Signal Transduction in Host Cells by a Glycosylphosphatidylinositol Toxin of Malaria Parasites

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Summary

In this study, we have identified a dominant glycolipid toxin of *Plasmodium falciparum*. It is a glycosylphosphatidylinositol (GPI). The parasite GPI moiety, free or associated with protein, induces tumor necrosis factor and interleukin 1 production by macrophages and regulates glucose metabolism in adipocytes. Deacylation with specific phospholipases abolishes cytokine induction, as do inhibitors of protein kinase C. When administered to mice in vivo the parasite GPI induces cytokine release, a transient pyrexia, and hypoglycemia. When administered with sensitizing agents it can elicit a profound and lethal cachexia. Thus, the GPI of *Plasmodium* is a potent glycolipid toxin that may be responsible for a novel pathogenic process, exerting pleiotropic effects on a variety of host cells by substituting for the endogenous GPI-based second messenger/signal transduction pathways. Antibody to the GPI inhibits these toxic activities, suggesting a rational basis for the development of an antiglycolipid vaccine against malaria.

Malaria has been recognized as a distinct disease since Vedic times because infection is characterized by periodic fevers (1). In *Plasmodium falciparum* and *P. malariae* infection (tertian and quartan malaria, respectively), these fevers arise through the synchronous release of parasite-derived "toxins" during the 48- or 72-h blood-stage developmental cycle (2). These in turn cause the release of endogenous pyrogens such as TNF and IL-1, which mediate the febrile response by acting upon the hypothalamus. In addition to involvement in this classical picture of uncomplicated clinical malaria in nonimmunes, there is evidence of a role for TNF in a wider spectrum of malaria-associated disease, including severe clinical malaria, various malarial pathologies, and even death due to the infection. A recent case-control study has shown a strong correlation between TNF and IL-1 levels in the sera of malarious individuals and the severity of the disease. Compared with clinically uncomplicated malaria, plasma TNF levels were twice as high in cerebral malaria survivors and 10 times elevated in those that died of the disease (3). Circulating TNF can also be detected at high levels in the sera of mice with lethal rodent malaria, the concentration increasing with parasitemia (4). In experimental models, administration of recombinant TNF mimics a variety of the pathologies often associated with more severe malarial disease. These include anemia, hypertriglyceridaemia, hypotension, accumulation of neutrophils in the pulmonary vasculature, diffuse intravascular coagulation, and abortion (5). TNF may also contribute to cerebral malaria by upregulating expression of the endothelial cell marker intercellular adhesion molecule 1, recognized by mature parasites during cerebral sequestration (6). Finally, the administration in vivo of specific neutralizing antibodies against TNF affords some protection to mice infected with lethal malaria (7). Although other cytokines and agents may well influence pathology and the clinical outcome of infection, it appears from this circumstantial and direct evidence that excess production of TNF in response to parasite toxins contributes to severe pathology and death in both rodent and human infections.

African trypanosomiasis shares certain pathophysiological features with malaria. It too is in part an immunopathological disease, or a form of systemic inflammation with a marked cachectic component. High levels of circulating monokines are found in association with the infection (8), and many of the syndromes that occur during the acute disease can also be induced experimentally by the administration of the endogenous pyrogens TNF and IL-1 (9).

Cocultivation studies have shown that the rodent malarias *P. berghei* and *P. yoelii* can induce TNF formation from macrophages in vitro (10). Furthermore, boiled extracts of these parasites are able to induce high levels of TNF in the sera of mice sensitized with bacterial agents (11). Beyond these studies, however, the specific biochemical identity of the postulated malarial toxins is unknown. In this study, we demonstrate that the glycosylphosphatidylinositol (GPI)[1] moieties

[1] *Abbreviations used in this paper:* GPI, glycosylphosphatidylinositol; PC-PLC, phosphatidylcholine-specific phospholipase C; PI-PLC, phosphatidylinositol-specific phospholipase C.

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covalently linked to the surface antigens of malaria parasites have the properties of such toxins, as the highly purified GPIs free of associated proteins are able to induce high levels of TNF and IL-1 from macrophages, and cause pyrexia and cachexia in vivo. The GPI moieties are also insulin mimetic, regulating glucose metabolism by adipocytes and inducing profound hypoglycaemia in vivo. We propose therefore that parasite GPI molecules exert a pleiotropic influence on a variety of host cells when released during merogony or cell death, by substituting for the endogenous GPI-based second messenger/signal transduction pathways of the host.

Materials and Methods

Reagents. Phospholipase A_2 from *Crotalus atrox* and bee venom (E.C. 3.1.1.4.) were obtained from Sigma Chemical Co. (St. Louis, MO) and Boehringer Mannheim Biochemicals (Mannheim, Germany), respectively. Phosphatidycholine-specific phospholipase C (*Bacillus cereus*) (E.C. 3.1.4.3.) and pronase were also obtained from both sources. Phosphatidylinositol-specific phospholipase C (PI-PLC) was obtained from Boehringer Mannheim Biochemicals. Protein G–Sepharose, Con A–Sepharose, polymyxin B–Sepharose, polymyxin B, dithiothreitol, *n*-octylthioglucopyranoside (n-otg), formic acid, TCA, 1,10-phenanthroline, PMSF, *p*-tosyl-L-lysine-chloromethylketone (TLCK), *N*-tosyl-L-phenylalaninechloromethylketone (TPCK), *p*-chloromercuriphenylsulphonic acid (p-CMPS), aprotinin, leupeptin, pepstatin, iodoacetamide, and *n*-ethyl-maleimide (NEM) were obtained from Sigma Chemical Co. Sephadex was from Pharmacia Fine Chemicals (Uppsala, Sweden). Analytical or HPLC-grade toluene, chloroform, diethyl ether, ethanol, methanol, and water were obtained from BDH and Waters Associates (UK). Recombinant murine TNF was from Genentech. mAb TN3.19.12 against murine TNF was the kind gift of Dr. Schreiber (University of Missouri) mAbs 111.4 and 113.1 against the MSP-1 (COOH-terminal domain) and MSP-2 surface antigens of *P. falciparum*, respectively, were the kind gift of Dr. Tony Holder (NIMR).

Animals. Adult CBA/Ca, BALB/c, and C3H/HeJ (LPS-hyporesponsive) mice were bred within the specific pathogen-free National Institute for Medical Research Animal Facility.

Malaria Parasite Culture. The T9.94 line of *P. falciparum* was grown in vitro by the method of Trager and Jensen (12). Synchronous development of parasites was maintained by a modification (13) of the sorbitol method of Lambros and Vanderberg (14). For the biosynthetic labeling of parasite proteins, [^{3}H]myristic acid (50 Ci/mmol) or [^{3}H]palmitic acid (50 Ci/mmol; Amersham International, Amersham, UK) was conjugated to defatted BSA in a molar ratio of 1:1 and added at a final specific activity of 10 μCi/ml, to RPMI 1640 cultures of 10^{10} parasites at the late trophozoite stage for 8–12 h. Washed parasites were harvested by 0.05% Saponin lysis and centrifugation in the cold at 15,000 *g* for 20 min, followed by storage at −70°C.

Purification of the 195-kD MSP-1 and 56-kD MSP-2 Antigens. Labeled malaria parasites at the mid to late schizont stage were lysed in 0.05% Saponin and centrifuged at 15,000 *g* for 20 min, as above. The pellet was extracted in 25 mM *n*-octyl-thioglucopyranoside (*n*-otg), 1% BSA, 1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 1 mM TPCK, 0.1 mM TLCK, 5 mM pCMPS, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM NEM, 150 mM NaCl, 5 mM iodoacetamide, by sonication on ice. The extract was clarified by centrifugation at 20,000 *g* for 30 min in the cold, and the supernatant decanted and loaded onto two immunoaffinity columns arranged in sequence, containing ~10 mg mAb 111.4 or 113.1, each crosslinked to protein G–Sepharose by glutaraldehyde (all procedures on ice). The protein extract was passed through the column at a rate of 0.3 ml/min. The columns were washed first with with 100 ml 10 mM n-otg, 1% BSA, 300 mM NaCl, followed by 100 ml 10 mM n-otg, 300 mM NaCl. Antigen was eluted from each column with four-column volumes of 10 mM n-otg, 200 mM glycine, pH 2.8. The pH of the eluate was neutralized with 2 M Tris. The purified proteins were dialyzed exhaustively against 25 mM Tris/HCl, pH 8.5, followed by lyophilization under vacuum. Protein concentration was determined by the method of Bradford (15). The proteins were reconstituted in a variety of buffers for biochemical analysis and in complete RPMI 1640 for use with viable cells. In some cases after reconstitution the proteins were further dialyzed against the buffer of choice, using dialysis membrane previously boiled exhaustively in 10 mM EDTA followed by boiling in 10 changes of double-distilled water.

SDS-PAGE. Parasite extract or purified proteins were boiled in reducing or nonreducing sample buffer and subjected to electrophoresis through a 4.5% stacking gel followed by 7.5 or 10% slab separation gels. Proteins were silver stained by the method of Oakley et al. (16), or stained with Coomassie brilliant blue. For autoradiography, gels were fixed in ethanol/acetic acid followed by DMSO and 20% PPO. Western blotting was carried out onto nitrocellulose paper via Tris/glycine/methanol. The paper was blocked with 1% BSA and exposed to polyclonal rabbit antisera raised against a 195-kD recombinant protein (kind gift of Dr. Tony Holder), followed by affinity-purified goat anti-rabbit Ig (γ chain specific) conjugated to alkaline phosphatase. After washing the color reaction was developed by the addition of substrate.

Purification of the GPI Anchor. To purify the intact COOH-terminal GPI free of associated protein or peptide fragments, we used the protocol developed by Roberts and Rosenberry (17, 18) for the purpose of purifying to homogeneity the GPI of acetylcholinesterase. Initially, to determine the relative molecular weight of the GPI, purified MSP-1 or MSP-2 were digested for 24 h with 2.5% (wt/wt) Pronase, at 50°C in 20 mM Sodium phosphate buffer, 1 mM Ca^{2+}, 0.05% TX-100, followed by addition of 0.25% (wt/wt) of fresh pronase and a further overnight incubation. The pronase-released GPI fragment was separated from undigested material by chromatography on a Sephacryl 400 column (1.5 × 80 cm) in 0.05% TX-100 in 20 mM phosphate buffer, pH 7.0. The UV absorbance at 280 nm of the flow-through was monitored (Uvicord SII) and recorded by a chart recorder (Rec 102; Pharmacia Fine Chemicals), and the elution position of radiolabeled peaks was determined with respect to blue dextran, protein markers, and $K_2Cr_2O_7$. Fractions containing the second radioactive peak were pooled, lyophilized, reconstituted in 88% formic acid, and rechromatographed on a 1-m LH-60 Sephadex column (1.5 × 100 cm) in absolute ethanol/88% formic acid in the ratio 3:1, over 24 h. The UV absorbance of the flow-through was monitored as before and the relative molecular weight of the eluted radioactive peaks was determined with respect to peptide markers (lysozyme, 14,300 M_r; aprotinin, 6,500 M_r; glucagon, 3,480 M_r; gramicidin, 1,850 M_r). The radioactive fraction was dried down in a Speedvac and resuspended by the addition of the buffer of choice and incubation in a sonicating water bath. In subsequent experiments, to purify the intact COOH-terminal GPI free of both detergent and associated protein or peptide fragments for use in vitro and in vivo, purified MSP-1 or MSP-2 in 10 mM n-otg were dialyzed against 100 mM NH_4HCO_3, 5 mM p-CMPS until free of detergent followed by pronase digestion as above without detergent, and extraction in butanol. The sample volume was reduced in a Speedvac, followed

by chromatography on a LH-60 Sephadex column as above. The radioactive fraction was dried down in a Speedvac and resuspended by the addition of 5 μl ethanol followed by buffer of choice and incubation in a sonicating water bath.

Stability of the Fatty Acid Linkage to the 195-kD Polypeptide. 0.5 ml chloroform/methanol was added to an appropriate volume of the purified radiolabeled proteins in water to give a ratio of 8:4:3. The pH of the aqueous phase was adjusted to 3 by the addition of HCl or glacial acetic acid. The mixture was vortexed and microfuged, the organic and aqueous phases removed, and the aqueous phase subject to two further washes with chloroform presaturated with water and methanol. Both aqueous and organic phases were assayed for tritium decay. In further experiments, labeled proteins were incubated in 50 mM NaOH or 50 mM NaCl in 90% ethanol for 20 min. The samples were divided into two aliquots, followed by the addition of acidified water (pH 2.8) and toluene extraction of one-half, and determination of radioactivity in the respective organic and aqueous phases. The remaining halves of the samples were precipitated in cold ethanol (−70°C), and the pellet was dissolved in 100 mM Tris/HCl, pH 8.0.

Treatment of Purified Lipoproteins with Phospholipase A_2. 100–1,000 U of phospholipase A_2 from *C. atrox* or bee venom was added to various concentrations of the purified lipoproteins in 0.5 M Tris/HCl, pH 7.5, 15 mM $CaCl_2$, 10 mM n-otg at 37°C overnight. The samples were split and some aliquots acidified to pH 3 with HCl and extracted into toluene, and the radioactivity in the organic and aqueous phases was determined. Other aliquots were subjected to electrophoresis in SDS-PAGE followed by transfer to nitocellulose and Western blotting with polyvalent rabbit anti-MSP-1, to determine the integrity of the protein determinants. Remaining aliquots were dialyzed extensively against complete RPMI 1640 and used in assays of TNF induction.

Treatment of Purified Lipoproteins with B. cereus Phospholipase C. 400 U of *B. cereus* phospholipase C was added to the purified native proteins in 100 mM phosphate buffer, 10 mM n-otg, and incubated with vigorous shaking at 37°C overnight. Aliquots of the digested material and control incubations in identical buffer without enzyme were removed for extraction with diethyl ether, and the organic and aqueous phases measured for tritium decay. Other aliquots of the PC-PLC-digested and control material were analyzed by SDS-PAGE, and the remaining material was dialyzed extensively against RPMI 1640 and used in assays in vitro.

Treatment of Purified Lipoproteins with PI-PLC. Staphylococcus aureus PI-PLC was added to purified MSP-1 and MSP-2 in 100 mM phosphate buffer, 0.5% NP-40, 5 mM dithiothrietol at 37°C overnight, followed by acidification to pH 3 with HCl and extraction in diethyl ether. Organic and aqueous phases were assayed for tritium decay.

Preparation of Macrophages. Mice were inoculated intraperitoneally with 2 ml 4% thioglycollate medium and the elicited peritoneal exudate cells were harvested by peritoneal lavage after 4 d, in HBSS containing 5 U/ml heparin and 5 μg/ml polymyxin B (Sigma Chemical Co.). The cells were washed twice in HBSS and numbers determined by hemocytometer counting in Turks' solution. Viability was determined by trypan blue exclusion. The cells were resuspended in RPMI 1640 + 10% endotoxin-free FCS to a concentration of 2.5×10^6/ml, and 0.1 ml aliquots dispensed into the wells of a 96-well plate. The cells were allowed to adhere for 3 h at 37°C in 5% CO_2 in air, after which an equal volume of medium containing 2 μg/ml indomethacin was added. After 30 min nonadherent cells were removed by washing with RPMI 1640 alone. The cells were then incubated overnight with test and control agents in duplicate in RPMI 1640 + 10% endotoxin-free FCS, and the culture supernatants were harvested for determination of TNF levels.

TNF Bioassay. Samples to be tested and a recombinant murine TNF of known specific activity were titrated twofold in medium containing actinomycin D (5 μg/ml), with a final volume of 100 μl, and their TNF levels were determined by measuring percentage cytotoxicity for L929 cells, as described (19). Assays were performed in 96-well plates, each well receiving 5×10^4 target cells at least 4 h before addition of samples. All plates contained both positive and negative controls (250 U TNF and medium alone, respectively). After overnight incubation at 37°C the wells were washed in PBS, methanol fixed, and stained with 1% crystal violet in water. After washing with PBS the wells received 33% acetic acid and the absorbance at 540 nm was determined in a Titertek Multiscan automated ELISA reader. In our assays we routinely observed close agreement between the manufacturers' reported specific activity of the recombinant TNF and the titrated lytic activity against the target L929 cells (standard curve). 1 U of activity is defined as that which causes 50% destruction of target cells, by comparison with the standard curve.

IL1 Capture ELISAs. IL-1 levels were determined by capture of ELISA (Genzyme, Boston, MA). Briefly, a standard curve was generated by the titration of mouse rIL-1, and test and control samples were incubated at 37°C for 40 min in 96-well plates precoated with monoclonal anti-IL-1, followed by washing and probing under the same conditions with biotinylated polyclonal anti-IL-1, followed by avidin-peroxidase. After addition and development of substrate, the plates were read by Titertek Multiscan automated ELISA reader.

Glucose Metabolism in Adipocytes. Adipocytes were prepared by collagenase digestion from the epidydimal fat pads of male Sprague-Dawley rats by the method of Rodbell (20). 2.25×10^4 cells were resuspended in polypropylene tubes with test agents in 500 μl Krebs-Ringer bicarbonate, 1% BSA, 5 mM [^{14}C]glucose, pH 7.4, for 2 h at 37°C, in 5% CO_2 in air. This concentration of glucose ensures that glucose transport is not rate limiting for oxidation or lipogenesis. Lipogenesis was measured by the method of Moody et al. (21), as the incorporation of label into toluene extractable lipids. Glucose oxidation was measured as the generation of $^{14}CO_2$ absorbed to hyamine hydroxide (20).

Results

Several major antigens of the malaria parasite are covalently linked to GPI, including the MSP-1 and MSP-2 antigens on the merozoite surface, currently under consideration as vaccine candidates. To investigate the biology of the malarial GPI moiety, *P. falciparum* parasites were metabolically labeled with [^{3}H]myristic or [^{3}H]palmitic acids, and these two proteins were affinity purified to homogeneity, by affinity chromatography against two columns arranged in sequence, each containing 10 mg of either mAbs 111.4 or 113.1 in the optimal steric configuration, obtained by crosslinking the Fc-bound mAbs to protein G–Sepharose via glutaraldehyde. The use of BSA and high salt in repeated washing of these columns served to abolish nonspecific retention of proteins. The eluted antigens appeared to be essentially 100% pure as judged by the relatively low levels or absence of contaminating bands on SDS-PAGE gels stained by sensitive silver staining protocols, or Coomassie brilliant blue (Fig. 1). Typically, yields of ~500 μg to 1.5 mg of each pure malarial protein were

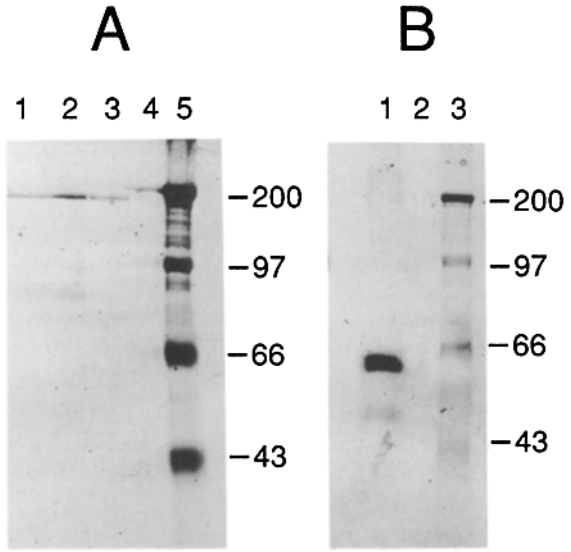

Figure 1. SDS-PAGE of *P. falciparum* MSP-1 and MSP-2 affinity purified to homogeneity. (*A*) Lanes *1–3* shows fractions eluted from a monoclonal 111.4 column (anti-MSP-1). Lanes *4* and *5* received a low and high concentration of molecular mass markers, respectively. (*B*) Lane *1* shows material eluted from a monoclonal 113.1 column (anti-MSP-2). Lane *2* received no sample and lane *3* received molecular mass markers, as indicated. Both gels were stained with Coomassie brilliant blue.

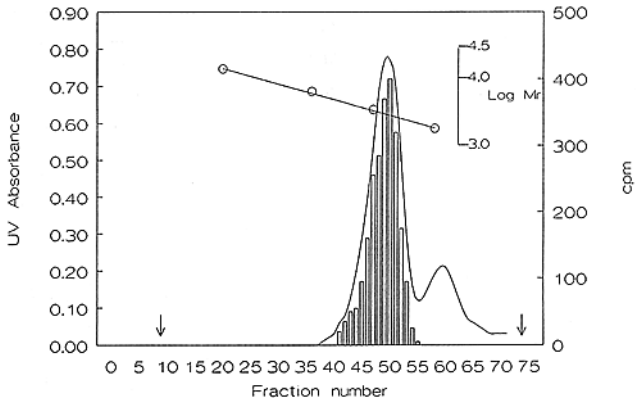

Figure 2. A280 Uvicord trace and cpm/fraction after LH-60 purification of [^{3}H]fatty acid–labeled malarial GPI. After Sephacryl fractionation of a pronase digest of MSP-2, the GPI peak was pooled, lyophilized, and redissolved in 88% formic acid. Ethanol was added to 75%, the samples were left at −70°C for 30 min, spun in a microfuge, and rechromatographed on a 1-m Sephadex LH-60 column equilibrated in ethanol/88% formic acid 3:1 for 24 h. Cherenkov counting in scintillation cocktail was undertaken on aliquots. Considerable overlap was observed between the migrating GPI and Triton X-100, as shown by UV absorbance. The peptide standards were: lysozyme, 14,300 M_r; aprotinin, 6,500 M_r; glucagon, 3,480 M_r; gramicidin, 1,850 M_r. The arrows mark the elution position of blue dextran and $K_2Cr_2O_7$, respectively.

obtained from 5–10 × 10^9 fatty acid–labeled schizonts, as determined by the Bradford method using BSA as a standard. Assuming 1 mol of GPI/mol of purified protein, the specific activity of incorporated [^{3}H]palmitic acid was found on various occasions to range from ~1.0 to 1.3 × 10^4 cpm/nmol GPI for MSP-1 and from 1.2 to 1.5 × 10^4 cpm/nmol GPI for MSP-2. Similar specific activities were obtained with [^{3}H]myristic acid.

After affinity purification and exhaustive dialysis, labeled proteins were extracted with chloroform/methanol followed by methanol/water–saturated chloroform. 97% of counts remained in the aqueous phase, indicating that the label was covalently linked to the protein. In various preparations, washing the proteins in cold acetone containing 5 mM cold myristic acid or palmitic acid resulted in the precipitation of between 85 and 97% of counts (data not shown). Incubation of both [^{3}H]myristic and [^{3}H]palmitic acid–labeled malarial proteins with 50 mM NaOH in ethanol for 30 min released essentially all of the counts into the organic phase in each case (data not shown), demonstrating that all of the label is ester linked to the protein, as previously reported for [^{3}H]myristic acid–labeled MSP-1 (22).

Purification of the COOH-terminal GPI Moiety. Following protocols used to generate the GPI moiety linked to the COOH-terminal amino acids of acetylcholinesterase, bacteriorhodopsin, and the membrane form VSG of *Trypanosoma brucei* (17, 18, 23), malarial antigens purified to homogeneity were dissolved in Triton X-100 and digested extensively with pronase, and the digested material was fractionated by molecular mass on a Sephacryl S-200 column. During control runs with undigested material, 100% of radiolabel appeared in the void volume of the column as determined by exclusion of blue dextran of 2 × 10^6 kD. After extensive pronase digestion, however, the label was associated with a more slowly migrating second peak, which was nonetheless close to the void volume. This peak, however, was not composed of neutral lipids or free fatty acids as it proved impossible to extract into toluene or chloroform (data not shown). A similar fractionation pattern has been reported for the GPI of acetylcholinesterase (17). Under these conditions, the hydrophobic GPI molecule will migrate in detergent micelles, giving an apparent molecular mass far in excess of the true value. To analyze the true molecular mass of the second fraction, and to free the sample of Triton X-100 and free peptides, the second peak was pooled, lyophilized, and redissolved in 88% formic acid. Ethanol was added to 75%, the samples were left at −70°C for 30 min, spun in a microfuge, and the supernatant rechromatographed under gravity on a 1-m Sephadex LH-60 column equilibrated in ethanol/88% formic acid (3:1) for 24 h. Percentage recoveries were usually very high, with 95–100% radiolabeled material migrating in a well-resolved peak covering 16.9–19.2% of the specific elution volume (Fig. 2). In different determinations the radiolabeled peak derived from pronase digestion of MSP-2 migrated with an apparent molecular mass of 2,950, 3,050, and 3,200 daltons, as compared with peptide markers. LH-60 has a fractionation range from ~10^2 to 10^4 daltons, and the column was well able to resolve the GPI peak from free fatty acids and free amino acids. When aliquots of intact (pronase-undigested) fatty acid–labeled MSP-2 were taken up in ethanol/88% formic acid and run on the LH-60 column, all proteins and label

eluted in the void volume. The Triton X-100 associated with the lyophilized GPI after Sephacryl fractionation migrated with considerable overlap of the GPI fractions as shown by the UV profile. To generate GPI free of contaminating detergent and peptides for use in vivo and in vitro, the fatty acid–labeled proteins were dialyzed until free of detergent before pronase digestion, and were then extracted in butanol and fractionated by LH-60. The tritium-containing fractions in the ~3-kD peak eluting from the LH-60 column were dried down by Speedvac and taken up in 2 μl ethanol, to which was added the aqueous buffer of choice. As expected, repeated extractions in chloroform/methanol failed to release counts into the organic phase, but essentially 100% of labeled material could be extracted into butanol. Similar patterns of fractionation and organic solvent extraction were obtained when the GPI was biosynthetically labeled by the incorporation of tritium-labeled sugars (data not shown).

Enzymatic Deacylation of the GPI. To reproduce the findings of previous studies, and to generate deacylated native protein for structure/activity studies, we treated the purified, labeled MSP-1 and MSP-2 lipoproteins with phospholipases. When [^{3}H]palmitic acid–labeled MSP-1 and MSP-2 were taken up in 0.5 M Tris/HCl, 15 mM $CaCl_2$, pH 7.5, 10 mM n-otg, followed by incubation at 37°C with 200 U of *C. atrox* phospholipase A_2 for 4 h, ~50% of counts were released into the organic phase (Table 1). No further counts were released by prolonging the incubation period or increasing the enzyme concentration to 1,000 U. Under similar conditions, however, and in contrast to a previous report (22), we were unable to obtain release of label from [^{3}H]myristic acid–labeled malarial proteins. The enzymatic cleavage of lipid from the acylated protein by phospholipases did not affect the integrity of the protein structure as evidenced by the ability to detect the intact protein with unaltered strength of signal by polyvalent antisera in Western blotting (data not shown).

In contrast to the membrane from variant surface glycoprotein (mfVSG) of *T. brucei*, the malarial structures were insensitive to PI-PLC from either trypanosomes or *Staphylococcus aureus*. In further contrast to the mfVSG GPI (23), myristic acid–labeled malarial GPI could be cleaved 100% by *B. cereus* phospholipase C. A previous study has shown that *B. cereus* PLC preparations (Sigma Chemical Co.) contain predominantly a phosphatidylcholine-specific PLC (23). As the *B. cereus* enzyme is inactive against mfVSG, and the malarial GPI appears insensitive to either *S. aureus* or trypanosomal PI-PLCs, the activity we observed may result from a contaminating PI-PLC that discriminates among malarial and trypanosomal GPIs, or from the ability of the PC-PLC itself to deacylate the malarial GPI. As these enzymes have positional specificity it is clear from these data that the malaria GPI differs from the *T. brucei* structure.

Table 1. *Chemical and Enzymatic Deacylation of the Highly Purified [^{3}H]Fatty Acid–labeled MSP-1 and MSP-2 Proteins*

	195		56	
	Myristic	Palmitic	Myristic	Palmitic
NaOH	100.5%	100%	104%	97.9%
PLA_2	0.2%	47.5%	2.1%	50.3%
PIPLC	0%	0.3%	0.9%	0%
PCPLC	96.2%	ND	99.8%	ND

After treatment with chemicals or enzymes, samples were extracted with organic solvents, and the percentage release of labeled fatty acid was determined by scintillation counting, as described in Materials and Methods.

Induction of TNF and IL-1 by Malarial Antigens and the Purified GPI. To determine whether the GPI-anchored surface antigens of parasites were able to induce the production of TNF and IL-1 from macrophages, highly purified MSP-1 and MSP-2 were dialyzed exhaustively against RPMI 1640. The absence of detergent and cytotoxic agents in the dialyzed samples was confirmed by a variety of methods, including incubation of aliquots with viable cells overnight followed by microscopic examination of morphology and trypan blue exclusion assays. The dialyzed labeled proteins were added to cultures of thioglycollate-elicited peritoneal macrophages from CBA/Ca or LPS-hyporesponsive C3H/HeJ mice overnight. The macrophage supernatants derived from these incubations were titrated further in RPMI 1640 containing 5 μg/ml actinomycin D and added to cultures of L929 cells for the determination of TNF levels. Low concentrations of purified native proteins of malaria were able to elicit high levels of TNF from macrophages (Table 2). To ensure that the release of TNF from macrophages was not the result of adventitious contamination of the samples or procedure by LPS, a number of stringent controls and precautions were undertaken. For example, the LPS-hyporesponsive mouse strain C3H/HeJ was fully able to respond to malarial antigens with the production of TNF (Table 2). In further experiments, 1-ml aliquots of purified native MSP-1 and MSP-2 proteins dialyzed against RPMI 1640 were mixed with 100-μl volumes of mAbs 111.4 or 113.1 bound to CNBr-activated Sepharose (stored routinely in sterile RPMI 1640) for 1 h at room temperature, followed by microfuge centrifugation. Immunoprecipitation of the native antigens in this manner reduced by ~90% the amount of TNF induced by the MSP-1 and MSP-2 preparations (Table 2). In addition, as shown below, TNF induction was sensitive to phospholipases that do not affect LPS. Finally, we have observed that polymyxin B, at concentrations sufficient to neutralize high levels of LPS, was unable to inhibit TNF induction by parasite GPI (data not shown). However, we have viewed the use of polymyxin B as an anti-LPS reagent with caution, since this antibiotic is a known inhibitor of protein kinase C, and in addition we have observed that polymyxin B–agarose binds to and precipitates the labeled GPI (data not shown).

sn-1, 2-Diacylglycerol Required for Cytokine Induction. To test the hypothesis that the covalently associated posttranscriptional glycolipid modification of the native proteins is responsible for the induction of cytokines by native antigen, native

Table 2. *Induction of TNF and IL-1/by Malarial Antigens in Vitro*

Agent	TNF/2 × 10^5 macrophages				IL-1/2 × 10^5 macrophages
	1*	2‡	3	4	5
			U		*pg*
MSP-1 (5 μg)	282	217	409	137	–
MSP-1 + 50 mM NaCl	198	–	–	–	–
MSP-1 + 50 mM NaOH	0	–	–	–	–
MSP-1 immunoprecipitated	12	19	–	–	–
MSP-1 + PLA_2	–	–	33	–	–
MSP-1 + PLC	–	–	–	3	–
MSP-2 (4.5 μg)	148	186	320	109	251
MSP-2 + 50 mM NaCl	133	–	–	–	–
MSP-2 + 50 mM NaOH	0	–	–	–	–
MSP-2 immunoprecipitated	28	22	–	–	66
MSP-2 + PLA_2	–	–	38	–	38
MSP-2 + PLC	–	–	–	0	13
MSP-2 GPI (250 ng)	408	354	–	–	842
MSP-2 GPI + 5 μM staurosporine	–	4	–	–	0

After purification, treatment, and dialysis against RPMI 1640, antigens were added to 2 × 10^5 CBA/Ca or C3H/HeJ thioglycollate-elicited peritoneal macrophages per well. Quantitative deacylation of [^{3}H]fatty acid–labeled malarial antigens was confirmed by organic solvent extraction of subaliquots. Remaining aliquots were dialyzed extensively against complete RPMI 1640 and used in assays of TNF induction. Antigens were immunoprecipitated by mixing with 10% by volume of specific mAbs (111.4 or 113.1) covalently conjugated to CNBr-activated Sepharose, stored under sterile conditions in RPMI 1640 with 100 μg/ml Polymyxin B. TNF levels were determined by titration on L929 cells.

* Experiment number.

‡ C3H/HeJ macrophages.

proteins were deacylated by a variety of methods and the influence of these treatments on the ability of the antigens to induce TNF was determined. Native proteins subject to mild base hydrolysis were unable to induce TNF from macrophages (Table 2). Similarly, native proteins exposed to PLC and PLA_2, with quantitative loss of the appropriate fatty acid demonstrated in aliquots by organic solvent extraction, lost their TNF-inducing capability (Table 2). Thus, the intact *sn*-1,2-diacylglycerol is required for TNF formation. These treatments, however, did not affect the protein structure of the molecules as judged by migration in SDS-PAGE and sensitivity to detection in Western blots by polyclonal rabbit antisera to MSP-1 or a monoclonal anti-MSP-2 (data not shown).

To determine whether the GPI moiety alone was sufficient to elicit cytokine production from macrophages in vitro, the COOH-terminal GPI fragment was added to macrophages in twofold serial dilutions. This titration revealed the highly purified GPI moiety to be more efficient in eliciting both TNF and IL-1 from macrophages than the native protein-GPI complex, when compared on a molar basis (Fig. 3 and Table 2).

Malarial GPI Substitutes for the Second Messenger of Insulin. Mammalian glycosylphosphatidylinositol is proposed to be the second messenger mediating signal transduction in response to insulin (24). Since hypoglycaemia and hypertriglyceridaemia are common pathophysiological conditions associated with both malaria and trypanosome infection, we sought to determine whether the malarial GPI is insulin mimetic. GPI-linked surface proteins and the purified GPI were

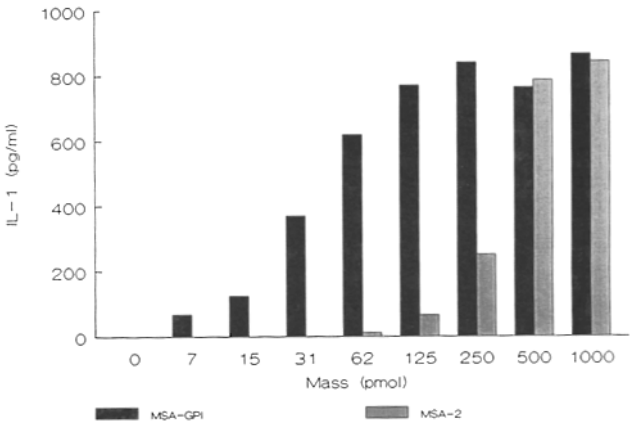

Figure 3. Titration of IL-1 production by the GPI. Various concentrations of purified MSP-2 GPI and native MSP-2 antigen in RPMI 1640 were added to 2 × 10^5 thioglycollate-elicited peritoneal macrophages overnight and the level of IL-1 determined by monoclonal capture ELISA (Genzyme).

indeed sufficient to induce a five- to sixfold increase in both triglyceride lipogenesis and glucose oxidation by adipocytes, similar to that induced by insulin in this and previous (25, 26) studies (Table 3). As previously reported (25), partial analogues of GPI such as mannose and inositol monophosphate inhibited lipogenesis by insulin. These agents were able to a similar degree to block the insulin-mimetic activity of the parasite GPI.

Pathophysiological Activities of the GPI In Vivo. To determine whether these activities on host cells in vitro can occur in vivo, thioglycollate-primed mice were inoculated intraperitoneally with 12 μg purified malarial GPI in saline. There was a pronounced and prolonged drop in blood glucose concentration (Fig. 4 *A*). The animals showed clear signs of a transient pyrexia (ruffled fur, cold extremities, listless behavior), and occasionally diarrhea, but did not die as a result of this treatment. However, the sensitivity of mice to the lethal effects of TNF may be markedly increased by exposure to a variety of bacterial agents, or to D-galactosamine (27). This phenomenon has already been used to demonstrate the existence of TNF-inducing agents in malarial extracts (11). In various experiments between 80 and 100% of mice died when receiving malaria GPI 30 min after exposure to D-galactosamine, although none died when exposed to either agent alone (Fig. 4 *B*). We wished to determine whether the observed cachexia and mortality were indeed due to the action of TNF, and, since TNF itself may cause a drop in blood glucose levels (15), to exclude the possibility that the observed hypoglycemia was merely a secondary consequence of TNF induction. Accordingly, administration of a TNF-neutralizing mAb rescued all or the majority of mice from GPI-induced cachexia and death, but did not greatly influence the development of hypoglycaemia (Fig. 4, *A* and *B*). The induction of TNF-independent hypoglycemia by extracts of *P. yoelii*–infected and uninfected erythrocytes has recently been reported elsewhere (28).

Table 3. *Induction of Lipogenesis and Glucose Oxidation by the Malarial GPI*

Agent	Metabolism of [^{14}C]glucose	
	$^{14}CO_2$	[^{14}C]-labeled lipids
None	121 ± 19	72 ± 8
Mannose	116 ± 8	66 ± 10
IMP	143 ± 24	59 ± 4
Insulin (100 μU)	603 ± 56	289 ± 17
Insulin + 2.5 mM mannose	177 ± 9	74 ± 11
Insulin + 2.5 mM IMP	355 ± 46	148 ± 30
MSP-1	498 ± 23	204 ± 18
GPI (2.5 μg)	670 ± 72	412 ± 62
GPI (0.25 μg)	551 ± 15	395 ± 27
GPI (0.25 μg) + 2.5 mM mannose	183 ± 33	97 ± 11
GPI (0.25 μg) + 2.5 mM IMP	209 ± 29	125 ± 16

Assays of glucose metabolism by adipocytes in response to insulin, parasite GPI, and inhibitors were carried out as described in Materials and Methods. Values shown are the means ± SE of quadruplicate determinations, and are expressed as pmol/10^6 cells ($^{14}CO_2$ production), and nmol/10^6 cells (^{14}C-labeled lipids). IMP, inositol monophosphate.

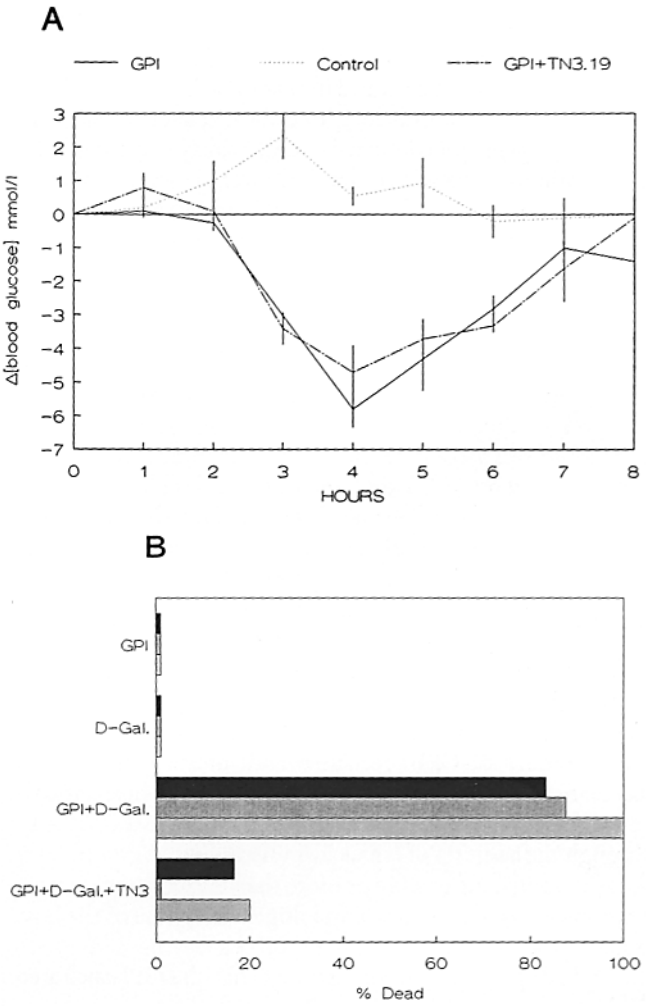

Figure 4. Malaria GPI induces TNF release and hypoglycaemia in vivo. Thioglycollate-primed mice received either PBS alone or 18 mg D-galactosamine in PBS intraperitoneally. After 30 min some received 12.5 μg, purified malarial GPI (time 0). Some groups also received 250 μg mAb TN3.19.12 against murine TNF (kind gift of Dr. Schreiber, University of Missouri), in 200 μl PBS intraperitoneally. (*A*) Blood glucose levels were determined by Reflolux S blood glucose meter (Boehringer Mannheim Diagnostics), and are expressed as the change in blood glucose concentration in mmol/liter ± 1 SE (*B*) In three separate experiments, deaths were scored after 48 h.

Discussion

The GPI molecules linked to surface antigens of the parasite are glycolipid toxins eliciting several pathophysiological responses associated with acute and severe malaria infection,

including hypoglycemia and the excess production of TNF, with attendant pyrexia and cachexia. This TNF-mediated toxicity can be lethal in sensitized individuals. Since each of these conditions has a counterpart in acute trypanosomiasis, this novel pathogenic process may be more widely associated with eukaryotic parasitism. Parasite GPI molecules may also contribute to other forms of cellular dysfunction found in both infections, such as the polyclonal activation of lymphocytes. The simplest explanation for our observations is that parasite GPI molecules exert an influence on host cells when released during merogony or cell death, by substituting for the endogenous second messenger/signal transduction pathway, which utilizes glycosylphosphatidylinositol and its derivatives to regulate protein kinase C and Ca^{2+} levels. In support of this view induction of both cytokines by the GPI requires an intact *sn*-1,2-diacylglycerol, and is blocked by the PKC inhibitor staurosporine (Table 2). Our demonstration that a single purified GPI species is strongly insulin mimetic substantially validates the proposed (24) role of inositolphosphoglycans in insulin signal transduction.

In this study we have concentrated on the mature-form GPI associated with the surface antigens of the parasites, simply to ensure the biochemical identity and purity of out material. It should be noted, however, that the parasites may contain a variety of GPI precursors and intermediates not in association with proteins (29), and glycosyl-phosphatidylinositols of this type, differing in polarity and hydrophilicity through the addition of ethanoalamine and sugar groups, may actually contribute equally or more than GPI-anchored membrane proteins to the pathophysiological response of the host when released during merogony or cell death.

We have previously demonstrated that the GPI-anchored (30) native circumsporozoite (CS) protein of malaria sporozoites is a thymus-independent antigen, capable of eliciting antibody production from primed B cells alone without a requirement for cognate T cell help (31). Full-length recombinant protein analogues lacking the GPI anchor, however, do not have this ability and behave as typical T-dependent protein antigens. This circumstantial evidence has lead us to propose that the GPI moiety on the native CS protein contributes to thymic independence by providing a mitogenic or comitogenic signal to B cells (31, 32). If this reasoning is correct, the malarial GPI might also contribute to the polyclonal activation of lymphocytes as a mitogen or comitogen, the existence of which in malarial infections was originally proposed by Greenwood and Vick (33).

In keeping with our propositions concerning B cell activation and the T independence of the GPI-anchored CS protein, we have recently shown that immunization of mice with highly purified malaria GPI prepared from the mature MSP-2 leads to an serological anti-GPI response with T-independent features (i.e., IgM dominated and without anamnestic characteristics). These anti-GPI sera are able even at high titration to neutralize TNF induction and lipogenesis by both the heterologous MSP-1 antigen and whole parasite extracts (our unpublished data). Although it is not possible to exclude the possibility that molecules unrelated to the GPI may also contribute to TNF induction and hypoglycaemia in the in vivo response to infection, these data support the view that the GPI may be the dominant pyrogen/toxin of malaria parasites. In addition, the same data raise the possibility that antibodies that neutralize the toxic activities of this molecule may be acquired after exposure to parasites and may mediate tolerance or acquired clinical immunity to disease. Since these antibodies are apparently T independent in nature any clinical immunity they might provide would wane fast in the absence of reinfection, as is thought to be the case with clinical immunity to malaria. The question of the existence of an antitoxic immunity has recently been reviewed by Playfair et al. (34). The identification of the GPI molecule as a parasite toxin and the development of methods to measure an anti-GPI serological response should allow further experimental and epidemiological studies to assess the role of the GPI and anti-GPI antibodies in both the clinical response and clinical immunity to infection. For example, it should be possible in appropriately defined case-control studies to determine whether the hyperactive malarial splenomegaly and clinically severe or cerebral malaria syndromes are associated with states of physiological hyperresponsiveness to the GPI, and correspondingly whether a condition of hyporesponsiveness is associated with the phenomenon of childhood "tolerance" to malaria.

The possibility of developing an "antidisease" vaccine against malaria has already been suggested (34). Both high TNF levels and hypoglycaemia are associated with poor prognosis in severe malaria infections (35). Our data raise the prospect of providing clinical protection against malarial disease, either by passive transfer of neutralizing anti-GPI monoclonal or polyclonal antibodies, or by immunization against the GPI or various nontoxic analogues. Notwithstanding technical problems and the need to avoid autoimmune reactions, the identification of the biochemical nature of this toxin and our observations concerning the neutralization of TNF induction and lipogenesis by anti-GPI antibodies may help to provide a rationale for the development of an antiglycolipid vaccine against malaria.

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