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Specific antibody-dependent cellular cytotoxicity in human malaria

J. BROWN & M. E. SMALLEY *Medical Research Council Laboratories, Fajara, The Gambia*

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SUMMARY

A micromethod for the study of specific antibody-dependent cellular cytotoxicity (ADCC) in human malaria is described, using cultured, asexual *Plasmodium falciparum* parasites as viable target cells. Lymphocytes from children with acute malaria, uninfected immune adult Gambians and adult Gambians infected with *P. falciparum* were capable of killing *P. falciparum in vitro* in the presence of malaria antibody. A parasite growth-promoting factor, produced by lymphocytes in non-immune serum and at a lymphocyte–parasite ratio of 10:1, in immune serum, was found to produce three-fold increases in growth of *P. falciparum*. The mechanisms by which ADCC may occur are also discussed.

INTRODUCTION

Effective host resistance to malaria is thought to involve a cell-mediated phase (Phillips, 1970; Phillips *et al.*, 1970; Weinbaum, Evans & Tigelaar, 1976) during which antibody-dependent cellular cytotoxicity (ADCC) by monocytes and lymphocyte subpopulations may play an important role (Coleman *et al.*, 1975; Greenwood, Oduloju & Stratton, 1977; McDonald & Phillips, 1978).

Specific killing of *P. berghei*-infected red blood cells by mononuclear cells in the presence of specific antibody has been demonstrated in mice (Coleman *et al.*, 1975) yet evidence of ADCC in human malaria is limited to the results of a non-specific assay in which chicken erythrocytes were haemolysed in the presence of rabbit anti-chicken RBC antibody by lymphocytes from children with acute malaria (Greenwood *et al.*, 1977).

The introduction by Trager & Jensen (1976) of continuous culture of the asexual cycle of *P. falciparum* has allowed the development of a simple microassay, used in this study, for investigation of ADCC in human malaria.

PATIENTS AND METHODS

Patients

(a) Fifteen healthy uninfected adult Gambians aged between 25 and 50 years, wholly or partially immune to *P. falciparum* malaria.

(b) Twenty-five Gambian children with acute malaria aged between 3 months and 6 years, with parasitaemias from 1 to 6%.

(c) Two adult Gambians aged 20 and 21 years with low-grade *P. falciparum* infections (<0·25%).

Correspondence: Mr J. Brown, Medical Research Council Laboratories, PO Box 273, Fajara, The Gambia.

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Methods

Six millilitres of blood was taken from each subject. Serum from 1 ml of blood was used as a supplement for the culture medium and for subsequent malaria antibody determination. The remainder of the blood was heparinized (10 iu/ml blood) and the mononuclear cells separated on a Ficoll/Hypaque gradient after centrifugation at 400 ***g*** for 15 min. Ninety-five per cent of the cells removed were lymphocytes and when tested by trypan blue dye exclusion 98% were viable. The remaining 5% were monocytes and neutrophils.

An adaptation of the microcultivation technique used by Smalley (1976) was employed in all experiments. Parasites grown in continuous culture for several months were used as the target and were adjusted to a starting parasite density of 0·3% by dilution in normal European group O erythrocytes at a haematocrit of 3% in RPMI (GIBCO, Paisley, Scotland) containing 10% human serum. The parasite suspension was then dispensed in 100-μl aliquots into a 96-well, sterile, flat-bottomed microtitre tray. The appropriate number of lymphocytes were added and the cultures were then incubated at 37°C using the candle jar technique of Jensen & Trager (1977), the medium being replaced daily. Films were taken every second day and stained with Giemsa.

The systems tested were:

(1) Lymphocytes + homologous serum. Control—homologous serum without lymphocytes.

(2) Lymphocytes + non-immune European serum. Control—European serum without lymphocytes.

Lymphocyte–parasite ratios of 10:1, 20:1 and 50:1 were examined.

The degree of ADCC was determined by assessment of the rate of parasite multiplication in the presence of lymphocytes and serum, compared with parasite growth in serum alone. Ten thousand erythrocytes were counted in each case.

Antibody to *P. falciparum* was measured against a schizont antigen using a double-diffusion gel technique (McGregor & Williams, 1978).

The statistical method used was Wilcoxon's rank sum test.

RESULTS

Children

Fig. 1 shows that at a lymphocyte–parasite ratio of 10:1 in homologous serum there was a significant increase in the growth of the parasite at the 5% level compared with the serum control. There was no difference in growth at a ratio of 20:1, but at a ratio of 50:1 lymphocytes from children with acute malaria produced a significant decrease in parasite growth in homologous serum ($P<0{\cdot}01$) compared with growth in serum only.

In non-immune European serum, a significant increase in parasite growth was observed at lymphocyte–parasite ratios of 10:1, 20:1 and 50:1 ($P<0{\cdot}01$ in each case), compared with the serum control.

Adults

When lymphocytes from immune adult Gambians were grown in homologous serum (Fig. 3) at a lymphocyte–parasite ratio of 10:1 there was a significant increase in growth at the 5% level over the serum control. At a ratio of 20:1 there was no difference but at 50:1 there was a significant inhibition of growth ($P<0{\cdot}01$).

When lymphocytes from immune adults were cultured in non-immune European serum there were significant increases in growth at lymphocyte–parasite ratios of 10:1 ($P<0{\cdot}01$), 20:1 ($P<0{\cdot}01$) and 50:1 ($P<0{\cdot}05$) compared with the European serum controls.

Table 1 shows that after 6 days parasite growth in childrens' sera without lymphocytes was 14·3 times the starting parasite density, whereas there was no increase on day 2 and an inhibition of growth on day 4 when lymphocytes were added at a 50:1 lymphocyte–parasite ratio. In adults there was no increased growth on day 2, a slight increase on day 4 and a reduction on day 6 in homologous serum only. The addition of fifty lymphocytes per parasite produced marked killing of the parasite on day 2.

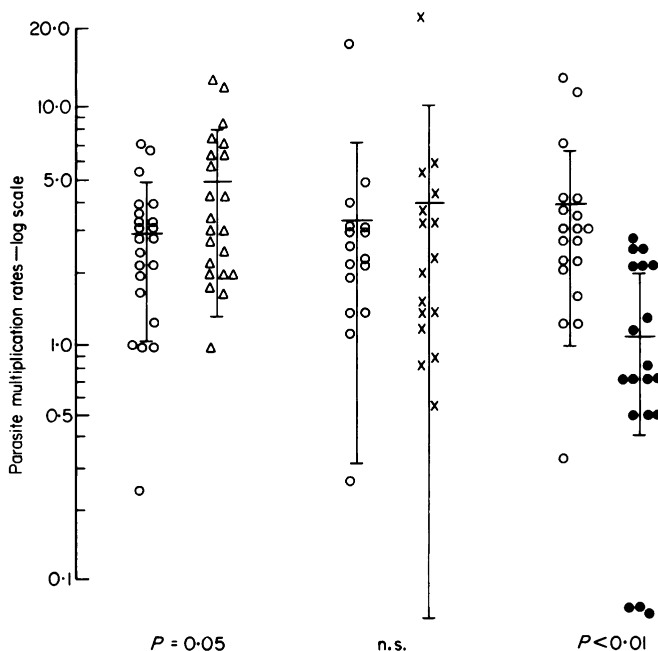

Fig. 1. Children with acute *P. falciparum* malaria. Parasites in homologous serum—2-day cultures. (o) Serum only; lymphocyte–parasite ratios: (▵) 10:1, (×) 20:1, (•) 50:1.

Table 2 shows that there was effective ADCC at a lymphocyte–parasite ratio of 10:1 in both individuals during infection. However, after recovery 2 weeks later, killing of the parasite was evident only at a ratio of 50:1.

Experiments carried out to compare ADCC in 25% serum compared with 10% serum showed no significant differences.

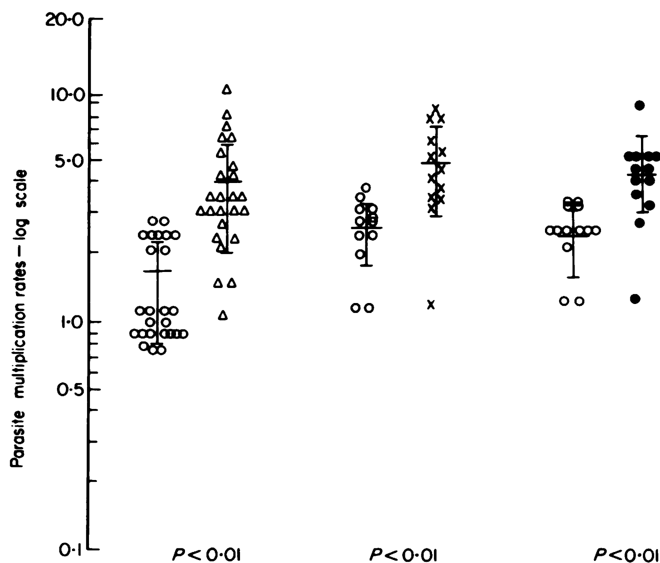

Fig. 2. Children with acute *P. falciparum* malaria. Parasites in non-immune European serum—2-day cultures. (o) Serum only; lymphocyte–parasite ratios: (▵) 10:1, (×) 20:1, (•) 50:1.

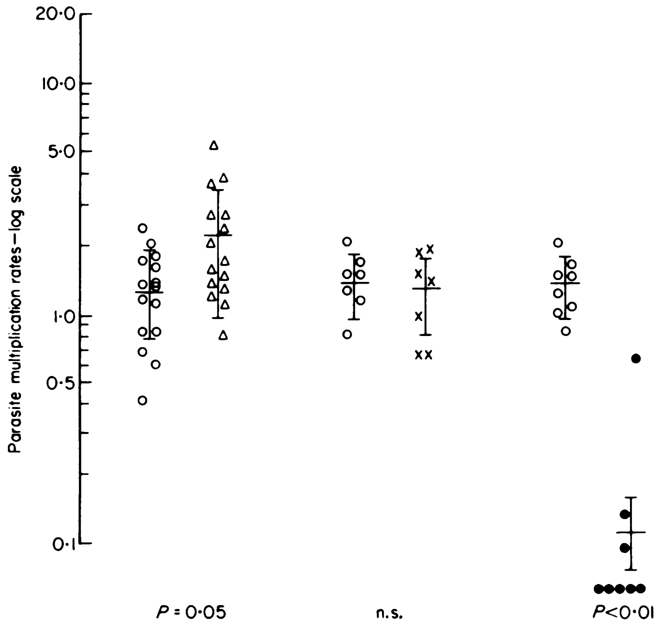

Fig. 3. Uninfected adult Gambians. Parasites in homologous serum—2-day cultures. (○) Serum only; lymphocyte–parasite ratios: (△) 10:1, (×) 20:1, (●) 50:1.

Three children (13%) showed increased ADCC at the 10:1 ratio, eleven (65%) at 20:1 and fourteen (93%) at 50:1 (Table 3). Each child showing increased killing at these levels had detectable malaria antibody. Eighteen children (72%) who had antibody failed to show ADCC at 10:1. Four children (24%) who had malaria antibody failed to show killing of the parasite at 20:1. Four children (17%) who had no demonstrable malaria antibody failed to show activity at a lymphocyte–parasite ratio of 10:1, two (12%) failed to show killing at 20:1 as did one (17%) at 50:1.

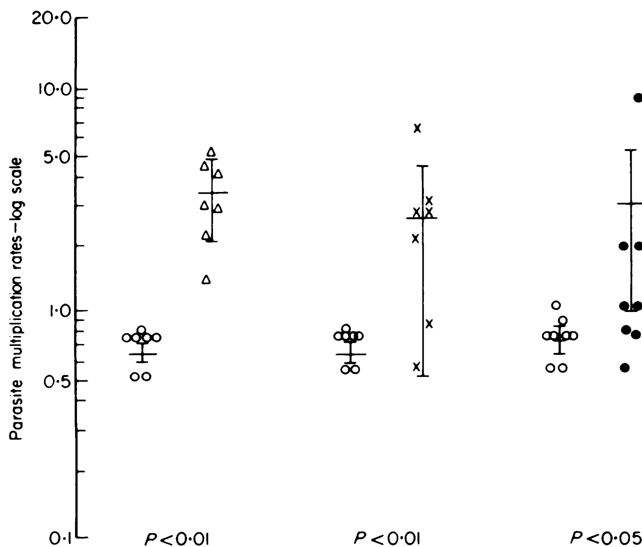

Fig. 4. Uninfected adult Gambians. Parasites in non-immune European serum—2-day cultures. (○) Serum only; lymphocyte–parasite ratios: (△) 10:1, (×) 20:1, (●) 50:1.

Table 1. Parasite multiplication in serum versus serum plus lymphocytes

	Day 2	Day 4	Day 6
Children			
Serum only ($n=11$)	$2{\cdot}6\pm1{\cdot}5$	$6{\cdot}6\pm3{\cdot}8$	$14{\cdot}3\pm5{\cdot}5$
Serum + lymphocytes ($n=16$)	$1{\cdot}0\pm0{\cdot}75$	$0{\cdot}3\pm0{\cdot}2$	Dead
Adults			
Serum only ($n=13$)	$1{\cdot}0\pm0{\cdot}5$	$1{\cdot}4\pm1{\cdot}4$	$0{\cdot}47\pm0{\cdot}67$
Serum + lymphocytes ($n=9$)	$0{\cdot}15\pm0{\cdot}3$	$0{\cdot}1\pm0{\cdot}4$	Dead

Mean parasite multiplication rates over 6 days in homologous serum and homologous serum plus lymphocytes (lymphocyte–parasite ratio 50:1) in adult Gambians and Gambian children with acute malaria.

Table 2. ADCC during and after infection

Patient	Serum control	Lymphocyte–parasite ratios 10:1	20:1	50:1
During infection				
K.C.	2·5	0·4	<0·01	<0·01
K.D.	3·3	0·16	<0·01	<0·01
After infection				
K.C.	1·0	1·7	1·0	0·66
K.D.	1·75	2·7	1·7	0·2

Parasite multiplication rates after 2 days in two young adult Gambians, during and after infection with *P. falciparum*. Parasites in homologous serum.

Table 3. Lymphocyte killing of *P. falciparum*—dependence on malaria antibody

Lymphocyte–parasite ratio	No. tested	No. showing killing: With Ab	No. showing killing: Without Ab	No. showing no killing: With Ab	No. showing no killing: Without Ab
Children					
10:1	25	3	0	18	4
20:1	17	11	0	4	2
50:1	15	14	0	0	1
Adults					
10:1	15	0	0	15	0
20:1	7	4	0	3	0
50:1	8	8	0	0	0

ADCC in relation to the presence of malaria antibody in uninfected adult Gambians and Gambian children with acute malaria. Two-day cultures.

In the immune adults without malaria, none of the patients tested showed ADCC at 10:1, although all had malaria antibody. Of seven adults tested at 20:1, four (57%) showed killing of the parasite and three (43%) did not, although all had antibody to malaria. At a ratio of 50:1 all eight (100%) adults tested demonstrated ADCC and all had malaria antibody.

DISCUSSION

Significantly increased ADCC to malaria parasites was demonstrated at a lymphocyte–parasite ratio of 50:1 in non-infected adults and in children with acute malaria (Figs 1 and 3). However, individuals from each group showed increased ADCC at ratios of 10:1 and 20:1. Killing was, without exception, dependent on the presence of malaria antibody.

Transient protection in malaria by passive transfer of immune serum has been shown in humans (Cohen, McGregor & Carrington, 1961) and in rats (Phillips & Jones, 1972). However, the most effective passive transfer of immunity in murine malaria requires both serum and cells (Phillips, 1970: Cottrell, Playfair & De Souza, 1978). In our experiments the parasite-killing effects of adult serum was evident by day 6; with addition of lymphocytes enhanced killing was demonstrable by day 2. In children, however, the addition of lymphocytes seemed to be a more important factor in killing the parasite, as parasite replication was reduced from 14·3 times the starting parasite density after 6 days to 0·3 times the parasite density on day 4 when the appropriate number of lymphocytes were added (Table 1).

It is possible that those children who exhibited increased ADCC at lymphocyte–parasite ratios of 10:1 and 20:1 possessed higher levels of malaria immunity than those whose cells reacted only at a ratio of 50:1. Such a view is supported by the observation that at a ratio of 50:1 cells from immune adults showed significantly greater killing capacity ($P<0{\cdot}01$) than did cells from infected children (Figs 1 and 3). It may be that the level at which ADCC occurs in an individual is an indication of protective immunity. Further experiments, employing a more detailed dose–response curve, of varying cell–parasite ratios will demonstrate whether or not this assay is a measure of protective immunity in human malaria.

T lymphocytes with IgG Fc receptors and non-T non-B lymphocytes ('null' cells) have been described as effector cells in ADCC against chicken erythrocytes (Li Shen *et al.*, 1979) and against *P. berghei*-infected RBCs (Coleman *et al.*, 1975). However, Phillips and his co-workers (1970) demonstrated that T lymphocytes had no cytotoxic effect on erythrocytes containing malaria parasites. Brown (1971) suggested that the role of T lymphocytes in malaria immunity was related to the production of protective antibody. Measurement of malaria antibody levels on culture supernatants, at the beginning of a culture and after death of the parasite due to ADCC with a T cell-depleted culture control, could possibly confirm this.

Monocytes (McDonald & Phillips, 1978) and polymorphonuclear neutrophil leucocytes (PMN) (Brown & Smalley, 1980) have also been implicated as cytotoxic cells to malaria-infected erythrocytes. It is unlikely that PMN acted as killer cells in the experiments described in this paper, since killing of *P. falciparum*-infected erythrocytes by neutrophils *in vitro* is independent of antibody (Brown & Smalley, 1980). Therefore non-specific killing of the parasite by polymorphs would also have occurred in non-immune European serum. Since there was 3% monocyte contamination of lymphocyte preparations some contribution to killing by monocytes cannot be discounted.

Greenwood *et al.* (1977) described increased non-specific ADCC in children with acute malaria associated with increased numbers of 'null' cells. After recovery, a decrease in killing was accompanied by a return to normally low 'null' cell levels. Two adult Gambians in these experiments, studied during and after infection with *P. falciparum*, showed five times more killing during infection than on recovery. Our observations agree with Greenwood *et al.* (1977) and suggest that the increase in ADCC against *P. falciparum*-infected erythrocytes is due to increased numbers of 'null' cells and that these cells contribute to the elimination of asexual *P. falciparum* parasites. It appears that serum on its own containing adult levels of malaria antibody is more effective in killing the parasite than serum from children and that in adults the addition of lymphocytes merely hastens the process. However, in infected adults far fewer effector cells are needed to kill the parasite than in infected children.

At lymphocyte–parasite levels of 10:1, 20:1 and 50:1 in European serum, in both adults and children, and at 10:1 in homologous serum, there were significant increases in parasite growth over the controls. The reasons for this phenomenon remain unknown and require further investigation. Nevertheless, this may prove to be an important finding in that the isolation of a soluble lymphocyte factor promoting parasite growth could prove invaluable in the *in vitro* production of high yields of asexual parasites.

Finally, since it is unlikely that the children and adults studied had past experience of only one strain or variant of *P. falciparum* and while their lymphocytes were yet able to kill the parasite *in vitro* in homologous serum, a degree of cross-protection between strains and variants of *P. falciparum* malaria seems likely.

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