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Natural cytotoxicity for *Plasmodium berghei in vitro* by spleen cells from susceptible and resistant rats

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SUMMARY

The susceptibility of 30-day-old rats to *Plasmodium berghei* infection has traditionally been ascribed to the higher levels of circulating blood reticulocytes for which *P. berghei* has a predilection. However, spleen cells soon develop natural cytotoxicity for *P. berghei* which may account, in part, for the increased natural resistance of older rats. Spleen cells from normal 30- or 50-day-old rats were cultured overnight with erythrocytes parasitized by *P. berghei* and then injected into MF1 mice. Six days later, the percentage parasitaemia was determined and the extent of killing by the spleen cells *in vitro* determined. Spleen cells from 50-day-old resistant rats were found to be four times better at killing *P. berghei in vitro* than those from 30-day-old susceptible rats. Antibody-dependent cell cytotoxicity (ADCC) was, at best, only a minor component. About 12% of total cytotoxicity was destroyed by pretreatment of spleen cells with monoclonal anti-Thy-1.1 antibody and complement. The possibility that natural cytotoxicity in these experiments is mediated by natural killer cells is discussed.

INTRODUCTION

The greater susceptibility of 30-day-old rats to *Plasmodium berghei* compared with rats of 50 days of age has long been ascribed to the higher level of reticulocytes in the younger rats because *P. berghei* has a predilection for reticulocytes. However, Zuckerman (1958) has shown that weanling rats are more susceptible to *P. vinckei* than mature rats; as *P. vinckei* invades all types of erythrocytes, it appears that age-related resistance to *P. vinckei* infection may have an immunological basis. During studies of antibody-dependent cell cytotoxicity (ADCC) mediated by spleen cells from 30- and 50-day-old rats against ^{51}Cr-labelled parasitized erythrocytes, Orago & Solomon (1986) found that there was no age difference in levels of splenic ADCC. However, when anti-*P. berghei* antibody was excluded from the *in vitro* assay, total splenic cell-mediated cytotoxicity (CMC) accounted for 50% of total cytotoxicity in spleen cells from 50-day-old rats, but was much lower in those from 30-day-old rats. This suggested that an antibody-independent natural cytotoxicity developed between 30 days and 50 days of age. Natural cytotoxicity (NC) of spleen cells from 30- and 50-day-old rats for *P. berghei in vitro* was compared by an 'infectivity assay'. After overnight culture, cells were injected into MF1 mice and 6 days later the percentage parasitaemia was assessed. Spleen cells from 50-day-old rats were found to be about four times more effective at killing *P. berghei in vitro* than those from 30-day-old rats when various effector/target cell ratios were compared. On the other hand, ADCC appeared to play a very minor role in killing *P. berghei*.

Abbreviations: ADCC, antibody-dependent cell cytotoxicity; CMC, cell-mediated cytotoxicity; FBS, fetal bovine serum; NC, natural cytotoxicity.

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MATERIALS AND METHODS

Animals

Outbred Sprague Dawley rats originally obtained from Charles River Ltd (Margate, Kent) were taken from a colony maintained in our animal house. *Plasmodium berghei* was passaged weekly in 30-day-old rats. Six-week-old outbred female MF1 mice from our closed colony were used for the infectivity assay. *Plasmodium berghei* (NK 65) was kindly supplied by Dr D. Walliker of the Institute of Animal Genetics, University of Edinburgh, Edinburgh.

*Anti-*P. berghei *serum, spleen cell preparation and treatment with anti-Thy-1.1 antibody*

The preparation of anti-*P. berghei* serum and its measurement by the ELISA technique, spleen cells from 30- or 50-day-old rats and treatment of spleen cells with anti-Thy-1.1 antiserum have been described by Orago & Solomon (1986).

Target cells

Rat erythrocytes (1×10^8, 20–25% parasitized with *P. berghei*) were injected intraperitoneally into 30-day-old rats. Seven days later (when the parasitaemia was about 20%) the rats were bled by cardiac puncture into phosphate-buffered saline, pH 7·2 (PBS), containing 100 units of heparin/ml. Parasitized erythro-

cytes were washed twice in PBS and made up to 4×10^7 cells/ml RPMI medium containing 2·0 g/l sodium bicarbonate supplemented with 10% v/v FBS (Flow Laboratories Ltd, Irvine, Ayrshire), 200 μg/ml streptomycin and 400 μg/ml penicillin G ('supplemented medium').

Culture conditions for natural cytotoxicity

Rat erythrocytes from infected rats with about 20% parasitaemia were used as target cells, and 2×10^7 such erythrocytes were suspended in 0·5 ml supplemented medium. Various numbers of effector spleen cells in 0·5 ml supplemented medium from either 30- or 50-day-old Sprague Dawley rats and 0·5 ml supplemented medium were added. Effector/target cell ratios were expressed as effector/total red cells. In experiments testing for ADCC the 0·5 ml medium was replaced by rat anti-*P. berghei* anti-serum (diluted 1/100) and the target cells stood for 30 min at 20° with intermittent shaking before the addition of spleen cells. The cell mixtures were cultured for 17 hr at 37° in loosely capped bottles in a 5% CO_2 gassed incubator.

Viability of P. berghei *in culture*

After overnight culture of 5×10^6 parasitized erythrocytes with approximately five times as many non-parasitized erythrocytes (20% parasitaemia) cells were diluted and 5×10^5 parasitized erythrocytes injected into mice. Six days later the percentage parasitaemia was 5·7%. When an identical dose of parasitized erythrocytes was injected into mice after only 2 hr *in vitro* (for washing and counting) the resultant parasitaemia 6 days later was 4%. This showed that the culture conditions reasonably maintained viability of *P. berghei*.

Infectivity assay

Five 6-week-old MF1 mice were each injected intraperitoneally with an aliquot (0·4 ml) of the cultures of spleen cells and parasitized erythrocytes. First, PBS (2·5 ml) was added to each culture (1·5 ml) in order to reduce the dose of parasitized erythrocytes to 5×10^5 per mouse. This provided a surplus of parasitized erythrocytes in case of leakage immediately after injection. Any mice showing leakage were killed. Six days later the mice were tail-bled and thin blood smears taken, fixed and stained with Geimsa (BDH Chemicals Ltd, Poole, Dorset). Percentage parasitaemias were determined by two observers, each counting 500 erythrocytes.

The degree of killing of *P. berghei in vitro* can be determined from the reduction in percentage parasitaemia. Different numbers of parasitized erythrocytes were cultured overnight in supplemented medium alone and diluted as above before injection into MF1 mice. A standard curve of parasites surviving in culture after 17 hr *in vitro* shows a close linear relationship with percentage parasitaemia at 6 days after infection of MF1 mice (Fig. 1). This enables the results to be finally expressed as 'number of parasites killed'.

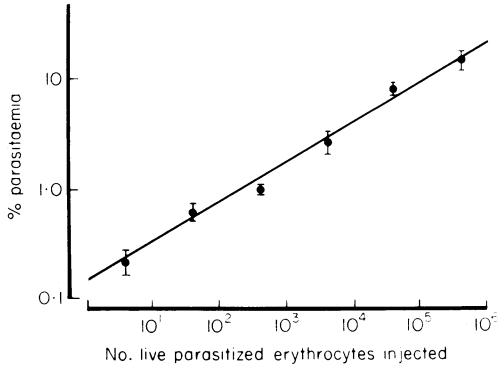

Figure 1. Survival of different numbers of erythrocytes parasitized with *P. berghei* after culture for 17 hr at 37° *in vitro* measured by injection into mice and assessing the percentage parasitaemia 6 days later. Each point represents the mean parasitaemia of 10 mice ± one SE.

Statistics

Standard errors (SE) of means were calculated for all values and statistical significance between means calculated by Students' *t*-test.

RESULTS

Natural cytotoxicity

Comparison of NC of spleens from 30- and 50-day-old rats at E/T ratios of 1 : 1 or greater show a similar degree of killing ranging from 75 to 100% (Table 1). However, at lower E/T ratios spleen cells from the younger age group of rats are clearly less cytotoxic for *P. berghei* than those from 50-day-old rats. There is no statistically significant killing (72%) by 30-day-old rat spleen cells until the E/T ratio is 1:2, whereas 50-day-old rat spleen cells can kill 76·0–91·2% *P. berghei* at an E/T ratio of 1 : 5 (Table 1). Probit analysis (Litchfield, 1949) revealed a median lethal (90%) E/T ratio of 1 : 1 for spleen cells from 30-day-rats and 1 : 4 for 50-day-old rats.

ADCC in the infectivity assay

Specific anti-*P. berghei* antiserum at a dilution of 1/50 killed 94·3% *P. berghei in vitro* but when diluted 1/100 there was no killing (Table 2). Accordingly, a dilution of 1/100 antiserum was used to coat parasitized erythrocytes before the overnight culture began. No increased killing due to antibody was found in a series of experiments with 30-day-old rat spleen cells at E/T ratios of 1 : 1–1 : 20. However, in two out of three such experiments with spleen cells from 50-day-old rats there was a slight but significant increase in the number of *P. berghei* killed in the presence of specific antibody (3×10^3–1×10^2 and $1{\cdot}3 \times 10^3$–40) (Table 2).

Four-hour culture for the infectivity assay

We have used a 16-hr period for the infectivity assay to parallel the same period used in the ADCC and CMC assays based on ^{51}Cr release (Orago & Solomon, 1986). When a 4-hr incubation period was used in the infectivity assay, killing only occurred at a much higher E/T ratio (50:1) (Table 3).

Treatment of spleen cells with anti-Thy-1.1 IgG

Pretreatment of normal 50-day-old rat spleen cells with monoclonal rat anti-Thy-1.1 IgG and complement resulted in the destruction of only a minor population of cells cytotoxic for *P. berghei* as 87·5% *P. berghei* was killed compared to virtually

Table 1. Natural cytotoxicity mediated by spleen cells from 30- and 50-day-old rats against *P. berghei in vitro*

	Age of rats							
	30 days*				50 days*			
E/T ratio	Control culture (no. parasitized erythrocytes)	No. parasites surviving	% killed	*P*	Control culture (no. parasitized erythrocytes)	No. parasites surviving	% killed	*P*
1:10	5×10^4	1×10^5	0		1×10^5	5×10^4	50·0	>0·05
1:5	1×10^5	1×10^5	0		$2{\cdot}5\times10^5$	6×10^4	76·0	<0·01
	5×10^4	7×10^4	0		9×10^4	1×10^4	89·0	<0·001
					9×10^4	8×10^3	91·2	<0·001
1:3	$1{\cdot}7\times10^4$	8×10^3	53	>0·05	$3{\cdot}2\times10^4$	$1{\cdot}3\times10^3$	96·3	<0·01
1:2	6×10^4	$1{\cdot}7\times10^4$	72	<0·05	$2{\cdot}5\times10^5$	$1{\cdot}8\times10^4$	93·0	<0·001
1:1	8×10^5	2×10^5	75	<0·05	$2{\cdot}2\times10^5$	3×10^4	86·0	<0·01
	6×10^4	6×10^3	90	>0·05	6×10^5	3×10^3	99·5	<0·001
	6×10^4	4×10^3	93	<0·01	$1{\cdot}3\times10^4$	$1{\cdot}4\times10^3$	89·2	<0·001
	5×10^4	1×10^2	99·8	<0·001				
2:1	$7{\cdot}5\times10^4$	4×10^3	95	>0·05			ND†	
	6×10^4	7	99·9	<0·001				
3:1	2×10^5	2×10^4	90	<0·001			ND	
5:1	2×10^5	0	100					
	6×10^4	6	99·9	<0·001	3×10^5	2×10^1	99·9	<0·001

When *P. berghei* was incubated for 1 min *in vitro* with 30- or 50-day-old rat spleen cells (E/T = 5:1) no killing occurred.

* Two rat spleens were used in each experiment.

† ND, not determined.

Table 2. ADCC and natural cytotoxicity mediated by spleen cells from 30- and 50-day-old rats against *P. berghei in vitro*

	Age of rats						
	30 days			50 days			
E/T ratio	Antibody dilution	No. parasites surviving	% killed	E/T ratio	Antibody dilution	No. parasites surviving	% killed
—	—	$2{\cdot}8\times10^5$					
—	1/50	$1{\cdot}6\times10^4$	94·3				
—	1/100	8×10^5	0	—	1/100	7×10^5	0
1:1	—	1×10^5	75	1:1	—	3×10^3	99·6
1:1	1/100	1×10^5	75	1:1	1/100	1×10^2*	99·9
—	1/100	6×10^4	0				
1:1	—	4×10^3	93				
1:1	1/100	3×10^3	95				
—	1/100	$1{\cdot}3\times10^4$	0	—	1/100	$3{\cdot}5\times10^4$	0
1:3	—	$6{\cdot}3\times10^3$	53	1:3	—	$1{\cdot}3\times10^3$	96·3
1:3	1/100	$2{\cdot}4\times10^4$	0	1:3	1/100	4×10^1*	99·9
—	1/100	$1{\cdot}3\times10^4$	0	—	1/100	9×10^4	0
1:5	—	$1{\cdot}7\times10^4$	0	1:5	—	1×10^4	89·0
1:5	1/100	$2{\cdot}0\times10^4$	0	1:5	1/100	1×10^4	89·0
—	1/100	$2{\cdot}3\times10^4$	0				
1:20	—	4×10^4	0				
1:20	1/100	$2{\cdot}3\times10^4$	0				

Two rat spleens were used in each experiment.

* $P<0{\cdot}01$.

Table 3. Natural cytotoxicity of 30-day-old rat spleen cells against *P. berghei* in a 4-hr culture

E/T ratio	No. parasites surviving	% parasites killed
None	3×10^5	0
8·3:1	6×10^5	0
17:1	3×10^5	0
50:1*	7×10^0	99·99

* Incubation for 1 min *in vitro* at this ratio did not kill any *P. berghei*.

Table 4. Some rat spleen cells cytotoxic for *P. berghei* express Thy-1 antigen

Culture contents	% parasitaemia	No. parasites surviving	% parasites killed
P. berghei only	21·3	8×10^5	0
P. berghei + spleen cells (E/T = 2·5:1)	0·2	2	99·99
P. berghei + spleen cells (E/T = 2·5:1) + anti-Thy-1 antibody	3·5	6×10^3	99·25
P. berghei + spleen cells (E/T = 2·5:1) + anti- Thy-1 antibody + complement	9·5	1×10^5*	87·50

* Significantly reduced ($P < 0.001$).

complete killing in the controls (Table 4). This suggests that only about 12·5% of cells cytotoxic for *P. berghei* bear Thy-1.1 antigen.

DISCUSSION

The higher susceptibility of young rats to *P. berghei* infection has for a long time been thought to be solely due to the predeliction of *P. berghei* for reticulocytes, which are at a higher level in the blood of 30-day-old rats than older rats (Singer, Hadfield & Lakonen, 1955). Rats are born physiologically and immunologically immature (Solomon, 1981) and even by 30 days of age have not yet achieved an adult-type blood cell pattern as the proportion of reticulocytes is still quite high (12·2%) but falls to near adult level (2·1%) by 50 days of age. Earlier observations (Singer *et al.*, 1955; Zuckermann, 1957, 1970) that *P. berghei* preferentially parasitizes reticulocytes have been confirmed by Orago & Solomon (1986). Splenectomy of mice lowered both the number of blood reticulocytes and parasitaemia (Singer, 1954), but splenectomized mice died from *P. berghei* infection (Zuckerman & Yoeli, 1954), suggesting that the spleen rather than the level of peripheral reticulocytes was important in providing protective immunity to *P. berghei* infection. Despite its predilection for reticulocytes, *P. berghei* will also infect mature erythrocytes (Ott, 1968; Bungener, 1979). We have found that when all the reticulocytes were parasitized in young rats, *P. berghei* would then begin to parasitize mature erythrocytes (Orago & Solomon, 1986). In the virtual absence of reticulocytes, *P. berghei* will infect mature erythrocytes in susceptible strains of mice and cause rapid death (Greenberg, 1956; Ott, 1968; J. B. Solomon, unpublished observations). Also, Zuckerman (1958) showed that weanling rats were more susceptible to *P. vinckei* infection than mature rats. As *P. vinckei* invades all types of erythrocytes of varying maturity, this was suggestive of a developing immunological defence mechanism.

Murine cells cytotoxic for *P. yoelii in vitro* have been studied by Taverne, Dockrell & Playfair (1982) by an infectivity assay. White blood cells belonging to the monocyte–macrophage series were found to be the most cytotoxic cells. These had acquired the ability to kill the parasite before becoming fully differentiated into macrophages. Cytotoxic cells were adherent to plastic and phagocytic, whereas lymphocytes were relatively ineffective. There was evidence for greatly increased killing in the parasiticidal test when a low dilution of high titred specific antiserum was added. In contrast, we have found ADCC mediated by rat spleen cells to be of minor cytotoxic importance in our infectivity test. In a series of experiments with spleen cells from 30-day-old rats we detected no ADCC by the infectivity assay. This is in sharp contrast to the results of Orago & Solomon (1986) using a ^{51}Cr-release method for ADCC, who found that specific antibody was more effective at increasing cytotoxicity in spleen cells from normal and infected 30-day-old rats compared to those from 50-day-old rats. In the experiments of Taverne *et al.* (1982) even a dilution of only 1/4 specific anti-*P. yoelii* serum did not kill the parasite *in vitro*, whereas in our experiments specific antibody had to be diluted 100-fold in order not to have killing by antibody alone. In addition, rat spleen cells appear to be much more cytotoxic for malarial parasites than mouse spleen cells, which required E/T ratios of 100:1 in contrast to our experiments with E/T ratios of 1:1 or less.

Susceptibility to *P. berghei* infection in rats does not appear to be due to defective macrophage activation. Superoxide anion production by stimulated macrophages in spleen cells from 30- and 50-day-old rats was found to be similar. Also, there was no age difference in the levels of increased superoxide anion production following infection of young and older rats (McGowan & Solomon, 1981). Young rats infected with *P. berghei* showed better evidence of activation (reduction of 5′-nucleotidase activity) of their splenic and peritoneal macrophages than older rats (Tomney & Solomon, 1981). Moreover, the chemiluminescent response of splenic macrophages (stimulated with sodium caseinate) from 30-day-old rats is as great as the response of the macrophages of older rats (J. B. Solomon, unpublished results).

Thy-1.1-positive cytotoxic cells only account for a minor population of NC cells (Table 4). Rat T cells only weakly express Thy-1.1 antigen (in contrast to murine T cells) and also share certain common markers with NK cells. Rat NK and ADCC effector cells showed identical marker heterogeneity when tested with monoclonal antibodies W3/13 and OX8 (Cantrell, Robins & Baldwin, 1983). The monoclonal IgG1 antibody (MRC OX7) that we used recognizes a membrane glycoprotein typical of rat T-helper cells. Zoller & Matzku (1983) found no Thy-1.1 on rat splenic NK cells but it was expressed by pre-NK cells.

Our recent investigations with beige mice have suggested that natural killer (NK) cells might be implicated in preventing

high parasitaemias during the early stages of infection with *P. berghei* (Solomon, Forbes & Solomon, 1985). NK cells increase in the spleen of rats between 30 days and 50 days of age and may account for the resistance of some of the older rats to malarial infection. When natural cytotoxicity (due to NK cells) was measured by allogeneic lymphocyte cytotoxicity, it was found to be weak in 3–4-week-old rats but had developed to adult levels by about 6 weeks of age (Heslop & McNeilage, 1983).

Our *in vitro* culture of *P. berghei* with spleen cells has shown quite clearly that spleen cells from naturally resistant (50-day-old rats) are four times more cytotoxic to *P. berghei* than those from susceptible 30-day-old rats. This agrees well with the four-fold increase in total CMC per spleen between 30 days and 50 days of age found by Orago & Solomon (1986). How much this cytotoxic activity (CMC and NC) contributes to total natural immunity *in vivo* is not known. Nevertheless, this is the first time that at least some of the age-related natural immunity in this rat model for childhood malaria has been shown to have an immunological basis.

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