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Antibodies against phosphatidylinositol and inositol monophosphate specifically inhibit tumour necrosis factor induction by malaria exoantigens

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SUMMARY

The active component of the exoantigens of malarial parasites which stimulates macrophages to secrete tumour necrosis factor (TNF) has been shown to depend upon a phospholipid, the activity of which was blocked by phosphatidylinositol (PI) and inositol monophosphate (IMP) in competitive inhibition studies. Antisera made against the exoantigens of *Plasmodium yoelii*, which inhibited their induction of TNF, were found by an ELISA assay to contain antibody against several other phospholipids. However, the inhibitory antibody was removed specifically by adsorption with liposomes containing PI, but not other phospholipids. Furthermore, PI was the only phospholipid in non-liposomal form which induced the production of inhibitory antisera. Mice immunized with IMP, but not inositol, also produced inhibitory antisera. When incorporated into liposomes several other phospholipids did give rise to inhibitory antibodies but, in contrast to the antisera against parasite exoantigens, PI and IMP, the inhibitory activity was removed by adsorption with heterologous phospholipid liposomes, suggesting that it was directed against a common determinant, presumably the phosphate ester head group. Inhibitory antibodies in the antisera tested were predominantly IgM and titres were not increased after repeated injections. Antisera raised against PI, IMP or the cross-reacting phospholipid liposomes also inhibited TNF secretion by macrophages stimulated by exoantigens of the human parasites *P. falciparum* and *P. vivax*, but not by bacterial lipopolysaccharide. These findings confirm our conclusion that exoantigens from these different species contain phosphate bound to inositol in their TNF-inducing moiety.

INTRODUCTION

The nature of the products of malarial parasites that might be responsible for the release of tumour necrosis factor (TNF) during infection is of considerable importance since high concentrations of this cytokine in the circulation have been shown to be associated with the severity of the disease in patients with *Plasmodium falciparum* malaria,[1,2] and especially with death from cerebral malaria.[3] Among its many properties, TNF has the ability to cause various changes in vascular endothelium. For example, it can increase the expression of adhesion molecules such as ICAM-1,[4] one of the molecules that may be concerned in the attachment of parasitized erythrocytes to the endothelium of brain capillaries, an event that has been associated with the development of cerebral malaria.[5] The possible involvement of TNF in the illness and pathology of malaria has been well reviewed by Clark and his co-workers.[6]

We have shown that exoantigens liberated from parasitized erythrocytes induce macrophages to secrete TNF *in vitro*[7–10] and *in vivo*[11] and are toxic, in that they can kill mice made hypersensitive to TNF by pretreatment with D-galactosamine.[11] Antibody made against the exoantigens blocks the production of TNF and mice immunized with them are protected from death. The antibody induced against *P. yoelii* is T independent and cross-reacts with *P. berghei* and *P. falciparum*[12] and also with *P. vivax*.[13] We have argued that immunization with such exoantigens,[14] perhaps in modified or detoxified form,[15] might form the basis of an anti-disease vaccine.

The induction of TNF by parasite exoantigens depends upon a phospholipid component and its presence was also found to be necessary for the induction of inhibitory antiserum.[16] This component, which we have recently shown in

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competitive inhibition studies to be alike in the exoantigens of *P. yoelii*, *P. falciparum* and *P. vivax*, contains phosphate and inositol.[15] An understanding of the specificity of the antibodies that inhibit the induction of TNF by the exoantigens is clearly important and could be of practical value. We report here the results of investigations into the nature of the phospholipid structures and their derivatives which give rise to the inhibitory antibodies.

MATERIALS AND METHODS

Mice

Outbred females at least 6 weeks old were used (Tuck No 1; A. Tuck & Sons, Battlesbridge, Essex, U.K.).

Rodent parasites

The YM lethal variant of *P. yoelii* (obtained from D. Walliker, Edinburgh University, Edinburgh, U.K.) was used.[17] Mice were injected intravenously with 10^4 parasitized erythrocytes, and parasitaemia was determined from blood films stained with Giemsa.

Exoantigen preparations

P. yoelii. Soluble antigens were prepared as previously described[16] by incubating 10^8 washed parasitized erythrocytes/ml in phosphate-buffered saline (PBS) overnight on a roller at 37°. Next day supernatants were collected, centrifuged, boiled for 5 min, centrifuged again, passed through a 0·2 μm Millipore filter (Flow Laboratories, Irvine, Ayrshire, U.K.) and stored at 4°.

P. falciparum. These were kindly provided by Dr D. Kwiatkowski of Oxford University, Oxford, U.K. They were derived from schizont-enriched cultures incubated in minimum essential medium without serum at 37° for 24 hr. The medium was collected, centrifuged again and the supernatants obtained were passed through a membrane filter and stored at 4°.

P. vivax.[13] These were kindly provided by Professor K. Mendis, University of Colombo (Sri Lanka). Erythrocytes obtained from three patients infected with *P. vivax* when the parasites had become schizonts were washed and concentrated to about 80% parasitaemia. They were suspended in PBS without serum at 1×10^8 infected erythrocytes/ml and incubated at 37° for 24 hr on rollers. Supernatants were then collected, passed through a 0·2 μm Millipore filter, boiled for 5 min, centrifuged again and the supernatants obtained were pooled and stored at 4°.

Since TNF-inducing activity was not associated with protein but was enhanced by its removal,[16] all exoantigen preparations were routinely incubated for 24 hr at 37° in 10 μg/ml of pronase E (Sigma, Poole, Dorset, U.K.), boiled and dialysed against PBS. No protein was then detectable by BioRad assay (< 1 μg/ml). Before use, they were mixed with polymyxin B-agarose (Sigma) to remove any endotoxin, centrifuged, passed through a Millipore filter and stored at 4°.

Preparation of liposomes

Multilamellar dehydration–rehydration vesicles were prepared as previously described[18] by mixing phospholipids and cholesterol (10 mg of each phospholipid and equimolar cholesterol) in $CHCl_3$/methanol (4:1 v/v). Following rotary evaporation of the solvents, the dry film was suspended in 2 ml of water, sonicated and then dehydrated by lyophilization. Controlled rehydration with 1 ml of PBS was carried out, the liposomes formed were washed thrice with PBS and finally suspended in 1 ml of PBS.

Antisera

Groups of mice were injected i.p. with 0·5 ml of an exoantigen preparation or with 2 mg/mouse of liposomes or 200 μg of phospholipids diluted first in ethanol, then in PBS or inositol monophosphate (IMP) dissolved in PBS. They were bled 10–12 days later and sera from three or four mice were pooled and heat inactivated at 56° for 30 min. They were titrated for their ability to block the induction of TNF by parasite exoantigens by mixing equal volumes of serial dilutions of antiserum with a constant dilution of an exoantigen preparation before addition to peritoneal macrophages. Titres are defined as the reciprocal of the dilution that reduced the amount of TNF produced by 50%. Antisera were depleted of IgG or IgM on isotype-specific agarose columns (immobilized anti-mouse immunoglobulins, Calbiochem, La Jolla, CA); sequential passage through the two columns removed all inhibitory activity.

Adsorption with liposomes

Antisera were diluted 1/20 or 1/50 and incubated for 1 hr at room temperature with 200 μg of the liposomes, which were then deposited by centrifugation; this was repeated two or three times.

Stimulation assays using peritoneal macrophages

The cells were prepared as described previously.[16] Briefly, they were collected from mice given 1 ml of 4% thioglycollate (Difco, East Molesey, U.K.) i.p. 3–5 days previously, using Hanks' balanced salt solution (HBSS) (Flow Laboratories) containing 1 U/ml of heparin and 5 μg/ml of polymyxin B (Sigma). Washed cells were suspended in 5% foetal calf serum (FCS) in RPMI-1640 containing polymyxin B, at 1×10^7 viable cells/ml and then 0·1 ml volumes were dispensed into wells of 96-well microtitre plates (Flow Laboratories). The medium over adherent cells that had been incubated for 30 min with an equal volume of medium containing 2 μg/ml indomethacin (Sigma) was replaced by 0·2 ml volumes of dilutions of the stimulants to be tested in RPMI-1640 containing polymyxin B [except in lipopolysaccharide (LPS) controls] and the cultures were incubated overnight. Next day, supernatants were collected and assayed for TNF (see below); they were stored in medium containing 5% FCS and 1 μg/ml of emetine (Sigma). Cultures incubated with several concentrations of LPS (phenol extract of *Escherichia coli* 055:B5; Sigma) and with medium alone were included in every experiment as positive and negative controls for the capacity of the macrophages to yield TNF.

TNF assay

Samples were assayed colorimetrically by their cytotoxicity for L929 cells as described previously.[16] Serial dilutions were tested in duplicate, in 0·1 ml volumes/well, in RPMI containing 1 μg/ml emetine. One unit is defined as that causing 50% cell destruction.

ELISA assays

Microtitre plates (Nunc Immunoplate Polysorp, Roskilde, Denmark) were coated overnight at 4° with 50 μl/well of solutions containing 5 μg/ml of phospholipid in ethanol. They

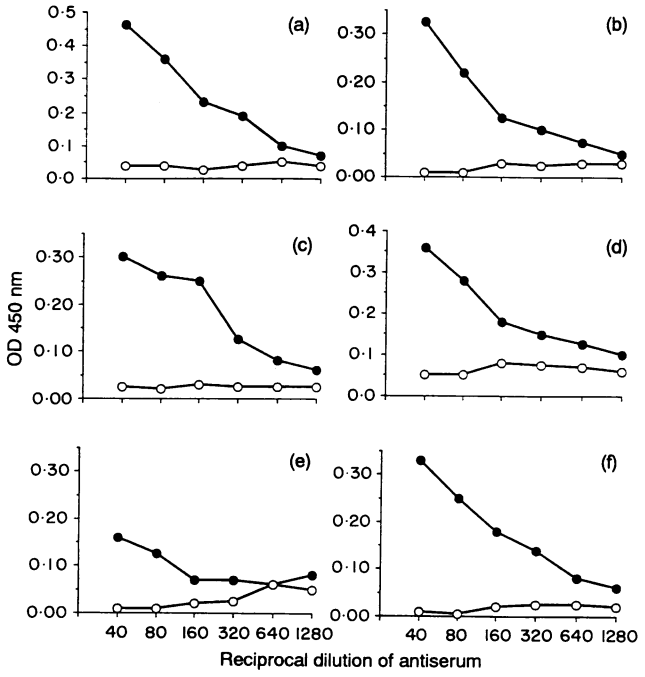

Figure 1. Antibody against common phospholipids in antisera made against *P. yoelii* exoantigens. Results of titrations by ELISA of an antiserum against *P. yoelii* exoantigens (●) compared with a control serum made against a normal mouse red blood cell supernatant (○), using plates coated with phosphatidylcholine (a), cardiolipin (b), phosphatidylserine (c), phosphatidic acid (d), phosphatidylethanolamine (e) and phosphatidylinositol (f).

were then blocked with 10% FCS in PBS and incubated with serial dilutions of antiserum diluted in 2% FCS in PBS. They were washed and developed with peroxidase-conjugated rabbit anti-mouse Ig (Dako-Immunoglobulins, Glostrup, Denmark) and *O*-phenylenediamine/H_2O_2 (Sigma) as substrate and the OD 450 nm was read on a Dynatech Microelisa Autoreader.

Other reagents

For liposome preparation: phosphatidylinositol (PI), from soya bean (Sigma), phosphatidylcholine (PC), Grade 1 from egg yolk (Lipid Products, Redhill, Surrey, U.K.), phosphatidylserine (PS), monosodium salt from bovine spinal cord (Lipid Products), phosphatidylethanolamine (PE), Grade 1 from egg yolk (Lipid Products), phosphatidic acid (PA), Grade 1 from egg yolk (Lipid Products), and cardiolipin (CL) from bovine heart (Sigma).

Otherwise, inositol monophosphate and the phospholipids used after dilution first in ethanol and then in PBS were obtained from Sigma. They were: PI (ammonium salt from bovine liver), PC (from egg yolk and bovine brain), PS (sodium salt from bovine brain), PE (from bovine brain), PA (sodium salt from egg yolk lecithin) and CL (from bovine heart).

RESULTS

We had found previously that the induction of TNF by parasite exoantigens depended upon a phospholipid component and that this TNF-inducing activity could be blocked both *in vitro* and *in vivo* by antisera raised against active preparations.[12,16] To see if antibody against various common phospholipids could be

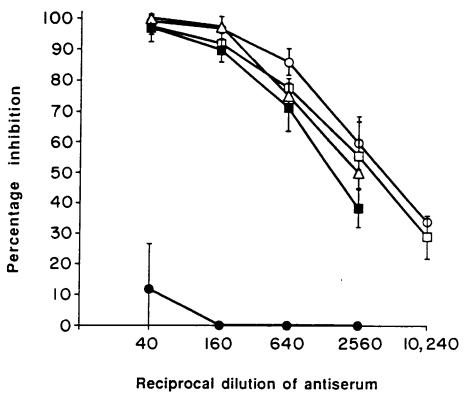

Figure 2. Titrations of specific antisera against *P. yoelii* exoantigens for inhibition of TNF induction by the antigens, before and after adsorption with various preparations of liposomes. Liposomes used for adsorption: none (○); PC (□); PA (△); PS (■); PI (●). The figures are means ± SD from titrations done with three different samples of antiserum made against the exoantigens before and after at least two cycles of adsorption with 1 mg/ml of the liposome preparations. In the absence of antiserum the exoantigens induced the production of the order of 11,200 U/ml of TNF.

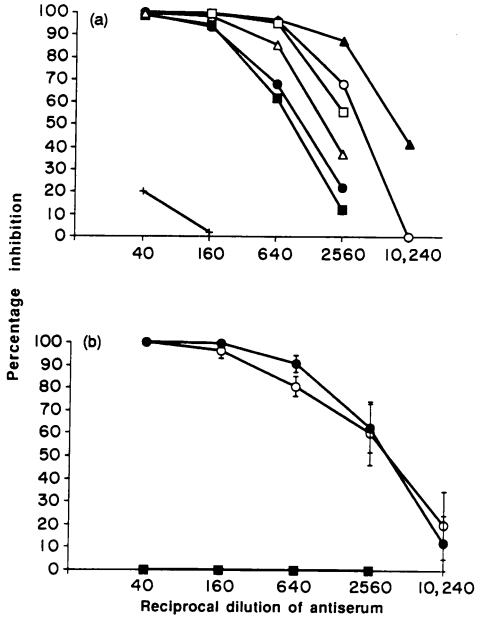

Figure 3. Inhibition of TNF induction by *P. yoelii* exoantigens by specific antiserum, compared with antisera made against various phospholipids in liposomes and against PI derivatives in non-liposomal form. (a) Antisera made against the exoantigens and against phospholipids in liposomes: *P. yoelii* exoantigens (▲); PI (○); CL (□); PA (△); PC (●); PS (■) and normal mouse serum (×). The figures are means obtained from at least two pools of antiserum in each case made on different occasions against different preparations of liposomes, except in the case of CL. In the absence of antiserum the exoantigen controls induced the production of 25,600–102,000 U/ml of TNF. (b) Antisera made against non-liposomal PI and its derivative IMP: PI salt (○); IMP (●) and inositol (■). The figures are means ± SD obtained with at least six different pools of antiserum. In the absence of antiserum the exoantigen induced the production of the order of 12,800 U/ml of TNF.

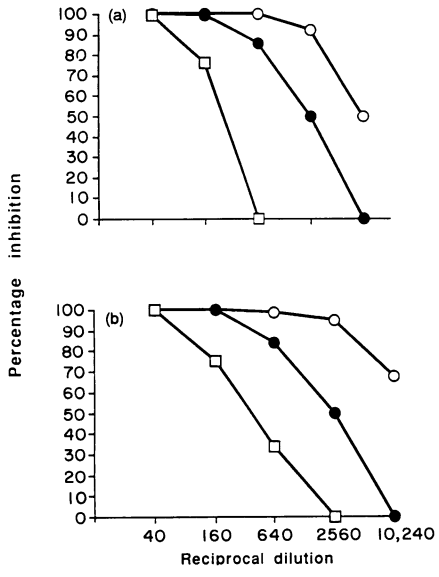

Figure 4. Titrations of IgG and IgM fractions of antisera against phosphatidylcholine liposomes and against inositol monophosphate for inhibition of TNF induction by *P. yoelii* exoantigens. (a) Antiserum against PC liposomes. The results shown are from one experiment representative of two; (b) antiserum against IMP: original antiserum (○); IgM (●); IgG (□). In the absence of antiserum the exoantigen controls induced TNF production of the order of 25,600 U/ml.

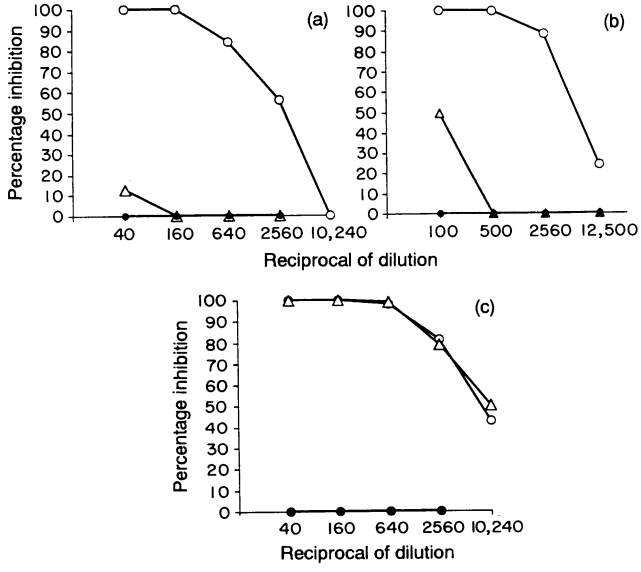

Figure 5. Titrations of antisera against cardiolipin and phosphatidylserine liposomes before and after adsorption with PC and PI liposomes, compared with antiserum against inositol monophosphate, for inhibition of TNF induction by *P. yoelii* exoantigens. (a) Antiserum against CL liposomes; (b) antiserum against PS liposomes; (c) antiserum against IMP. Liposomes used for adsorption: none (○); PC (△); PI (●). The results shown are from one experiment representative of two in the case of (a) and (c). In the absence of antiserum the exoantigens induced the production of the order of 4667 U/ml of TNF.

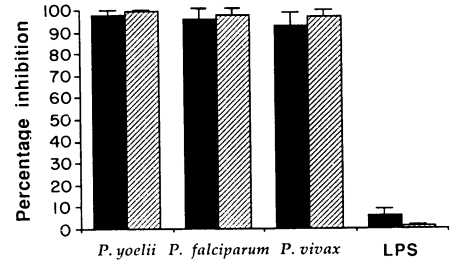

Figure 6. Inhibition of TNF induction by exoantigens of *P. yoelii*, *P. falciparum* and *P. vivax* and by LPS by antisera against liposomes and inositol monophosphate. Means ± SD obtained with antisera against liposomes (pooled results from samples made against CL, PC and PI liposomes) (■) and with antisera against IMP (▩). At least three pools were tested in each case. The antisera were tested at 1/200 final dilution against exoantigen preparations and against 200 ng/ml of LPS all of which stimulated the secretion of the order of 25,600 U/ml of TNF in the absence of antiserum.

detected in antiserum raised against these exoantigens, antisera made against exoantigens of *P. yoelii* were titrated by ELISA and compared with antiserum made against a control normal mouse erythrocyte supernatant (Fig. 1). With all the compounds tested, more Ig binding was observed in the test antiserum than in the control.

We have shown that exoantigen induction of TNF can be blocked specifically by competitive inhibition by PI and IMP (though not by inositol itself), but not by PC, PS, PE, PA or CL.[15] To investigate the specificity of the inhibitory activity of antisera against the exoantigens, we tried to adsorb this activity with the various phospholipids in the form of liposomes. No loss of activity was detected when they were adsorbed with liposomes composed of PC, CL, PS, PE or PA, but all the activity was removed by adsorption with PI lipsomes (Fig. 2).

Inhibitory activity of antisera made against liposomes

To investigate the possibility that phospholipids that cross-reacted with the antibodies present in antisera made against the parasite exoantigens might themselves give rise to inhibitory antisera, mice were immunized with liposomes composed of various phospholipids with cholesterol. Antisera raised against liposomes of PI and CL were found to possess inhibitory activity of the same order as that of specific antiserum made against the exoantigens themselves (titres of 5120–10,240) and those against PA, PC and PS were also markedly inhibitory (titres of ~640–2560); normal mouse serum had no significant activity (Fig. 3a). PC liposomes prepared without cholesterol stimulated the production of antisera which had inhibitory titres of the same magnitude as those obtained with liposomes containing cholesterol, indicating that the activity was directed against the phospholipid itself and not against the sterol (data not shown).

Antisera made against phospholipids and derivatives in non-liposomal form

When mice were immunized with various phospholipids in non-liposomal form, by injecting aliquots diluted into PBS, no inhibitory activity was detected in serum from those given PC, PS, PE or CL. By contrast, mice injected with PI in this form produced highly inhibitory antiserum (Fig. 3b). Furthermore,

mice given the related molecule IMP, which contains inositol but no diacyl glycerol, also produced inhibitory antisera of similar activity: those given inositol itself did not. Indeed, groups of mice given immunizing doses of IMP of 1 mg, 200 μg, 40 μg and 8 μg per mouse yielded antisera of the same titre; only when the dose was 1·6 μg/mouse was the titre lower (data not shown).

Since antibodies against phosphatidylinositol phosphate block the cytotoxicity of diphtheria toxin by reacting with the target cell membrane,[19] it was possible that the anti-phospholipid antisera were blocking the production of TNF similarly, by binding to receptors for the exoantigen on the surface of the macrophage. However macrophages pretreated with a 1/20 dilution of antiserum against PC or PI liposomes or against IMP or non-liposomal PI and then washed before stimulation with serial dilutions of *P. yoelii* exoantigens did not secrete any less TNF than untreated controls, indicating that the antisera were inhibitory because they reacted with the exoantigens themselves rather than with the macrophage.

Immunoglobulin isotypes responsible for inhibition

Fractionation of antisera made against PC liposomes and against IMP revealed that, like the inhibitory activity of antisera against the exoantigens,[12] most activity was present in the IgM, although some was clearly present in the IgG fraction (Fig. 4). As was found with the antisera against the exoantigens,[12] the titres of antisera made against PC liposomes and against IMP were not increased by repeated injection: when collected 12 days after one injection or after three injections at fortnightly intervals, the titres were 1280 for both samples made against PC and 5120 against IMP.

Specificity of antiphospholipid antibodies

Since it appeared that antibodies against phospholipid liposomes were inhibiting TNF induction by the parasite exoantigens by cross-reacting with a common component such as the phosphate head group, adsorption experiments were again performed to investigate the specificities of the various kinds of inhibitory antisera. Antisera made against liposomes of CL and of PS, molecules which also have diacyl glycerol in common but which differ in their polar head groups, were almost entirely depleted of inhibitory activity when adsorbed with PC liposomes and were totally depleted by adsorption with PI liposomes (Fig. 5a, b). The specificity of the inhibitory activity of antisera made against IMP, an unacylated molecule with a different head group, was, however, quite different. Whereas adsorption with PI liposomes again removed all the activity, adsorption with PC liposomes was without effect (Fig. 5c). This was consistent with our previous findings in competitive inhibition experiments in which the TNF-inducing activity of parasite exoantigens was blocked by PI and IMP, but not by PC, and suggested that antisera made against IMP possessed the same specificity as antisera against *P. yoelii* exoantigens.

Inhibition of parasite exoantigens from other species and differentiation from LPS

The TNF-inducing elements of the exoantigens of *P. falciparum* and *P. vivax* appear to embody the same phospholipids as *P. yoelii*, in that they cross-react serologically[12,13] and their activity is inhibited by detoxified exoantigens of *P. yoelii* and by PI and IMP.[15] To confirm these similarities, and again to distinguish the exoantigens from LPS, antisera against liposomes and against IMP were titrated for their ability to inhibit TNF production in response to exoantigens of *P. falciparum* and *P. vivax*, compared with *P. yoelii* and LPS, all diluted to contain similar activities. Percentage inhibition was calculated for a 1/200 final dilution of each sample of antiserum and pooled results obtained for antisera against three kinds of liposome and against three pools of IMP antiserum are illustrated (Fig. 6). All the antisera inhibited the activity of *P. yoelii*, *P. falciparum* and *P. vivax* equally: none had any significant effect on LPS.

DISCUSSION

The main conclusion from this work is that antibodies inhibiting the induction of TNF by the parasite exoantigens fall into two categories: (1) those that appear to recognize phosphatidylinositol specifically and (2) those that react more broadly with phosphate-containing molecules. Thus, the antibody in antisera made against parasite exoantigens belongs to the first group. This was evident from adsorption experiments with a variety of liposomes which showed that only those composed of phosphatidylinositol clearly and reproducibly removed the inhibitory activity and is consistent with our earlier inference that the active portion of the TNF-inducing molecule comprises covalently bound inositol and phosphate.[15] It is also supported by the surprising finding that mice immunized simply by one injection of inositol monophosphate generated inhibitory antibody. Again this antibody was specific, because it was not removed by adsorption with liposomes made of phosphatidylcholine but was removed by liposomes containing phosphatidylinositol. In competitive inhibition studies, exoantigen induction of TNF was blocked by both PI and IMP, but not by other phospholipids or monophosphate saccharides tested.[15] There is, however, an anomaly that we cannot explain. We reported previously that the presence of a lipid component of the exoantigens appeared to be necessary for the induction of inhibitory antibody, since antigen preparations deacylated by treatment with dilute NaOH or digested with lipases were not effective,[16] yet IMP, a molecule without acyl groups, acts as an excellent, highly specific, purified immunogen that induces inhibitory antibody that is active against exoantigens from the rodent parasites and from two species of human malarial parasites, *P. falciparum* and *P. vivax*. Antigens with repeating structures, a category which excludes IMP, are well known to give rise to IgM antibodies, and phosphate-containing molecules are known to give rise to antibodies of broad specificity that cross-react with other phosphate-containing molecules,[20] which makes the results obtained with IMP and the specificity of inhibition, the more remarkable. It was also striking that when administered in non-liposomal form, the only phospholipid that elicited inhibitory antibody was PI. However, although immunization with non-liposomal cardiolipin, for instance, did not elicit inhibitory antibody, it did elicit antibody which bound to CL, though not to other phospholipids, in an ELISA assay (data not shown). We do not know in what physical form the phospholipids exist when diluted in buffer, whether they occur as micelles that might be presented to B cells as repeating structures or whether, when injected, they bind to host proteins which act as carrier

molecules. Nevertheless, this inhibitory antibody elicited by the PI salt was specific (although that elicited by PI liposomes, in the one experiment tested, was not—data not shown).

The second group includes inhibitory antibodies elicited by immunization with liposomes composed of any of a range of phospholipids; these had a broad specificity. They were removed non-specifically by adsorption with liposomes containing a heterologous phospholipid, namely phosphatidylcholine, as well as by those containing phosphatidylinositol. It would appear that the cross-reacting antibodies are directed against a shared epitope, probably the phosphate ester head group common to the different phospholipids. As mentioned above, antibodies to phospholipids are known to contain a phosphate-binding subsite in the antigen-combining site that can result in cross-reactivity with many phospholipids and with soluble phosphorylated compounds; it is said to be difficult to obtain highly specific polyclonal antisera to some acidic phospholipids.[20] This cross-reactivity would explain why the specific antisera raised against parasite exoantigens reacted with a number of phospholipids (by ELISA) and possibly why DNA-binding antibodies[21] and antibodies that bind to cardiolipin[22] are found in the serum of patients with malaria. It will obviously be of interest to study the levels and specificity of such antibodies in the sera of malaria patients with varying degrees of immunity.

While investigating the production of antibodies to lipid A, Schuster and co-workers[23] reported that the injection of liposomes into rabbits did not elicit antibodies against phospholipids unless the liposomes contained lipid A, and this observation seems to be generally accepted by others. Furthermore, Banerji and Alving[24] did not obtain a primary immune response to PS or PA after a single immunization of rabbits with liposomes of these phospholipids containing lipid A. Our liposomes did not stimulate macrophages to secrete TNF, suggesting that they were not detectably contaminated with LPS, and when made in the presence of polymyxin B, which would bind to any contaminating lipid A and neutralize its biological activity,[25] they still stimulated mice to produce inhibitory antibody (data not shown). In addition, the antibody responses we obtained to phospholipids in liposomes (without inclusion of lipid A), as well as to PI and IMP, resembled those elicited by the parasite exoantigens in that repeated immunization did not significantly increase their inhibitory activity, which was predominantly associated with the IgM fraction, and there was no evidence of isotype switching (data not shown). Mice are significantly less sensitive to the lethal effects of endotoxin than rabbits (or people) and it is possible that they respond differently to phospholipids.

We have been pursuing the idea that immunization with modified parasite exoantigens which would protect individuals who then develop malaria from the effects of the excessive production of TNF might form the basis of an anti-disease vaccine.[14] Our findings now raise the question as to whether such immunization might in fact induce harmful autoimmune reactions, since anti-phospholipid antibodies, some of which cross-react with DNA, are associated with autoimmune diseases such as systemic lupus erythematosis. However, no pathological consequences have been reported in phase I and II trials of drug-containing liposomes[26] and experimental animals given repeated injections of phospholipids are known not to develop autoimmune disease. We are currently investigating the other potential stumbling block, namely the apparent T independence of the TNF-triggering antigens[12] and the consequent lack of a memory response; preliminary experiments suggest that this may be overcome by conjugation to suitable protein carriers.

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