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A Monoclonal Antibody That Recognizes Phosphatidylinositol Inhibits Induction of Tumor Necrosis Factor Alpha by Different Strains of *Plasmodium falciparum*

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The clinical symptoms of human malaria are mediated, at least in part, by the release of tumor necrosis factor alpha (TNF) by monocytes and macrophages. We have found that lysates of *Plasmodium falciparum*-infected erythrocytes stimulate the secretion of TNF from human mononuclear cells, and we have generated several immunoglobulin M monoclonal antibodies (MAbs) that inhibit the induction of TNF by such lysates. Here we describe the properties of MAb 5AB3-11, which causes dose-dependent inhibition of the TNF-inducing factors derived from *P. falciparum*-infected erythrocytes, with a 50% reduction in TNF secretion at nanomolar concentrations (1 to 2 μg/ml). The inhibitory effect appears to be malaria specific in that the induction of TNF by either lipopolysaccharide or lipoteichoic acid is not affected. MAb 5AB3-11 binds to liposomes containing phosphatidylinositol but not to other phospholipid liposomes, showing that it recognizes a phosphatidylinositol-like epitope. MAb 5AB3-11 inhibits the induction of TNF by whole lysates from several strains of *P. falciparum* which originated from different parts of the tropics, indicating that all of the major TNF-inducing factors derived from *Plasmodium*-infected erythrocytes contain a common epitope. A phosphatidylinositol-like epitope expressed by *Plasmodium*-infected erythrocytes may be a suitable immunological target for the prevention or treatment of severe malaria.

Tumor necrosis factor alpha (TNF) plays an important role in the pathology of human malaria. It has been shown to be a critical mediator of malaria fever, which is the most common clinical manifestation of *Plasmodium* infections. In patients infected with *Plasmodium vivax*, malarial fever paroxysms are associated with a sharp rise in plasma concentrations of TNF (12), and in patients infected with *P. falciparum*, an anti-TNF monoclonal antibody (MAb) reduces fever (17). Clinical studies have also shown that fatal cerebral malaria is associated with very high TNF levels, suggesting that TNF has a role in the pathophysiology of this condition (11, 13, 16). Experimental studies have revealed several potential mechanisms for this association, including TNF-induced upregulation of endothelial cell adhesion molecules that mediate parasite sequestration (8) and possibly cytokine-induced generation of nitric oxide from endothelium (14).

These observations raise the possibility that strategies to prevent excessive TNF production might be of value in the prevention or treatment of cerebral malaria, which presently has a case fatality rate of around 15% (9). In this paper, we consider the feasibility of immunologically inhibiting the critical parasite-derived components that are responsible for stimulating the host TNF response. This approach has previously been used in the treatment of septic shock caused by gram-negative bacteria. Gram-negative bacteria contain lipopolysaccharide (LPS), which is a powerful stimulant of TNF production. A MAb that binds to lipid A, the biologically active component of LPS, has been shown to inhibit TNF production by LPS derived from different bacteria in vitro (10) and to protect mice challenged with gram-negative bacteria (18).

We have previously shown that *Plasmodium*-infected erythrocytes stimulate TNF production by macrophages (6). Since *Plasmodium*-infected erythrocytes contain factors that are functionally similar to endotoxin (LPS), we refer to them as malaria toxins. When mice are immunized with *Plasmodium* lysates or with phosphatidylinositol (PI), they produce immunoglobulin M (IgM) antibodies which inhibit TNF secretion in response to malaria toxins (3, 4). We have recently found evidence that a similar type of IgM antibody response is produced by humans exposed to malaria (2). To extend our understanding of the immune response to the biologically active components of the malaria toxins, we have raised a number of inhibitory murine MAbs. Here we describe the properties of MAb 5AB3-11, which was raised after immunization with PI conjugated to keyhole limpet hemocyanin (KLH). We have used this MAb to address the question of whether the TNF-inducing properties of different strains of parasite can be attributed to a single type of biologically active moiety.

MATERIALS AND METHODS

Immunization and fusions. Groups of (C57BL × BALB/c)F1 female mice at least 10 weeks old were immunized with either lysates of *P. falciparum*-infected erythrocytes, or PI (from bovine liver; Sigma) conjugated to KLH (Calbiochem), using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide methiodide (Sigma) as previously described (5). Both of these immunization regimens had been shown to stimulate the production of inhibitory antibodies (5). Three to 5 days after the third injection, spleens were removed from a mouse from each group and teased apart under sterile conditions. Cell suspensions were collected and resuspended with mouse myeloma NS-1/1-Ag4-1 cells growing in log phase at a ratio of 5:1. Fusion was performed with 50% (wt/vol) polyethylene glycol 1500 (Boehringer Mannheim) according to standard procedures. As soon as hybridoma growth was macroscopically

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visible, supernatants were screened for the ability to inhibit TNF production by human peripheral blood mononuclear cells stimulated with lysates of *P. falciparum*-infected erythrocytes, as described below. Cloning of positive cultures was carried out by limiting dilution in the presence of 15% fetal calf serum containing 10% EL4 conditioned medium (Boehringer Mannheim). Two further rounds of cloning by limiting dilution were carried out to ensure the clonality of the hybridomas described here.

Purification of MAbs. Hybridoma supernatants or control preparations of normal mouse serum diluted 1:10 were spun at 2,000 × *g* and filtered. They were then applied to an isotype-specific affinity column containing goat anti-mouse IgM bound to agarose (Sigma). The columns were washed extensively with 0.1 M glycine HCl at pH 5 to remove contaminants and then with sterile phosphate-buffered saline (PBS). The IgM fraction was eluted with 0.1 M glycine HCl at pH 2.5, immediately neutralized with 100 mM Tris buffer at pH 8, concentrated to 1 mg/ml with 100-kDa Centricon filters (Amicon), dialyzed against PBS, filtered, and stored at 4°C prior to testing. Protein concentrations were determined by Micro bicinchoninic acid protein assay kit (Pierce). Several control IgM MAbs were purified from hybridoma supernatants without inhibitory activity, and one was obtained from Sigma.

Parasite preparations. For immunization and screening of hybridoma supernatants, we used R29, a cloned laboratory line of *P. falciparum* derived from the Brazilian isolate IT14/4/25. This was cultured in human group O erythrocytes, using RPMI 1640 containing 2 g of glucose per liter and 10% A^+ human serum (22). Preparations of mature trophozoites and schizonts at a parasitemia of ~10% were harvested by centrifugation at 10,000 × *g* for 10 min. The supernatant was removed, and the remaining pellet was lysed by the addition of 4 volumes of 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 the pellet in PBS and then in a mixture of methanol-chloroform-water (8:4:3). Where indicated in the text, partially purified preparations were digested further with 100 μg of mixed proteases (pronase E; Sigma) per ml.

To investigate the inhibitory activity of MAbs against different parasite strains, we compared R29 with three other lines derived from IT 14/4/25 (ITO4, A4, and C28) (20), as well as Malayan Camp, the Gambian strain FCR3A2, the Thai strain T996, and a Kenyan wild isolate (Kilifi 0303).

TNF production assay. Blood from healthy European volunteers was collected in heparinized containers and mixed with an equal volume of saline before mononuclear cells were isolated on Lymphoprep (Nyegaard). Peripheral blood mononuclear cells were washed three times, resuspended in RPMI 1640 containing 1% A^+ human serum, and dispensed into flat-bottomed, 96-well microtiter plates at 10^5 cells per well. After 1 h at 37°C, nonadherent cells were removed by gentle washing, and then inhibitors were added followed by stimulants, all diluted in RPMI 1640, to a total volume of 200 μl. Titrations of LPS from *Escherichia coli* O55:B5 or lipoteichoic acid from *Streptococcus pyogenes* (Sigma) were used as positive controls. After overnight incubation in 5% CO_2 at 37°C, the supernatants were harvested and assayed for TNF by an enzyme-linked immunosorbent assay (ELISA), as described previously (16).

For certain experiments we used the human monocyte-macrophage cell line Mono Mac 6 (19). Mono Mac 6 cells were grown in RPMI 1640 supplemented with 10% fetal calf serum. Before use, cells were washed, resuspended in the same medium supplemented with 100 ng of phorbol myristate acetate per ml, and dispensed in 96-well microtiter plates at 5 × 10^4 cells per well. After the cells were left to adhere for a minimum of 1 h, the medium was removed, and first inhibitors and then stimulants were added, all diluted in RPMI 1640, to a total volume of 200 μl. After incubation overnight in 5% CO_2 at 37°C, supernatants were harvested and tested for TNF by ELISA.

When appropriate, TNF secretion in the presence of MAbs is expressed as a percentage of TNF levels obtained in control experiments without antibody. This allows us to pool data obtained from several experiments carried out on different days.

Adsorption of MAb by liposomes. Multilamellar dehydration-rehydration vesicles were produced by a dehydration-rehydration procedure, as previously described (15). Purified MAbs were incubated for 1 h at room temperature with 200 μg of different liposomes per ml, which were then deposited by centrifugation. The supernatant was filtered through a 0.2-μm-pore-size Spinex centrifuge filter unit (Costar) before testing.

Adsorption of MAb by erythrocytes. Venous blood from eight different donors was collected into heparin and separated on Lymphoprep (Nyegaard). Erythrocytes were washed three times in endotoxin-free normal saline and resuspended at 10^9/ml in RPMI. Purified MAbs were added at 50 μg/ml and incubated for 2 h at 37°C. The erythrocytes were then removed by centrifugation at 10,000 × *g* for 10 min, and the supernatant was tested for inhibitory activity.

Binding of MAbs to macrophages. Human mononuclear cells were allowed to adhere in RPMI 1640 containing 1% A^+ human serum at 10^5 cells per well in flat-bottomed, 96-well microtiter plates. The antibodies to be tested were added and diluted in RPMI 1640, and plates were incubated at 37°C. After 1 h, the antibodies were removed, cells were gently washed, and stimulants were added (all diluted in RPMI 1640) to a total volume of 200 μl.

RESULTS

IgM MAbs that inhibit TNF secretion by malaria toxins. Hybridomas secreting inhibitory antibodies were grown from the fusion of spleen cells from two mice immunized with parasite lysates and from two mice immunized with a conjugate of PI and KLH (Table 1). MAbs 4BB5-30-A and 4BC5-13-A originated from mice immunized with parasite lysates, and the other MAbs originated from mice immunized with conjugates of PI and KLH. All of the inhibitory MAbs were of the IgM isotype, and full activity was retained after the IgM fraction had been purified on isotype-specific columns. Control IgM MAbs and IgM antibodies isolated from normal mouse serum did not affect TNF production. No protein contaminants were detected when the purified MAb preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue.

MAb 5AB3-11 was chosen for further characterization since the hybridoma from which it was derived showed rapid growth and stable antibody secretion. It inhibited malaria toxin-induced TNF secretion in a dose-dependent fashion. When the MAb was titrated against a toxin preparation which induced near-maximal levels of TNF secretion for this assay (8,500 pg/ml), we found that MAb concentrations of 1 to 2 μg/ml reduced TNF levels by 50% (Fig. 1A). When *P. falciparum* lysates were titrated in the presence and absence of MAb 5AB3-11 at 25 μg/ml, it was found that 50 times as much toxin was needed to stimulate a given level of TNF when the MAb was present (Fig. 1B).

TABLE 1. Inhibitory effect of MAbs on TNF secretion induced by factors derived from *P. falciparum*-infected erythrocytes[a]

Antibody	TNF (pg/ml)	SD
None	7,254	577
Control IgM MAb	7,875	334
IgM from normal mouse serum (500 μg/ml)	7,684	570
5CD1-2[b]	7,481	533
7BD4-2[b]	7,473	592
4BC5-13-A[c]	1,247	229
7AC4-17[b]	892	197
7FB6-7[b]	863	238
4BB5-30-A[c]	806	232
5FC2-8-A[b]	789	193
7BC6-2-SA-7[b]	571	151
5AB3-11[b]	457	198

[a] Amounts of TNF secreted by human mononuclear cells incubated with TNF-inducing factors from *P. falciparum*-infected erythrocytes in the presence of 25 μg of various IgM MAbs or IgM isolated from normal mouse serum per ml. Values shown are the means and standard deviations from quadruplicate wells. Mononuclear cells incubated in the absence of any stimulant gave a mean TNF level of 78 pg/ml: this background has been subtracted from the above values.
[b] From mice immunized with KLH-PI conjugates.
[c] From mice immunized with lysed *P. falciparum*-infected erythrocytes.

The inhibitory effect of MAb 5AB3-11 is not due to binding to macrophages. To show that the inhibition caused by MAb 5AB3-11 is due to the antibody binding to epitopes on malaria toxins, we sought to exclude the alternative possibility that the MAb bound to macrophage receptors. We showed that pretreatment of mononuclear cells with MAb 5AB3-11 did not affect toxin-induced TNF secretion compared with untreated cells, cells pretreated with a control IgM MAb, or cells treated with a 1:100 dilution of normal mouse serum (Fig. 2). Furthermore, MAb 5AB3-11 did not bind to either phorbol myristate acetate-activated Mono Mac 6 cells or human mononuclear cells in adsorption experiments (data not shown).

MAb 5AB3-11 does not inhibit LPS- or lipoteichoic acid-induced TNF secretion. To see if the inhibitory effect of MAb 5AB3-11 is specific, we tested its ability to inhibit TNF secretion from LPS- or lipoteichoic acid-stimulated cells. We found that the presence of 25 μg of MAb 5AB3-11 per ml did not affect amounts of TNF secreted by cells incubated with serial dilutions of either LPS (Fig. 3A) or lipoteichoic acid (Fig. 3B).

MAb 5AB3-11 binds to PI liposomes. We had previously shown that antibodies in polyclonal antisera which inhibit the induction of TNF by malaria toxins bind specifically to liposomes containing PI. To see if MAb 5AB3-11 also bound to PI liposomes, adsorption experiments were performed. MAb 5AB3-11 did not bind to liposomes containing phosphatidylserine, phosphatidylcholine, or cardiolipin but did bind to liposomes containing PI (Fig. 4). The possible confounding effect of liposomal breakdown products in this experiment was ruled out by showing that liposomes treated with a control IgM MAb did not affect TNF secretion (data not shown). Although MAb 5AB3-11 binds to a phospholipid that is a constituent of normal cell membranes, we found that it did not bind to erythrocytes isolated from a range of different donors (data not shown).

MAb 5AB3-11 inhibits TNF stimulation by whole parasite lysates. As parasitised erythrocytes may contain various TNF-inducing components, we investigated the ability of the MAb to inhibit different parasite preparations, including whole lysates. MAb 5AB3-11 inhibited the induction of TNF by preparations of whole parasitized cells that had been either

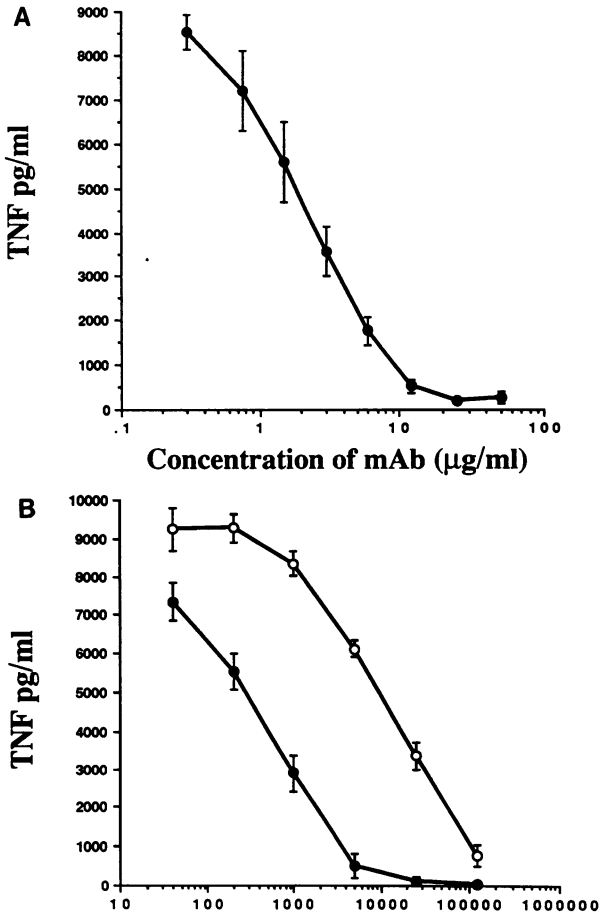

FIG. 1. Inhibitory effect IgM MAb 5AB3-11 on malaria toxin-induced TNF secretion. (A) Titration of MAb 5AB3-11 against malaria toxin-induced TNF secretion: yield of TNF (mean and standard deviation for triplicate wells) from human mononuclear cells incubated with malaria toxins and different concentrations of MAb 5AB3-11. (B) Titration of *P. falciparum* lysates in the presence or absence of MAb 5AB3-11: yield of TNF (mean and standard deviation for triplicate wells) from human mononuclear cells incubated with serial dilutions of *P. falciparum* lysates incubated in the presence (●) or absence (○) of 25 μg of MAb 5AB3-11 per ml.

water lysed or sonicated. It also inhibited the induction of TNF by partially purified preparations that had been pronase digested (Table 2). All preparations used were from the R29 strain of *P. falciparum*.

Cross-reactivity of the major malaria toxins in different strains of *P. falciparum*. It has been observed that different strains of *P. falciparum* vary in the ability to stimulate TNF secretion (1). To see if different strains of *P. falciparum* produce different TNF-inducing toxins, we tested MAb 5AB3-11 to see if it could inhibit TNF induction by whole lysed parasites from several laboratory and wild strains of *P. falciparum*. MAb 5AB3-11 was found to reduce TNF secretion in response to all strains of *Plasmodium* parasite tested (Fig. 5).

DISCUSSION

We have identified several MAbs that inhibit the induction of TNF by malaria toxins. All of the inhibitory MAbs were of

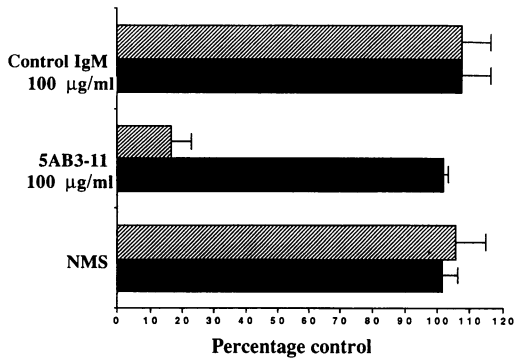

FIG. 2. The inhibitory effect of MAb 5AB3-11 is not due to binding to macrophages. Shown is the yield of TNF (mean and standard deviation) from human mononuclear cells stimulated with malaria toxins in the presence of either 100 μg of MAb 5AB3-11 per ml, 100 μg of an isotype control per ml, or a 1:100 dilution of normal mouse serum (NMS). Hatched bars show the effect of adding antibodies at the same time as the toxin. Black bars show the effect of preincubating mononuclear cells with the antibodies, which were then washed off immediately before the toxin was added. Values shown are pooled data from a minimum of nine experiments, expressed as a percentage of control preparations incubated in serum-free media.

the IgM isotype. Control IgM MAbs and IgM antibodies isolated from normal mouse serum were not inhibitory, showing that the inhibition was not a general property of this isotype. No protein contaminants were detected when the purified IgM preparations were analyzed by SDS-PAGE, indicating that the inhibitory effect was mediated by antibody.

Only 1 to 2 μg of MAb 5AB3-11 per ml was required to reduce malaria toxin-induced TNF production by 50%, showing that it was active at a nanomolar concentration. We note that a similar concentration of a MAb that recognizes the lipid A portion of LPS is required to inhibit LPS-induced TNF secretion (10). However, this provides only a limited measure of antibody activity in that the inhibitory activity of MAb 5AB3-11 could be overcome by increasing the concentration of malaria toxins. Since we do not know the relationship between the concentration of malaria toxins and TNF production, we cannot calculate the amount of toxin bound by a given concentration of MAb. A better measure of the inhibitory activity of the antibody is provided by the data in Fig. 1B, which shows that 50 times as much toxin was needed to stimulate a given level of TNF when 25 μg of MAb 5AB3-11 per ml was present.

MAb 5AB3-11 did not inhibit TNF production in response to either LPS or lipoteichoic acid, excluding the possibility that the MAb was nonspecifically inhibitory or toxic to cells. The inhibitory activity could not be explained by the MAb binding to macrophage receptors, as cells pretreated with this MAb responded to the subsequent addition of malaria toxins. Furthermore, the MAb did not bind to activated Mono Mac 6 cells or to human mononuclear cells in adsorption experiments. These observations suggest that MAb 5AB3-11 acts by binding to critical epitopes on the TNF-inducing factors derived from malaria-infected erythrocytes.

Although the TNF-inducing activity of malaria toxins is resistant to extensive digestion with mixed proteases, the malaria toxins seem to be associated with a protein component (7). However, we have two reasons to believe that MAb 5AB3-11 does not bind to protein epitopes on the malaria toxins. First, we showed that MAb 5AB3-11 inhibited partially

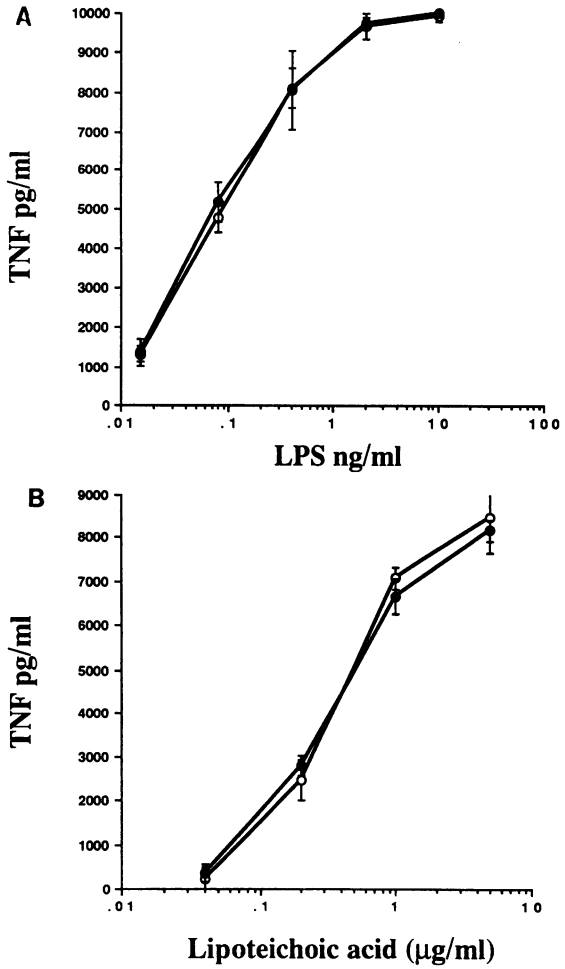

FIG. 3. MAb 5AB3-11 does not inhibit TNF secretion induced by LPS or lipoteichoic acid. Shown is the yield of TNF (mean and standard deviation for triplicate wells) secreted from human mononuclear cells incubated with serial dilutions of either (A) LPS or (B) lipoteichoic acid incubated in the presence (●) or absence (○) of 25 μg of MAb 5AB3-11 per ml.

purified preparations that had been extensively digested by mixed proteases, indicating that it was not binding a protein epitope. Second, the MAb was raised from mice immunized with a conjugate of PI and KLH. Immunization with KLH alone did not result in inhibitory antiserum, showing that the inhibitory antibodies were directed towards the PI component (5). These results are consistent with our finding that MAb 5AB3-11 bound to liposomes containing PI. We conclude that this PI-like epitope is either closely associated with or constitutes a part of the active moiety of malaria toxins. This explanation is compatible with previous findings which suggest that the malaria toxins contain a PI component required for TNF-inducing activity (3, 21). Although PI is a constituent of normal membranes, we found that MAb 5AB3-11 did not bind to human erythrocytes from different donors, to human mononuclear cells, to fibroblasts, or to Mono Mac 6 cells (data not shown) and conclude that MAb 5AB3-11 recognizes a PI-like epitope that is not exposed on the surface of normal cells. This may be explained by the fact that PI appears to be mainly located on the cytoplasmic side of the plasma membrane.

MAb 5AB3-11 originated from a mouse immunized with a

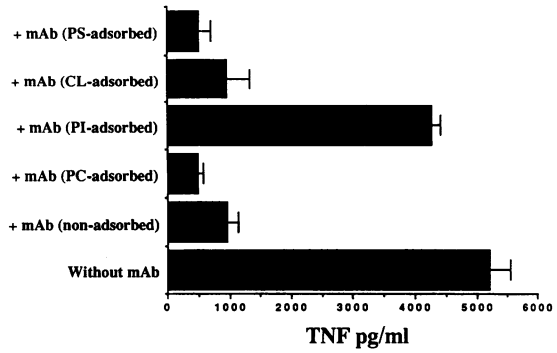

FIG. 4. MAb 5AB3-11 binds to liposomes containing PI. Shown are amounts of TNF (mean and standard deviation of triplicate wells) from human mononuclear cells stimulated by malaria toxins in the presence of 25 μg of MAb 5AB3-11 per ml after adsorption with a panel of liposomes. Abbreviations: PS, phosphatidylserine; CL, cardiolipin; PC, phosphatidylcholine.

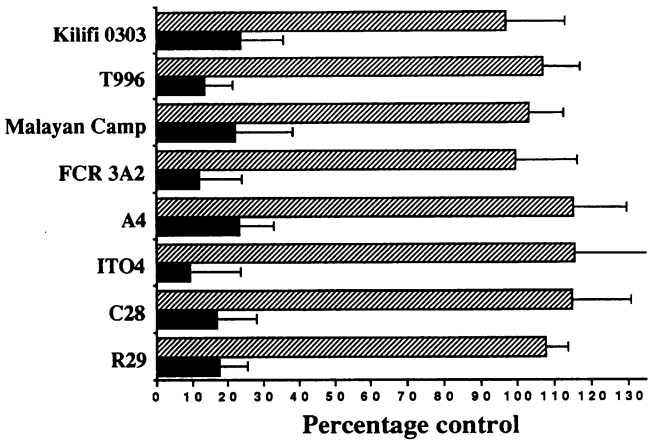

FIG. 5. MAb 5AB3-11 inhibits TNF secretion induced by different *P. falciparum* isolates. Shown are yields of TNF secreted by human mononuclear cells incubated with strains of *P. falciparum* which originated from different parts of the tropics in the presence of 25 μg of MAb 5AB3-11 per ml (■) or a control MAb (▨). Values shown are means and standard deviations from at least six experiments expressed as a percentage of control experiments incubated in serum-free medium.

KLH-PI conjugate. Inhibitory MAbs were also raised after immunization with lysates of *P. falciparum*-infected erythrocytes. In preliminary studies these MAbs showed the same properties as MAb 5AB3-11; namely, they did not inhibit TNF secretion induced by LPS or lipoteichoic acid and bound specifically to PI liposomes (data not shown). Furthermore, the properties of MAb 5AB3-11 are similar to those observed for the inhibitory antibodies that are found in either polyclonal antisera raised to malaria toxins (3) or sera from malaria patients (2).

In most of our experiments we used partially purified malaria toxins that we believe contain the major TNF-inducing component in *P. falciparum*-infected erythrocytes. MAb 5AB3-11 also inhibited the induction of TNF by whole parasitized cells that had been either water lysed or sonicated, showing that within the R29 strain of *P. falciparum* all major TNF-inducing factors contain a common PI-like epitope. Furthermore, MAb 5AB3-11 inhibited the induction of TNF by different laboratory strains of *P. falciparum* originating from Malaya, Brazil, The Gambia, and Thailand. It also inhibited TNF-induction by wild isolates from Kenya and The Gambia (data not shown). These results consolidate our conclusion based on studies using polyclonal antisera that the major TNF-inducing toxins from different *Plasmodium* parasites contain an antigenically conserved epitope (3, 4). The conservation of an active moiety in different *Plasmodium* strains and species is analogous to the conservation of the active moiety of LPS, lipid A, from gram-negative bacteria. IgM MAbs, directed against lipid A, inhibit cytokine production by LPS from different bacteria (10).

This study concentrates on the ability of MAb 5AB3-11 to modulate TNF secretion by human monocytes in response to toxins of *P. falciparum*-infected erythrocytes. We have focused on TNF because it appears to be a critical mediator of malaria fever (17) and because there is both clinical and experimental evidence that excessive TNF production may be an important factor in the pathogenesis of cerebral malaria. However, it is important to recognize that other pyrogenic cytokines such as interleukin-1, interleukin-6, and lymphotoxin are also released in malaria; furthermore, cell types other than the monocyte-macrophage series, particularly lymphocytes and endothelial cells, may be involved in cytokine production during this infection. MAbs against major cytokine-inducing toxins provide an essential tool in investigating the molecular pathogenesis of infection and may lead to new forms of treatment for life-threatening complications of malaria.

TABLE 2. Inhibitory effect of MAb 5AB3-11 on TNF secretion induced by different preparations of *P. falciparum*[a]

P. falciparum prepn	% TNF secreted (95% confidence interval)	
	5AB3-11	Control IgM
Water-lysed pellet	19 (15–23)	103 (97–109)
Sonicated water-lysed pellet	22 (15–28)	106 (97–115)
Partially purified	16 (13–20)	108 (101–115)
Partially purified and pronase digested	9 (6–12)	103 (95–111)

[a] Yields of TNF secreted by human mononuclear cells or Mono Mac 6 cells incubated with preparations from *P. falciparum*-infected erythrocytes (R29 strain) in the presence of MAb 5AB3-11, or a control IgM MAb, at 25 μg/ml. Values represent the means of at least six experiments expressed as a percentage of control preparations incubated in medium only.

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