INFECTION AND IMMUNITY, Aug. 1994, p. 3086–3091
0019-9567/94/$04.00+0

Vol. 62, No. 8

Inhibitory Immunoglobulin M Antibodies to Tumor Necrosis Factor-Inducing Toxins in Patients with Malaria

C. A. W. BATE* AND D. KWIATKOWSKI

Department of Paediatrics, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom

Received 14 March 1994/Returned for modification 14 April 1994/Accepted 4 May 1994

Cytokines such as tumor necrosis factor alpha (TNF) appear to play an important role in the pathogenesis of malaria. We have previously shown that TNF is produced in response to substances released at schizont rupture, which we have called malaria toxins. In mice these toxins stimulate a T cell-independent antibody response, generating short-lived immunoglobulin M (IgM) antibodies that inhibit the TNF-inducing activity of the toxins. We report here that a similar antibody response is seen in humans. Serum from a European adult infected with *Plasmodium falciparum* inhibited the induction of TNF by malaria toxins derived from *P. falciparum*-infected erythrocytes. We found that IgM antibodies were responsible for the inhibitory activity. These inhibitory antibodies could not be detected in convalescent-phase serum collected from the same patient 6 weeks later or in sera from healthy European and African controls. The antibodies appeared to be malaria specific in that they inhibited TNF induction by a variety of *P. falciparum* isolates but failed to inhibit TNF induction by bacterial lipopolysaccharide or lipoteichoic acid. The inhibitory antibodies bound to liposomes containing phosphatidylinositol but not other phospholipids. Serum from a European adult infected with *P. vivax* also inhibited the activity of toxins derived from *P. falciparum*-infected erythrocytes, and this too was mediated by IgM antibodies which were malaria specific and bound to phosphatidylinositol liposomes.

Several lines of evidence indicate that tumor necrosis factor alpha (TNF), a potent endogenous pyrogen (10), is a major mediator of the fever that classically accompanies human malaria. Malaria fever occurs when a large number of erythrocytic schizonts rupture (11), and in vitro studies have shown that schizont rupture stimulates human mononuclear cells to release TNF (16). Paroxysms of fever in vivax malaria are associated with a sharp rise in circulating TNF levels (13), and monoclonal antibodies against TNF have been shown to reduce fever in children with cerebral malaria (18). There is now considerable interest in the parasite toxins that stimulate host monocytes and macrophages to secrete TNF (5). Parasite lysates contain a major TNF-inducing component whose active moiety depends on phospholipid (7), and there is evidence that this may be a glycosylinositolphospholipid-like structure (2, 6, 22).

It is commonly observed that some individuals in malaria-endemic areas tolerate high levels of parasitemia without fever or other symptoms of infection (23). This suggests that the pyrogenic response is somehow attenuated in individuals who are frequently reinfected with malaria. We have previously observed that mice immunized with parasite lysates develop antibodies that inhibit TNF induction by malaria toxins (3). In the present study we have investigated whether such antibodies are produced in the course of human malaria.

MATERIALS AND METHODS

Sera. Sera from patients with malaria were tested for their ability to inhibit TNF production by human monocytes in response to malaria toxins in vitro. Before testing, sera were heat inactivated at 56°C, centrifuged at 10,000 × *g* for 10 min, and filtered through a 0.2-μm-pore-size Spinex centrifuge filter unit (Costar).

Patient details. Patient A was a 30-year-old European man returning from a 3-week visit to Zaire, who presented to hospital with a 24-h history of fever. He gave a history of malaria fever while resident in Zaire 5 years previously. On the present occasion he was found to have >1% asexual forms of *Plasmodium falciparum* and no other apparent cause of fever. He was treated successfully with oral quinine. An acute-phase serum sample was obtained on admission, and convalescent-phase samples were obtained 3 and 6 weeks later.

Patient B was a 36-year-old European woman who had been resident in Vanuatu for 3 years. She had returned to the United Kingdom 9 months previously, and during this period she had suffered several episodes of fever associated with *P. vivax* parasitemia. These episodes were treated with chloroquine, but the infection was not eradicated with primaquine because she was pregnant. Four months after delivery she presented with 5 days of tertian fever and *P. vivax* parasitemia. An acute-phase serum sample was taken prior to radical antimalarial treatment.

Control sera were obtained from three European and three Gambian adults. All were healthy individuals who were free of malaria parasitemia at the time of sampling.

Parasite preparations. *P. falciparum* was cultured in human erythrocytes of blood group O by using RPMI 1640 supplemented with 2 g of glucose per liter and 10% human serum, essentially as described by Trager and Jensen (24). Cultures containing mature trophozoites and schizonts at ~10% parasitemia were centrifuged at 10,000 × *g* for 10 min, and the supernatant was removed. The pellet was lysed by the addition of 4 volumes of sterile endotoxin-free water and stored at −20°C prior to testing. For most experiments we used partially purified preparations of these lysates, from which nonstimulatory components had been removed by washing in phosphate-buffered saline (PBS) and methanol-chloroform-water (8:4:3) (22a). Except where otherwise stated, the parasite strain was

* Corresponding author. Mailing address: Department of Paediatrics, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom. Fax: 44 865 222626. Electronic mail address: DPK @ VAX. OX. AC. UK.

R29; this is a laboratory line derived from the Brazilian isolate IT 14/4/25, and it has been previously shown to have high TNF-inducing activity (1). One set of experiments compared R29 with other parasite strains. These included three other lines derived from IT 14/4/25 (ITO4, A4, and C28) (21), Malayan Camp, a Gambian strain (FCR3A2), a Thai strain (T996), and a Kenyan wild isolate (Kilifi 0303).

TNF stimulation assay. For most experiments we used the human monocyte cell line Mono Mac 6 (20). Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum. Before assay they were washed, resuspended in the same medium supplemented with 100 ng of phorbol myristate acetate per ml, and dispensed at 5×10^4 cells per well into 96-well microtiter plates. They were left for a minimum of 1 h to adhere before the supernatant was carefully removed. The sera to be tested were serially diluted in RPMI 1640 and added at 100 μl per well; this was followed by addition of 100 μl of the diluted malaria toxin. As positive controls for TNF stimulation, we used titrations of lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 or lipoteichoic acid from *Streptococcus pyogenes* (Sigma). After overnight incubation in 5% CO_2 at 37°C, supernatants were harvested and assayed for TNF by enzyme-linked immunosorbent assay as described previously (7).

For certain experiments we used human peripheral blood mononuclear cells to confirm the results obtained with Mono Mac 6 cells. Heparinized blood from healthy adult volunteers was mixed with an equal volume of normal saline, and mononuclear cells were isolated on Lymphoprep (Nyegaard). They were washed three times, resuspended in RPMI 1640 plus 1% human serum, and dispensed at 10^5 cells per well into flat-bottomed 96-well microtiter plates. After the cells had been allowed to adhere for 1 h at 37°C, nonadherent cells were removed by gentle washing in serum-free RPMI 1640, and then diluted sera followed by diluted toxins were added to a total volume of 200 μl. TNF production was assayed after overnight incubation as above.

When pooling results from different experiments, we allowed for day-to-day variation in cell sensitivity of Mono Mac 6 cells by expressing TNF inhibition as a percentage of the control value rather than as absolute values.

Determination of Ig isotypes. Heat-inactivated, spun, and filtered sera (500 μl) were diluted 1/10 in RPMI 1640 and applied to a 1-ml affinity column containing goat anti-human immunoglobulin G (IgG) or anti-human IgM bound to agarose (Sigma). The columns were washed with 20 column volumes of sterile PBS, and antibodies were eluted with 0.1 M glycine HCl (pH 2.5), immediately neutralized with 1.0 M Tris buffer (pH 8), dialyzed against PBS, and returned to the original volume.

Adsorption of sera by liposomes. Multilamellar dehydration-rehydration vesicles were kindly supplied by G. Gregoriadis, School of Pharmacy, London University. Sera diluted 1/50 in RPMI 1640 were incubated for 1 h at room temperature with 2 mg of the liposomes per ml, which were then deposited by centrifugation. The remaining supernatant was filtered through a 0.2-μm-pore-size Spinex centrifuge filter unit.

Adsorption of sera by Mono Mac 6 cells. Mono Mac 6 cells were harvested, washed, and incubated for 2 h in RPMI 1640 supplemented with 10% fetal calf serum and 100 ng of phorbol myristate acetate per ml. They were then washed twice in RPMI 1640 and resuspended at 10^7/ml in RPMI 1640. Sera were added to the cell suspension at a final concentration of 1/50 and incubated for 1 h at 37°C. Cells were deposited by centrifugation at $800 \times g$ for 10 min, and the supernatant was collected, filtered, and tested.

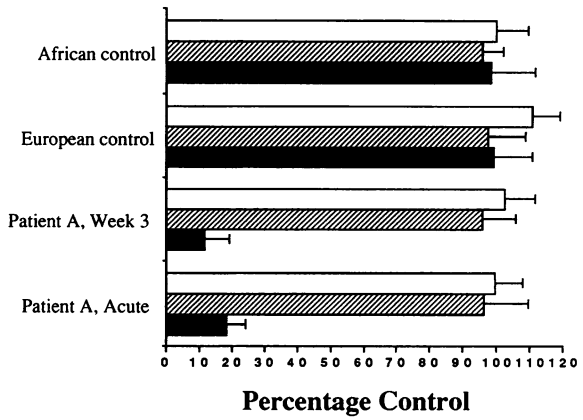

FIG. 1. Inhibition of malaria toxin-induced TNF secretion by sera from a European adult infected with *P. falciparum*. Levels of TNF secreted by stimulated Mono Mac 6 cells in the presence of a 1:100 dilution of sera from patient A and healthy controls are shown. Values are means and standard deviations for a minimum of six experiments, expressed as a percentage of results of control experiments without serum. Acute-phase and week 3 convalescent-phase sera inhibited TNF induction by malaria toxins (■) but not by LPS (▨) or lipoteichoic acid (□). Also shown are pooled data from three European and three African adults.

RESULTS

Inhibitory activity of sera from a patient infected with *P. falciparum*. Sera from patient A, infected with *P. falciparum*, were tested for their ability to inhibit TNF secretion. Both at the time of diagnosis and 3 weeks later these sera inhibited the ability of semipurified malaria toxins to stimulate TNF production by Mono Mac 6 cells (Fig. 1). This inhibitory activity appeared to be malaria specific in that these sera did not alter the amounts of TNF secreted by cells in response to LPS or lipoteichoic acid. Control sera from three healthy European and three healthy African adult donors did not affect the amounts of TNF induced by malaria toxins, LPS, or lipoteichoic acid.

To compare levels of inhibitory activity, we retested these sera in serial dilutions. The serum sample collected at 3 weeks (approximately 2 weeks after parasite clearance) was more inhibitory than that collected at diagnosis, but serum obtained at 6 weeks (i.e., approximately 5 weeks after parasite clearance) did not contain inhibitory activity (Fig. 2).

Ig isotypes responsible for inhibition. To determine whether specific antibodies were responsible for this inhibitory activity, we isolated Igs from the sera by affinity chromatography. Most of the inhibitory activity was found in the IgM fraction (Table 1). Although roughly similar amounts of Ig were isolated from European and African control sera, as measured by protein concentration, these did not affect TNF production. The purified IgM that inhibited the malaria toxins had no effect on LPS-induced TNF secretion.

Inhibitory antibodies do not bind to activated Mono Mac 6 cells. To confirm that the inhibition was due to a direct interaction between antibodies and the malaria toxins, we sought to exclude the alternative possibility that the antibodies act indirectly by binding to macrophage receptors for the toxin. This was done in two ways (Table 2). First, we attempted to adsorb the inhibitory antibodies by incubation with phorbol myristate acetate-activated Mono Mac 6 cells: sera retained their inhibitory activity after preincubation with Mono Mac 6 cells. Second, we attempted to block macrophage receptors by

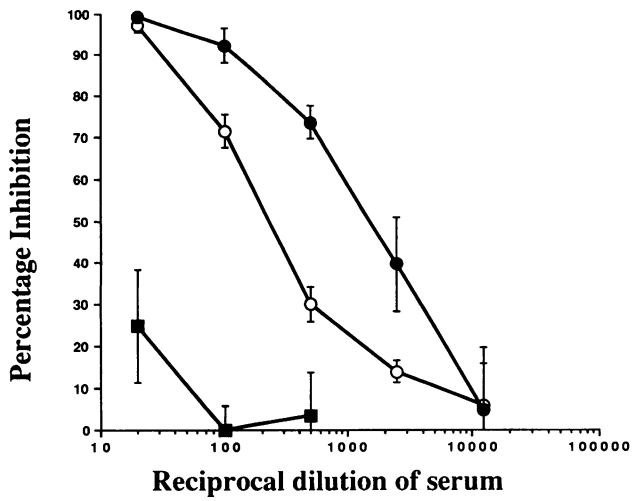

FIG. 2. Titration of the inhibitory activity in human malaria sera. The yields of TNF (mean and standard deviation) from triplicate wells of Mono Mac 6 cells stimulated by malaria toxins in the presence of dilutions of the sera from patient A taken during the acute illness (○) and in convalescent-phase serum collected 3 weeks (●) and 6 weeks (■) later are shown.

incubating Mono Mac 6 cells with inhibitory serum, which was then washed off before the toxin was added. Cells pretreated in this way with inhibitory sera showed the same level of TNF response to malaria toxins as did the controls.

Adsorption of inhibitory activity on phospholipid liposomes. We have previously observed that the inhibitory antibodies in mouse antisera raised to malaria toxins specifically bind to liposomes containing phosphatidylinositol (PI) (2). To see if the inhibitory antibodies in human sera were also PI specific, we performed adsorption experiments. All the inhibitory activity in the acute-phase serum and in the 3-week-postinfection convalescent-phase serum was removed by pretreatment with PI liposomes, whereas incubation with phosphatidylserine (PS), phosphatidylcholine (PC), or cardiolipin (CL) liposomes did not affect the inhibitory activity (Fig. 3). Untreated liposomes or liposomes treated with either control European or control African sera did not affect TNF secretion in response to malaria toxins, showing that liposome breakdown products were not responsible for inhibition (data not shown).

TABLE 1. Specific inhibition of malaria toxin-induced TNF secretion by Igs isolated from the sera of patient A and from European and African controls

Serum sample	Isotype	% Yield after stimulation with[a]:	
		Malaria toxins	LPS
Patient A			
Acute phase	IgG	89.8 ± 11	100.4 ± 13.3
	IgM	18.6 ± 7.9	100.3 ± 6.4
Week 3	IgG	79.6 ± 23.5	102.3 ± 9
	IgM	14 ± 6.3	98 ± 11.9
African adults	IgG	94.7 ± 13.2	ND
	IgM	98.4 ± 7.3	ND
European adults	IgG	102.6 ± 8.8	ND
	IgM	96.9 ± 6	ND

[a] Yields of TNF secreted by Mono Mac 6 cells stimulated by either malaria toxins or LPS in the presence of purified immunoglobulins equivalent to a 1:100 dilution of human serum. Values are means and standard deviations of results of a minimum of six experiments, expressed as a percentage of results of control experiments without serum. Serum samples from three European and three African adults were tested independently, and the results were pooled.

TABLE 2. Inhibitory antibodies in sera from humans with malaria do not react with activated Mono Mac 6 cells

Source of serum	% Inhibitory effect of[a]:		
	Unmodified serum applied with toxin	Serum adsorbed with Mono Mac 6 cells	Mono Mac 6 cells pretreated with serum
No serum	100.0 ± 3.8	99.7 ± 5.3	100.0 ± 11.7
Patient A			
Acute phase	20.8 ± 7.6	18.3 ± 7.1	93.0 ± 3.9
Week 3	12.0 ± 5.5	11.0 ± 3	99.7 ± 5.2

[a] Level of TNF secreted by Mono Mac 6 cells stimulated by malaria toxins. Inhibitory effects of a 1:100 dilution of serum applied to Mono Mac 6 cells with the toxin, a 1:100 dilution of serum tested after it had been adsorbed against activated Mono Mac 6 cells, and Mono Mac 6 cells tested after preincubation for 1 h with a 1:100 dilution of serum which was washed off before the addition of toxin are given. Values are means and standard deviations of results of a minimum of six experiments, expressed as a percentage of results of control experiments without serum.

Cross-reactivity of the major malaria toxins in *P. falciparum* isolates. It has been observed that different strains of *P. falciparum* vary in their ability to stimulate TNF production by human peripheral blood mononuclear cells (1). To explore the possibility of major antigenic differences among the TNF-inducing toxins of different strains, we compared whole-parasite lysates from a range of *P. falciparum* isolates. The acute-phase serum from patient A inhibited all the strains tested (Table 3).

Inhibitory activity in the serum from a patient infected with *P. vivax*. We tested serum obtained from a patient infected with *P. vivax* to see if it contained antibodies that inhibited malaria toxins derived from *P. falciparum*-infected erythrocytes. This serum inhibited the secretion of TNF by cells stimulated by malaria toxins but did not affect LPS- or lipoteichoic acid-induced TNF secretion (Fig. 4). The inhibitory activity was similar to that found in patient A and was mediated by IgM antibodies. Furthermore, the inhibitory activity was removed by pretreatment with PI liposomes but not with other phospholipid liposomes (Fig. 5). This serum also inhibited TNF induction by a range of different *P. falciparum* isolates (data not shown).

DISCUSSION

We have found that malaria-infected individuals produce IgM antibodies that inhibit TNF-inducing malaria toxins. Inhibitory activity was present during the acute illness and 3 weeks later (approximately 2 weeks after the clearance of parasites from the blood) but was undetectable 6 weeks after infection. We found that the inhibitory activity was specific for malaria and did not inhibit the TNF-inducing activity of bacterial LPS, but it was effective against a wide variety of *P. falciparum* isolates. The antibodies bound to liposomes of PI but not other phospholipids. Thus the antibody response in humans is similar to that described in mice, which produce IgM antibodies that specifically inhibit malaria toxins and bind to PI liposomes (2).

These observations could be interpreted as a primary immune response, except that both of the patients studied here had previously experienced malaria and patient B was known to have had several episodes of malaria fever in the previous year. The absence of an inhibitory IgG response up to 6 weeks after the acute episode in patient A also goes against this

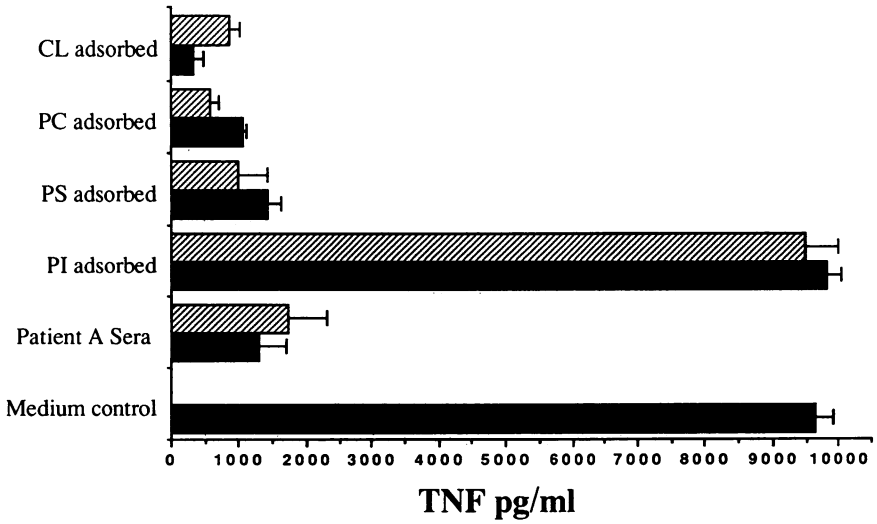

FIG. 3. The inhibitory activity of human malaria sera specifically bind to PI liposomes. The yield (mean and standard deviation) of TNF from triplicate assays of Mono Mac 6 cells stimulated by malaria toxins in the presence of a 1:100 dilution of acute-phase serum (■) or week 3 convalescent-phase serum (▨) after adsorption with a panel of liposomes is shown.

interpretation. An alternative explanation is that, like mice, humans mount a transient, T cell-independent antibody response to toxins released during malaria infection. This might explain why we failed to detect inhibitory IgG antibodies in three Gambian adults, all of whom who would be expected to have had several episodes of malaria in the past.

The cross-reactivity that we have observed is consistent with our previous findings in mice (4). Antibodies in the serum of patient A, who had been in Zaire, were capable of inhibiting TNF induction by a panel of *P. falciparum* isolates originating from Malaya, The Gambia, Brazil, Thailand, and Kenya. All these *P. falciparum* isolates were also inhibited by antibodies in serum from patient B, who was infected with *P. vivax* (data not shown), demonstrating that the response is cross-reactive between species as well as between strains.

The apparent antiphospholipid nature of such antibodies suggests that they might originate from the CD5-positive pool of B cells (8). The role of these short-lived IgM antibodies in infection is poorly understood. In the context of a long-lived infection such as malaria, they could explain why untreated individuals become increasingly tolerant of parasitization, i.e., why the level of parasitemia necessary to cause fever tends to rise as the infection progresses (14). However, there is evidence that TNF and fever are important antiparasitic mechanisms, particularly in the early phase of infection, and it might not benefit the host to ablate this response totally (15). The fact that both of the patients that we studied had fever despite the presence of inhibitory antibodies is consistent with the idea that these antibodies act to down-regulate the TNF response rather than to abolish it completely. This down-regulatory effect could be of particular importance in preventing excessive TNF production, which is thought to be a factor in the pathogenesis of cerebral malaria (9, 12, 17). In interpreting these data, it is important to recognize that pyrogenic cytokines

TABLE 3. Inhibitory effect of serum on TNF induction by different *P. falciparum* isolates

Isolate	% of control with serum sample from[a]:		
	European adults	African adults	Patient A (acute phase)
R29	103.5 ± 13.9	98.5 ± 6.9	19.3 ± 3.2
ITO4	102.7 ± 12.8	109.1 ± 8.2	11.0 ± 10.7
A4	105.6 ± 12.5	100.3 ± 10.7	19.4 ± 7.3
C28	102.0 ± 11.3	96.2 ± 8.4	17.9 ± 6.2
Malayan Camp	101.6 ± 12.1	94.4 ± 8.3	13.6 ± 5.6
FCR 3A2	100.5 ± 4.2	112.4 ± 10.3	17.3 ± 6.9
T996	101.2 ± 8.6	108.4 ± 16.6	24.2 ± 8.8
0303	100.1 ± 10.2	99.4 ± 5.7	13.8 ± 3.3

[a] Yield of TNF secreted by human peripheral blood mononuclear cells, stimulated by *P. falciparum* isolates of different origin, in the presence of a 1:100 dilution of human serum. Values are means and standard deviations of results of at least four experiments expressed as a percentage of results of control experiments without serum. Serum samples from three European and three African adults were tested independently, and the results were pooled.

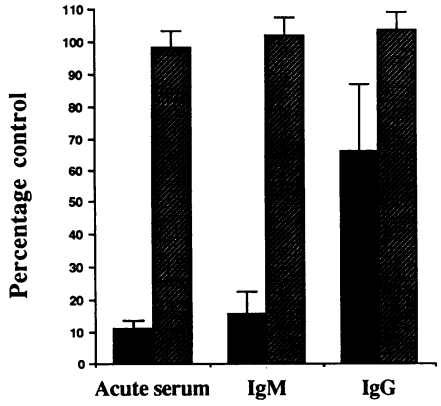

FIG. 4. Inhibition of malaria toxin-induced TNF secretion by serum and isolated Igs from a European adult infected with *P. vivax*. The yields of TNF secreted by Mono Mac 6 cells stimulated by *P. falciparum*-derived malaria toxins (■) and LPS (▨) in the presence of a 1:100 dilution of acute serum from patient B (infected with *P. vivax*) and isolated IgM and IgG from that serum are shown. Values given are means and standard deviations for a minimum of four experiments, expressed as a percentage of control experiments without serum.

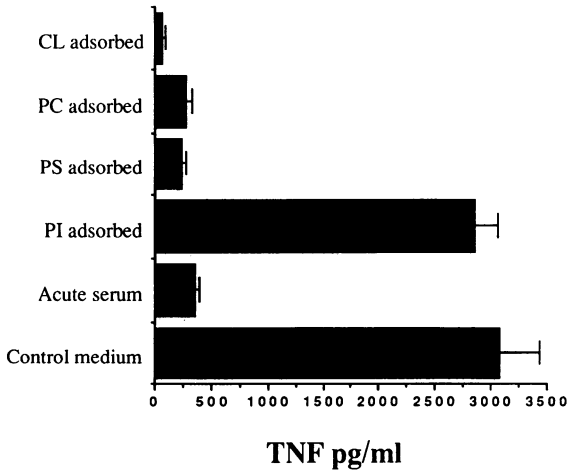

FIG. 5. The inhibitory activity of serum from a *P. vivax*-infected patient specifically binds to PI liposomes. The yields (mean and standard deviation) of TNF from triplicate assays with Mono Mac 6 cells stimulated by malaria toxins in the presence of a 1:100 dilution of acute-phase serum from a *P. vivax*-infected patient before and after adsorption with a panel of liposomes are shown.

other than TNF (such as interleukin-1 and -6) and sources of cytokine production other than monocytes may also influence the clinical manifestations of malaria. We have focused here on TNF production by monocytes because of the clear evidence that TNF is a critical mediator of malaria fever and because cells of the monocyte/macrophage lineage are a dominant source of TNF production, but clearly the effects of antitoxic antibodies on other cytokines and other cell types merit further investigation.

It has been proposed that inhibitory antibodies against malaria toxin might provide the basis for an antidisease vaccine (19). The transience of the inhibitory effect that we have observed in European malaria patients and the lack of detectable inhibitory antibodies in three Gambian adults suggest that a sustainable immune effect may be difficult to achieve. It is worth noting, however, that although these Gambian adults would undoubtedly have been infected with malaria several times, they were resident in a periurban area of relatively low transmission. We cannot exclude the possibility that an inhibitory IgG response develops in areas of more intense transmission and that this may explain the high levels of clinical tolerance that are sometimes observed in these circumstances. Further studies are needed to determine whether antibodies that inhibit TNF-inducing toxins influence the level of morbidity in malaria-endemic areas.

ACKNOWLEDGMENTS

We thank Richard Allan and Pauline Beattie for culturing the parasites and Ian Scragg for providing semipurified malaria toxins. We are grateful to G. Gregoriadis for preparing liposomes. The parasite line 0303 was kindly provided by Peter Warn.

This work was supported by the Tropical Disease Research Programme of the World Health Organization and the Medical Research Council. D.K. is an MRC Senior Clinical Fellow.

REFERENCES

1. **Allan, R. J., A. Rowe, and D. Kwiatkowski.** 1993. *Plasmodium falciparum* varies in its ability to induce tumor necrosis factor. Infect. Immun. **61**:4772–4776.
2. **Bate, C. A. W., J. Taverne, H. Z. Bootsma, R. C. S. Mason, N. Skalko, G. Gregoriadis, and J. H. L. Playfair.** 1992. Antibodies against phosphatidylinositol and inositol monophosphate specifically inhibit TNF production by malaria exoantigens. Immunology **76**:31–35.
3. **Bate, C. A. W., J. Taverne, A. Dave, and J. H. L. Playfair.** 1990. Malaria exoantigens induce T-independent antibody that blocks their ability to induce TNF. Immunology **70**:315–320.
4. **Bate, C. A. W., J. Taverne, N. D. Karunaweera, K. N. Mendis, D. Kwiatkowski, and J. H. L. Playfair.** 1992. Serological relationship of tumor necrosis factor-inducing exoantigens of *Plasmodium falciparum* and *P. vivax*. Infect. Immun. **60**:1241–1243.
5. **Bate, C. A. W., J. Taverne, and J. H. L. Playfair.** 1988. Malarial parasites induce TNF production by macrophages. Immunology **64**:227–231.
6. **Bate, C. A. W., J. Taverne, and J. H. L. Playfair.** 1992. Detoxified exoantigens and phosphatidylinositol derivatives inhibit tumor necrosis factor production by malaria exoantigens. Infect. Immun. **60**:1894–1901.
7. **Bate, C. A. W., J. Taverne, E. Roman, C. Moreno, and J. H. L. Playfair.** 1991. TNF induction by malaria exoantigens depends on phospholipid. Immunology **75**:129–135.
8. **Casali, P., and A. L. Notkins.** 1989. Probing the human B-cell repertoire with EBV: polyreactive antibodies and CD5+ B lymphocytes. Annu. Rev. Immunol. **7**:513–535.
9. **Clark, I. A.** 1987. Cell-mediated immunity in protection and pathology of malaria. Parasitol. Today **3**:300–305.
10. **Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, and J. V. O'Connor.** 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin-1. J. Exp. Med. **163**:1433–1450.
11. **Golgi, C.** 1889. Sul ciclo evolutio dei parassiti malarici nella febbre terzana: diagnosi differenziale tra i parassiti endoglobulari malarici della terzana e quelli della quartana (on the cycle of development of malarial parasites in tertian fever: differential diagnosis between the intracellular malarial parasites of tertian and quartan fever) Arch. Sci. Med. **13**:173–196. Extracts reprinted in B. H. Kean, K. E. Mott, and A. J. Russell (ed.), Tropical medicine and parasitology: classic investigations, vol. 1 (1978). Cornell University Press, Ithaca, New York.
12. **Grau, G. E., T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel, and P.-H. Lambert.** 1989. Tumor necrosis factor and disease severity in children with falciparum malaria. N. Engl. J. Med. **320**:1586–1591.
13. **Karunaweera, N. D., G. E. Grau, P. Gamage, R. Carter, and K. N. Mendis.** 1992. Dynamics of fever and serum levels of tumor necrosis factor are closely associated during clinical paroxysms in Plasmodium vivax malaria. Proc. Natl. Acad. Sci. USA **89**:3200–3203.
14. **Kitchen, S. F.** 1949. Symptomatology, p. 966–1045. *In* M. F. Boyd. (ed.), Malariology, vol. 2. The W. B. Saunders, Co., Philadelphia.
15. **Kwiatkowski, D.** 1991. Cytokines and anti-disease immunity to malaria. Res. Immunol. **142**:707–712.
16. **Kwiatkowski, D., J. G. Cannon, K. R. Manogue, A. Cerami, C. A. Dinarello, and B. M. Greenwood.** 1989. Tumor necrosis factor production in falciparum malaria and its association with schizont rupture. Clin. Exp. Immunol. **77**:361–366.
17. **Kwiatkowski, D., A. V. S. Hill, I. Sambou, P. Twumasi, J. Castracane, K. R. Manogue, A. Cerami, D. R. Brewster, and B. M. Greenwood.** 1990. TNF concentration in fatal cerebral, nonfatal cerebral, and uncomplicated Plasmodium falciparum malaria. Lancet **336**:1201–1204.
18. **Kwiatkowski, D., M. E. Molyneux, S. Stephens, N. Curtis, N. Klein, P. Pointaire, M. Smit, R. Allan, D. R. Brewster, G. E. Grau, and B. M. Greenwood.** 1993. Anti-TNF therapy inhibits fever in cerebral malaria. Q. J. Med. **86**:91–98.
19. **Playfair, J. H. L., J. Taverne, C. A. W. Bate, and J. B. de Souza.** 1990. The malaria vaccine: anti-parasite or anti-disease? Immunol. Today **11**:25–27.
20. **Pradines-Figueres, A., and C. R. H. Raetz.** 1992. Processing and secretion of tumor necrosis factor α in endotoxin-treated mono mac 6 cells are dependent on phorbol myristate acetate. J. Biol. Chem. **267**:23261–23268.

21. **Roberts, D. J., A. G. Craig, A. R. Berendt, R. Pinches, G. Nash, K. Marsh, and C. I. Newbold.** 1992. Rapid switching to multiple antigenic and adhesive phenotypes in malaria. Nature (London) **357:**689–692.
22. **Schofield, L., and F. Hackett.** 1993. Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites. J. Exp. Med. **177:**145–153.
22a. **Scragg, I. G., et al.** Unpublished data.
23. **Sinton, J. A.** 1938. Immunity or tolerance in malarial infections. Proc. R. Soc. Med. **31:**1298–1302.
24. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673.