

Plasmodium falciparum Varies in Its Ability To Induce Tumor Necrosis Factor

RICHARD J. ALLAN,¹ ALEXANDRA ROWE,² AND DOMINIC KWIATKOWSKI^{1*}

Molecular Infectious Disease Group¹ and Molecular Parasitology Group,² Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom

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Tumor necrosis factor (TNF) has a variety of protective and pathological actions in human malaria. We report that different laboratory lines of *Plasmodium falciparum* which were derived from a single wild isolate (IT 4/25/5) varied widely in their ability to stimulate TNF production by human mononuclear cells. In the cloned line R29 we observed that subcultures selected for high rosetting frequency gave significantly higher levels of TNF stimulation than subcultures with low rosetting frequency, indicating that TNF induction can vary within populations that have originated from a single genotype. These results raise the possibility that the clinical severity of malaria is partly determined by the TNF-inducing activity of the infecting strain of parasite.

Falciparum malaria causes much illness and death in the tropics. However, fatal complications (primarily severe anemia and cerebral malaria) occur in only a small proportion of infections: in Gambian children this is estimated to be on the order of 1% (10). Why some children but not others develop severe complications of malaria is unclear.

During the course of infection host mononuclear cells secrete tumor necrosis factor (TNF) and other cytokines which may contribute to the pathology of malaria in a variety of ways (6, 13). TNF is an endogenous pyrogen (7) which mediates fever in children with falciparum malaria (16). Though a moderate increase in TNF production appears to be a normal and possibly beneficial host response in this infection, excessive production may be deleterious. This is suggested by clinical studies that have documented a significant correlation between circulating TNF levels and disease severity in patients with cerebral malaria (9, 12, 15) as well as by experimental studies showing that TNF is an important mediator of cerebral pathology in mice infected with *Plasmodium berghei anka* (8).

Thus, it is possible that the severity of malaria is determined, at least in part, by the ability of the infecting strain of parasite to induce TNF production by the host. As little is known about the variability of TNF induction between different strains, we have investigated five cultured lines of *Plasmodium falciparum*, including three of clonal origin, for evidence of strain specificity in TNF induction.

MATERIALS AND METHODS

Standardized TNF-inducing parasite preparations. Parasites were grown in erythrocytic culture by conventional methods (22). Erythrocytes were obtained from a single donor of blood group O and suspended in RPMI 1640 supplemented with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (both from ICN-Flow) plus 5 mM glucose and 10% human serum pooled from European donors of blood group A. Cultures were maintained in a mixture of 1% oxygen, 3% carbon dioxide, and 96% nitrogen at 37°C and were synchronized by intermittent treatment with 5% sorbitol (18). Parasite stage and density were

determined by counting at least 1,000 erythrocytes on a Giemsa-stained thin film.

When synchronized cultures reached the early schizont stage at 7 to 10% parasitemia, they were sedimented at 500 × *g* for 7 min and the parasitemia was adjusted to 5% by adding uninfected erythrocytes. Three aliquots each containing 25 μl of packed cells were added to 1 ml of minimal essential medium (MEM; from ICN-Flow) in sterile 1.5-ml microcentrifuge tubes. Identical control preparations with uninfected erythrocytes from the same donor were made on each occasion. These preparations were then incubated at 37°C in sealed containers with a gas mixture of 1% oxygen, 3% carbon dioxide, and 96% nitrogen. After 18 h the tubes were centrifuged for 10 min at 10,000 × *g*. The supernatant and pellet were separated and stored at -20°C. Prior to testing for TNF-inducing activity, the pellet was thawed and lysed by mixing with four times the pellet volume of sterile pyrogen-free water; it was then reconstituted to 1 ml with MEM.

Measurement of TNF-inducing activity. Blood from a single European donor of blood group A was collected with 1 U of sterile preservative-free heparin per ml. Mononuclear cells were separated by density gradient centrifugation on Lymphoprep (Nycomed), washed three times with MEM, and dispensed into flat-bottomed 96-well microtiter plates at 2 × 10⁵ cells per well. Lysed pellets and supernatants of the parasite preparations were added to the wells at a final concentration of 1:20 in 200 μl of MEM supplemented with 1% heat-treated serum from a single European donor of blood group AB⁺. The plates were incubated at 37°C in 5% CO₂. At 18 h the supernatants were harvested and assayed for TNF.

TNF was measured by enzyme-linked immunosorbent assay as previously described (14). In summary, 96-well Maxisorb plates (Nunc) were coated with a murine monoclonal antibody to human TNF (CB0006; Celltech) in carbonate buffer. After washing wells with phosphate-buffered saline plus 0.2% Tween 20 (PBS-T), samples were added. The plates were then incubated at 37°C for 2 h, washed with PBS-T, incubated for 1 h with rabbit anti-human TNF immunoglobulin G (Endogen), washed with PBS-T, and then incubated for 45 min with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase. After a final wash with PBS-T, *p*-nitrophenylphosphate in diethanolamine buffer

* Corresponding author. Electronic mail address: DPK@VAX.OX.AC.UK.

TABLE 1. Levels of TNF produced by human mononuclear cells in response to different parasite preparations

Parasite line	n	TNF induced [geometric mean pg/ml (95% CI)] by:		Growth rate [geometric mean % (95% CI)] ^a
		Parasite pellet	Parasite supernatant	
R29	17	2,324 (1,660–3,253)	1,229 (867–1,741)	5.6 (5.2–6.1)
ITO4	18	42 (28–62)	30 (20–44)	5.1 (2.2–11.7)
A4	19	999 (497–2,009)	174 (85–356)	3.7 (2.8–5.0)
C24	6	1,396 (534–3,648)	375 (111–1,267)	2.9 (1.9–4.5)
Control erythrocytes ^b	22	53 (42–67)	34 (23–50)	

^a Rate of multiplication over a 48-h cycle, starting with a synchronized preparation of rings at 1% parasitemia, estimated from three experiments.

^b Preparations of uninfected erythrocytes were processed in parallel with the parasite preparations.

was added and color development was measured at 405 nm (reference, 450 nm) with a Titertek MCC340 plate reader. TNF concentrations were estimated with reference to recombinant human TNF standards (Bissendorf) which were included on each plate.

Parasite strains. The initial experiments were carried out with four laboratory lines of *P. falciparum* (ITO4, A4, C24, and R29) derived from the Brazilian isolate IT 4/25/5, kindly provided by David Roberts. The history of these lines is described in detail elsewhere (20). In summary, a series of positive selection experiments gave rise to the rosetting strain ITOR and the endothelial binding strain ITO4. By micromanipulation of single cells, R29 was subcloned from ITOR. A4 was subcloned from ITO4 and is antigenically indistinguishable from ITO4. C24 was subcloned from A4 and is antigenically distinct from A4.

Later experiments also used an unrelated rosetting strain derived from Malayan Camp (kind gift of Russell Howard).

Selection of rosetting and nonrosetting parasites. A rosette is defined as two or more uninfected erythrocytes adherent to a parasitized erythrocyte (3). Rosetting frequency (the percentage of mature trophozoites and schizonts involved in rosettes) was assessed by fluorescence microscopy of cells suspended in RPMI with 20 µg of ethidium bromide per ml. One hundred parasitized erythrocytes were counted in duplicate samples for each culture studied.

Cultures with a high rosetting frequency were separated into high-rosetting and low-rosetting subcultures by the following method. The culture was synchronized by using sorbitol and when at the mature trophozoite stage was sedimented at 500 × g for 10 min, adjusted to 50% hematocrit in RPMI 1640, and mixed with an equal volume of plasmagel in a centrifuge tube. After being allowed to settle at 1 × g for 15 min at 37°C, the supernatant (enriched for nonrosetting parasites) and pellet (containing uninfected erythrocytes and rosetting parasites) were separated and washed in RPMI. Aliquots were taken to determine rosetting frequency and to measure TNF-inducing activity. By returning the high-rosetting subpopulation to culture, it was possible to repeat this process through several subsequent cycles.

RESULTS

TNF induction by ITO-related strains. As shown in Table 1, the four ITO-derived parasite lines showed marked differences in their ability to induce TNF production (analysis of variance, $P = 0.0005$). These differences considerably exceeded the day-to-day variation observed in the TNF-inducing activity of individual lines (Fig. 1). Uninfected erythrocyte controls showed little TNF-inducing activity. Lysed pellets contained more TNF-inducing activity than superna-

tants, but different parasite lines ranked similarly by both methods. ITO4, which had by far the lowest level of TNF-inducing activity, had a growth rate in culture similar to those of the other three lines (Table 1).

Rosetting experiments. Since the highest level of TNF-inducing activity was found in the rosetting strain R29, we explored the relationship between rosetting frequency and TNF-inducing activity.

We began by excluding a trivial explanation, namely, that the presence of rosettes might have increased the number of erythrocytes taken up in a 25-µl aliquot of packed cells. Aliquots (10 µl) of four rosetting parasite preparations and four nonrosetting preparations were treated with 100 µg of fucoidan (which disrupts R29 rosettes) per ml and counted automatically (Coulter Counter ZM). These experiments revealed no difference in the number of erythrocytes present in rosetting and nonrosetting preparations (data not shown).

We selected for high-rosetting and low-rosetting subpopulations of R29 and a second unrelated rosetting strain, Malayan Camp. Frozen stabilates of R29 and Malayan Camp were each divided into five aliquots and simultaneously established in culture by using a single erythrocyte donor. Each culture was sorbitol synchronized and grown for three complete cycles before separation into rosetting-enriched and rosetting-depleted subcultures by plasmagel flotation at the mature trophozoite stage. An aliquot of each subculture was adjusted to 5% parasitemia by adding uninfected erythrocytes and divided into triplicate aliquots to determine TNF-inducing activity and rosetting frequency as described in Materials and Methods. By repeating this process through four consecutive cycles, we generated 19 pairs of high- and low-rosetting R29 subcultures and 15 such pairs of Malayan Camp subcultures (Fig. 2). High-rosetting subcultures of R29 had a mean rosetting frequency of 82.2% and elicited a geometric mean TNF response of 433 pg/ml (95% confidence interval [CI], 310 to 623 pg/ml) compared to 165 pg/ml (95% CI, 95 to 287 pg/ml) for the low-rosetting subcultures, which had a mean rosetting frequency of 2.1%. This difference was statistically significant ($P = 0.01$ by two-tailed paired *t* test). Malayan Camp showed less difference in rosetting frequency between high- and low-rosetting subcultures (39.2 versus 4.7%), and in this case the TNF-inducing activity of the high-rosetting parasites (geometric mean, 193 pg/ml; 95% CI, 140 to 265 pg/ml) was somewhat lower than that of the low-rosetting parasites (mean, 370 pg/ml; 95% CI, 229 to 600 pg/ml), though this difference was not statistically significant.

DISCUSSION

These data indicate that different strains of *P. falciparum* can vary widely in their ability to stimulate TNF production

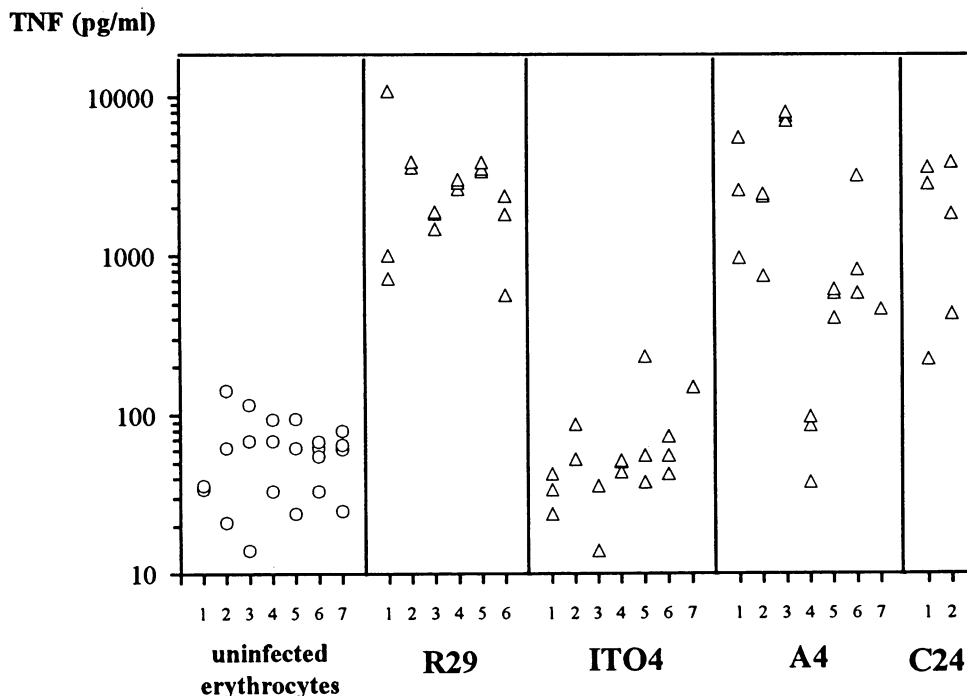


FIG. 1. Amount of TNF produced by human peripheral blood mononuclear cells stimulated by resuspended pellets of lysed parasitized erythrocytes and control preparations of uninfected erythrocytes. All four parasite lines are derived from a single wild isolate. Each point represents a single parasite lysate; numbers along the x axis represent preparations made on different days. All lysates were tested simultaneously by using a single mononuclear cell donor.

by the host. As TNF mediates malaria fever (16) and is implicated in the pathogenesis of cerebral malaria (6), these differences may be of considerable clinical significance.

We were concerned to exclude two possible sources of experimental error. First, traces of endotoxin contamination

can spuriously stimulate TNF production: this was ruled out by the consistently low levels of stimulation we observed in control preparations of uninfected erythrocytes; moreover, all parasite lines were cultured simultaneously by using a single erythrocyte donor and common reagents. Second,

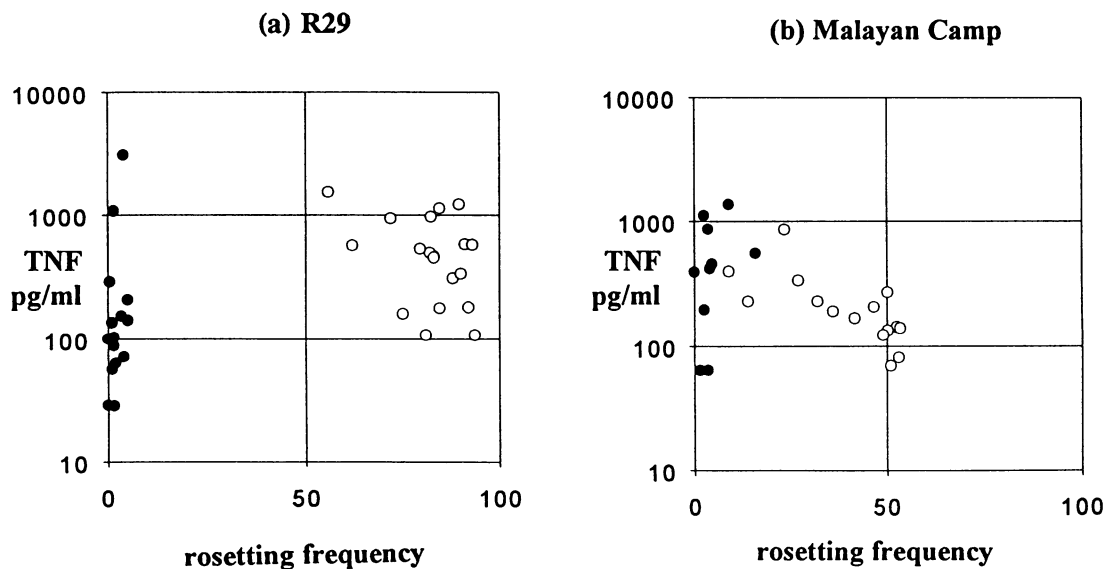


FIG. 2. TNF produced by human peripheral blood mononuclear cells stimulated by resuspended pellets of lysed parasitized erythrocytes. The parasite lines used were R29 (a) and Malayan Camp (b). Subpopulations of high and low rosetting frequency were segregated by plasmagel flotation: closed circles represent buoyant subpopulations, and open circles represent nonbuoyant subpopulations. Each point represents a single parasite lysate. All lysates were tested simultaneously by using a single mononuclear cell donor.

overall levels of TNF responsiveness vary between individuals and within individuals tested on more than one occasion, as is illustrated by the different levels of TNF induced by R29 in two separate experiments shown in Fig. 1 and 2, respectively. However, the differences between parasite lines that we have observed cannot be explained by variability in mononuclear cell responsiveness, since within each experiment, the different parasite preparations were tested simultaneously on mononuclear cells from a single donor. Subsequent experiments using other donors have confirmed that ITO4 consistently gives lower levels of stimulation than A4, C24, and R29 (data not shown).

The marked difference in TNF stimulation between ITO4 and its subclones A4 and C24 is interesting. Roberts and colleagues have shown that subclones of ITO4 are phenotypically diverse in their ability to adhere to endothelial receptors and to form rosettes, while their serological studies of surface antigen expression indicate that antigenic switching occurs at the extraordinarily high rate of 2% per generation (20). The present data indicate that the TNF-inducing ability of the parasite is a further phenotypic characteristic that can vary within a single infecting strain of parasite. This appears to occur independently of variation in cytoadherence and surface antigen phenotype in that A4 resembles ITO4 in both these characteristics yet induces much higher levels of TNF stimulation, whereas A4 and C24, which are antigenically distinct, show roughly similar levels of TNF stimulation (20).

In the ITO-derived lines that we studied, the highest levels of TNF stimulation were obtained from a rosetting parasite of clonal origin, R29. When high-rosetting and low-rosetting subpopulations of R29 were segregated by plasmagel flotation, we found that high rosetting frequency was significantly associated with high TNF-stimulating activity. This may be of some clinical importance, as plasma TNF levels (9, 15) and parasite rosetting frequencies (3) are both significantly higher in children with cerebral malaria than in those with uncomplicated malaria fever. It has been proposed that rosetting may contribute to cerebral pathology by impeding vascular flow (11). The present observations suggest that rosetting may also be related to the TNF-stimulating activity of the parasite. However, the relationship between rosetting frequency and TNF-stimulating activity that we observed in R29 was absent in Malayan Camp, a different (and unrelated) rosetting strain. This is intriguing because the rosetting characteristics of Malayan Camp differ from those of R29 in terms of response to certain monoclonal antibodies (to CD36 and to the plasmodial antigen PfHRP1), suggesting that different rosetting mechanisms may operate in the two strains (4, 20a). In summary, our observations indicate that TNF-inducing activity is not linked to the rosetting phenomenon per se but that in R29 the putative rosetting ligand and the TNF-inducing toxin seem to be coexpressed.

The molecular basis of these differences in TNF stimulation remains to be elucidated. There is now a considerable amount of circumstantial evidence suggesting that TNF-inducing components of parasitized erythrocytes include a lipid moiety (1), and there is evidence indicating that this may be a glycosyl-phosphatidylinositol anchor such as that associated with the major merozoite surface antigen MSP-1 (21). It is worth noting that small glycosylinositolphospholipids are abundant in the membrane of *Leishmania* promastigotes and that there is significant strain variation in the carbohydrate composition of these glycosylinositolphospholipids, rather analogous to the variation in core structure of bacterial endotoxin (19). Such structural variation could

conceivably underlie the strain differences in TNF-inducing ability that we describe here, and formal elucidation of the structure of the malaria toxin is an important priority in the investigation of this phenomenon. It is also possible that our observations reflect strain differences in expression of the toxin. Such differences could arise during synthesis or processing of the toxin, but they may also be due to later events which influence the ability of the toxin to induce a host TNF response once it is released from the rupturing schizont.

TNF is involved both in host defense and in the pathology of malaria (6). Recently attention has been focused mainly on its possible role in the pathogenesis of cerebral malaria, with particular interest in its role in the upregulation of endothelial adhesion molecules, such as ICAM-1, which mediate parasite cytoadherence to the vascular endothelium (2, 17). It has also recently been proposed that excessive TNF production in cerebral blood vessels may stimulate high levels of nitric oxide production from cerebral endothelium and thereby disrupt neurotransmission in the surrounding brain (5). Though an excessive TNF response may cause severe pathology, it is important to recognize that life-threatening complications of malaria occur in only a small proportion of infections, and a moderate TNF response may provide an important aspect of host defense for the majority of infected individuals (13). Thus, it is likely that there exists an optimal level of TNF response which is sufficient to provide protection without inducing severe pathology. The present data indicate that the propensity of *P. falciparum* to induce a host TNF response may vary considerably between strains and even within a single infecting strain of parasite. Field studies are now needed to establish whether this variability plays a major part in determining the clinical outcome of infection.

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