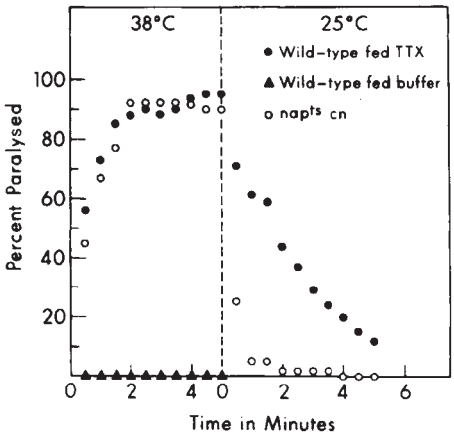

Fig. 4 Effects of temperature on wild-type flies fed sublethal doses of tetrodotoxin. Canton-S wild-type flies (3–6 days post-eclosion) were placed in vials containing two 2.5-cm filter paper circles wet with 150 μl 20 mM citrate buffer (*p*H 4.8) alone (controls) or with buffer containing 10 μg ml^{-1} (31.4 μM) tetrodotoxin (TTX). After 24 h at 21 °C this dose of TTX caused 75–90% lethality. Survivors were selected that maintained good locomotor activity and could still climb the walls of the glass culture vial. Control and treated flies were transferred to clean vials and exposed to 38 °C for 5 min. The number of flies paralysed was recorded at 30-s intervals. After 5 min, flies were transferred to 25 °C and the rate of recovery from paralysis was recorded. The paralysis of nap^{ts} mutants (genetically marked with an eye colour mutation *cn*) was determined simultaneously as a control.

recovery of the nap^{ts} mutant are shown for comparison. Although toxin-fed wild-type flies became paralysed with kinetics similar to the nap^{ts} mutant, the recovery from paralysis was slower in the toxin-fed wild-type than in the mutant. Reasons for this are unknown. Nevertheless, a reduction in number of functional channels is sufficient to produce a temperature-induced paralytic phenotype.

Using ligand binding, we have identified a subset of temperature-sensitive paralytic mutants with abnormal saxitoxin-binding phenotypes. Mutations affecting the saxitoxin-binding site will be useful for determining the genetic relationship between this and other distinct pharmacological sites of the sodium channel. Such mutants can also be used to study the developmental regulation of sodium channels, and the general role of cell excitability in development.

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Evidence for immunological cross-reaction between sporozoites and blood stages of a human malaria parasite

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Malaria parasites (*Plasmodium* spp.) show a complex pattern of development in the mammalian host and many studies support the view that the surface of the sporozoite, injected by the mosquito, has no antigens in common with the erythrocytic stage of development[1]. For example, immunization with the erythrocytic parasites generates antisera with negligible titre by indirect immunofluorescence to the sporozoite surface[2–4]. Although monoclonal antibodies prepared against erythrocytic stages were reported to show cross-reaction to the sporozoite stage[5], this appeared to be due to cytoplasmic antigens exposed by the method of sporozoite preparation[3,5], and in *Plasmodium knowlesi*, a cDNA clone coding for the circumsporozoite antigen, the major protein of the sporozoite surface, showed no hydridization to mRNA isolated from the erythrocytic stages[6]. Here, however, we present evidence for an antigenic determinant shared by the sporozoite surface and the erythrocytic stages of the human malaria parasite, *P. falciparum*. Moreover, our studies show that the antigen(s) elicit a strong immune response in man.

Monoclonal antibodies against the cultured erythrocytic stages of *P. falciparum* Kl, a Thai isolate[7], were tested by indirect immunofluorescence microscopy against *P. falciparum* sporozoites. One of the 22 tested, McAb 5.1, gave a strongly positive reaction to the sporozoite preparation (Fig. 1*a*). The titre of the McAb 5.1 ascites fluid was very high (1 in 10^6 dilution) and equally active on sporozoites and erythrocytic stages. The other monoclonal antibodies tested did not stain sporozoites at a dilution of 1:100 (Fig. 1*b*).

Both air-dried and glutaraldehyde-fixed sporozoites are stained by indirect immunofluorescence with McAb 5.1. The latter method of preparation only allows antibody to bind to surface determinants[3] and the clear microscope image it provides is strong evidence that the antigen is located at the sporozoite surface. These results should be compared with indirect immunofluorescence of the erythrocytic parasites. Here the 5.1 epitope appears to be associated with the parasitophorous vacuole and parasite-derived inclusions in the infected red blood cell (ref. 7 and Fig. 1*c*). Another monoclonal antibody, 7.7, gives an identical immunofluorescence pattern[7] to McAb 5.1 in infected red blood cells but does not react with the sporozoite stage (data not shown). Different immunofluorescence patterns are obtained on infected red blood cells with other monoclonal antibodies, for example McAb 7.3[7] (Fig. 1*d*).

The 5.1 antigen can be clearly detected in SDS extracts of parasites prepared by saponin lysis of infected red blood cells. The proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2, lanes 1, 2), transferred to nitrocellulose, probed with McAb 5.1 and the antigen visualized by enzyme-linked immunosorbent assay (ELISA)[8] (Fig. 2, lane 4). A single band carries the 5.1 epitope. It migrates as a protein of molecular weight (M_r) 23,000. The antigen can be partially

purified from Nonidet P-40 (NP40) extracts of parasitized erythrocytes on a McAb 5.1–Sepharose column, despite low affinity of the antibody for its antigen (which complicated earlier studies[7]). This preparation (Fig. 2, lanes 3, 5) also shows a single band carrying the epitope migrating slightly faster (M_r 22,000) than that in the unpurified extracts, perhaps because the protein is cleaved during purification.

The 5.1 antigen is parasite-encoded. NP40 extracts from parasites metabolically labelled with ^{35}S-methionine were analysed by affinity chromatography and gel electrophoresis. The separated proteins were visualized by autoradiography (Fig. 2, lanes 6, 7). A unique, labelled protein band (M_r 22,000) is retained by the McAb 5.1 affinity column (lane 7).

We now present evidence that the 5.1 epitope is determined by the primary amino acid sequence of the antigen molecule. *P. falciparum* mRNA[9] can be translated in rabbit reticulocyte extracts[10] into proteins which are not processed or modified. The McAb 5.1–Sepharose column traps several of the *in vitro* products nonspecifically (Fig. 2, lane 9). These are also trapped by other monoclonal antibody–Sepharose columns (see lane 10). However, the McAb 5.1 column retains a unique protein, M_r 24,500 (lane 9). No other column (3/3 tested) retains this protein (see lane 10). It is reasonable to conclude that it carries the 5.1 epitope in the amino acid sequence. Note that the primary transcript appears larger than the mature antigen, suggesting that it is processed or modified *in vivo*.

These results lead to the important conclusion that the 5.1 epitope of the sporozoite and erythrocytic stages is not a common modification of two entirely different proteins, but the same amino acid sequence(s) found in two proteins made at different stages of the life cycle.

The 5.1 antigen is recognized by human sera from areas (Nigeria or Gambia) endemic for *P. falciparum* (Fig. 3). Total parasite proteins and proteins immunoadsorbed on McAb 5.1–Sepharose were subjected to SDS-PAGE (Fig. 3*a*). They were transferred to nitrocellulose and probed by ELISA. For comparison we studied proteins immunoadsorbed by McAb 7.7 which gives the same immunofluorescence pattern as McAb 5.1. Shown in Fig. 3, the following antibody probes were used: *b*, a mixture of monoclonal antibodies 5.1 and 7.7; *c*, sera (pooled) from individuals living in an area endemic for *P. falciparum* malaria; and *d*, serum from an individual never exposed to *P. falciparum*.

The bands revealed by endemic sera (Fig. 3*c*) and monoclonal antibodies 5.1 and 7.7 (Fig. 3*b*) are specific. No bands are seen with the nonimmune serum (Fig. 3*d*) or with other monoclonal antibody controls (data not shown). Figure 3*b* shows the 5.1 antigen (M_r 22,000, 22K). It is quite distinct from the 7.7 antigen (33K). Both appear together in the total parasite protein (Fig. 3*b*, lane 1) and separately in the purified antigen preparations (lanes 2, 3). The pooled human sera (Fig. 3*c*) have no detectable antibodies against the purified 7.7 antigen (Fig. 3*c*, lane 3). By contrast, the purified 5.1 antigen is very strongly recognized (lane 2). Note that the pooled human sera detect many antigens amongst the total parasite proteins (lane 1) but there is one particularly strong band (arrow). A comparison of antigen band positions and intensities in tracks 1 and 2 of Fig. 3*b* and *c* suggests that this is the 5.1 antigen. In fact, Sepharose-bound 5.1 antigen reacts with as much as 1% of the antibodies in these pooled sera (data not shown).

We conclude that a major immunogenic protein of the erythrocytic stage of *P. falciparum* contains an antigenic determinant also present in a protein at the surface of sporozoites. In this connection we were interested to learn that Coppel and coworkers[11] have identified a 220K protein of the erythrocytic stages with an immunofluorescence pattern similar, if not identical, to that of the 5.1 antigen. Remarkably, it has random repeats of an 11 amino acid sequence. This repetitive feature has also been described in the major circumsporozoite protein of *P. knowlesi*[6].

The blood stage antigen bearing the 5.1 epitope appears highly immunogenic; why then has immune cross-reaction to the sporozoite surface not been detected experimentally? One explanation, with important implications, is that the 5.1 antigen becomes strongly recognized by human sera only through long-term continual exposure to the antigen, as experienced by individuals living in areas endemic for malaria. Experimental tests for stage cross-reactivity of monoclonal antibodies or antisera, raised by short-term immunization protocols, would not reveal this epitope.

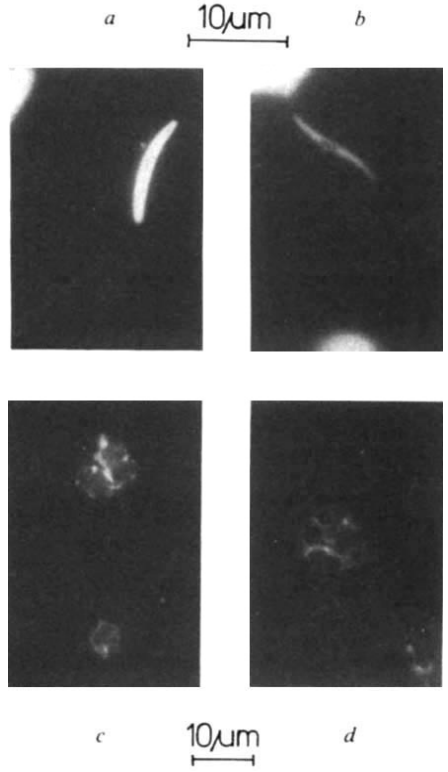

Fig. 1 Indirect immunofluorescence staining of *P. falciparum* microscope preparations using monoclonal antibodies. *a, b*, Glutaraldehyde-fixed sporozoites collected in Thailand (a gift of Dr Ruth Nussenzweig). *c, d*, Air-dried thin blood smears of an asynchronous *in vitro* culture[13] of *P. falciparum* (isolate Kl from Thailand[14]). The parasitaemia was 5%.
Methods: All slides were fixed in acetone for 5 min. The staining was carried out at room temperature in a moist chamber as detailed in ref. 7. The slides were incubated with monoclonal antibodies for 30 min, stained with fluorescein isothiocyanate-conjugated polyvalent rabbit anti-mouse immunoglobulin (Miles-Yeda) and counterstained with Evans blue (0.1% in phosphate-buffered saline (PBS)). The slides were examined by fluorescence microscopy after mounting in 50% glycerol. *a, c*, Monoclonal antibody 5.1 was used in the primary incubation; *b, d*, for comparison, the staining produced by a different monoclonal antibody called 7.3 (see ref. 7). Bound antibody appears as a vivid green fluorescence (*a, c, d*) whereas parasites which do not bind antibody (*b*) appear red. Photographs were taken at ×1,000 magnification using Kodak Tri-X Pan film.

It is interesting that McAb 7.7 recognizes an epitope on a totally different molecule from McAb 5.1 despite the fact that they cannot be distinguished by immunofluorescence microscopy. It is particularly striking that the 22K protein of McAb 5.1 is very immunogenic in man whilst the 33K protein of McAb 7.7 elicits no detectable immune response. It is also relevant that, unlike the 7.7 epitope, that of 5.1 is not present in all strains of *P. falciparum*[7].

Natural immunity to malaria of individuals living in endemic areas is only fully acquired by adolescence[12]. An antigen to which an immune response is only raised very slowly therefore may be of importance in the development of immunity. Such an antigen would be of particular importance if it were present on both the sporozoite and erythrocytic stages of the parasite.

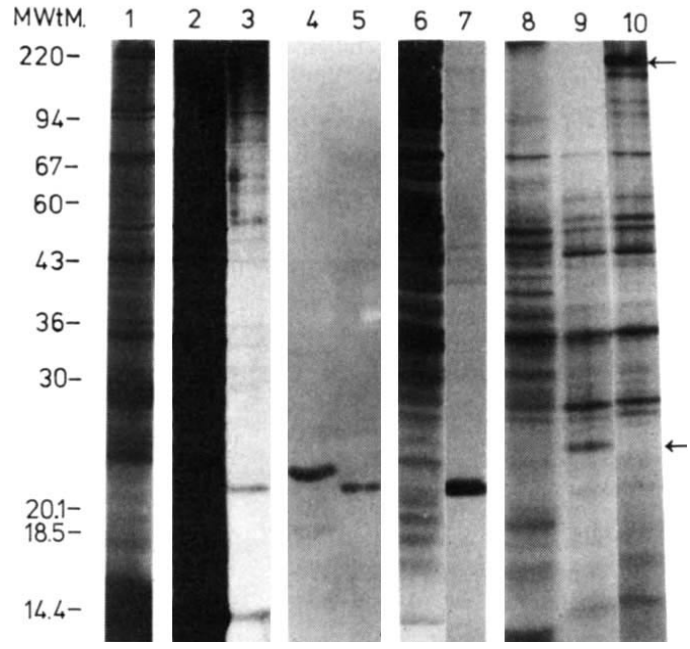

Fig. 2 Characterization of 5.1 antigen. Proteins were separated by SDS-PAGE[15] on 10% gels. Lane 1 contains total parasite protein stained with Coomassie brilliant blue R. Lanes 2 and 3 contain, respectively, total parasite protein and partially purified 5.1 antigen visualized by silver staining[16]. The position of 5.1 antigen is revealed in lanes 4 and 5 by Western blotting[8] of total parasite proteins (lane 4) and partially purified antigen (lane 5) and detection by an ELISA procedure[8]. Lanes 6–10 show autoradiographed, ^{35}S-methionine-labelled proteins. Total parasite proteins (lane 6) and 5.1 antigen (lane 7) were metabolically labelled in culture. Lane 8 shows total parasite proteins synthesized by *in vitro* translation of *P. falciparum* mRNA. From the total *in vitro* translation products 5.1 antigen (lane 9) has been partially purified on a 5.1 monoclonal antibody affinity column. For comparison, lane 10 shows the unrelated antigen, P190, recognized by McAb 7.3 (ref. 7), prepared in a similar way. Specifically adsorbed bands in the two lanes are arrowed.

Methods: The proteins in lanes 1 and 2 were prepared from an asynchronous *in vitro* culture[13] of *P. falciparum* (5×10^9 erythrocytes; 5% parasitaemia), washed once in PBS and lysed in 0.1% saponin in PBS at 4 °C. Released parasites were washed three times in PBS before pelleting and freezing at −70 °C until required. For electrophoresis, the pellet was solubilized by boiling in gel sample buffer. Lane 2 was deliberately overloaded to allow detection of the antigen after Western blotting (see lane 4 below), since the 5.1 antigen constitutes a minor proportion of the total proteins (compare lanes 2 and 3 with 4 and 5). 5.1 antigen (lane 3) was prepared by affinity chromatography. An asynchronous parasite protein extract, solubilized with NP40 (BDH)[17], was applied to a 5.1 monoclonal antibody affinity column. This was prepared by binding McAb 5.1 (purified from ascites fluid by $(NH_4)_2SO_4$ precipitation and DEAE ion-exchange chromatography) to CNBr-activated Sepharose 4B (Pharmacia). After applying the extrct the column was washed briefly in the extraction buffer[17], before elution of bound proteins in 8 M urea (4 °C). Proteins of lanes 2 and 3 were transferred to nitrocellulose paper (lanes 4, 5) by Western blotting[8] and the position of 5.1 antigen was revealed by ELISA[8]. The nitrocellulose blot was first probed with McAb 5.1 (ascites fluid at a 1 in 200 dilution), then a polyvalent rabbit anti-mouse IgG (serum at a 1:500 dilution) and finally with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) (at a 1:1,000 dilution). The conjugated enzyme was reacted with *o*-dianisidine dihydrochloride (Sigma) and hydrogen peroxide, giving a brown colour at the position of 5.1 antigen. The parasite proteins in lanes 6 and 7 were labelled metabolically with ^{35}S-methionine in an asynchronous culture[13] of *P. falciparum* by the method of Deans *et al.*[18]. Proteins of a total NP40 extract (see above) of the labelled culture appear in lane 6 (3,000 c.p.m. of trichloroacetic acid (TCA)-precipitable material). Lane 7 shows protein purified by the McAb 5.1 affinity column (see above) from the labelled parasite extract (3×10^6 c.p.m. of TCA-precipitable material). The parasite proteins in lanes 8, 9 and 10 were synthesized in a reticulocyte lysate *in vitro* translation system[10] supplemented with ^{35}S-methionine and *P. falciparum* poly(A)$^+$ mRNA from asynchronous cultures[9]. Lane 8 shows total *in vitro* translation products (5,000 c.p.m. of TCA-precipitable material). Lane 9 shows proteins adsorbed from the total *in vitro* translation products (5×10^6 c.p.m. of TCA-precipitable material) on a McAb 5.1 affinity column (see above). Lane 10 shows the translation products adsorbed in the same way, by an affinity column coupled with a different monoclonal antibody, McAb 7.3 (ref. 7). The gel was processed for fluorography[19], dried and autoradiographed (lanes 6 and 10 for 3 weeks and lanes 7, 8 and 9 for 1 week). Molecular weight markers (Pharmacia): ferritin (220,000), phosphorylase *b* (94,000), albumin (67,000), catalase (60,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), ferritin (18,500) and α-lactalbumin (14,400).

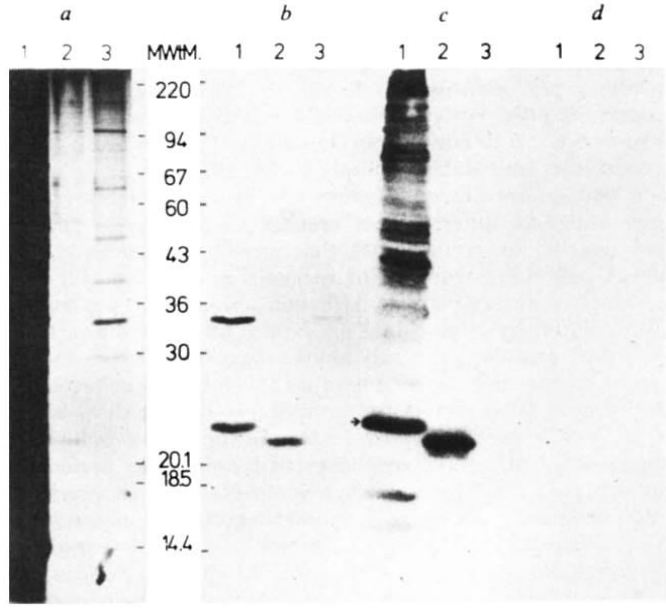

Fig. 3 Recognition of 5.1 antigen by human sera. In all lanes proteins were separated by SDS-PAGE[15] on 10% gels. In *a*, the proteins were visualized by silver-staining[16]. Lane 1 is total parasite protein (see Fig. 2). This lane is overloaded to enable antigens to be seen after Western blotting (see below). Lane 2 is partially purified 5.1 antigen (see Fig. 2). Lane 3 is partially purified 7.7 antigen prepared by affinity chromatography using a 7.7 monoclonal antibody affinity column. (Methods in Fig. 2.) *b*, *c*, *d*, The proteins of *a* transferred to nitrocellulose by the Western blotting procedure[8]. These blots were then probed with antibody by the ELISA procedure, as described for Fig. 2. Blot *d* was initially probed with human serum (1:200 dilution) from an individual never exposed to *P. falciparum* malaria. Blot *c* was initially probed with human sera (1:200 dilution) obtained by pooling 50 serum samples from pregnant women living in Nigeria, an area endemic for malaria. Both blots *c* and *d* were then probed with horseradish peroxidase-conjugated goat anti-human IgG (Sigma) (1:1,000 dilution). Blot *b* was probed first with the monoclonal antibodies 5.1 and 7.7 (1:200 dilution) and then probed using polyvalent rabbit anti-mouse IgG (1:500 dilution) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000 dilution). For all blots *b*, *c* and *d*, the position of bound antibody was determined using the enzymic colour reaction as detailed in Fig. 2. Molecular weight markers are as in Fig. 2.

Our studies raise two major questions: are the antibodies against the 5.1 antigen in endemic sera protective, and, do the majority of them cross-react with sporozoites? Since we are now able to extract significant amounts of the 5.1 antigen from erythrocytic parasites, it should be possible to investigate these questions.

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Lymphocyte suppression in leprosy induced by unique *M. leprae* glycolipid

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Leprosy remains a significant medical and social problem in many developing countries. The varied forms of the disease form a spectrum[1]. At one pole, tuberculoid leprosy, patients develop high levels of cell-mediated immunity which results in the killing and clearing of bacilli in the tissues. At the lepromatous pole, patients exhibit a selective immunological unresponsiveness to antigens of *Mycobacterium leprae* so that the organisms inexorably multiply in the skin. We have suggested that in lepromatous leprosy one or a small number of unique antigenic determinants present on *M. leprae* might induce specific suppressor cells that inhibit the reactivity of helper T-cell clones capable of recognizing other specific or cross reactive determinants[2]. Although unique epitopes have been identified by monoclonal antibodies on a small number of *M. leprae* proteins[3], the only unique species of antigen present in *M. leprae*, and not on any other species of mycobacteria so far examined, is a phenolic glycolipid (gly-I)[4]. We show here that this unique antigen of *M. leprae* is capable of inducing suppression of mitogenic responses of lepromatous patients' lymphocytes *in vitro* and provide evidence that the suppressor T cells recognize the specific terminal trisaccharide moiety.

It remains unclear why the vast majority of people exposed to *M. leprae* develop no clinical disease, and why only a minority who do develop clinical disease become immunologically unresponsive to antigens of the organisms. Lepromatous patients who are anergic to antigens of *M. leprae* frequently show cell-mediated immunity to antigens of the tubercle bacillus although all known species of protein and glycoprotein antigens in *M. leprae* are either serologically identical or cross-reactive with those of BCG[5]. Some experimental support *in vitro* for the existence of lepromin-induced suppressor activity of mononuclear cells from lepromatous leprosy patients has appeared. Adherent cells from lepromatous patients, presumably monocytes, have been found to suppress mitogen and antigen responses *in vitro*[5–8]. Lepromin-induced suppressor T cells have also been implicated in some circumstances. We have previously reported that 84% of patients with lepromatous leprosy have a T cell bearing the OKT5/OKT8 phenotype which can be induced by lepromin to suppress responses of the patients or normal donors lymphocytes to the mitogen, concanavalin A (Con A)[6]. These suppressor cells (T_S) are active in patients with lepromatous and borderline leprosy, but not tuberculoid leprosy or in normal donors. A high percentage (approximately 50%) of the T8 cells from lepromatous patients expressed the activation markers, Fc IgG receptors and HLA-DR antigens[9]. Depletion of these T8 cells in a third of the patients with lepromatous leprosy permitted the remaining T cells to respond vigorously to lepromin, indicating that, in some patients, specifically reactive T cells were present, but unable to respond in the presence of the T suppressor cells.

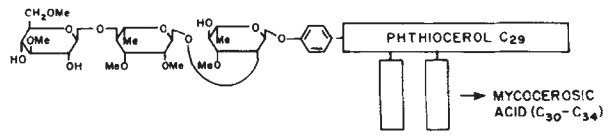

Fig. 1 Schematic structure of *M. leprae* phenolic gly-I. It is composed of 3,6-di-*O*-methylglucose, 2,3-di-*O*-methylrhamnose, 3-*O*-methylrhamnose linked to phenol-dimycocerosyl phthiocerol.

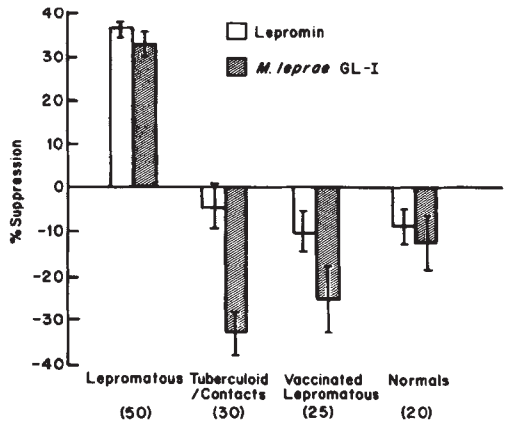

Fig. 2 Suppression of mitogenic responses of peripheral blood lymphocytes of leprosy patients and normals to Con A in the presence of *M. leprae* specific phenolic gly-I and Dharmendra lepromin. Peripheral blood mononuclear cells were obtained from heparinized blood by Ficoll-Hypaque centrifugation. The suppression of mitogenic response of lymphocytes to Con A, induced by lepromin and phenolic glycolipid, was assayed as described earlier[2]. Briefly, 2×10^5 lymphocytes were cultured in triplicate in RPMI 1640 containing 10% heat inactivated pooled human AB serum with (1) no additions, (2) 2.5 μg ml^{-1} Con A, (3) Con A plus Dharmendra lepromin (1:10), 0.5 μg ml^{-1} *M. leprae* phenolic gly-I in the form of liposomes or control liposomes. Liposomes were prepared by the method of Six *et al.*[12]. A mixture containing 2.0 mg sphingomyelin, 0.73 mg cholesterol, 0.065 mg dicetylphosphate and 0.23 mg *M. leprae* gly-I in 125 μl of Tris-NaCl buffer (*p*H 8.0) was sonicated for 1 h with glass beads. The cultures were labelled on day 2 with 1 μCi ^{3}H-thymidine (specific activity 5 Ci mmol^{-1}) per well and harvested 18 h later. The data were analysed by analysis of variance and Duncan's multiple range test. Significance was accepted at the $P<0.05$ level. Numbers in parenthesis represent the number of subjects studied in each group.