SHORT COMMUNICATION

Penetration of Maturing Red Blood Cells by *Toxoplasma gondii*

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Penetration of *Toxoplasma gondii* tachyzoites was studied *in vitro* using murine erythroid cells at different stages of development. *Toxoplasma gondii* penetrated nucleated erythroblasts and macroreticulocytes from foetal mouse liver and the circulating erythrocytes of foetal, neonatal or severely anaemic adult mice. Immature reticulocytes were more susceptible to penetration than mature ones, indicating that some change in their membrane properties occurred during maturation. The present results confirmed our previous finding that the major erythrocyte membrane-specific proteins do not prevent erythrocyte penetration since these proteins are known to be present in the reticulocyte membrane.

INTRODUCTION

Toxoplasma gondii is an intracellular parasite causing human and animal toxoplasmosis. Studies *in vitro* have shown that all mammalian and avian culture cells so far tested are susceptible to *T. gondii* penetration (Doran, 1973) with the exception of mammalian erythrocytes. Elucidation of the mechanism by which erythrocytes escape penetration would provide an important insight into the mechanism of entry by the parasite. We have previously shown that murine erythroleukaemia cells transformed by Friend virus (FL cells), when induced to synthesize erythrocyte membrane-specific proteins and haemoglobin, are readily penetrated by *T. gondii* to the same extent as uninduced cells (Tanabe *et al.*, 1978). Thus erythrocyte membrane-specific proteins are not responsible for the resistance to penetration. Since induced FL cells correspond to late erythroblasts, erythroid cells must presumably become resistant to parasite penetration at some later stage in their maturation.

In the present study, penetration by *T. gondii* was examined in more advanced erythroid cells obtained from the liver and peripheral blood of foetal or neonatal mice and also in erythrocytes from adult mice rendered anaemic either by daily bleeding, by phenylhydrazine injection or by infection with rodent malaria.

METHODS

Parasites. Suspensions containing *Toxoplasma gondii* (RH strain) tachyzoites freed from host cells were obtained by filtering the peritoneal exudate of infected mice through a cellulose powder column (Tanabe *et al.*, 1978).

Animals. Two strains of mice, 2 to 4 months old, were used: C57B1/6 HeMs (from the National Institute of Genetics, Mishima, Japan and maintained by brother-sister mating in our department) and CD-1 mice (from Charles River, Japan). The animals were mated overnight from 16:00 to 10:00, and the first day of gestation was considered to begin on the following morning.

Preparation of erythroid cells. Embryos removed from pregnant mice were bled from their necks after trans-section, and the blood was collected in phosphate-buffered saline (PBS, pH 7.2). The blood was washed twice with PBS by centrifugation (400 g, 5 min) and the pellet was suspended in Eagle's minimum essential medium containing Earle's salts (MEM) to give 10^8 red blood cells ml^{-1} . After bleeding, the foetal livers were dissected in PBS and liver cell preparations were made by gently squeezing the organs between frosted glass slides in PBS at room temperature. The dissociated cells were filtered through a 200-mesh stainless-steel screen, washed twice with PBS (400 g, 5 min) and suspended in MEM (5×10^7 cells ml^{-1}). Red blood cells from neonatal mice, collected by carotid bleeding or by cardiac puncture, and those from adult anaemic mice were also examined. Reticulocytosis was induced in adult mice either (i) by daily bleeding of 0.1 ml from the tail for 10 or 15 d, (ii) by six daily subcutaneous injections of phenylhydrazine (1.3 mg in 0.1 ml distilled water for each mouse) or (iii) by inoculation with 10^6 erythrocytes parasitized by *Plasmodium chabaudi* (a self-resolving malaria parasite) (see Tanabe *et al.*, 1977). At 1 d after the last bleeding, 2 d after the last phenylhydrazine injection or at various intervals after *P. chabaudi* infection, red blood cells were collected by cardiac puncture and tested for susceptibility to *T. gondii* penetration. Reticulocyte counts were made on red blood cells after staining with New Methylene Blue (Chroma, West Germany).

Assay for T. gondii penetration into erythroid cells. Erythroid cell suspensions containing 10^8 cells ml^{-1} in MEM (0.2 ml) were mixed with 1.8 ml MEM containing 15 % (v/v) foetal calf serum (Gibco) and transferred to Falcon plastic culture dishes (35 $\times$ 10 mm). *Toxoplasma gondii* tachyzoites (2×10^7 in 0.2 ml MEM) were then inoculated into the cultures; cell smears were made after 4 and 8 h incubation (37 °C, in 5 % CO_2 /95 % air) and stained with Wright or Giemsa stain. To assess the penetration by *T. gondii*, 3000 host cells were examined and the results were expressed as the percentage of cells containing parasites.

Electron microscopy. After 4 h incubation of erythroid cells with parasites, the mixture was centrifuged (400 g, 5 min) and the supernatant was discarded. The pellet was fixed with 2.5 % (w/v) glutaraldehyde and 2 % (w/v) paraformaldehyde in 0.1 M-phosphate buffer (pH 7.4) and post-fixed in 1.0 % (w/v) OsO_4 in 0.1 M-cacodylate buffer. The samples were dehydrated with an ethanol/acetone series and embedded in Epon 812. Thin sections were examined (Hitachi-11DS electron microscope) after staining with uranyl acetate.

RESULTS AND DISCUSSION

Foetal liver erythroid cells of C57B1/6 mice were removed on day 13 or 14 of gestation, when non-erythroid hepatic cells were relatively few in number (Tarbutt & Cole, 1970), and incubated *in vitro* with *T. gondii* tachyzoites for 4 h. Examination of Giemsa-stained smears revealed that penetration occurred in all varieties of erythroid cells: proerythroblasts, basophilic erythroblasts, poly- and orthochromatophilic erythroblasts, yolk sac-derived nucleated erythrocytes and enucleated liver reticulocytes.

Circulating red blood cells from foetuses at various stages of development (C57B1/6 mice) were also incubated with *T. gondii*; the percentage of red blood cells penetrated by parasites was high for cells taken on day 15 and rapidly decreased thereafter (Table 1; Fig. 1*a, c*). However, *T. gondii* was still able to penetrate cells from embryos taken just before birth (day 20) and those from neonatal mice (up to day 9, and at certain other ages up to day 20) although the penetration rate, expressed as the percentage of cells containing parasites, was low compared with that found in embryonic red cells, and generally decreased as neonatal mice aged (Table 1). The penetration rate of red blood cells from embryonic or neonatal CD-1 mice was nearly identical to that of C57B1/6 mice (results not shown).

Red blood cells of adult C57B1/6 mice, rendered anaemic in various ways, were also tested for penetration by *T. gondii* (Table 1). Red blood cells from mice bled daily were considerably resistant to penetration, whereas those from phenylhydrazine-treated mice were to some extent penetrated by parasites. On the other hand, *T. gondii* penetrated red cells from mice with low (day 15) but not high (day 7) *P. chabaudi* parasitaemia. When mice had recovered from the infection (day 20 or 24), the penetration rate was reduced or not detectable.

Since polychromatophilic erythrocytes (PCE) are known to be immature reticulocytes (Perrotta & Finch, 1972), differential counts of PCE were made on Wright-stained smears after incubation with *T. gondii*. The numbers of parasitized PCE were expressed as a percentage of the total number of parasitized cells (i.e. PCE plus non-PCE; Table 1). It is

Table 1. Penetration of red blood cells by *Toxoplasma gondii*

Red blood cells (2×10^7) from embryonic, neonatal or anaemic adult mice were incubated with 2×10^7 *T. gondii* tachyzoites for 4 or 8 h. Penetration rates, i.e. the percentages of cells containing parasites, were determined in cell smears.

Mice	Day	Reticulo-cytosis (%)	Penetration rate (%)		PCE* (%)	Parasites within PCE† (%)
			4 h	8 h		
Embryos	15	100	8.60	9.40	66	89
	16	99	1.80	2.80	42	87
	17	96	1.80	3.20	41	76
	18	97	1.40	1.30	25	71
	19	81	0.35	0.90	29	52
	20	56	0.50	0.45	16	51
Neonates	0	37	0.30	0.30	16	38
	1	41	0.15	0.17	6	33
	2	31	0.15	0.40	4	20
	3	42	0.40	0.53	6	28
	4	26	0.10	0.40	5	14
	5	37	0.20	0.35	7	27
	7‡	24	0.10	0.20	4	17
Adults Bled daily	10	27	0	0.07		
	15	24	0.03	0.03		
Phenylhydrazine-injected		88	0.20	0.27	17	50
<i>P. chabaudi</i> -infected	7 (51.0 %)	1	0	0		
	15 (1.5 %)	68	0.75	1.27	35	84
	20 (0.1 %)	55	0.23	0.20	10	83
	24 (0.0 %)	8	0	0		

* PCE, Polychromatophilic erythrocytes.

† Number of parasitized PCE expressed as a percentage of the total number of parasitized cells (i.e. PCE plus non-PCE).

‡ Penetration was found in erythrocytes from neonates at certain other ages up to day 20, but the penetration rate was always less than 0.20 %.

§ Plasmodial parasitaemia.

clear that while the number of PCE decreased considerably with age in both foetal and neonatal mice, the percentage of parasites found within them was always higher than the proportion of PCE relative to all blood cells. This was also true for the red cells from adult anaemic mice (Table 1). Thus, *T. gondii* apparently penetrated immature reticulocytes in preference to mature reticulocytes or erythrocytes. However, electron micrographs (Fig. 1*a, b*) revealed that parasites were present in red cells with few or no ribosomes, i.e. mature reticulocytes or erythrocytes. [The red cell in the upper left of Fig. 1(*a*) contains many ribosomes.] After entry into erythrocytes, the parasites seemed somewhat degenerate (Fig. 1*b*). Although Schupp *et al.* (1978) have recently reported invasion of erythrocytes by *T. gondii* *in vitro*, some of their photographs clearly show that the penetrated host cells are reticulocytes rather than erythrocytes, since the host cells contain many ribosomes.

The present study showed that immature reticulocytes were more susceptible to *T. gondii* penetration than mature reticulocytes, but the parasite was still able to penetrate pre- and post-natal erythrocytes with few or no ribosomes. The only fully resistant red cells were the mature erythrocytes of normal adult mice. Electron microscopy (Aikawa *et al.*, 1977) and biochemical experiments (Lycke *et al.*, 1975) have clearly demonstrated that *T. gondii* closely interacts with the host cell membrane. Lycke *et al.* (1975) extracted a penetration-enhancing factor which presumably acts enzymically on the host cell membrane to assist parasite penetration. Thus, a study of changes in membrane properties during erythroid cell maturation may provide an explanation for the failure of *T. gondii* to enter mature mammalian erythrocytes.

Fig. 1. Electron and photomicrographs of peripheral erythrocytes from embryonic (day 17; *a*, *c*) and neonatal (day 4; *b*) mice after 4 h incubation with *Toxoplasma gondii* tachyzoites. Bar marker representst 1 μ m in (*a*, *b*) and 10 μ m in (*c*).

The immature red cells of embryonic or neonatal mice and severely anaemic mice are known to be released into the peripheral blood in the form of macroreticulocytes (Grüneberg, 1942; Scribner *et al.*, 1968; Ganzoni *et al.*, 1969). They remain macrocytic after entry into the circulation for a relatively long period. Walter *et al.* (1978) have demonstrated that reticulocytes produced in severe anaemia gave rise to erythrocytes with abnormal membrane surface properties. Shattil & Cooper (1972) have shown that the macroreticulocytes lose substantial amounts of membrane lipids during maturation. They could not, however, detect a difference between the macroreticulocyte and erythrocyte membrane in terms of lipid composition and cholesterol/phospholipid ratio, of which the latter is thought to influence the microviscosity of cell membranes (Shinitzky & Inbar, 1974). There is also evidence that the protein composition of the reticulocyte membrane is very similar to that of the erythrocyte membrane, which contains the major membrane-specific proteins, such as spectrin (Fairbanks *et al.*, 1971) or glycophorin (Marchesi *et al.*, 1972). The present findings thus confirm our previous conclusion (Tanabe *et al.*, 1978) that it is not these membrane proteins that are responsible for the resistance of mature mouse erythrocytes to penetration by *T. gondii*, but changes in membrane properties during macroreticulocyte maturation. Clarification of these changes is clearly important for understanding the mechanism of entry into the host cell. In particular, membrane components or substances which actively participate in host cell-*Toxoplasma* interaction must be looked for.

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