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Lymphoproliferative responses to a merozoite surface antigen of *Plasmodium falciparum*: preliminary evidence for seasonal activation of CD8$^+$/HLA-DQ-restricted suppressor cells

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SUMMARY

We have investigated the phenotype of human lymphocytes responding to a defined *Plasmodium falciparum* malaria antigen *in vitro*. Cells were obtained from the peripheral blood of malaria-immune donors from an endemic area of West Africa and were tested for proliferation in response to cloned fragments of a merozoite surface protein (PfMSP1). Depletion and inhibition studies indicated that the majority of proliferating cells were CD4$^+$ and restricted by HLA-DR or -DQ. A proportion of responding cells appeared to be CD8$^+$, but their response was dependent on help from CD4$^+$ cells. In two donors there was evidence that low responses could be enhanced by removal of CD8$^+$ cells and/or blocking of antigen presentation by anti-HLA-DQ antibodies. This phenomenon was observed in cells collected during the wet (malaria transmission) season but not in cells collected from the same individual during the dry season.

Keywords malaria PfMSP1 immunoregulation HLA-DQ

INTRODUCTION

In malaria-endemic areas, clinical immunity to malaria is acquired in an age-dependent fashion related to the level of malaria transmission. Complete sterilizing immunity is rarely achieved and periodic re-infection occurs throughout life. Both cell-mediated and antibody-mediated mechanisms are believed to contribute to clinical immunity, but can also contribute to the pathology of malaria infection. For example, macrophages—activated by T cell-derived interferon-gamma (IFN-γ)—produce tumour necrosis factor-alpha (TNF-α) which may result in damage to the host as well as the parasite (reviewed in [1]). Similarly, unregulated activation of B cells can lead to hypergammaglobulinaemia and hyper-reactive malarial splenomegaly [2]. Thus, in the face of persistent antigenic stimulation, the antimalarial immune response must be carefully regulated to maintain the delicate balance between protection from infection and avoidance of immune-mediated pathology.

It has been proposed [3] that the primary function of particular subsets of T cells is to down-regulate immune responses to antigens which cannot be eliminated from the body, such as autoantigens and intracellular parasites, and there is evidence to suggest that cellular immune responses to malarial antigens are down-regulated in individuals with persistent long-term exposure to *Plasmodium falciparum* or *P. vivax* [4,5]. Peripheral blood mononuclear cells (PBMC) from a proportion of apparently immune individuals are non-responsive to crude or partially purified schizont extracts, but non-responsiveness can be reversed by removal of CD8$^+$ cells from the PBMC population [6–9]. It has also been suggested that blocking HLA-DQ molecules on the surface of antigen-presenting cells will reverse non-responsiveness to malaria antigens [7]. A similar phenomenon of HLA-DQ-associated T cell non-responsiveness has been described for the human immune response to *Schistosoma japonicum* soluble egg antigen [10], *Mycobacterium leprae* [11,12] and streptococcal cell wall antigens [13].

We have examined the response of human peripheral T cell populations to a defined antigen of *P. falciparum*, the major merozoite surface antigen (PfMSP1). We were able to show that depletion of CD8$^+$ cells or blocking of HLA-DQ increased the proliferative response to PfMSP1 in some non-responding donors, but this effect was seen only during the malaria transmission season when recent exposure to parasite antigens might be expected.

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SUBJECTS AND METHODS

Subjects

Nine adults (three female, six male, age range 18–55 years) were recruited from a rural village in the Gambia where malaria is

Table 1. Lymphoproliferative responses to PfMSP1 proteins and phytohaemagglutinin (PHA)

	Dry season					Wet season				
Donor	Unstim.	pME1	pME6	pME14	PHA	Unstim.	pME1	pME6	pME14	PHA
7SC	334	1478	2328	375	73 580	450	5715	6975	4455	50 580
50OS	**351**	**2854**	**1867**	**1566**	**38 505**	425	4080	2040	1445	29 070
107FK	**161**	**1215**	**1201**	**859**	**59 103**	**287**	**2066**	**3329**	**5855**	**24 710**
1MS	126	1439	747	19 302	22 415	311	2768	5536	7153	30 167
28BC	99	949	2260	349	46 352	2140	7918	4708	19 902	17 976
122IS	115	14 119	10 087	291	35 029	454	3042	3314	1998	23 789
69JC	123	5882	833	181	58 536	**106**	**625**	**541**	**625**	**25 546**
79AJ	96	11 853	9620	758	107 741	128	358	269	256	35 456
82AS	193	23 601	12 178	687	50 161	**529**	**3862**	**3227**	**3333**	**28 883**

Geometric mean ct/min. Values which are underlined are those where the SI (*versus* the appropriate fusion partner control) is > 3. Values shown in bold indicate that the donor was parasitaemic at the time the blood was collected.

seasonally endemic (peak transmission occurs from July to November after the annual rains; point prevalence parasitaemia is approximately 30% in adults at the end of the transmission season [14]). All the subjects were long term residents of the village and had been exposed to *P. falciparum* malaria throughout their lives. Blood samples were collected at the end of the dry season, after several months of little or no malaria transmission, and again at the end of the malaria transmission season.

Venous blood samples were collected into a sterile heparinized container. Thick and thin blood films were stained with Giemsa's stain and examined for malaria parasites. Two of the nine donors had low levels of parasitaemia at the end of the dry season (approximately five asexual stage parasites/μl); three donors were infected at the end of the transmission season (2–7 asexual stage parasites/μl).

Batches of pooled serum were screened for HIV-1 and HIV-2 seropositivity; all pools were negative.

Antigens

PfMSP1 proteins were derived from the Wellcome strain of *P. falciparum* [15]. Proteins pME1, pME6 and pME14 were derived as βgalactosidase or TrpE′ fusion proteins from recombinant *Escherichia coli* expression vectors [16], purified by solubility fractionation or affinity chromatography and freeze dried. Freeze-dried proteins were reconstituted in sterile PBS and diluted in culture medium. pME1 represents the N-terminal half of PfMSP1 (blocks 3–11 according to the scheme of Tanabe *et al.* [17]), pME6 represents block 3 plus part of block 4 (i.e. is contained within pME1), and pME14 represents blocks 10–16 towards the C-terminus of the molecule.

The mitogen, phytohaemagglutinin (PHA; Difco, Detroit, MI) was used as a positive control for cell viability.

Preparation of PBMC populations

PBMC were isolated from heparinized blood by density gradient centrifugation (Lymphoprep, Nygaard, Norway). Washed cells were suspended in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, 30 mM HEPES, 0·22% sodium bicarbonate and 10% non-immune human serum.

$CD4^+$ and $CD8^+$ T cells were selectively removed from mixed PBMC preparations using anti-CD4 or anti-CD8-coated magnetic beads (Dynal, Oslo, Norway). Briefly, aliquots of washed mononuclear cells were suspended in complete culture medium at a concentration of 10^6 cells/ml. Antibody-coated magnetic beads were added to the cell suspension and incubated at room temperature for 1 h. Beads, with specifically bound $CD4^+$ or $CD8^+$ lymphocytes, were drawn to the side of the tube using a magnet and the supernatant (together with the unbound cell population) removed into a clean tube. The depleted cell preparation was washed and resuspended to its original volume in complete culture medium. The efficiency of depletion was determined by immunofluorescence and flow cytometry.

Lymphocyte proliferation assays

PBMC, or depleted cell populations, were aliquoted into round-bottomed, 96-well microtitre plates (100 μl/well). Purified PfMSP1 proteins and fusion partner controls (final concentration 1 μg/ml) or PHA (2 μg/ml) were added to triplicate wells to form a final culture volume of 200 μl/well. Optimal antigen concentration was determined by titration experiments (data not shown). Control wells were cultured in the absence of any antigen or mitogen. Cultures were incubated at 37°C in 5% CO_2 for 7 days. Eighteen hours before harvesting, cells were pulsed with 1 μCi ^{3}H-thymidine (Amersham, Aylesbury, UK). Cellular incorporation of ^{3}H-thymidine was measured by liquid scintillation counting. Proliferative responses were calculated as the geometric mean (gm) ct/min of triplicate wells. Stimulation indices (SI) are calculated as the gm ct/min of triplicate antigen-stimulated wells divided by the gm ct/min of appropriate (fusion partner) control wells.

Antibodies to HLA class II antigens

Antigen presentation via HLA class II antigens was selectively blocked using MoAbs directed against monomorphic epitopes of HLA-DR or HLA-DQ (Leu-10) (Becton Dickinson, San Jose, CA). Antibodies were diluted in complete culture medium and added to cell cultures on day 0. Optimal antibody concentration was determined by titration (data not shown).

RESULTS

Lymphoproliferative responses to PfMSP1

Lymphoproliferative responses to recombinant PfMSP1 antigens are shown in Table 1. In the dry season, cells from all nine

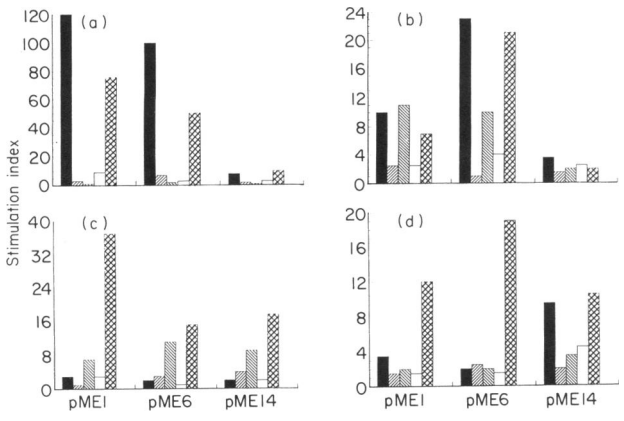

Fig. 1. Effect of depletion of $CD4^+$ or $CD8^+$ T cells, or additon of anti-DR or anti-DQ antibodies, on the proliferative response (SI) to PfMSP1 proteins in two donors. Responses of the other seven donors were not significantly different from the examples shown in (a) and (b); no significant seasonal changes, other than those shown, were observed. (a, b) Dry season study. (c, d) Wet season study. (a, c) Donor no. 79. (b, d) Donor no. 82. ■, Untreated; ■, $CD4^+$-depleted; ▨, $CD8^+$-depleted; □, with anti-DR antibody; ▩, with anti-DQ antibody.

donors tested gave positive proliferative responses (defined as SI > 3) to pME1 and pME6, but only two responded to pME14. In the wet season 8/9 donors responded to pME1 and pME6, but four donors responded to pME14.

Patent parasitaemia during the wet season was accompanied by decreased proliferative responses to PfMSP1 in two donors (nos 69 and 82; Table 1). The responses of one donor with patent parasitaemia in the dry season (no. 50) were not noticeably lower in comparison with responses of the same individual in the wet season. One donor (no. 107) was parasitaemic on both occasions. Cells from one donor (no. 79) showed a much lower response in the wet season than in the dry season, although this donor was not apparently infected on either occasion.

Phenotype of responding cells

Immunofluorescence staining revealed that anti-CD4-coated magnetic beads removed approximately 99% of $CD4^+$ cells, but that anti-CD8-coated beads removed only 57% of $CD8^+$ cells. A further round of depletion improved the $CD8^+$ depletion efficiency to approximately 75%. $CD4^+$-depleted or $CD8^+$-depleted cells were cultured for 7 days with pME antigens and the proliferative response of the depleted population compared with the response of the intact population (Fig. 1).

Proliferative responses of PHA-stimulated cultures were not significantly affected by the treatment regime (data not shown), indicating that the treatment was not intrinsically harmful to the cells. In all donors responses to PfMSP1 were completely ablated by depletion of $CD4^+$ cells. Responses were slightly decreased after $CD8^+$ depletion in some donors, indicating that some of the responding cells are $CD8^+$, although these cells did not appear to proliferate in the absence of $CD4^+$ cells.

In the dry season there was no indication that responses to any of the PfMSP1 antigens were enhanced by removal of $CD8^+$ cells. However, in the wet season responses of cells from one of the nine donors (no. 79) were clearly enhanced after removal of $CD8^+$ cells (Fig. 1). The responses of cells from this donor showed a markedly lower proliferative response to PfMSP1 antigens in the wet season than in the preceding dry season (Table 1).

Effect of blocking antigen presentation by HLA class II molecules

Antibodies to HLA-DR markedly inhibited the proliferative response to all the malaria antigens in all donors, but had no significant effect on the proliferative response to PHA (data not shown). Anti-DQ antibodies had a more variable effect, but it was clear that a proportion of the proliferating cells were DQ-restricted (Fig. 1).

In the dry season there was no evidence that anti-DQ antibodies enhanced the proliferative response to any of the antigens. However, in the wet season the proliferative responses of cells from two donors (nos 79 and 82) were clearly enhanced after blocking of antigen presentation via HLA-DQ.

DISCUSSION

The notion that persistent infections eventually lead to down-regulation of the anti-parasitic immune response, with consequent reduction of immune-mediated pathology, is not a new one [18], although the mechanisms by which such immune modulation may occur are still rather unclear. In the case of parasitic helminths, which are typically long lived but which do not multiply within a single host, selective immunological tolerance via induction of specific T cell anergy appears to be a satisfactory solution [19,20]. The problem in infections such as malaria is maintaining the balance between controlling the infection (keeping parasitaemia below levels at which clinical symptoms appear) and avoiding the pathological consequences of persistent immune activation. In this case, a more dynamic concept of immune regulation may be appropriate.

Previous studies have indicated that in some malaria-immune donors proliferative responses to crude schizont antigen preparations can be enhanced by removal of the $CD8^+$ lymphocyte population from the culture system [6–9], but the effect of $CD8^+$ depletion on responses to defined malaria antigens has not been investigated. We have examined the phenotype of cells proliferating in response to one such antigen, PfMSP1. The responding cell population appears to be predominantly $CD4^+$ and HLA-DR-restricted, but a smaller population of $CD8^+$ cells—which may be HLA-DQ restricted—can also respond.

Although there was no consistent difference in proliferative responses to PfMSP1 antigens between the two seasons, responses of several donors were noticeably lower in the wet season than in the dry season. At the end of the dry season, all donors responded to the related constructs pME1 and pME6, but only two donors responded to pME14. The response to pME14 in the non-responders could not be enhanced by removal of $CD8^+$ cells or blocking of HLA-DQ molecules. In contrast, at the end of the wet season, the response to pME1 and pME6 (but not to the fusion partner controls) in the one non-responding donor (no. 79) could be enhanced by either removal of $CD8^+$ cells or blocking of HLA-DQ. There was a similar but

less marked effect on the response to pME14. Anti-DQ antibodies also slightly enhanced the response to PfMSP1 proteins (but not fusion partner controls) in one other donor (no. 82), where the response in the wet season was significantly lower than in the dry season. In this case, removal of $CD8^+$ cells had no effect.

Taken together, these results suggest that ($CD8^+$) DQ-restricted cells may be activated during periods of malaria transmission to down-regulate the cellular proliferative response to PfMSP1. Seasonal variation in the suppressive effects of $CD8^+$ cells has been reported previously [8] ($CD8^+$ cells isolated from malaria-exposed donors during the malaria transmission season had suppressive activity whereas cells isolated in the dry season did not), but the only other study of DQ-mediated suppression of responses to malaria antigens [7] was conducted in an area of year-round malaria transmission where such seasonal changes would not be expected.

DQ-mediated suppression of lymphoproliferation does not appear to be a universal phenomenon. In this study, the effect was observed in only two of nine donors. The effect was not observed in two other donors (nos 122 and 69), whose responses were also lower in the wet season than in the dry season. Similarly, anti-DQ antibodies enhanced the proliferative response to *Myco. leprae* in 4/22 patients in one study [11] and in 1/18 patients in another study [21]. It is possible that individuals expressing different class II alleles may recognize a different array of T cell epitopes with varying susceptibility to DQ-mediated suppression. In the case of malaria, many of the crucial cellular interactions occur in the deep vasculature of the spleen and liver, and it is possible that cells isolated from the peripheral blood at any one time represent only a subset of the total malaria-reactive T cell pool. Although not directly related to patent malarial parasitaemia in the donor, the fact that DQ-mediated suppression was evident only during the malaria transmission season suggests that recent exposure to infection may lead to alterations in the composition of the circulating T cell population.

In this study we have confirmed that $CD8^+$ and/or DQ-restricted cells can down-regulate proliferative responses to malaria antigens and have demonstrated these effects using purified, defined antigens rather than crude parasite extracts. Whilst far from conclusive, these data tend to support the notion that the observed response to PfMSP1 is the net result of activation of a variety of cell populations leading to either stimulation or suppression of T cell-mediated immunity. Further studies are required to elucidate the mechanism of T cell regulation in individuals with repeated, long term exposure to malaria.

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REFERENCES

1 Clark IA, Cowden WB. Roles of TNF in malaria and other parasitic infections. In: Aggarwal BB, Vilcek JT, eds. Tumor necrosis factors: structures, regulation and functions. New York: Marcel Dekker, 1990:365–407.

2 Crane GG. Hyper-reactive malarious splenomegaly (tropical splenomegaly syndrome). Parasitol Today 1986; **2**:4–9.

3 Bloom BR, Salgame P, Diamond B. Revisiting and revising suppressor T cells. Immunol Today 1992; **13**:131–6.

4 Riley EM, Allen SJ, Wheeler JG *et al.* Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity. Parasite Immunol 1992; **14**:321–37.

5 Goonewardene R, Carter R, Gamage CP, Del Giudice G, David PH, Howie S, Mendis KN. Human T cell proliferative responses to *Plasmodium vivax* antigens: evidence of immunosuppression following prolonged exposure to endemic malaria. Eur J Immunol 1990; **20**:1387–91.

6 Riley EM, Jobe O, Whittle HC. $CD8^+$ T cells inhibit *Plasmodium falciparum* induced lymphoproliferation and gamma interferon production in cell preparations from some malaria-immune individuals. Infect Immun 1989; **57**:1281–4.

7 Mshana RN, McLean S, Boulandi J. *In vitro* cell-mediated immune responses to *Plasmodium falciparum* schizont antigens in adults from a malaria-endemic area: $CD8^+$ T lymphocytes inhibit the response of low responder individuals. Int Immunol 1990; **2**:1121–32.

8 Theander TG, Hviid L, Abu-Zeid YA, Abdulhadi NH, Saeed BO, Bayoumi RAL, Jensen JB. Activation of $CD8^+$ suppressor cells during the malaria season. Immunol Infect Dis 1993; **3**:97–102.

9 Goonewardene R. Cellular immune responses to *Plasmodium vivax* malaria. PhD thesis, University of Colombo, Sri Lanka; 1993.

10 Ohta N, Edahiro T, Ishii A, Yasukawa M, Hosaka Y. HLA-DQ controlled T cell response to soluble egg antigen of *Schistosoma japonicum* in humans. Clin Exp Immunol 1990; **79**:403–8.

11 Ottenhoff THM, Walford C, Nishimura Y, Reddy NBB, Sasazuki T. HLA-DQ molecules and the control of *Mycobacterium leprae*-specific T cell nonresponsiveness in lepromatous leprosy patients. Eur J Immunol 1990; **20**:2347–50.

12 Salgame P, Convit J, Bloom BR. Immunological suppression by human $CD8^+$ T cells is receptor dependent and HLA-DQ restricted. Proc Natl Acad Sci USA 1991; **88**:2598–602.

13 Sasazuki T, Kikuchi I, Hirayama K, Matsushita S, Ohta N, Nishimura Y. HLA-linked immune suppression in humans. Immunol 1989; **S2**:21–24.

14 Koram K, Erunkulu F, Armstrong J, Riley E, Whittle HW. A malaria survey in a rural area of The Gambia. West African J Med 1993; in press.

15 Holder AA, Lockyer MJ, Odink KG *et al.* Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. Nature 1985; **317**:270–3.

16 Holder AA, Sandhu JS, Hillman Y, Davey LS, Nicholls SC, Cooper H, Lockyer MJ. Processing of the major merozoite surface antigens of *Plasmodium falciparum*. Parasitol 1987; **94**:199–208.

17 Tanabe K, MacKay M, Goman M, Scaife J. Allelic dimorphism in a surface antigen gene of the malaria parasite isolated from *Plasmodium falciparum* merozoites. J Mol Biol 1987; **195**:273–87.

18 Mitchell GF. Effector cells, molecules and mechanisms in host-protective immunity to parasites. Immunol 1979; **38**:209–23.

19 Maizels RM, Lawrence RA. Immunological tolerance: the key feature in human filariasis. Parasitol Today 1991; **7**:271–6.

20 Stadecker MJ. The role of T cell anergy in the immunomodulation of schistosomiasis. Parasitol Today 1992; **8**:199–204.

21 Li S-G, De Vries RRP. HLA-DQ molecules may be products of an immune suppression gene responsible for *Mycobacterium leprae*-specific nonresponsiveness. Int J Leprosy 1989; **57**:554–6.