J. clin. Path. (1960), **13**, 118.

THE RELATIONSHIP OF SERUM GAMMA-GLOBULIN CONCENTRATION TO MALARIA AND SICKLING

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(RECEIVED FOR PUBLICATION NOVEMBER 9, 1959)

The serum protein patterns of groups of Nigerians including 98 children partially protected by "daraprim" against malaria, and 113 unprotected children, have been studied.

The protected children have a significantly lower concentration of serum γ globulin than the unprotected children from the age of 12 months onwards.

In children, sicklers have a significantly higher serum γ globulin than non-sicklers.

It is suggested that sickling protects against malaria by enhancing the antibody response against the malaria parasite.

Surveys of serum protein patterns in different populations have shown that some communities have a higher mean concentration of serum γ globulin than others (van Oye and Charles, 1951; Holmes, Stanier, Semambo, and Jones, 1951; Arens and Brock, 1954; Vera and Roche, 1956; Edozien, 1957; Rawnsley, Yonan, and Reinhold, 1956; Nuñez Montiel, 1957; Bakker, Bliek, and Luyken, 1957; Curnow, 1957). Race, nutrition, and immune antibody response to certain endemic infections are some of the factors which are thought to be of significance in producing these differences. Of the endemic diseases malaria has been most frequently associated with high serum γ globulin. Holmes, Stanier, and Thompson (1955) in Uganda compared the protein patterns of three groups of Africans who are exposed to different rates of malaria transmission and suggested that a causal relationship may exist between malaria incidence and serum γ globulin level. McGregor, Gilles, Walters, Davies, and Pearson (1956) investigated a group of children, half of whom were protected for three years from malaria by weekly doses of chloroquine: they found significant differences in the γ globulin levels, the unprotected children having higher serum γ globulin than the protected children. In rats, Woodruff (1957) observed a significant increase in serum γ globulin six months after infection with *P. berghei*. In the present study the relationship between γ globulin and malaria prophylaxis is further examined in a group of Nigerian children after varying periods of protection.

Allison (1954) postulated that the sickle-cell trait afforded protection against subtertian malaria and that this advantage of heterozygote AS individuals over homozygous AA individuals was responsible for the maintenance of a high sickling rate in many populations in spite of the fact that homozygous SS individuals are continually being eliminated. There has been much controversy over the validity of Allison's conclusions and the mechanism by which this protection may be produced. A serious limitation in the investigation of this hypothesis is the difficulty of isolating a biological response of the body to the presence of the malaria parasite which can be measured and compared in sicklers and non-sicklers. Much of the work which has been done so far is centred around comparisons of infection rates, erythrocyte parasite densities, and infant mortality rates from malaria in sicklers and non-sicklers. Having established that one of the effects of malaria in a hyperendemic area is to produce a high mean serum γ globulin concentration, we consider that a study of the latter in sicklers and non-sicklers may throw further light on the relationship of sickling to malaria. We have therefore included in this study an examination of the relationship of sickling to serum γ globulin concentration.

Materials

The human subjects studied are (1) 350 Nigerians and 50 European adults at Ibadan (including 200 Nigerians and 40 Europeans previously reported (Edozien, 1958)); (2) 25 adults from Ilora, 35 miles

from Ibadan; (3) 40 patients with kwashiorkor aged 1½–2½ years from the villages near Ibadan; and (4) 211 children living at Imesi, a village 100 miles from Ibadan.

These 211 children are part of a group arising from 400 consecutive births in the village. The group has been studied from early in the mothers' pregnancies and it is hoped to follow the children through to their fifth birthday. One half of the mothers bearing these children were treated with pyrimethamine ("daraprim"), 50 mg. monthly, and the child when born has been given the same treatment, except that the children received 25 mg. a month. Daraprim tablets contain some lactose, and control tablets were made consisting entirely of lactose. The control tablets were used on the other half of the mothers and their offspring. The two tablets are indistinguishable in taste. If a mother or a child develops fever in either group they are given a single dose of chloroquine in the quantity advised by Covell, Coatney, Field, and Jaswant Singh (1955). The "daraprim" and control tablets were administered by the nursing sister who is resident in the village; the majority of the mothers have attended very well for their tablets and many of them have not missed one of their child's monthly tablets over two years.

Liver function tests on the lines previously described (Edozien, 1958) have been used to exclude liver disease in all the subjects included in this study.

Blood was collected by femoral vein puncture from the children and from an antecubital vein in adults. Part of each sample was placed in an oxalated bottle for the sickling test and paper electrophoresis of haemoglobin and the rest allowed to clot. The sera were separated on the day of collection, and the protein analyses on most of the specimens were performed on the same day. Sera which could not be handled on the day of collection were stored in the refrigerator at 4° C. and processed the following day.

Methods

Total Proteins.—The biuret method of Wolfson, Cohn, Calvary, and Ichiba (1948) was used.

Paper Electrophoresis of Proteins.—The method of Flynn and de Mayo (1951) was used. Serum, 6 µl., was applied to strips of Whatman No. 1 filter paper 2.5 cm. wide. Electrophoresis was for 18 hours at a constant current density of 0.5 ma. per strip and room temperature 23° C. ± 1° C. Barbitone buffer of *p*H 8.6 and ionic strength 0.083 was used. The papers were dried for 30 minutes at 110° C., then stained for 10 minutes in 0.75% azocarmine B (Gurr) in 50% methanol and 10% acetic acid and finally washed in successive changes of 10% acetic acid until a clear background was obtained. After drying at 110° C. for 15 minutes the papers were scanned in the "chromograph," an automatic reflectance scanner produced by Joyce, Loebl and Co., Newcastle, England. The protein fractions were computed as percentages of the total protein by dividing the area under each peak by the total area. By applying these percentages to the total serum protein the value of each fraction was obtained. No correction factors were applied: duplicate analyses show that values obtained in this way did not differ from values obtained by transmission densitometry with application of a globulin correction factor of 1.6 as previously reported (Edozien, 1958).

Sickling.—Slide sickling using 2% sodium metabisulphite was performed.

Paper Electrophoresis of Haemoglobin. — The samples were prepared for electrophoresis according to the method of Singer, Chernoff, and Singer (1951). Quantities, each of 25 µl., of an approximately 10% haemoglobin solution were applied from a micropipette to sheets of Whatman No. 3MM paper 20 × 36 cm., five samples and a control AS preparation being applied to one sheet. Barbitone buffer *p*H 8.6 and ionic strength 0.05 was used. Electrophoresis was for 10 to 12 hours at 230 volts and temperature 23° C. ±1° C. in a Shandon vertical tank. At the end of the run the sheets were dried in a hot air oven at 110° C. for 15 minutes.

Results

The Imesi subjects have been divided into two groups. Group A contains 98 children who received regular prophylactic doses of "daraprim" from birth. Group B is the control group, containing 113 children who did not get prophylactic antimalaria therapy although some of them were treated with chloroquine when they had fever. Within each group, the children have been further subdivided into three age groups: 4½–12 months, 12½–18 months, 18½–26 months. Table I shows the age distribution and haemoglobin genotype of the 211 children. In the analyses of the data, three children with γ globulin values of 2.55, 2.89, and 2.93 g./100 ml. have been excluded because these figures are well outside the range of all the other values and may be the result of unsuspected illness at the time the blood was collected.

TABLE I
HAEMOGLOBIN GENOTYPE OF THE 211 IMESI CHILDREN

	Age (Mth.)	AA	AS	SS	AC	CC	SC	Total
Protected	4½–12	35	9	1	0	0	0	45
	12½–18	16	5+1*	0	2	0	0	24
	18½–26	19	7	1	2	0	0	29
Unprotected	4½–12	32	3	2	2	0	0	39
	12½–18	30+1*	5	0	0	0	0	36
	18½–26	28+1*	8	0	1	0	0	38
	Total	161	39	4	7	0	0	211

* Excluded from analysis of data.

In Table II the serum proteins of protected AA and unprotected AA children are compared in order to show the effect of malaria prophylaxis on the serum proteins. It will be observed that there

are no significant differences in albumin, α_1 globulin, α_2 globulin, and β globulin concentrations between protected and unprotected children. Up to the age of 12 months no significant difference is found between the γ globulin concentrations of the two groups. At 12½–18 months and at 18½–26 months, however, there is a statistically significant difference ($0.02>P>0.01$ in either case) between the γ globulin concentrations of the two groups, the protected children having a significantly lower mean concentration of γ globulin.

Comparison of the serum proteins of unprotected AA and unprotected AS children and of protected AS and protected AA children are shown in Tables III and IV respectively. The mean γ globulin for the 13 unprotected AS children for the period 12–26 months of life is 1.79 ± 0.34 g./100 ml. as compared with 1.58 ± 0.32 g./100 ml. for the unprotected AA children of the same age. This difference is statistically significant ($0.05>P>0.02$). The difference between AA and AS individuals almost disappears if protected AS and protected AA children in the period 12–26 months are compared: the serum γ globulin for the protected AS children is 1.41 ± 0.28 g./100 ml. as compared with 1.35 ± 0.30 g./100 ml. for the AA children.

TABLE II
EFFECT OF MALARIA PROPHYLAXIS ON SERUM PROTEINS

	4½–12 Months (67)				12½–18 Months (46)				18½–26 Months (47)			
	Protected AA (35)		Unprotected AA (32)		Protected AA (16)		Unprotected AA (30)		Protected AA (19)		Unprotected AA (28)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Total proteins*	6·80	0·48	6·82	0·55	6·99	0·55	6·95	0·55	6·80	0·52	6·97	0·49
Albumin	3·25	0·40	3·18	0·35	3·29	0·38	3·14	0·38	3·23	0·34	3·25	0·37
α_1 globulin	0·30	0·15	0·32	0·13	0·29	0·13	0·35	0·15	0·29	0·11	0·29	0·12
α_2 ,,	1·07	0·28	1·02	0·21	1·10	0·26	0·87	0·25	0·99	0·19	0·92	0·18
β ,,	0·95	0·25	1·01	0·17	0·96	0·12	1·00	0·16	0·95	0·17	0·95	0·21
γ ,,	1·23	0·32	1·29	0·35	1·35	0·35	1·59	0·37	1·34	0·28	1·56	0·31

* All values expressed in g./100 ml.

TABLE III
SERUM PROTEINS OF UNPROTECTED AS AND AA INDIVIDUALS

	4½–12 Months				12½–18 Months				18½–26 Months			
	AS (3)†		AA (32)		AS (5)		AA (30)		AS (8)		AA (28)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Total proteins*	6·80	0·64	6·82	0·55	6·94	0·38	6·95	0·55	7·40	0·23	6·97	0·49
Albumin	3·39	0·38	3·17	0·35	3·19	0·40	3·14	0·38	3·23	0·18	3·25	0·37
α_1 globulin	0·24	0·11	0·32	0·13	0·26	0·12	0·35	0·15	0·42	0·09	0·29	0·12
α_2 ,,	0·88	0·14	1·02	0·21	0·87	0·18	0·87	0·25	0·97	0·12	0·92	0·18
β ,,	0·98	0·23	1·02	0·17	0·89	0·18	1·00	0·16	0·95	0·14	0·95	0·21
γ ,,	1·31	0·69	1·29	0·35	1·73	0·25	1·59	0·37	1·83	0·40	1·56	0·31

* All values expressed in g./100 ml. serum. † Figures in brackets represent the number of individuals investigated.

TABLE IV
SERUM PROTEINS OF PROTECTED AA AND AS CHILDREN

	4½–12 Months (44)				12½–18 Months (32)				18½–26 Months (26)			
	AA (35)		AS (9)		AA (16)		AS (5)		AA (19)		AS (7)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Total proteins*	6·80	0·48	6·70	0·39	6·99	0·55	6·96	0·39	6·80	0·52	6·90	0·31
Albumin	3·25	0·40	3·36	0·18	3·29	0·38	3·28	0·27	3·23	0·34	3·27	0·21
α_1 globulin	0·30	0·15	0·24	0·08	0·29	0·13	0·30	0·09	0·29	0·11	0·29	0·08
α_2 ,,	1·07	0·28	0·96	0·14	1·10	0·26	1·00	0·13	0·99	0·19	0·93	0·18
β ,,	0·95	0·25	0·95	0·18	0·96	0·12	0·98	0·15	0·95	0·17	0·95	0·16
γ ,,	1·23	0·32	1·19	0·39	1·35	0·35	1·40	0·39	1·34	0·28	1·42	0·23

* Values expressed in g./100 ml.

The analyses of the data from the Ibadan and Ilora subjects are presented in Table V and the serum protein changes in kwashiorkor are shown in Table VI. These results show that (1) the serum albumin of the group of Nigerian professional people is higher than that of Nigerians of lower economic status and approaches very close to the value in Europeans. (2) The mean serum gamma globulin of the professional group, although less than that of the non-professional group, is still significantly higher than the mean for Europeans. (3) The serum albumin of the village adults is only slightly lower than the level found in urban Nigerians who have the same or slightly better economic means, but the γ globulin level is very significantly higher. (4) Severe protein malnutrition (kwashiorkor) profoundly reduces the albumin level, but the changes in γ globulin are not significant. The conclusion drawn from these observations is that in health the serum albumin level is influenced by the nutritional status of the individual, and that the serum γ globulin, although subject to changes in socio-economic conditions, is independent of nutrition.

TABLE V

ANALYSIS OF DATA FOR IBADAN AND ILORA SUBJECTS

	Europeans in Ibadan (50)		Nigerian Professionals (50)		Non-professional Nigerians in Ibadan (300)		Ilora Adults (25)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Total proteins	6·92	0·53	7·27	0·47	6·87	0·40	8·24	0·60
Albumin	4·05	0·30	3·90	0·32	3·40	0·31	3·20	0·41
α_1 globulin	0·32	0·03	0·25	0·08	0·23	0·08	0·28	0·10
α_2 ,,	0·52	0·05	0·51	0·12	0·51	0·13	0·62	0·16
β ,,	0·83	0·12	0·80	0·18	0·68	0·18	0·97	0·19
γ ,,	1·20	0·21	1·81	0·31	2·05	0·35	3·17	0·45

TABLE VI

SERUM PROTEINS IN PATIENTS WITH KWASHIORKOR AND IN HEALTHY CHILDREN

	Healthy Unprotected Children Aged 18–26 Months (36)	Kwashiorkor Patients Aged 1½–2½ Years (40)	
	Mean (g./100 ml.)	Mean (g./100 ml.)	% of Normal Mean
Total proteins ..	7·06±0·50	4·13±0·64	58·5
Albumin	3·24±0·36	1·28±0·37	39·5
α_1 globulin ..	0·31±0·13	0·27±0·11	87·1
α_2 ,, ...	0·95±0·15	0·53±0·14	55·8
β ,, ...	0·95±0·18	0·41±0·19	43·2
γ ,, ...	1·62±0·38	1·64±0·51	101·2

Discussion

In discussing the results obtained in this survey we are fully conscious of the limitations imposed by our methods. The dosage scheme for "daraprim" was not intended to render the subjects completely parasite-free all the time. The village was organized by one of us (D.C.M.) for another clinical research project, and this dosage schedule was adopted because, while rendering the children free of parasites for most of the month, it still permitted a sufficient number of days of exposure to parasites to induce the development of natural immunity against malaria. Full details of infection rates and parasite densities in children protected according to this dosage schedule will be published elsewhere. This dosage scheme had the advantage that it cut down visits to the clinic to a minimum and this was responsible for keeping the number of defaulters to a negligible proportion. We believe that it is reasonable to presume that any differences found between the serum γ globulin levels of the two groups of children would have been greater had the children in group A been rendered free of parasites at all times.

Our data show that children who have received regular prophylactic doses of antimalarial drugs have lower mean γ globulin concentrations than children who have not been protected against malaria. The inference is that a high mean γ globulin concentration is caused, at least in part, by a response of the body to malaria parasites. We are, however, unable to indicate from our results the full extent to which malaria alone can account for the excess γ globulin in the serum of Nigerians, because our subjects were not completely parasite-free at all times. McGregor *et al.* (1956), who used weekly doses of chloroquine, found that the γ globulin values of protected Gambian children were still higher than in European children. Vera and Roche (1956), working in Caracas, Venezuela, where malaria infestation is at present a rarity, have observed an excess of γ globulin in the serum of negroes as compared with white subjects. Rawnsley *et al.* (1956), in a study of 45 Caucasoid and 45 North American negro blood donors of comparable social status in whom liver disease had been excluded, found a significantly higher concentration of serum γ globulin in the negro group. Schofield (1957), from a study of the serum protein pattern of West African students in London, reported that the γ globulin concentration did not fall to normal European values after several years of continuous residence in the United Kingdom. Bakker *et al.* (1957) reported high

γ globulin values in malaria-free natives of the Netherlands New Guinea. It is clear from these studies that there are other additional factors which contribute to the production of high γ globulin values in a community.

Our results support the view that nutrition is not an important factor in producing the hypergammaglobulinaemia in Africans (Holmes *et al.*, 1955; Rawnsley *et al.*, 1956; Comens, 1957). Curnow (1957) also found high γ globulin levels in Australian aborigines who are said to live on high-protein diets. Van Oye and Charles in 1956 re-examined the Africans of the Belgian Congo who were first investigated in 1951, and, despite improvements in nutrition standards which were represented in other blood values, no change was found in the serum globulin (van Oye and Charles, 1956).

Sickling may be a contributory factor since sicklers unprotected from malaria have a significantly higher concentration of γ globulin than unprotected non-sicklers. But, whereas hypergammaglobulinaemia appears to be universal in Africans, only 20–25% of adults in the Ibadan district sickle, hence sickling alone cannot explain all the facts.

Differences in the intensity of malaria transmission, together with the extent and efficiency of malaria control measures such as the use of mosquito nets and antimalarial drugs, may be partly responsible for differences in γ globulin level in groups of Nigerians. It must also be borne in mind that the standard of general sanitation and the quality of the water supply vary enormously, so that contact with other organisms such as water-borne pathogenic bacteria and parasites may be other contributory factors.

When all the evidence available at present is considered it points to the probability that the capacity to maintain a high serum γ globulin concentration in health is an inherited characteristic which has been selected for the protection it offers against malaria and possibly other endemic diseases. The actual levels found on examination depend on whether or not the individuals are in repeated contact with the organisms which cause the disease: thus Africans in hyperendemic malaria areas have higher values than Africans living in non-malarious areas, while the latter have higher values than Caucasoids living in comparable socio-economic circumstances.

Regarding the mechanism by which plasmodial growth can produce this effect, the suggestion has been made that the high γ globulin value is due to liver damage caused by malaria. A recent review of hepatic function tests in Nigerians (Edozien, 1958) has, however, failed to reveal any evidence of hepatic damage in symptom-free Nigerians even though they have high γ globulin values. The excess γ globulin was shown to have the flocculation properties of normal γ globulin, unlike the globulin in the serum in cases of hepatic disease, which behaves differently.

Holmes *et al.* (1955) have suggested a humoral mechanism. Our results are in agreement with this view. The response begins at about the age of 1 year. It is a reversible response, the γ globulin concentration decreasing when the stimulus is removed or diminished. However, until it becomes possible to measure quantitatively the amount of specific immune antibody in serum, we are unable to say categorically whether the excess γ globulin represents antibody or whether it is a by-product formed during the development of immunity to malaria.

The views currently expressed about the mechanism by which sickle haemoglobin protects against malaria relate principally to the existence of factors in the red blood corpuscles of carriers of the sickle cell trait which make it difficult for malaria parasites to establish themselves in sufficient numbers to cause serious illness (Allison, 1957; Raper, 1959). One hypothesis has been that sickle haemoglobin is unsuitable for the metabolism of the malaria parasite. Another theory is that changes occurring within the red blood corpuscles due to parasite metabolism cause the infected cells to sickle and are thereby removed from the circulation before the parasites can fully establish themselves (Miller, Neel, and Livingstone, 1956). In these, as in other hypotheses which have been put forward, the sickle haemoglobin, by preventing the establishment of malaria parasites in the red blood corpuscle, would be expected, like "daraprim," to be associated with a lower γ globulin concentration. Our results show that this is in fact not the case.

We therefore conclude that any protection which sickling may afford against malaria is not due to an inability of the malaria parasite to multiply and develop in the red blood corpuscle (a point confirmed by Raper's cultivation experiments). Our data, on the other hand, suggest that sickling may be associated with an altered or enhanced antibody response against the malaria parasite, since unprotected AS children have significantly higher mean γ globulin concentration than unprotected AA children of the same age, while the difference beween protected AS and protected AA children is not significant.

It would appear, therefore, that in the African the high serum γ globulin concentration to malaria and sickling are both directed to the same purpose, namely, protection against malaria and possibly other endemic diseases, but, while the γ globulin response has become universal, sickling has been limited because the homozygous SS state is lethal.

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