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SPECKLED ANTINUCLEAR FACTOR IN AFRICAN SERA

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SUMMARY

Speckled antinuclear factor reacting with rat liver nuclei was found in a high proportion of sera from apparently healthy inhabitants of Western Nigeria, Northern Nigeria, Liberia and Uganda. A significant relationship was found between the occurrence of the antibody in Nigerian sera and the presence of high levels of malaria antibody and of high levels of IgM. Speckled antinuclear factor was also found in the sera of CBA mice infected with *Plasmodium berghei*. These findings suggest that the speckled antinuclear factor found in African sera may be a cross-reacting antibody to a nuclear component of malaria parasites. The demonstration of speckled antinuclear factor in the serum of a subject who has been resident in part of tropical Africa cannot be taken as a reliable indication of the presence of a connective tissue disease.

INTRODUCTION

Speckled antinuclear factor (ANF) can be demonstrated in the sera of a proportion of patients with connective tissue diseases but it is rarely found in the sera of healthy Caucasian subjects. During the course of an investigation into the incidence of serological abnormalities in the sera of Nigerian patients with rheumatoid arthritis (Greenwood & Herrick, 1970), we found that sera from many of these patients gave bright speckled staining of rat liver nuclei in an indirect immunofluorescent test. However, when sera from healthy Nigerian control subjects were examined, it was found that a similar proportion of these normal sera also contained speckled ANF. This paper records the prevalence of this antibody in sera from apparently healthy Africans and describes some of its characteristics.

MATERIAL AND METHODS

Sera

Sera from 324 Western Nigerians were studied. These sera were collected during the course of an epidemiological survey into the prevalence of rheumatoid arthritis carried out in two

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villages in Western Nigeria by one of us in conjunction with Dr A. S. Muller and Dr H. A. Valkenburg of the University of Leiden. Most of the subjects from whom serum was taken had a number of parasitic infections. Fifty-eight sera collected during a similar survey in Liberia were also examined, together with forty-five sera collected during a malarial survey in Northern Nigeria and eighteen sera obtained from healthy Ugandan Africans.

Sera from eleven adult CBA mice experimentally infected with the rodent malaria parasite *Plasmodium berghei yoelii* (Landau & Killick-Kendrick, 1966) were also tested for speckled ANF.

Immunofluorescence

All sera were tested for the presence of speckled ANF by an indirect immunofluorescent technique using cryostat liver sections obtained from adult Wistar or black-hooded rats as substrate. Some sera were also tested on other rat tissues, on sections of rabbit liver and kidney, human liver and thyroid and on films of human polymorphonuclear leucocytes treated by freezing and thawing (Elling, 1967).

For routine tests sera were diluted 1:10 and a monospecific goat anti-human IgG conjugate (kindly provided by the Blood Group Reference Laboratory) was used. In some experiments a conjugate prepared from a rabbit anti-human IgM antiserum was used. When mouse sera were tested a rabbit anti-mouse globulin conjugate (Nordic Pharmaceuticals Ltd, Tilburg) was employed.

Sera with known ANF activity were tested for complement-fixing ANF by an immunofluorescent technique. A drop of test serum and a drop of fresh guinea-pig serum were mixed and applied to a rat liver section for 1 hr at room temperature. After washing in Coons saline the section was treated with a rabbit anti-guinea-pig globulin conjugate (Nordic Pharmaceuticals Ltd, Tilburg) which had been adsorbed with heat-inactivated human serum. A section treated with test serum and heat-inactivated guinea-pig serum was used as a control.

The presence of malaria antibodies was assayed by an indirect immunofluorescent technique (Voller, 1964). *Plasmodium falciparum* in human blood films was used as the antigen and anti-whole human γ-globulin conjugate (Wellcome Reagents Ltd, Beckenham) was employed.

Immunoglobulin estimations

Quantitative immunoglobulin estimations were carried out by a radial immunodiffusion method (Fahey & McKelvey, 1965) using monospecific sheep anti-human immunoglobulin antisera (kindly provided by the Department of Experimental Pathology, The University of Birmingham). Results of immunoglobulin determinations are expressed as a percentage of the immunoglobulin content of the Medical Research Council pooled normal human serum standard.

Fractionation procedures

Gel filtration of two sera was carried out using Sephadex G-200 (Pharmacia, Uppsala). Mercaptoethanol reduction was carried out according to the method of Osler, Mulligan & Rodriguez (1966). Samples treated with phosphate buffered saline and then with iodoacetamide were used as controls.

RESULTS

Prevalence of speckled ANF in African sera

When tested at a dilution of 1:10, 11% of 324 sera from Western Nigeria gave bright speckled staining of rat liver nuclei (Fig. 1). A further 39% of these sera gave weak speckled staining which was readily overlooked. Homogeneous staining of rat liver nuclei was given by only one serum. Variations in the prevalence of speckled ANF in Nigerian sera in relation to variations in age and sex were studied. Speckled ANF was found more frequently in the sera of 114 female Nigerians (59%) than in the sera of 210 male Nigerians (45%) and slightly more frequently in older subjects than in children. The highest incidence of positive tests (72%) was found in women of the child-bearing age (25–34 years old).

Speckled ANF was also found in twenty-eight of forty-five sera from Northern Nigeria (62%), in thirty-nine of fifty-eight Liberian sera (67%) and in five of eighteen sera from healthy Ugandans (28%). In contrast, speckled ANF was found in only one of fifty sera from healthy English adults when the same technique was employed.

Nature of speckled antigen

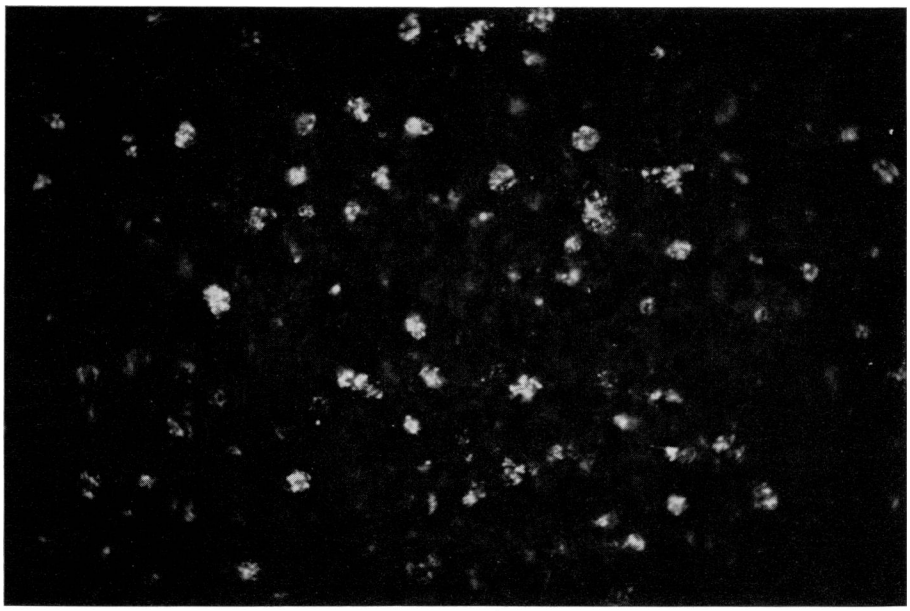
Freshly cut sections from snap frozen rat liver were found to be the most satisfactory substrate for demonstrating the presence of speckled ANF in Nigerian sera. Some weakly positive sera failed to show speckled staining on sections prepared from rat liver which had been kept at −20°C for more than a few days. Some weakly positive sera also showed speckled staining on liver sections prepared from some rats but not from others. Constant results were obtained with more active sera.

Ten sera which gave bright speckled nuclear staining when tested initially on rat liver sections were tested on a variety of other tissues. Speckled staining of a similar appearance to that seen with rat liver was observed in sections of rat stomach and kidney, rabbit liver and kidney, human thyroid and liver and calf thyroid. When human blood films were used as substrate speckled staining of mononuclear cells and polymorphonuclear leucocytes was seen but the speckled character of the staining was less readily discernible than when tissue sections were used as substrate.

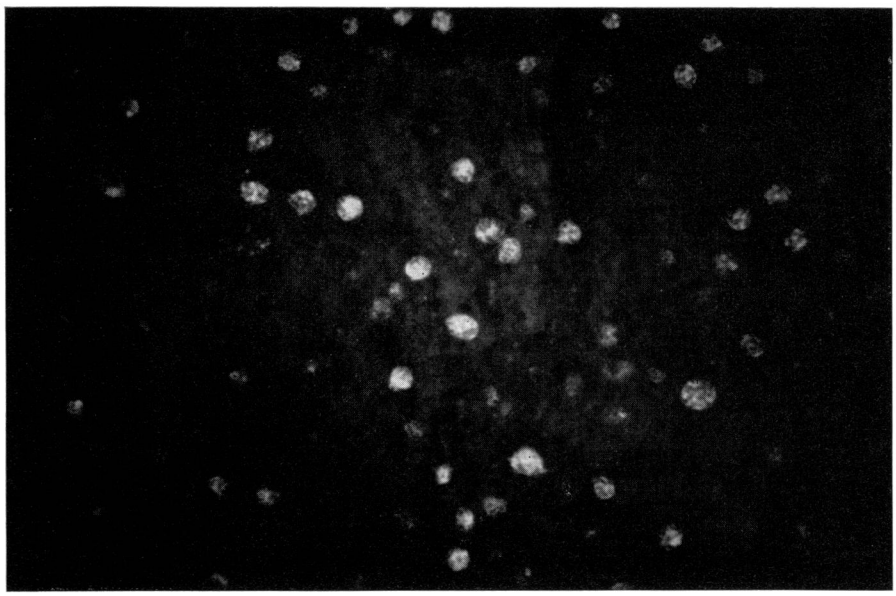
The nature of the reactive antigen in rat liver sections was investigated by elution and absorption experiments. In these experiments six Nigerian sera containing speckled ANF and six sera from English patients with connective tissue diseases containing speckled ANF were tested in parallel. In each experiment identical results were obtained with both the Nigerian and English sera. It was found that the speckled antigen was completely removed by washing in phosphate buffered saline at pH 8·0 for 30 min but that the antigen was not removed by washing in unbuffered saline (pH 4·5–5·5). Fixation in alcohol did not destroy the antigen but distorted the speckled nature of positive staining. Incubation of rat liver sections with a 0·1% solution of desoxyribonuclease in 0·003M $MgSO_4$ did not remove the antigen. It was not possible to test the activity of ribononuclease as the antigen was eluted by the solvent, phosphate buffered saline. The speckled ANF was not absorbed from either Nigerian or English sera by absorption with calf thymus desoxyribonucleic acid or nucleoprotein.

Nature of antinuclear antibody

Antibody titres were determined for ten Nigerian sera that had given bright speckled



(a)



(b)

FIG. 1. Speckled staining of rat liver nuclei given by (a) serum from a healthy 35-year-old Nigerian woman, (b) serum from a 40-year-old English female patient with Sjögren's syndrome.

staining of rat liver nuclei on initial testing. Titres of 1:500 (two sera), 1:200 (five sera) and 1:100 (three sera) were obtained. In no instance was the pattern of nuclear staining observed to change during the course of dilution.

TABLE 1. Effect of treatment with 6-mercaptoethanol on the titre of speckled ANF of six Nigerian sera

Serum	Control	Treated with 6-mercaptoethanol
1	1:500	1:250
2	1:250	1:100
3	1:100	1:100
4	1:100	1:50
5	1:50	1:50
6	1:50	1:20

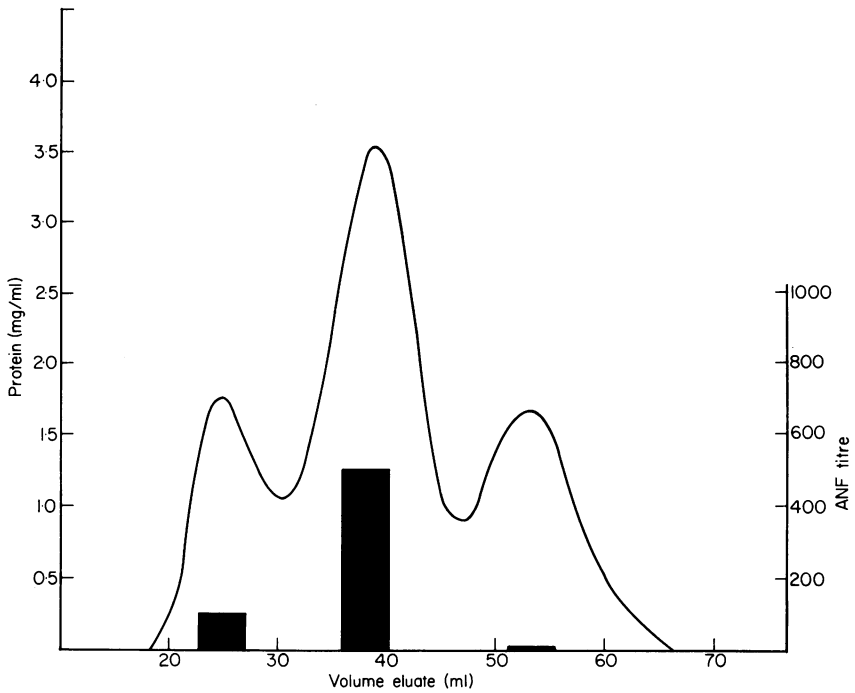


FIG. 2. Gel filtration on Sephadex G-200 of a Nigerian serum containing speckled ANF. Fractions from each protein peak tested for ANF activity on rat liver sections.

Experiments were carried out to determine the immunoglobulin class of the speckled ANF present in Nigerian sera. A monospecific anti-human IgG conjugate was used routinely but ten sera giving bright staining with this conjugate were also tested using a monospecific anti-human IgM conjugate. All ten sera gave speckled staining of rat liver nuclei when tested at a dilution of 1:5 with the latter conjugate. The effect of mercaptoethanol reduction on the speckled ANF titre of six Nigerian sera was studied. A serum sample

treated with phosphate buffered saline was used as a control. The speckled ANF titres obtained for the paired samples using a conjugate with anti-human IgG and anti-human IgM activity are indicated in Table 1. Mercaptoethanol reduction resulted in a fall in the speckled ANF titre of four of the six sera. Finally, two sera were fractionated on Sephadex G-200. Speckled ANF was demonstrated in the IgM and IgG peaks of both sera (Fig. 2).

Ten Nigerian sera containing speckled ANF were tested for complement-fixing ANF by an immunofluorescent technique described above. Eight of the ten sera gave speckled staining of the rat liver nuclei when tested by this method. Pretreatment of the guinea-pig serum by heating at 56°C for 30 min greatly reduced the intensity of the staining. No staining was observed in controls from which the guinea-pig serum had been omitted.

Relationship of the presence of speckled ANF to immunoglobulin levels

The presence of speckled ANF was related to the IgG and IgM levels of 100 Nigerian sera. The mean IgM level of sixty-eight sera containing speckled ANF (mean 355%, standard deviation 150%) was significantly higher than the mean IgM level of thirty-two negative sera (mean 270%, standard deviation 135%) ($t = 2{\cdot}8$, $P = <0{\cdot}01$). The mean IgG level of the sera containing speckled ANF (mean 290%, standard deviation 95%) was slightly higher than the mean level of sera giving a negative test (mean 260%, standard deviation 85%) but this difference is not statistically significant.

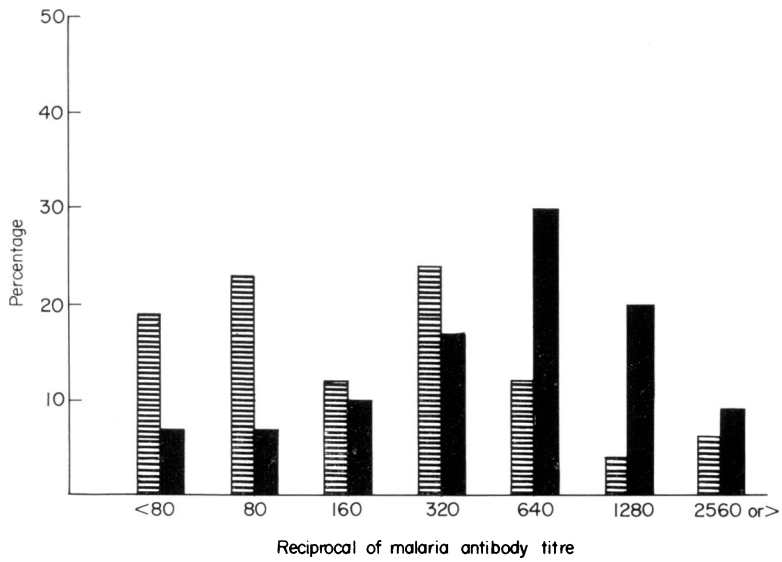


FIG. 3. Fluorescent malaria antibody titres of seventy-seven Nigerian sera containing speckled ANF (solid columns) and fifty-two Nigerian sera without ANF activity (hatched columns).

Relation of speckled ANF in Nigerian sera to parasitic infections

No significant difference was found in the prevalence of speckled ANF in sera taken from the inhabitants of two villages in Western Nigeria. One of the villages, Igbo-Ora, is situated in open savannah, the other, Isheri, within the tropical rain forest. As a consequence of their different ecology the pattern of parasitic infection occurring in these two villages is markedly different (Greenwood, 1970). Malaria, however, is holoendemic in both areas.

The pattern of parasitic infection present in twenty-three subjects from these two villages whose sera contained speckled ANF in high titre was compared with the pattern of parasitic infection present in the villages as a whole. Parasitological evidence of infection with schistosomiasis, loiasis or onchocerciasis was found no more frequently among the individuals with a high titre of speckled ANF than among other members of the villages. The incidence of splenomegaly in the twenty-three subjects with a strongly positive test for speckled ANF (40%) was slightly higher than the incidence of splenomegaly in twenty-three age and sex matched controls (25%) but this difference is not statistically significant.

Malaria antibody levels were measured in 129 Nigerian sera using an indirect immunofluorescent technique. The mean malaria antibody level of seventy-seven sera containing speckled ANF was significantly higher than the mean malaria antibody level of fifty-two sera in which speckled ANF could not be demonstrated ($t = 4{\cdot}1$, $P = <0{\cdot}01$) although there was a considerable degree of overlap between the antibody levels of the two groups of sera (Fig. 3).

Speckled ANF in CBA mice infected with Plasmodium berghei yoelii

Sera from eleven 1-year-old CBA mice, diluted 1:10, were tested for speckled ANF before infection and 2 or 3 weeks after infection with *P. berghei yoelii*. Speckled ANF was not seen in any of the pre-infection sera but sera from eight mice contained speckled ANF 2 or 3 weeks after infection. The two sera giving the brightest staining were titred out; end-point titres of 1:100 and 1:50 were obtained. The pattern of nuclear staining given by the positive mouse sera was similar to that given by Nigerian sera. Positive mouse sera failed to give any staining when rat liver sections were washed in phosphate-buffered saline for 30 min prior to testing.

DISCUSSION

Antinuclear factor is occasionally demonstrated in the sera of healthy Europeans by immunofluorescent techniques, especially when neat serum is used, but it is an uncommon finding. Prevalence figures for the occurrence of ANF in normal Caucasian sera vary according to the age of the population surveyed and according to the fluorescent technique employed, but most series report an incidence of less than 5% when tissue sections are employed (Feltkamp, 1966). At the M.R.C. Rheumatism Unit, Taplow, Ward, Johnson & Holborow (1964) found an incidence of less than 3% in healthy controls when sera were tested at a dilution of 1:10 and when calf thyroid was used as substrate. Little attention has been paid to the pattern of nuclear staining given by the ANF occasionally found in the sera of healthy Europeans. Svec & Viet (1967) report that the ANF present in elderly normal Caucasians usually gives homogeneous staining and our experience is in agreement with this finding. The demonstration of speckled ANF at high titre in 11% of sera from apparently healthy Nigerians and at a lower titre in a further 39% is thus a significant finding and indicates that speckled ANF must be added to the increasing list of immunological changes recorded in the sera of apparently healthy members of tropical populations (Greenwood, Herrick & Voller, 1970).

Few studies of the prevalence of autoantibodies in African populations have yet been carried out. Shaper *et al.* (1968) record a high incidence of thyroid antibodies and gastric parietal cell antibodies in Ruandan Africans but do not comment on the occurrence of ANF

in this population. Our demonstration of speckled ANF in small groups of sera from Northern Nigeria, Liberia and Uganda suggests that this antibody may be found widely throughout tropical Africa.

The nuclear antigen reacting with the speckled ANF found in the sera of Caucasian patients with connective tissue diseases is soluble in phosphate buffered saline (Beck, 1961) and is probably a glycoprotein (Lachmann & Kunkel, 1961). Our absorption and elution experiments suggest that the speckled ANF present in Nigerian sera was reacting with a similar or identical antigen. Speckled ANF has been demonstrated in both IgG and IgM serum fractions of patients with connective tissue diseases (Gonzales & Rothfield, 1966) although Bonomo, Tursi & Dammacco (1965) related speckled staining specifically to the IgM fraction. The speckled ANF present in Nigerian sera was found in both IgG and IgM serum fractions. Dorsch *et al.* (1966) have shown that the pattern of nuclear staining of the ANF present in patients with connective tissue diseases is markedly influenced by dilution of the serum. However, it was found that the ANF present in Nigerian serum always gave a speckled staining pattern regardless of the serum dilution. Conversion of speckled to homogeneous staining was never observed during any of the absorption and elution experiments or during the fractionation procedures.

The possibility that the occurrence of speckled ANF in Nigerian sera was related to the presence of a parasitic infection was considered. No epidemiological evidence was found to suggest any relationship between the development of the antibody and the presence of schistosomiasis, onchocerciasis or loiasis. However, a significant correlation was found between the presence of speckled ANF in Nigerian sera and high titres of malaria antibody. A relation to malaria infection could also explain the correlation found between the presence of speckled ANF and high IgM levels, for it has been shown that in Ugandan sera (Shaper *et al.*, 1968) and Nigerian sera (unpublished data) high IgM levels are associated with high titres of malaria antibody. The view that the presence of speckled ANF in African sera was related to malaria infection received further support from the finding that CBA mice infected with *P. berghei yoelii* developed a similar antibody.

Infection with malaria could lead to the development of speckled ANF in several ways. The antibody might be produced as a non-specific consequence of the marked increase in immunoglobulin production occurring during malaria infection. However, it is also possibly that the speckled ANF found in African sera is a cross-reacting antibody directed primarily against one of the breakdown products of the nucleus of the malaria parasite. It has recently been demonstrated that extracted malaria antigen contains nucleoproteins and possibly glycoprotein (Turner & McGregor, 1969) and antibody development to one or more of these nuclear components could readily occur during natural malaria infection. Such an antibody would not necessarily give fluorescent staining of the intact nucleus of the malaria parasite. It may be possible to prove whether the speckled ANF found in African sera is a cross-reacting antibody to a malaria component by absorption experiments with extracted malaria antigens. If this interpretation of the mode of development of speckled ANF in African sera proves to be correct, it raises the interesting possibility that the similar antibody found in patients with connective tissue diseases might also be a cross-reacting antibody directed primarily against a nuclear component of another micro-organism.

The finding of speckled ANF in a high proportion of sera from apparently healthy Africans indicates that the demonstration of the antibody cannot be used as a reliable diagnostic feature of connective tissue disease among the indigenous population of parts

of tropical Africa. The diagnostic value of demonstration of the antibody in the sera of individuals who have lived in other parts of the tropics should be approached with caution until more is known about its geographical distribution and its relationship to past malarial infection.

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