorded in infected males (40.4 + 5.9).

Relationship between PBA, RBC sensitization and anaemia – In order to study the role of PBA in the phenomenon of RBC sensitization by Ig and C3d and their relevance in the development of the malaria anaemia we looked for the correlation between 1) the levels of activation of Ig (G, A or M) SC and those of RBC sensitization; 2) the levels of IgSC activation and that of anaemia and 3) the levels of RBC sensitization and that of anaemia. The only statistically significant relationships were: a) a positive one between the numbers of IgGSC/10^6 cells and the amount of C3d-RBC in *Pf*MI and between the numbers of IgMSC/10^6 cells ($r = 0.27$ $P < 0.05$) and the amount of IgG-RBC in *Pf*MI ($r = 0.34$ $P < 0.02$) and b) a negative one between the amount of IgG-RBC and the hematocrite values in *Pv*MI ($r = 0.22$ $P < 0.05$). However, when *Pf*MI individuals were classified according to their degree of RBC sensitization in "high" or "low" RBC-sensitization groups (above or below the mean calculated for MI) the mean Ht level was significantly lower in the "high" (> 4,5%) C3d-RBC (25.8 + 13.9) or "high" (> 13%) IgG-RBC (35.7 + 7.3) groups than in "low" C3d-RBC (38.3 + 7.5 – $t = 6.2$ $P < 0.001$) or IgG-RBC (39.1 + 5.9 $t = 2$ $P = 0.05$) groups.

In the same way *Pv*MI with "high" IgG-RBC levels or "high" (> 6.5%) IgM-RBC levels groups presented respectively higher numbers of IgASC (393 + 556) and of IgMSC (159 + 242) per 10^6 cells than *Pv*MI in the low levels groups (70 + 139 and 42 + 65 respectively $P < 0.001$ and $P < 0.05$).

DISCUSSION

The present report clearly shows for the first time that malaria infected individuals (MI) living in areas where malaria is endemic presented a high degree of PBA as indicated by the two or three fold increase in the mean number of cells spontaneously secreting IgG (IgGSC) or (IgMSC) when compared to NMI from the same region. However this increase, recorded for the subjects concerned by this study, is relatively low when compared to the seven fold increase in the numbers of IgGSC presented by non-immune individuals who contracted malaria during first visits to endemic regions in Amazonia (Banic, Galvão-Castro & Daniel Ribeiro, manuscript in preparation). The meaning of these differences is not known but one tentative explanation is that immune individuals could be less susceptible to the PBA properties of *Plasmodium* than non-immune individuals and this idea is in agreement with our recent observation that individuals with higher levels of Abs directed against the main epitope of the circumsporozoite protein of the sporozoite surface present a lower degree of PBA than those with low levels of these Abs (Oliveira-Ferreira et al., 1986).

Plasmodium extracts or supernatants of *in vitro* culture have been shown to possess the capacity of inducing lymphocyte proliferation *in vitro* (Greenwood, Oduloju & Platts-Mills, 1979; Ballet et al., 1981; Gabrielsen & Jensen, 1982) or even antibody synthesis *in vivo* (Freeman & Parish, 1978; Rosenberg, 1978; Burger, Daniel-Ribeiro & Ballet, manuscript in preparation) and in some cases the degree of the induced lymphocyte proliferation was related to the number of parasites in the *in vitro* culture or in the RBC preparations. In this study we have observed that the amount of IgGSC and of IgMSC in malaria infected individuals was related to the degree of parasitaemia. This is in accordance with our previous observation that in *P. yoelii* infected CBA mice the number of total IgSC rises simultaneously with the rise of the parasitaemia (Daniel-Ribeiro & Burger, manuscript in preparation) and taken together these data could represent an indirect evidence that the PBA in malaria infected individuals is at least partially a consequence of the mitogenic and PBA properties of the *Plasmodium* parasites as previously proposed (Greenwood, 1974).

In the present study no correlation could be detected between the Ht and Hb values on one hand and the parasitaemia on the other, showing once more that the malaria anaemia is not a direct consequence of RBC infection and destruction by *Plasmodium* parasites and this fact leads us to discuss the participation of immunological mechanisms in the development of the malaria anaemia.

One possible mechanism that could be involved is the sensitization of non parasitized RBC by antibodies and complement. We could in effect find a high degree of IgG (but not of IgM or C3d) sensitization of RBC in MI.

The demonstration of the presence of IgG on the membrane of RBC in malarious patients has already been done by Facer & Brown (1979) and Abdalla & Weatherall (1982) in Gambian children with *P. falciparum* malaria. These authors however have also reported a high prevalence of C3d-RBC bearing patients, a fact that we failed to observe in this study. Once more the reasons for this difference are not understood but one fact that must be emphasized is that those studies concerned only children and ours concerned only subjects above 14 years old and Abdalla & Wea-

therall (1982) could observe that IgG-RBC bearing individuals were a significantly older age group than those positive for C3d-RBC. It is possible that this relationship between the level of IgG-RBC sensitization and age would in fact reflect another one between the development of IgG-RBC and the acquisition of protective malarial immunity, since in autochtonous populations of endemic areas, as those studied by these authors, one can consider that the age reflects the degree of an individual's immunity. Indeed Abdalla & Weatherall (1982) have clearly shown that IgG-RBC positive (but not the negative) Gambian children as well as adults, presented schizont opsonizing antibody in their sera. One must however keep in mind that our population consisted of migrant people from non-malarious areas as well as individuals living in the endemic region for more than ten years. In these conditions the age does not necessarily reflect the degree of immunity and some other factor(s) (viral or other parasitic infections in childhood should be evoked to explain this difference in the pattern of erythrocyte sensitization between our population and those studied by Facer & Brown (1979) and Abdalla & Weatherall (1982). Finally, like these authors we could not find the sensitization of RBC by IgM reported by Rosenberg et al. (1973).

The data concerning the role of RBC sensitization by immunoglobulins in the development of the malaria anaemia are far from conclusive and even conflicting in many instances. In the present paper we found a positive significant relationship between the degree of sensitization of RBC by IgG and that of anaemia and although this relationship could not be demonstrated in *P. falciparum* MI the Ht mean value was significantly lower in *P. falciparum* MI with higher amounts of IgG-RBC and of C3d-RBC than in those with lower amounts of these sensitized erythrocytes. Our findings suggest that the RBC sensitization by immunoglobulin could be involved in the pathogenesis of anaemia. This idea is also shared by Facer & Brown (1979) who found that the direct antiglobulin test (DAT) was more frequently positive in anaemic than in non-anaemic *P. falciparum* infected children and by Oluyemi, Kelton & Blajchman (1983) who could also observe a positive correlation between the RBC associated IgG and the degree of anaemia. These findings however are not in accordance with those of Abdalla & Weatherall (1982) who could not detect such relationship.

The last point that merits to be discussed is the origin or the mechanism of formation of the erythrocyte bound IgG molecules. The fact that non-parasitized RBC can be sensitized by Ig allows us to consider that these Abs could in fact be auto-Abs and since PBA is known to induce auto-Ab synthesis (Hammarstrom et al., 1976) they could arise as a consequence of the malaria associated PBA phenomenon clearly demonstrated in the present paper. However, in spite of the observance of a positive relationship between the number of activated IgMSC and the degree of RBC sensitization by IgG (the only class of RBC bound Ig found) in *P. vivax* MI, no positive relationship could be found between the degree of activation of Ig secreting cells of a given class and that of sensitization of RBC by the same class of Ig. Moreover we have previously shown that a limited number of auto-Ags is involved in the autoimmune phenomena observed in malaria (Daniel Ribeiro et al., 1983) suggesting that the polyclonal activation of autoreactive cells is not a generalized (non specific) phenomenon. Auto-Abs could also appear as a result of a cross-reaction between RBC and parasites (Abdalla & Weatherall, 1982) and this idea could even implicate that anti-malaria protective immunity involves anti-erythrocyte autoimmunity as proposed by Jarra (1983). One other possibility is that the RBC bound Ig could in fact be anti-plasmodial Abs previously complexed to malarial Ags and passively adsorbed to the membrane of erythrocytes (Facer & Brown, 1979). The data reported in the present paper do not provide any direct evidence that permits a choice between these last hypotheses but the study of the specificity of the RBC associated Ig currently been undertaken in our laboratory could help to clarify this point.

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