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Anti-ssDNA and antinuclear antibodies in human malaria

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SUMMARY

The incidence of serum antinuclear antibodies and serum antibodies to single stranded (ss) and double stranded (ds) DNA was investigated following acute malaria in 58 Caucasians visiting tropical countries but resident in Britain and in 24 Ghanaians resident in Ghana. In Caucasians this infection was associated with a significant increase in the incidence of speckled antinuclear antibodies (38% compared to 3% in controls; $P < 0{\cdot}001$) and a significant rise in antibody levels against ssDNA (14% compared to 5%; $P < 0{\cdot}05$), but no rise in antibodies against dsDNA. Acute malaria in Ghanaians was associated with an incidence of 25% of antinuclear antibodies and 4% of antibodies to ssDNA; these were similar to those found in healthy Ghanaians who are chronically exposed to malaria. Antibodies against dsDNA were not detected. The incidence of antinuclear antibodies and levels of anti-ssDNA antibodies was higher in the Ghanaian healthy population than in normal Caucasians. These observations indicate that malaria is associated with the development of antinuclear and anti-ssDNA antibodies. Ghanaian patients with a tropical splenomegaly syndrome or with a nephrotic syndrome, both of which conditions are suspected of having a malarial aetiology, had serum levels of anti-ssDNA higher than healthy controls. This observation adds further circumstantial evidence to the role of malaria in causing anti-DNA antibodies.

INTRODUCTION

Antinuclear antibodies are a manifestation of autoimmunity and develop spontaneously in disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (Hughes, Cohen & Christian, 1971). Recently it has been reported that antinuclear antibodies develop during viral infections (Koffler *et al.*, 1971), bacterial infections (Roberts & Lewis, 1978) and parasitic infections of man such as malaria (Greenwood, Herrick & Holborow, 1970; Voller, O'Neill & Humphrey, 1972; Quakyi, 1980), schistosomiasis (Hillyer, 1971) and trypanosomiasis (Lindsley, Kysela & Steinberg, 1974).

The humoral response to malaria in humans is characterized by high serum levels of IgG and IgM antibodies (Turner & Voller, 1966; Rowe *et al.*, 1968). Only a small proportion of these antibodies is directed against malaria antigens (Curtain *et al.*, 1964) the remainder showing heterophile (Adeniyi-Jones, 1967), rheumatoid factor (Greenwood, Muller & Valkenburg, 1971) and antinuclear specificities (Greenwood *et al.*, 1970; Voller *et al.*, 1972). It has been suggested that

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the development of heterophile antibodies and autoantibodies in malaria might be a result of polyclonal B lymphocyte activation by parasite related mitogens (Greenwood, 1974).

The present study was designed to investigate the incidence and nature of antinuclear antibodies in Caucasians with a first attack of malaria, in healthy Ghanaians chronically exposed to malaria and in Ghanaians with an acute attack of malaria. Two other groups of Ghanaian subjects, the first with the tropical splenomegaly syndrome (TSS) and the second with a nephrotic syndrome, were also studied as in both disorders there is suggestive evidence of a malarial aetiology (Sagoe, 1970; Gilles & Hendrickse, 1963).

SUBJECTS AND METHODS

Sera from the following groups of subjects were studied.

(a) Ninety-two healthy adult Ghanaians living in a holoendemic malarious area and thus constantly exposed to malaria.
(b) Twenty-four adult Ghanaians living in the same area with a clinical attack of malaria, viz. headache, fever and rigors, and *P. falciparum* parasitaemia.
(c) Forty-seven Ghanaians with a nephrotic syndrome. Histology of a renal biopsy was available in 40 patients and showed a minimal change nephropathy (14), focal segmental glomerulosclerosis (eight), proliferative glomerulonephritis (eight), membranous glomerulonephritis (five), mesangiocapillary glomerulonephritis (three), and chronic interstitial nephritis (two).
(d) Eighteen Ghanaians with a clinical diagnosis of a tropical splenomegaly syndrome characterized by massive splenomegaly, anaemia and a high IgM level. Liver biopsies were not performed in these patients.
(e) Sixty-two healthy Caucasian blood donors who had never visited a malarial area.
(f) Fifty-eight Caucasians studied during a first attack of malaria. Thirty-one had *P. falciparum* parasitaemia, 24 *P. vivax* parasitaemia and three *P. ovale* parasitaemia. (These serum samples were kindly supplied by Dr A. Hall of the Hospital for Tropical Diseases, London.)

Immunofluorescent test for the detection of antinuclear antibodies. All sera were screened for antinuclear antibodies by the indirect immunofluorescent method on unfixed cryostat sections of rat liver (Johnson, Holborow & Dorling, 1978). Sera were tested at a dilution of 1:20 and 1:40. Bound immunoglobulin was detected with fluorescein isothiocyanate labelled sheep anti-human immunoglobulin (Wellcome Reagents). The working dilution of conjugate was established by block titration. Sections were read on a Reichert fluorescence microscope equipped for darkground illumination with a quartz–halogen lamp.

Double stranded DNA antibodies. Antibodies to double stranded (ds) DNA were detected using the *Crithidia luciliae* immunofluorescent technique (Aarden, de Groot & Feltkamp, 1975). The *C. luciliae* were stored, cultured and applied to multispot glass slides as described by Aarden *et al.* (1975). Sera were diluted 1:10 in phosphate-buffered saline (PBS) 0·14 M pH 7·4 and applied to the slides. The slides were incubated for 30 min at room temperature, washed in buffer for 30 min and incubated with FITC-labelled sheep anti-human IgG and IgM (Wellcome) for 30 min. They were then washed again and mounted in buffered glycerol pH 7·8 and the slides read by fluorescent microscopy and scored as positive and negative.

Single stranded DNA antibodies. Single stranded (ss) DNA antibodies were measured using a solid phase enzyme-linked immunoassay (Voller *et al.*, 1974; Grippenberg *et al.*, 1978). Single stranded DNA was prepared by heating dsDNA (highly polymerized calf thymus DNA type II, Sigma) in PBS 0·14 M pH 7·4 at 100°C for 15 min and chilling in an ice bath. Rabbit anti-human IgG was conjugated to alkaline phosphatase (Sigma VII) using glutaraldehyde. The enzyme-linked immunoassay was performed in Cooke disposable polystyrene microtitre plates (Cooke M129A, Dynatech Laboratories Ltd). Two hundred microlitres of ssDNA in a carbonate–bicarbonate buffer pH 9·6 at a concentration of 0·5 μg/ml was added to each well of the haemagglutination plate. This was incubated for 2 hr at 37°C in a moisture chamber. The contents of the plates were shaken out and the wells washed with PBS-Tween (0·14 M PBS, pH 7·5 + 0·05% v/v Tween 20), filled with the PBS-Tween and left for 3 min. The washing procedure was repeated twice. Sera were diluted

1:200 in PBS-Tween and 200 μl placed in each well. The plates were incubated for a further 2 hr at room temperature and then washed as before and emptied. Two hundred microlitres of anti-IgG alkaline phosphatase conjugate was added to each well and incubated for 16 hr at 4°C. After washing, 200 μl of the substrate *p*-nitrophenyl phosphate (Sigma 104) in a concentration of 1 mg/ml in a 10% diethenolamine buffer pH 9·8 containing 0·5 mM $MgCl_2$ was added to each well and the plates incubated for 30–60 min at room temperature. The enzyme reaction was stopped in each well by the addition of 50 μl of 3 M NaOH and the absorbance read at 405 nm in a micro-ELISA Spectrophotometer. The results were expressed as a percentage of a reference positive serum obtained from a patient with very active untreated SLE and used consistently throughout this study. A negative control serum was included on each plate.

Serum IgG. Serum IgG in Caucasians with malaria and in healthy Caucasians was measured by radial immunodiffusion into agar containing monospecific antiserum to IgG (Mancini, Carbonara & Heremans, 1965).

Statistics. Prevalence of ANA in patient populations was compared to that of the appropriate controls by χ^2 analysis. Titres of ssDNA antibodies in the patient groups were compared with the controls using an analysis of variance (Dunnett, 1964). Serum IgG levels in Caucasians with acute malaria and in healthy Caucasians were compared by means of Student's *t*-test.

RESULTS

Antinuclear antibodies (ANA) (Table 1)

The incidence of ANA was similar in healthy Ghanaians (30%), in Ghanaians with acute malaria (25%), and in Ghanaians with the nephrotic syndrome (34%) but in patients with TSS was significantly higher (56%) than that found in healthy Ghanaians ($0{\cdot}05 > P > 0{\cdot}02$). Thirty-eight per cent of the Caucasians with acute malaria had a positive ANA compared with 3% of the healthy controls ($P < 0{\cdot}001$). A coarse speckled nuclear staining pattern was obtained with all except seven positive sera. Two Ghanaian sera showed diffuse nuclear staining, two nodular and one nucleolar staining (all five had a nephrotic syndrome). The two ANA positive normal Caucasian controls gave a diffuse staining pattern.

Single stranded (ss) DNA antibodies (Fig. 1)

The mean ssDNA binding (ssDNAB) of healthy Ghanaians was $8{\cdot}57 \pm 5{\cdot}1\%$ (s.d.) giving an upper limit of normal of 19·0% and was significantly higher than the mean ssDNAB of healthy Caucasians, $3{\cdot}31 \pm 2{\cdot}4\%$, upper limit of normal 8·2% ($P < 0{\cdot}01$).

Eight out of 58 (14%) Caucasians with acute malaria had an elevated ssDNAB and the mean ssDNAB in this group as a whole was significantly higher than in normal Caucasians ($P < 0{\cdot}05$). The mean ssDNAB in Ghanaians with acute malaria did not differ significantly from that found in normal Ghanaians ($P > 0{\cdot}05$). Fifteen of 50 (30%) Ghanaian nephrotics had a raised ssDNAB and the mean ssDNAB titre was significantly higher than in healthy Ghanaians ($P < 0{\cdot}01$). Data from two patients with SLE and nephritis were excluded from the statistical analysis. Although the mean

Table 1. Titres of antinuclear antibodies

	No. of patients	−ve	1:20	1:40
Healthy Ghanaians	92	64	21	7
Acute malaria Ghanaians	24	18	0	6
Nephrotic syndrome Ghanaians	47	31	7	9
Tropical splenomegaly syndrome Ghanaians	18	8	0	10
Healthy Caucasians	62	60	2	0
Acute malaria Caucasians	58	36	20	2

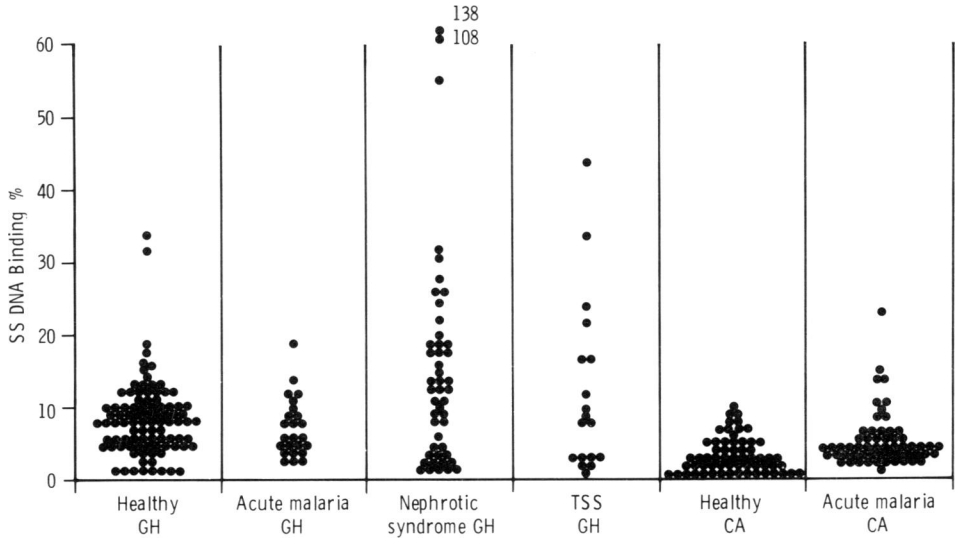

Fig. 1. Serum anti-ssDNA antibody levels in patients with malaria, tropical splenomegaly syndrome (TSS), nephrotic syndrome and in control subjects.

ssDNAB in Ghanaians with a TSS was higher than in the control group, this difference did not achieve statistical significance ($P > 0{\cdot}05$).

Double stranded (ds) DNA antibodies

Sera from only two patients in the study had antibodies to dsDNA. Both were Ghanaian females with clinical SLE and a nephrotic syndrome caused by a diffuse proliferative glomerulonephritis.

Serum IgG

The mean serum IgG in Caucasians with acute malaria was $17{\cdot}0 \pm 6{\cdot}6$ g/l (s.d.) and this was significantly higher than the mean serum IgG of $9{\cdot}0 \pm 1{\cdot}7$ g/l in healthy Caucasians ($P < 0{\cdot}001$). In Caucasians with acute malaria, there was no correlation between serum IgG and the levels of IgG antibody to ssDNA ($r = 0{\cdot}22$; $P > 0{\cdot}05$).

DISCUSSION

The demonstration of serum antinuclear and anti-ssDNA antibodies in Caucasians following a single attack of malaria strongly indicates that these antibodies were a result of malaria infection. Serum levels of IgG in these patients were significantly higher than in healthy controls but did not correlate with the titres of IgG antibody to ssDNA. There is a single report of anti-ssDNA antibodies in human malaria (Quakyi *et al.*, 1979) and of antibodies to DNA in rats infected with *P. berghei* malaria (Kreier & Dilley, 1969).

Healthy Ghanaians living in a holoendemic malarious area had a higher incidence of ANA and higher mean titres of anti-ssDNA antibodies than healthy Caucasians, who had not been exposed to malaria. We presume that the antinuclear and anti-ssDNA antibodies in healthy Ghanaians were due to previous infections by malaria. In 1970, Greenwood *et al.* reported a high incidence of a speckled antinuclear antibody detected by immunofluorescence in the sera of apparently healthy individuals living in malarious areas of Nigeria, Uganda and Liberia. A significant correlation was found between the occurrence of these antinuclear antibodies and the presence of high serum levels of malaria antibodies and they suggested that the development of antinuclear antibodies in these individuals was related to malaria infection. These observations were confirmed by Voller *et al.* (1972) who found a high incidence of antinuclear antibodies in a malarious area of Tanzania but not in an area of Tanzania in which malaria is rare. Experimental support for these observations comes

from reports that rats and mice infected with *P. berghei* malaria developed antinuclear and anti-DNA antibodies (Kreier & Dilley, 1969; Greenwood *et al.*, 1970).

The precise mechanism(s) leading to the development of antinuclear antibodies in malaria and other infections, and their role (if any) in tissue damage is not known. The anti-DNA antibodies reported in infections have been in most cases directed against ssDNA (Koffler *et al.*, 1971; Lindsley *et al.*, 1974), a notable exception being the report by Roberts & Lewis (1978) of circulating dsDNA–anti-dsDNA complexes in two patients with bacterial infections. Normal human B lymphocytes have DNA receptors (Bankhurst & Williams, 1975) and the development of anti-DNA antibodies may therefore be a normal host response to circulating DNA. It is probable that during infection host as well as parasite DNA is released into the circulation. Human anti-DNA antibodies cross-react with viral and bacterial as well as mammalian DNA (Koffler *et al.*, 1971) and are therefore likely to react with plasmodial DNA. Cellular destruction as in human leukaemia leads to circulating ssDNA (Koffler *et al.*, 1973) and the development of anti-ssDNA antibodies (Izui *et al.*, 1976); a similar sequence of events may occur in malaria.

It has been suggested that the development of high serum levels of IgM antibody directed at non-parasite antigens during malarial infections was due to polyclonal B lymphocyte activation by parasite related mitogens (Greenwood, 1974). Polyclonal B lymphocyte activators such as bacterial lipopolysaccharide induce the formation of autoantibodies including anti-DNA antibodies in mice (Fournie, Lambert & Miescher, 1974; Izui *et al.*, 1977a, 1977b). Culture supernatants from *P. falciparum* infected human red blood cells transform lymphocytes not only from individuals immune to malaria but also from individuals not previously exposed to malaria (Greenwood & Vick, 1975; Greenwood, Oduloju & Platts-Mills, 1979). In mice, *P. berghei* and *P. yoelii* infections lead to an increase in plaque forming cells (PFC) secreting IgM antibody to non-parasite antigens and injection of uninfected mice with supernatants of lysates of parasitized red blood cells also increased the background PFC (Freeman & Parish, 1978). These observations suggest that autoantibody formation in malaria may be due to polyclonal B lymphocyte activation.

In our study Ghanaians with acute malaria did not develop a rise in antinuclear and anti-ssDNA antibodies greater than that found in healthy Ghanaians. The previous exposure of the Ghanaian patients with acute malaria to this infection may explain the absence of a rise in serum levels of ssDNAB in contrast to the Caucasians. The B lymphocytes of individuals constantly exposed to malaria may be maximally activated by the putative parasite related mitogens and cannot therefore respond further to an acute infection.

The clinical significance of antinuclear and anti-ssDNA antibodies in malaria is not known. The observation that tissue damage in SLE may result from the deposition of DNA–anti-DNA immune complexes (Koffler *et al.*, 1971), raises the possibility that these complexes may play a similar role in malaria. Although immune complexes are detectable in the sera of patients with malaria (Houba *et al.*, 1976; Quakyi *et al.*, 1979) their antigen and antibody composition is not known.

Evidence for a malarial aetiology in patients with the TSS in Africa comes from the demonstration of high levels of IgM and malarial antibody titres in the serum of affected patients and the regression of the splenomegaly with anti-malarial treatment (Sagoe, 1970). The pathogenesis of TSS is not known and the role and nature of the raised serum levels of cryoglobulins found in this condition is unclear (Ziegler, 1973). The high incidence of antinuclear and anti-ssDNA antibodies in our subjects with a TSS raises the possibility that nuclear antigens and antibodies may be involved in immune complex formation in this disorder.

A high incidence of *P. malariae* parasitaemia is found in patients with a nephrotic syndrome in tropical Africa (Gilles & Hendrickse, 1963) and the demonstration by immunofluorescence of *P. malariae* antigens in the glomeruli of affected patients and the elution of antibody to *P. malariae* from affected glomeruli is strong evidence that *P. malariae* causes an immune complex mediated glomerulonephritis (Ward & Kibukamusoke, 1969; Houba *et al.*, 1971). Our observation of a raised incidence and levels of ssDNA antibodies in Ghanaians with a nephrotic syndrome raises the interesting possibility that the glomerulonephritis in these patients may be mediated in part by ssDNA-anti-ssDNA complexes.

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