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HETEROPHILE ANTIBODIES IN NIGERIAN SERA

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SUMMARY

A heterophile agglutinin was found at a titre of 1:4 or greater in 332 of 336 Nigerian sera investigated. The antibody was demonstrated to be an IgM macroglobulin. Although many of the sera tested had high IgM levels, only a slight correlation was found between titres of heterophile agglutinin and IgM levels. Absorption studies differentiated the Nigerian heterophile agglutinin from the antibodies seen in glandular fever and serum sickness. No correlation was found between the occurrence of high titres of heterophile agglutinin and infection with malaria, onchocerciasis, loaisis or schistosomiasis. None of the subjects investigated was known to have trypanosomiasis, a parasitic infection in which heterophile antibodies are known to occur.

INTRODUCTION

Antibodies agglutinating sheep red cells have been found at high titre in the sera of patients with glandular fever (Paul & Bunnell, 1932), serum sickness (Davidsohn, 1929) and African trypanosomiasis (Henderson-Begg, 1946; Houba & Allison, 1966), but they are rarely found in the sera of healthy Europeans. However, Henderson-Begg (1946), during the course of an investigation into the occurrence of heterophile agglutinins in trypanosomiasis, noted the occurrence of heterophile agglutinins at a titre of 1:28 or greater in 58% of his control group of 100 West Africans without trypanosomiasis. In 1967 Adeniyi-Jones reported the occurrence of an antibody specifically agglutinating tanned sheep red cells in the sera of a high proportion of healthy Nigerians.

During the course of an investigation into the occurrence of rheumatoid factor in Nigerians it was found that the serum of many healthy Nigerians agglutinated fresh sheep red cells. This paper describes the prevalence of this antibody and some of its characteristics.

MATERIAL AND METHODS

Sera

Serum samples from 336 Nigerians were investigated for the presence of heterophile agglutinins. Specimens were obtained from four groups of subjects:

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(a) Ninety-nine healthy male blood donors attending University College Hospital, Ibadan, Western Nigeria.

(b) Ninety-seven subjects from the village of Isheri which is situated in a region of tropical forest near Lagos. Blood samples were collected from these individuals during the course of a population survey into the prevalence of rheumatic diseases in Western Nigeria, undertaken in conjunction with Dr A. S. Muller and Dr H. A. Valkenburg of the University of Leiden.

(c) Sixty-eight subjects from the village of Igbo-Ora investigated during the same survey. Igbo-Ora is situated in the open savannah region of Western Nigeria.

(d) Seventy-two villagers from a rural area of Northern Nigeria investigated during the course of a malaria survey.

Nearly all members of the first three groups belonged to the Yoruba tribe. Many different tribal groups were represented among individuals in Group (d).

Serum samples from twenty-three monkeys (thirteen *Erythrocebus patas*, ten *Macacca mulatta*) infected with *Plasmodium cynomolgi* and *Plasmodium knowlesi*, respectively, were also tested for the presence of heterophile agglutinins.

Parasitological investigations

Parasitological data was collected from individuals in Groups (b), (c) and (d) at the time that serum was obtained. Blood films and urine samples were examined by standard techniques. Skin snips were taken from subjects in Groups (b) and (c) by Dr G. B. Wyatt and examined for the microfilariae of *Onchocerca volvulus*. Serum from randomly selected members of each group has subsequently been tested for the presence of malaria antibodies using a fluorescent technique (Voller, 1964). Blood films from Gambian patients with a *Plasmodium falciparum* infection were used as antigen.

Agglutination tests

Agglutination tests were carried out in Perspex trays. To 0·2 ml of two-fold serial dilutions of test serum was added 0·2 ml of 1% erythrocytes. Rodent and monkey erythrocytes were suspended in 0·2% foetal bovine serum. Sera were inactivated by heating at 56°C for 15 min before testing. The pattern of agglutination was read after the trays had stood at room temperature overnight. Agglutination titres refer to the serum dilution at the end-point before the addition of erythrocytes. Mean agglutination titres for groups of subjects have been expressed as a mean tube titre ($<1:4 = 0$; $1:4 = 1$; $1:8 = 2$, etc.).

Sheep red cells were tanned by addition of washed sheep red cells, drop by drop with constant stirring, to an equal volume of a 1:40,000 solution of tannic acid followed by incubation for 15 min at 37°C. The mixture was then centrifuged and the tanned sheep red cells washed three times in phosphate buffered saline.

Absorption experiments

A volume of 1·3 ml of test serum was absorbed with 0·5 ml of washed packed sheep cells at 37°C for 1 hr as recommended for absorption of heterophile agglutinins in the sheep cell agglutination test (SCAT) for rheumatoid factor (Ball, 1963). Serum samples diluted 1:4 in saline were also absorbed with 2 volumes of packed sheep red cells at 4°C overnight. Sera diluted 1:4 were absorbed overnight with guinea-pig kidney (Oxoid), ox cells (Oxoid), trypsinized human cells and human red cell stroma.

Human erythrocytes were trypsinized by the method employed by Kano, McGregor & Milgrom (1968). Ten volumes of 2% washed erythrocytes were mixed with 1 volume of 1% trypsin solution for 10 min at 37°C and the cells washed in saline. Human red cell stroma was prepared by adding fresh blood to a large volume of distilled water at 4°C saturated with carbon dioxide. The insoluble precipitate was washed three times in chilled distilled water.

Immunoglobulin studies

Zone electrophoresis was carried out in Pevikon (Shandon, London). Gel filtration was performed using Sephadex G-200 (Pharmacia, Uppsala) and ion exchange chromatography using DEAE-cellulose (Whatman, Balston Ltd, England).

Mercaptoethanol reduction was carried out by treatment of serum with an equal volume of 0·1 M mercaptoethanol in phosphate buffered saline at room temperature for 18 hr. Three volumes of 0·34 M iodoacetamide were then added and the serum dialysed against phosphate buffered saline (Osler, Mulligan & Rodriguez, 1966).

Immunoglobulin levels were determined by a modified Mancini method (Fahey & McKelvey, 1965) using sheep anti-human antisera kindly provided by the Department of Experimental Pathology, University of Birmingham. Results are expressed as a percentage of the pooled normal human serum standard provided by the Department of Biological Standards, Mill Hill.

Rheumatoid factor activity against rabbit γ-globulin and against human γ-globulin was assayed by the human erythrocyte agglutination test and a latex test respectively (Valkenburg, 1963).

Elution studies. Heterophile antibodies were eluted from coated sheep red cells by the method of Rubin (1963). Two volumes of ether were added to 1 volume of washed packed sheep red cells and one volume of normal saline. After mixing for 1 min the mixture was centrifuged at 3000 rev/min for 10 min. Agglutinin activity was present in the saline layer. Macroglobulin antibody was separated from haemoglobin by gel filtration.

RESULTS

Prevalence of heterophile antibodies

Three hundred and thirty-two of the 336 Nigerian sera tested were found to contain a heterophile agglutinin reacting with non-sensitized sheep cells (NSCAT-agglutinin) at a titre of 1:4 or greater. In contrast an NSCAT-agglutinin was found in only twenty-two of 200 serum samples submitted to the laboratory at the Canadian Red Cross Memorial Hospital, Taplow, for routine testing for rheumatoid factor. NSCAT-agglutinins were present in many Nigerian sera at a high titre (Fig. 1). No significant difference was found in titre distribution for males and females. A slight, but significant, fall in mean titre was found in relation to increasing age. Little difference was found in the occurrence of NSCAT-agglutinins in the four population groups studied (Table 1).

Characteristics of NSCAT-agglutinins

The effect of temperature on the agglutinating activity of twenty high titre sera was investigated. No significant difference in NSCAT-agglutinin titre was found when tests were carried out at 37°C, room temperature or 4°C. When tests were carried out without prior heat inactivation of test serum, haemolysis occurred.

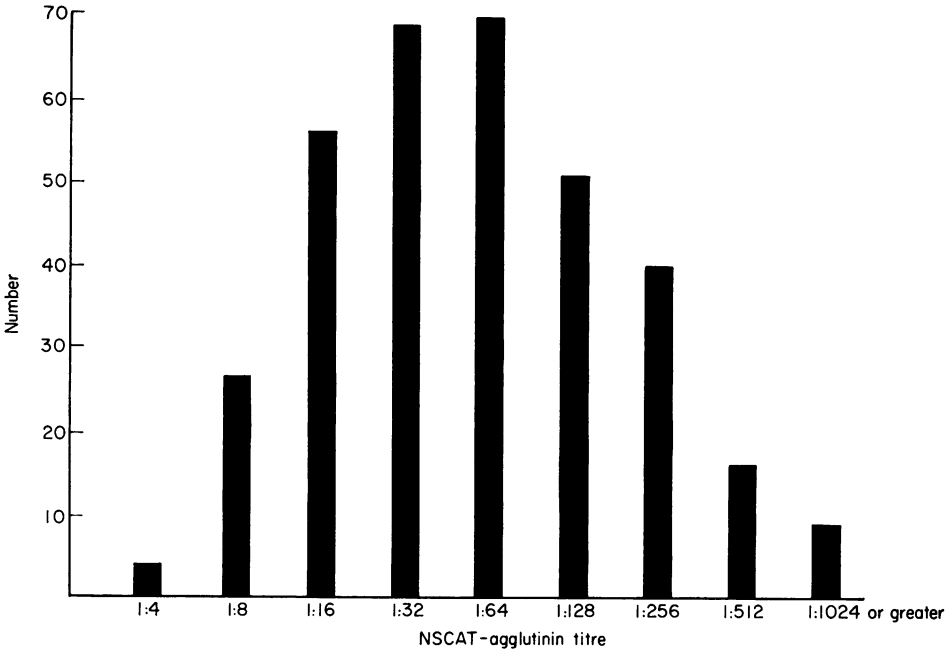

FIG. 1. Distribution of NSCAT-agglutinin titres in 336 Nigerian sera.

Six sera with high titres of NSCAT-agglutinin were fractionated by zone electrophoresis in Pevikon. In each case agglutinin activity was found to be restricted to the γ-globulin peak. Four sera were fractionated by gel filtration on Sephadex G-200. In each case NSCAT-agglutinin activity was found only in fractions from the first peak, shown to contain α_2 and γ-globulin on cellulose acetate electrophoresis and IgM on immunoelectrophoresis (Fig. 2). No activity was found in the IgG fraction of three sera fractionated on DEAE-cellulose. The NSCAT-agglutinin activity of ten sera was completely abolished by treatment with mercapto ethanol.

Relation of NSCAT-agglutinin activity to IgM levels

High IgM values were obtained for many of the 201 randomly selected sera tested. Sera with an NSCAT-agglutinin activity of 1:4 or less had a lower mean IgM level (mean 220%,

TABLE 1. NSCAT-agglutinins in four Nigerian communities

Age (years)	Northern Nigeria (72)	Ibadan (99)	Isheri (97)	Igbo-Ora (68)
5–14	4·0		3·9	5·1
15–24	4·4	4·2	3·1	4·0
25–34	4·3	3·9	3·4	4·1
35–44	3·0	3·9	3·9	3·7
45–54	2·8	2·8	3·5	3·3
>55	2·3		3·4	3·0

Results expressed as a mean tube titre. Number tested in parentheses.

standard deviation 150%) than sera with higher NSCAT-agglutinin titres (mean 315%, standard deviation 185%) but the overall correlation between the titre of NSCAT-agglutinin and IgM level was only slight (correlation coefficient = +0·15, standard error = 0·07).

Absorption of high titre sera with 2 volumes of packed sheep cells overnight resulted in a mean fall in IgM level of 25%. However, absorption with human erythrocytes, against which the ten sera studied had no agglutinin activity, resulted in a comparable fall in mean IgM level (21%).

NSCAT-agglutinins eluted from coated sheep red cells did not give a detectable level of IgM using the radial immunodiffusion technique. The lower limit at which IgM levels could be estimated by this technique was found to be approximately 5% of the normal human serum standard.

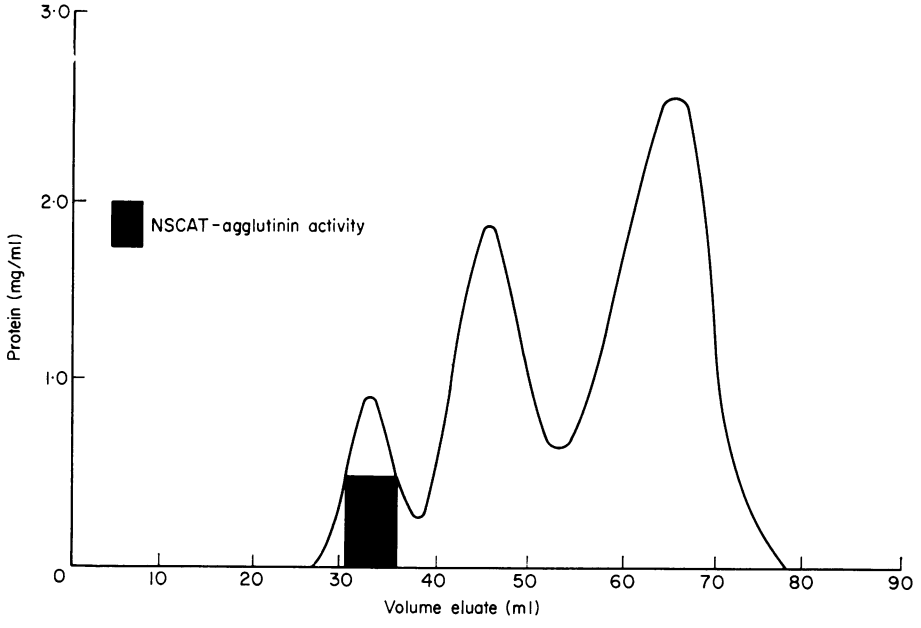

FIG. 2. Gel filtration on Sephadex G-200 of a Nigerian serum containing NSCAT-agglutinins.

The mean titre of heterophile agglutinin was determined for thirty sera containing rheumatoid factor and for thirty sera from age and sex matched control subjects from the same village. No difference in the mean tube titre was found (mean titre for rheumatoid factor positive subjects 4·0; mean titre for controls 3·8).

Cross-reactions of heterophile agglutinins

One hundred sera were tested for agglutinating activity against 1% fresh sheep red cells and against 1% tanned sheep red cells. A high degree of correlation in agglutinin titres was obtained (correlation coefficient = +0·86, standard error = 0·1). Agglutinin activity against fresh sheep red cells or against tanned sheep red cells was not completely removed from sera with a high titre of heterophile agglutinin by absorption of 1·3 ml of serum with 0·5 ml of sheep cells at 37°C for 1 hr. However, agglutinin activity against both fresh sheep red cells

and tanned sheep red cells was completely removed by prolonged absorption with large volumes of sheep cells or by repeated absorptions.

Twenty sera with varying NSCAT-agglutinin titres were tested for agglutinin activity against rat, rabbit, guinea-pig, ox, chicken, monkey and human erythrocytes. These tests were carried out at room temperature. No agglutination of ox or human erythrocytes was

TABLE 2. Pattern of agglutinating activity observed in Nigerian sera before and after absorption with sheep cells, guinea-pig kidney and ox cells

	Sheep	Rat	Rabbit	Guinea-pig
Unabsorbed sera	++	+++	+++	++
Sera absorbed with sheep erythrocytes	0	+	++	+
Sera absorbed with guinea-pig kidney	0	+	++	+
Sera absorbed with ox erythrocytes	++	+++	+++	++

0 = No agglutination; + = agglutination in low titre; ++ = agglutination in moderate titre; +++ = agglutination in high titre.

observed. Agglutination of chicken erythrocytes was produced by only one serum, to a titre of 1:8. Six of the twenty sera tested agglutinated monkey erythrocytes (*M. mulatta*). All twenty sera produced some agglutination of rabbit, rat and guinea-pig red cells. Several sera agglutinated rat and rabbit erythrocytes to a titre of greater than 1:5120. A significant correlation was found between the relative activity of each serum against sheep erythrocytes and against rodent erythrocytes. Agglutinin activity against rat and guinea-pig red cells was markedly reduced by overnight absorption with fresh sheep red cells and the agglutination titre to rabbit erythrocytes was moderately reduced.

TABLE 3. Prevalence of parasitic infections in 571 subjects studied in the villages of Igbo-Ora and Isheri

	Igbo-Ora	Isheri
Malaria	High	High
Onchocerciasis	High	Low
Schistosomiasis	High	Not found
Loiasis	Not found	High

Absorption with guinea-pig kidney had a similar effect to absorption with sheep erythrocytes (Table 2). Absorption with ox cells was without effect.

Absorption of test sera with fresh human erythrocytes, trypsinized human erythrocytes and human red cell stroma had no significant effect on the NSCAT-agglutinin titre.

Relation of NSCAT-agglutinins to parasitic infections

Although the pattern of parasitic infections occurring in the villages of Igbo-Ora and Isheri was markedly different (Table 3), little difference was found in titre distribution of NSCAT-agglutinins in the two villages. No difference was found in the mean NSCAT-agglutinins in the two villages. No difference was found in the mean NSCAT-agglutinin

titre of subjects with parasitological evidence of infection with malaria, *O. volvulus*, *Loa loa* and *Schistosoma haematobium* at the time that the serum sample was collected and the mean NSCAT-agglutinin titre of control groups from the same village matched for age and sex (Table 4).

Sera from twenty-three monkeys were tested for the presence of NSCAT-agglutinins before and on two or three occasions after infection with *P. knowlesi* or *P. cynomolgi*. One monkey was found to have an NSCAT-agglutinin at a titre of 1:64 before infection with malaria. In no case was a significant rise in NSCAT-agglutinin titre observed following infection.

TABLE 4. NSCAT-agglutinin titres, expressed as the mean tube titre, in Nigerian subjects with parasitic infections and in age and sex matched controls

	Parasitized subjects	Controls
Malaria	3·6 (19)	3·8 (19)
Onchocerciasis	3·8 (14)	3·5 (14)
Schistosomiasis	4·4 (7)	3·6 (7)
Loiasis	3·4 (22)	3·9 (22)

Numbers tested in parentheses.

DISCUSSION

Sera from apparently healthy individuals living in four areas of Nigeria were found to agglutinate the erythrocytes of sheep, rats, rabbits, guinea-pigs and monkeys. The close correlation found between agglutinin titres to fresh sheep red cells and to tanned sheep red cells, and the fact that activity to tanned sheep red cells could be completely removed by repeated absorption with fresh sheep erythrocytes, suggests that the heterophile antibody described in this paper is identical to the antibody directed specifically against tanned sheep red cells described in Nigerian sera by Adeniyi-Jones (1967). Adeniyi-Jones showed that agglutinin activity against tanned sheep red cells was absent from cord blood, detectable in 50% of infants and present at high titre in a considerable proportion of school-children. In the present study children under the age of 5 years were not investigated. High titres were found frequently in older children and young adults and a decrease in mean titre was found in old people.

The NSCAT-agglutinin found in Nigerian sera was demonstrated to be an IgM antibody and thus of the same immunoglobulin class as the heterophile agglutinin found in patients with glandular fever (Grubb & Swahn, 1958) and the NSCAT-agglutinin seen in human subjects and monkeys with trypanosomiasis (Houba & Allison, 1966; Houba, Brown & Allison, 1969). High levels of serum IgM have previously been noted among Africans in Nigeria (Turner & Voller, 1966), in the Congo (Michaux, 1966) and in the Gambia (Rowe *et al.*, 1968). In the present study 65% of the sera tested were found to have an IgM level greater than 200% of the MRC normal human serum standard. It therefore seemed possible that the presence of an IgM heterophile agglutinin in most of these sera could have been a non-specific manifestation of increased IgM production. The fact that only a slight correlation was found between the titre of heterophile agglutinin and the IgM level makes this unlikely. A rheumatoid factor-like IgM antiglobulin reacting with human but not rabbit

γ-globulin has been found in a high proportion of sera from adult Nigerians (to be published). No correlation was found between the presence of this factor and high titres of heterophile agglutinin, suggesting that different aetiological factors are involved in their production.

Agglutinin activity against fresh sheep red cells and tanned sheep red cells was completely removed by absorption of serum with fresh sheep erythrocytes, but large volumes of erythrocytes were required and sometimes repeated absorption was necessary to remove all activity. The low avidity of the heterophile antibody for sheep cells may explain why Adeniyi-Jones (1967) reported that the agglutinin to tanned sheep cells found in Nigerian sera could not be absorbed by fresh sheep red cells. This observation led her to suggest that the antibody was reacting with an antigen specific to the tanned sheep erythrocyte. Difficulty in achieving complete absorption of heterophile agglutinins from Nigerian sera renders fresh or tanned sheep red cells an unsuitable carrier for antigens in haemagglutination tests. Only one of twenty sera tested was found to agglutinate fowl erythrocytes and these might prove a more satisfactory antigen carrier in studies involving Nigerian sera.

The effect of absorption with guinea-pig kidney and ox cells differentiates the Nigerian NSCAT-agglutinin from the heterophile antibody seen in glandular fever and serum sickness. Agglutinin activity was not directed against the Forssmann antigen as agglutination of rat and rabbit erythrocytes occurred. The effects of absorption on the Nigerian NSCAT-agglutinin were similar to those found with the heterophile agglutinin found in monkeys with experimental trypanosomiasis (Houba *et al.*, 1969), although some reduction in agglutinin activity against rat and rabbit erythrocytes was observed after absorption with sheep red cells and with guinea-pig kidney. The NSCAT-agglutinin seen in experimental trypanosomiasis was absorbed by a *Trypanosoma rhodesiense* antigen. It has been suggested that NSCAT-agglutinins might have some diagnostic value in patients with trypanosomiasis (Houba *et al.*, 1969). However, the occurrence of high titres of NSCAT-agglutinins in a small proportion of apparently healthy Nigerians suggests that in Nigeria NSCAT-agglutinins are unlikely to be of value in the diagnosis of this condition. None of the subjects investigated was known to have trypanosomiasis.

In view of the established relationship between trypanosome infections and heterophile agglutinins, the possibility that the occurrence of NSCAT-agglutinins in Nigerian sera might be related to other parasitic infections was explored. The occurrence of complement fixing antibodies to human erythrocyte stroma (Mayer & Heidelberger, 1946) and of agglutinins to trypsinized human erythrocytes (Kano *et al.*, 1968) in subjects with malaria suggests that damage to the red cell by the malaria parasite may reveal erythrocyte antigens that are normally not exposed. It seemed possible that heterophile agglutinins might be produced in a similar way. However, no correlation was found between the titre of NSCAT-agglutinin and parasitological or serological evidence of malaria infection. Eluted NSCAT-agglutinin did not have any direct action against human erythrocytes parasitized with *P. falciparum*. Absorption experiments indicated that the NSCAT-agglutinin was distinct from the antibody reacting with human erythrocyte stroma and trypsinized human erythrocytes previously described in subjects with malaria.

Epidemiological data did not suggest any correlation between the occurrence of high titres of NSCAT-agglutinins and infection with onchocerciasis, loiasis and schistosomiasis. The parasitological data on which these observations are based must, however, be treated with caution as samples were examined for the presence of parasites on only one occasion.

Ascariasis and hookworm infections were probably prevalent in each of the population groups investigated, but data on the incidence of intestinal parasitic infections was not available.

No obvious relationship has been found between the occurrence of high titres of NSCAT-agglutinins in Nigerian sera and the presence of parasitic infections. Heterophile antibodies are frequently found in high titre in patients with glandular fever, a condition that is probably due to a virus infection. It is possible that widespread infection with a virus of low pathogenicity is responsible for the presence of heterophile agglutinins in the sera of healthy Nigerians.

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REFERENCES

ADENIYI-JONES, C. (1967) Agglutination of tanned sheep erythrocytes by serum from Nigerian adults and children. *Lancet*, **i**, 188.

BALL, J. (1963) Serological techniques. (a) Sensitised sheep cell agglutination test (SCAT). *The Epidemiology of Chronic Rheumatism* (Ed. by M. R. Jeffrey, J. H. Kellgren and J. Ball), Vol. 1, p. 328. Blackwell Scientific Publications, Oxford and Edinburgh.

DAVIDSOHN, I. (1929) Heterophile antibodies in serum sickness. *J. Immunol.* **16**, 259.

FAHEY, J.L. & MCKELVEY, E.M. (1965) Quantitative determination of serum immunoglobulins in antibody agar plates. *J. Immunol.* **94**, 84.

GRUBB, R. & SWAHN, B. (1958) Destruction of some agglutinins but not of others by two sulfhydryl compounds. *Acta path. microbiol. scand.* **43**, 305.

HENDERSON-BEGG, A. (1946) Heterophile antibodies in trypanosomiasis. *Trans. R. Soc. trop. Med. Hyg.* **40**, 331.

HOUBA, V. & ALLISON, A.C. (1966) M-antiglobulins (rheumatoid-factor-like), globulins and other gamma-globulins in relation to tropical parasitic infections. *Lancet*, **i**, 848.

HOUBA, V., BROWN, K.N. & ALLISON, A.C. (1969) Heterophile antibodies, M-antiglobulins and immunoglobulins in experimental trypanosomiasis. *Clin. exp. Immunol.* **4**, 113.

KANO, K., MCGREGOR, I.A. & MILGROM, F. (1968) Hemagglutinins in sera of Africans of Gambia. *Proc. Soc. exp. Biol. (N.Y.)*, **129**, 849.

MAYER, M.M. & HEIDELBERGER, M. (1946) Studies in human malaria. V. Complement-fixation reactions. *J. Immunol.* **54**, 89.

MICHAUX, J.L. (1966) Les immunoglobulines des Bantous à l'état normal et pathologique. *Ann. Soc. belge Med. trop.* **46**, 483.

OSLER, A.G., MULLIGAN, J.J., JR & RODRIGUEZ, E. (1966) Weight estimates of rabbit anti-human serum albumin based on antigen-binding and precipitin analyses: Specific hemagglutinating activities of 7S and 19S components. *J. Immunol.* **96**, 334.

PAUL, J.R. & BUNNELL, W.W. (1932) The presence of heterophile antibodies in infectious mononucleosis. *Amer. J. med. Sci.* **183**, 90.

ROWE, D.S., MCGREGOR, I.A., SMITH, S.J., HALL, P. & WILLIAMS, K. (1968) Plasma immunoglobulin concentrations in a West African (Gambian) community and in a group of healthy British adults. *Clin. exp. Immunol.* **3**, 63.

RUBIN, H. (1963) Antibody elution from red blood cells. *J. clin. Path.* **16**, 70.

TURNER, M.W. & VOLLER, A. (1966) Studies on immunoglobulins of Nigerians. I. The immunoglobulin levels of a Nigerian population. *J. trop. Med. Hyg.* **69**, 99.

VALKENBURG, H.A. (1963) Serological techniques. (b) Human erythrocyte agglutination test (HEAT) and (f) Latex fixation test (LFT). *The Epidemiology of Chronic Rheumatism* (Ed. by M. R. Jeffrey, J. H. Kellgren and J. Ball), Vol. 1, pp. 330 and 337. Blackwell Scientific Publications, Oxford and Edinburgh.

VOLLER, A. (1964) Fluorescent antibody methods and their use in malaria research. *Bull. Wld Hlth Org.* **30**, 343.