



*Vivax series:*

# The paroxysm of *Plasmodium vivax* malaria

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**The paroxysms of *Plasmodium vivax* malaria are anti-parasite responses that, although distressing to the human host, almost never impart serious acute pathology. Using plasma and blood cells from *P. vivax* patients, the cellular and noncellular mediators of these events have been studied *ex vivo*. The host response during a *P. vivax* paroxysm was found to involve T cells, monocytes and neutrophils, and the activity, among others, of the pyrogenic cytokines tumor necrosis factor  $\alpha$  and interleukin 2 in addition to granulocyte macrophage-colony stimulating factor. However, interferon  $\gamma$  activity, associated with serious acute pathogenesis in other studies on malaria, was absent. Induction of the cytokines active during a *P. vivax* paroxysm depends upon the presence of parasite products, which are released into the plasma before the paroxysm. Chemical identification of these natural parasite products will be important for our understanding of pathogenesis and protection in malaria.**

One of the oldest physiological responses to infection is the paroxysm – an acute fever that is typically preceded by chills and rigor. It occurs during various types of bacterial, viral and protozoal infection. While the initiating agent is the infecting organism, the events of a paroxysm (e.g. shivering and rising body temperature) are clearly activities of the host animal. These are complex physiological activities. But they are also very reproducible and have the hallmark of an evolved biological process. The evolutionary success of paroxysms can only be because, in the balance, their benefit to the host species has outweighed any associated harm.

Interest in the paroxysm of *Plasmodium vivax* malaria arises because the malarial paroxysm is a prominent and, indeed, a defining feature of this major group of human diseases [1–3] (Box 1). Moreover, *P. vivax* malaria offers probably the best available system for the study of paroxysm. It is predictable and reliable, sharp in duration, defined in character and carries a very low risk of serious outcome. It is thus accessible to ethical investigation in naturally infected human volunteers.

## *In vivo* studies on the paroxysm of *P. vivax* malaria

As was first demonstrated by Golgi in 1889 [1], a malarial paroxysm is preceded by the synchronous rupture of schizont-infected red blood cells. This observation led to the idea that malarial fever is the product of fever-inducing toxins (pyrogens) released by the parasites during schizont rupture. In 1904, in Vera Cruz, Mexico, an observation was made which seemed to confirm this view. Five ml of serum from blood collected from a *P. vivax* malaria patient at the height of a paroxysm was passed through a bacterial filter and transfused into the circulation of a healthy adult volunteer. Within half an hour of the transfusion, the volunteer experienced a typical, full-blown, *P. vivax* paroxysm, which peaked and resolved itself over a period of about six hours [4]. The experiment appeared to demonstrate the existence of circulating non-cellular and certainly filterable mediators that were sufficient in themselves to set in motion the full process of a malarial paroxysm in an uninfected human subject.

This experiment lacked controls and has never, apparently, been repeated. However, if the above interpretation is valid, then the following conclusions are also warranted: (1) the filterable components of blood at the time of a paroxysm must contain all of the elements needed to rapidly activate a paroxysm in a healthy and malarialogically naive individual; and (2) because 5 ml of serum introduced into the ~5 l of an adult human whole body blood volume represents a dilution of around one in 1000, the effects of the filterable mediators of a paroxysm can have little to do with their absolute concentration but much to do with the timing of their appearance. These findings accord well with our own more recent observations on *P. vivax* paroxysms in hospital patients in Sri Lanka [3]. While there was no relationship between the peak concentration of tumor necrosis factor (TNF)- $\alpha$  and peak body temperature during a paroxysm, a rather constant interval of 30–45 min was evident between the rise of TNF- $\alpha$  concentration and the rise of body temperature [3].

Direct evidence for the pyrogenic activity of TNF- $\alpha$  as an endogenous pyrogen during malaria infection was obtained by Kwiatkowski *et al.* [5]. In *P. falciparum* malaria patients who had been injected with anti-TNF- $\alpha$  antibody, body temperature was significantly lowered

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### Box 1. Clinical description of the paroxysms of *Plasmodium vivax* malaria

The paroxysms of *Plasmodium vivax* malaria typically recur at intervals of 48 h. It has been known for over a century that malarial paroxysms coincide with the rupture of malaria parasite schizont-infected red blood cells in a patients' circulation [1]. Schizont rupture exposes parasite material to the host immune and other physiological systems. In a natural, sporozoite-induced infection, the first two to three cycles of schizont rupture in the blood may be largely asymptomatic. Prominent paroxysms in non-immune individuals develop mainly following this period but, generally, when parasite densities are still less than one parasitized red blood cell per 10 000 uninfected red cells.

The detailed symptoms associated with paroxysms are specific to each species of malaria parasite [2]. In *Plasmodium falciparum*, paroxysms are usually poorly delineated febrile episodes that may extend irregularly over >24 h. Severe, and sometimes fatal, complications, including cerebral involvement and coma, can arise in non-immune individuals infected with *P. falciparum*. By contrast, *P. vivax* infections are almost always without serious risk or complication, and the paroxysms are typically short and sharply delineated within a period of <8 h [3]. Fever is one feature that is almost invariably present during a paroxysm. Any of its common accompaniments, chills, rigors and sweating, are less reliably so.

In a typical paroxysm in *P. vivax* malaria in a non-immune individual, the first symptom experienced is a chill, which lasts for up to one hour. A sufferer will invariably attempt to cover himself or herself for added warmth. Onset of the chill is closely followed by the beginning of the rigor with violent shivering and chattering of teeth, which might also continue for around one hour. During this period, body temperature rises sharply, reaching peak values of up to ~41°C within the first one to two hours after the onset of the chill. In the next period, at the height of the fever, the sufferer feels intensely hot, casts off bedclothes, and experiences often distressing discomfort. Within an hour or so, however, the temperature begins to fall again and is accompanied by profuse sweating. When compared with the rapid rise in temperature towards the start of a *P. vivax* paroxysm, its decline takes place slowly over several hours, normal temperature being restored usually between five to seven hours after the onset of the chill. These symptoms of a paroxysm could be accompanied by others, including headache, nausea and vomiting, and moderate to severe muscle, joint and back pain. Its aftermath, however, is commonly marked by a sensation of exhaustion and relief leading into deep sleep.

compared with a control group who had received a placebo treatment. Interestingly, the treatment did not reduce the risk of severe disease.

#### Ex vivo studies on *P. vivax* paroxysm

The following sections discuss the observations made upon blood plasma or whole blood cells collected from *P. vivax* patients at or around the time of a paroxysm.

#### Soluble and cellular mediators

Coinciding exactly with the period of a *P. vivax* paroxysm, there is an almost total loss of infectivity of the parasites to mosquitoes [6]. This *in vivo* effect can be reproduced *ex vivo*. When incubated for three hours in the presence of plasma collected during a *P. vivax* paroxysm, *P. vivax* gametocyte-infected blood from a nonparoxysmal patient loses between 80% and 90% of its infectivity to mosquitoes (i.e. there were 80–90% fewer oocysts in the mosquitoes when incubated in paroxysm plasma) [6]. This is shown by placing the paroxysm plasma-incubated, gametocyte-infected blood, or normal plasma-incubated controls, into a warmed, water-jacketed membrane chamber and feeding to mosquitoes. The mosquitoes are dissected seven days later and examined for the presence of oocysts on their midguts.

Using this *ex vivo* experimental protocol, we have investigated the cellular and noncellular components of blood that are active in gametocyte killing at the time of a *P. vivax* paroxysm. Cytokines and parasite products in plasmas collected before, during and after a paroxysm were neutralized by antibodies to cytokines or parasite products, and/or the cytokines and parasite products were added to the plasmas. Likewise, specific cell types, namely CD14<sup>+</sup> monocytes and CD2<sup>+</sup> T cells, were depleted from, or restored to, *P. vivax* gametocyte-infected blood. The combinations of gametocyte-infected blood and plasmas exposed to these treatments were then tested for infectivity to mosquitoes [6–8]. The following analysis arises

strictly from these *ex vivo* experiments; it is summarized in Box 2 and represented in Fig. 1.

(1) Parasite products are essential mediators of parasite killing during *P. vivax* paroxysms. As already indicated, plasma collected during a *P. vivax* paroxysm suppressed the infectivity of *P. vivax* gametocytes to mosquitoes. However, plasma collected from just before or just after a *P. vivax* paroxysm did not affect gametocyte infectivity [6]. The gametocyte-killing activity of *P. vivax* paroxysm

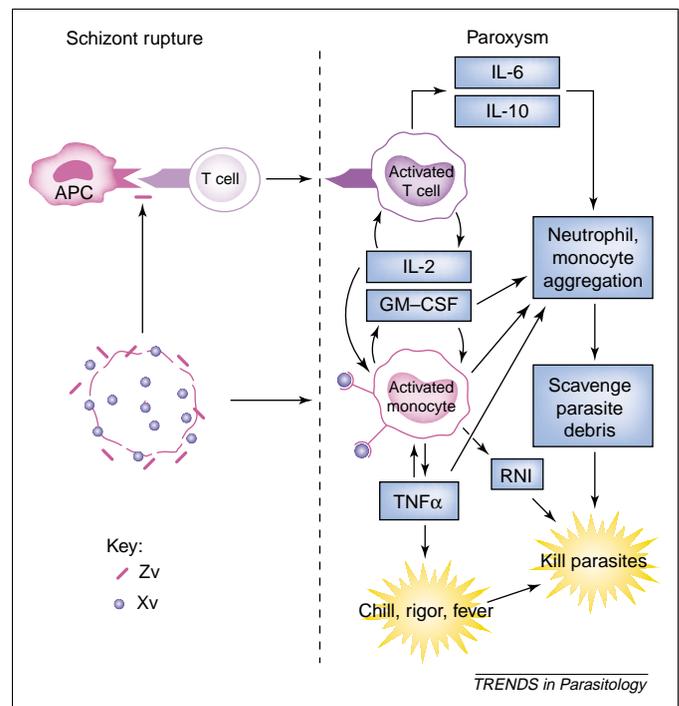


Fig. 1. Cellular and humoral events in the blood circulation leading to, and during, a paroxysm of *Plasmodium vivax* malaria. Abbreviations: APC, antigen-presenting cell; GM-CS, granulocyte macrophage-colony stimulating factor; IL, interleukin; RNI, reactive nitrogen intermediates; TNF, tumor necrosis factor; Xv, endotoxin-like parasite product of blood schizonts that activates monocytes; Zv, putative schizont rupture product with the properties of a 'superantigen' leading to the activation of T cells. See also Box 2.

## Box 2. Hypothesis for events, mediators and mechanisms of *Plasmodium vivax* paroxysm

The findings discussed here can be summarized in the following partial hypothesis of *Plasmodium vivax* paroxysm (see Fig. 1).

- (i) From the start of schizont rupture in a *P. vivax* infection, T cells begin to be activated by the parasite product which we have called factor  $Z_v$  and which, we suggest, has the properties of a superantigen. The activated T cells produce the cytokine interleukin (IL)-2, but at no point is interferon (IFN)- $\gamma$ , another T cell product, active during a *P. vivax* paroxysm.
- (ii) IL-2 induces production of granulocyte macrophage-colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)- $\alpha$  from monocytes leading, on one hand, to the induction of fever,

- and, on the other hand, together with the *P. vivax*-specific and paroxysm plasma-unique product, factor  $X_v$ , to the monocyte-dependent destruction of gametocytes and, probably also, of mature asexual blood stage parasites. These effects would be enhanced by highly elevated body temperature.
- (iii) Following the general destruction of mature blood stage parasites, monocytes and neutrophils scavenge parasite debris under the essential co-mediation of GM-CSF, TNF- $\alpha$ , IL-6 and IL-10, acting together with parasite products with chemical and antigenic properties compatible with those of  $X_v$ .

plasma was neutralized by serum from rabbits that had been immunized with extracts of *P. vivax* blood schizonts [7,8]. Extracts of blood schizonts of *P. vivax*, or indeed of *P. falciparum* (from *in vitro* cultures), restored the gametocyte-killing activity to post-paroxysm plasmas [7,8]. Material responsible for the gametocyte-killing activity both in the *P. vivax* paroxysm plasmas and in the schizont extracts of *P. vivax* and *P. falciparum*, was shown to be lipidic in nature and its activity resistant to boiling [7].

The active material released into *P. vivax* paroxysm plasma *in vivo* and the active material in the *in vitro* schizont extracts are, however, different. While the activity in the schizont extracts of both *P. vivax* and *P. falciparum* was neutralized by rabbit immune sera raised against the extracts of either species, the activity in *P. vivax* paroxysm plasma was neutralized only by immune serum against extracts of schizonts of *P. vivax* [7]. We will designate the *P. vivax* paroxysm-specific activity as factor X, and the biologically equivalent, but antigenically distinct, activity in the schizont extracts of both *P. vivax* and *P. falciparum*, factor S.

How can we explain how *P. vivax* schizont extracts in which S, but not X, is biologically active, nevertheless induce antibodies that neutralize factor X in *P. vivax* paroxysm plasma? We can do so by postulating that X is, in fact, present in *P. vivax* schizonts, but as a biologically inactive precursor,  $X^*$ . During the natural rupture of the blood schizonts, the inactive  $X^*$  would be processed and released into the plasma in its active form as factor X. S, however, is not released into the circulation at all during natural schizont rupture. While S appears to be functionally and antigenically equivalent in *P. vivax* and *P. falciparum*, X might well be antigenically *P. vivax*-specific. To accommodate the possibility, we will designate the *P. vivax* form,  $X_v$ .

(2) Parasite killing during *P. vivax* paroxysm depends upon the presence of host cytokines. Factors  $X_v$  and S are incapable of mediating gametocyte killing by themselves [6]; additional soluble mediators are essential. Through immune depletion and reconstitution experiments, we have shown that these are the cytokines TNF- $\alpha$ , interleukin (IL)-2 and granulocyte macrophage-colony stimulating factor (GM-CSF) [8]. There is nothing arbitrary about the identification of these cytokines for involvement in gametocyte killing during a *P. vivax* paroxysm. Those named are the cytokines whose activity has been experimentally demonstrated *ex vivo* to be essential and

sufficient to the mediation of gametocyte inactivation by *P. vivax* paroxysm plasma [8]. Other cytokines that were tested for activity in *P. vivax* paroxysm plasma were IL-1 $\alpha$  and IL-1 $\beta$ , IL-3, IL-4, IL-6 and interferon (IFN)- $\gamma$ . None of these had any detectable involvement in the gametocyte-killing activity of the paroxysm plasmas [6].

The absence of IFN- $\gamma$  activity during *P. vivax* paroxysm has particular resonance. With the same *ex vivo* protocols used here, we had previously found that IFN- $\gamma$  is potently active in gametocyte killing during infection crisis in Toque monkeys infected with *Plasmodium cynomolgi*—a close relative of *P. vivax* [9]. Unlike the paroxysm of *P. vivax* malaria, infection crisis in *P. cynomolgi* infections is a truly pathological and dangerous event. Had IFN- $\gamma$  been active in gametocyte killing in *P. vivax* paroxysm plasmas, there is no doubt that it would have been detected by our method [6]. Its absence during *P. vivax* paroxysm and its presence during *P. cynomolgi* infection crisis could reflect a crucial role for IFN- $\gamma$  in serious, acute pathology during malarial infection [10].

IFN- $\gamma$  activity has also been detected during *P. vivax* infections through the measurement of excreted neopterin, a product of IFN- $\gamma$ -activated macrophages [11]. This study was not designed, however, to follow IFN- $\gamma$  activity through the course of *P. vivax* paroxysms, and provides no direct evidence concerning IFN- $\gamma$  in this specific event. Nevertheless, levels and activity of IFN- $\gamma$  tended to increase with the magnitude of fever in the *P. vivax* patients. As higher fevers could reflect more-severe infection, this observation is reminiscent of the finding of IFN- $\gamma$  activity during the crises of *P. cynomolgi* infections in Toque monkeys, but not during their milder periods [10].

(3) Parasite killing during *P. vivax* paroxysm depends upon the presence of white blood cells. The cytokine-mediated and parasite-product-mediated inactivation of gametocytes during a *P. vivax* paroxysm is dependent upon the presence of white blood cells in the bloodmeal itself [12] and, more specifically, of CD14<sup>+</sup> monocytes [9,10]. Inhibition of inducible nitric oxide synthase also abolished gametocyte inactivation [12]. Thus, monocytes, activated under the effects of TNF- $\alpha$ , GM-CSF, IL-2 and parasite factor  $X_v$ , probably kill the parasites through the action of reactive nitrogen intermediates (RNIs), and possibly of other parasitocidal moieties (Fig. 1).

(4) The mediators of *P. vivax* paroxysm in its aftermath. As already noted, plasmas collected shortly after a paroxysm, 5–7 h after the temperature peak, no longer cause gametocyte inactivation. However, the addition of

*P. vivax* schizont extract, which contains factor S, the surrogate of factor X<sub>v</sub>, to post-paroxysm plasma restores its killing activity [8]. The addition of TNF- $\alpha$ , GM-CSF and/or IL-2, however, has no effect because it is not these, but the parasite product, factor X<sub>v</sub>, which is the missing essential ingredient [S.K. Wijesekera, PhD Thesis, 1997, University of Colombo]. Thus, in the immediate aftermath of a paroxysm, parasite factor X<sub>v</sub> has been eliminated from the circulation, but the essential cytokines, including GM-CSF and TNF- $\alpha$ , are still present and active.

However, although still biologically active, these cytokines are physiologically inert in the sense that they are no longer pyrogenic. This observation is, once again, consistent with it being the kinetics of appearance of the cytokines and not simply their presence or concentration that governs the host's temperature response to these cytokines.

#### *Induction of the mediators of the paroxysm of P. vivax malaria*

Malarial fever was, until recently, thought to be the result of the interaction of schizont rupture-derived, endotoxin-like material with blood monocytes to produce the endogenous pyrogen, TNF- $\alpha$  [13–19]. While *in vitro* extracts of schizonts of different *Plasmodium* spp. all induce TNF- $\alpha$  *in vitro* from human or murine white blood cells [20], the effect is not due solely to their interaction with monocytes. The *in vitro* induction of TNF- $\alpha$  by *Plasmodium* schizont extracts involves T cells and, moreover, IFN- $\gamma$  is also produced [21,22].

If only because of the presence of IFN- $\gamma$ , these *in vitro* reconstructions cannot be taken as valid representations of a *P. vivax* paroxysm. The IFN- $\gamma$ -inducing material which is present in *in vitro* extracts of schizonts of *P. vivax* and *P. falciparum*, and which we will designate factor Y, cannot be assumed to be among the active parasite products that are released before a *P. vivax* paroxysm. Similar to S, but unlike X<sub>v</sub>, factor Y is antigenically crossreactive between *Plasmodium* spp. [20]. Similar to both S and X<sub>v</sub>, Y is heat-stable and lipidic in nature [16,23].

The *in vitro* systems described above do, nevertheless, have a parallel with the initiating events of a *P. vivax* paroxysm; both are T-cell-dependent. In relation to *P. vivax* paroxysm, this was shown by the involvement of IL-2 in the paroxysm plasma-mediated inactivation of gametocytes [8], for IL-2, as far as appears to be known, is produced only by cells of the T-cell family. Moreover, the removal of CD2<sup>+</sup> T-cells from *P. vivax*-infected blood from shortly before the start of a paroxysm increased its infectivity to mosquitoes [S.K. Wijesekera, op. cit.].

From the time of schizont rupture to the start of a *P. vivax* paroxysm is only one to two hours [2]. T-cell activation by the pathway of classical major histocompatibility complex (MHC)-restricted antigen presentation, however, usually requires days to become effective. It would seem very unlikely that such a process could meet the rapid schedule of a malarial paroxysm. There is, however, a form of antigen presentation to T cells that proceeds much more rapidly. It is the MHC presentation of superantigen. Superantigens bridge between the V $\beta$  region of a T-cell receptor and a MHC molecule on an antigen-presenting cell. The specificity of a superantigen is for an entire T-cell V $\beta$  family. Consequently, all the

accessible cells of that T-cell family, typically one in 10–20 of all T-cells in the body, can be immediately activated. This is the process by which toxic shock, a condition characterized by the rapid onset of fever [24,25], is precipitated in response to certain staphylococcal and streptococcal infections [24,26].

Superantigen activity has been demonstrated during malarial infection [27], and might account for the activation and involvement of T cells during a *P. vivax* paroxysm (Fig. 1). We shall designate the putative parasite-derived material responsible for this activity, as factor Z. Superantigens are generally protein. As such, Z would probably be antigenically species-specific, and could, therefore, be re-designated Z<sub>v</sub>.

#### *P. vivax malaria paroxysm-associated aggregation of white blood cells*

In addition to the T-cell-dependent, monocyte-mediated inactivation of gametocytes during the paroxysm of *P. vivax* malaria, we have identified two other cellular phenomena that are tightly associated with paroxysm. One of these is the aggregation of white blood cells in the presence of *P. vivax* paroxysm plasma [S.K. Wijesekera, op. cit.; D. Wanasekera, unpublished]. Similar to gametocyte inactivation, white blood cell aggregation is mediated by paroxysm plasma, but not by plasmas collected from before a paroxysm, or in the immediate post-paroxysmal period. As in the case of gametocyte killing, the presence of CD14<sup>+</sup> monocytes is essential for white cell aggregation to occur. However, visual inspection of the aggregates shows that the main participating cell types are neutrophils. We suggest that the significance of the phenomenon might be as a scavenging activity by phagocytic cells, especially neutrophils, to remove parasite debris from the circulation in the aftermath of schizont rupture (Fig. 1).

A specific set of host cytokines, which is similar, but not identical, to that involved in gametocyte killing during a *P. vivax* paroxysm, is involved in paroxysm-associated aggregation of white blood cells. Thus, TNF- $\alpha$ , GM-CSF, but not IL-2, and, in this case, also IL-6 and IL-10, together with parasite products are all essential plasma mediators of white cell aggregation (Fig. 1). Other cytokines were tested for, including IFN- $\gamma$ , which, once again, was notable by its absence.

Immune serum against extracts of schizonts of *P. vivax* abrogated white cell aggregation by *P. vivax* paroxysm plasma [D. Wanasekera, unpublished]. Immune serum against extracts of schizonts of *P. falciparum* did so slightly less effectively. The physical chemical properties of the white blood cell-aggregating material in *P. vivax* paroxysm plasma are similar to those of factor X<sub>v</sub> (Fig. 1) being lipidic and its activity stable to boiling [D. Wanasekera, unpublished].

Prospects for the full chemical characterization of the parasite-derived material responsible for the cell aggregating activity in *P. vivax* paroxysm plasmas are relatively good because the assay by which it is defined, white blood cell aggregation itself, is easily managed.

#### *$\gamma\delta$ T cells and the paroxysm of P. vivax malaria*

We have recorded one further cellular phenomenon during the paroxysm of *P. vivax* malaria. It is a sudden rise in the

numbers of circulating  $\gamma\delta$  T-cells on the day of a paroxysm [28]. On days when no paroxysm has been experienced, the  $\gamma\delta$  T-cells return to their normal, very low, levels.

The functions of  $\gamma\delta$  T-cells have been mysterious for some time. In experimental observations on  $\gamma\delta$  T-cells during malarial infections, *in vivo* [28–32] and *in vitro* [33–40], most speculation has been that they contribute to parasite-killing and host pathogenesis. Recently, however, evidence from observations of other conditions suggests that  $\gamma\delta$  T-cells mediate healing, in response, for example, to tissue damage [41] and by contributing to the deconstriction (opening up) of airways during asthma attacks [42].

We have argued that the paroxysm of malaria is primarily an antiparasitic response and not a pathogenic one. Nevertheless, there is certainly harmful potential to malarial paroxysms including the damaging consequences of the repeated release of cytokines such as TNF- $\alpha$  into the circulation, as first suggested by Clark [43,44]. Could the appearance of large numbers of  $\gamma\delta$  T-cells during a *P. vivax* paroxysm be part of a healing response to this potentially harmful event?

#### Characterization of the parasite-derived mediators of *P. vivax* paroxysm

Much work has been done to identify parasite material with the properties of factor Y. A phosphatidyl inositol-containing moiety from malaria blood schizonts, identified by Bate *et al.* [16,45,46], has the properties that define factor Y. Glycosylphosphatidylinositol (GPI), a moiety which forms a hydrophobic anchor to several proteins of blood stage schizonts of malaria parasites, has been identified by Schofield *et al.* [17,47]. The *in vitro* properties of *Plasmodium* GPI are also compatible with those of factor Y. It has been proposed that *Plasmodium* GPI could constitute the ‘malaria toxin’ [17,47]. The properties of GPI, however, do not appear to correspond to those of either  $X_v$ , or  $Z_v$ , the biologically active parasite products released during the paroxysm of *P. vivax* malaria. Both  $X_v$  and  $Z_v$  are eligible for the role of ‘malaria toxin’ as defined by Golgi [1] and Rosenau *et al.* [4]. Their chemical identities remain, however, to be defined.

#### Conclusions

The mediators and mechanisms of *P. vivax* paroxysm have been discussed here based on observation and experiment on *P. vivax* patients, and on material collected directly from them. The event of *P. vivax* paroxysm and the mediators and processes that we have identified in association with it all lack acute serious pathogenic features. In particular, the activity of one specific host mediator, IFN- $\gamma$ , and of one class of parasite products, GPI, have not registered prominently in any of our analyses of *P. vivax* paroxysm plasmas. Both IFN- $\gamma$  and GPI have been associated elsewhere with serious acute pathogenesis during malarial infection.

It is apparent that the term ‘malaria toxin’ is now being used in what might be two distinct contexts. The original definition was of a parasite product involved in initiating an essentially nonpathogenic process, the paroxysm of malaria, and more particularly of the ‘benign’ malarias, such as *P. vivax*. More recently, it has been used, by

implication, as parasite product(s) that initiate(s) severe pathology. The two situations are very different and possibly unrelated. Until the parasite products involved in these different kinds of event have been chemically characterized, their roles in the effects of malarial infection will not be understood.

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