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Activation by malaria antigens renders mononuclear cells susceptible to HIV infection and re-activates replication of endogenous HIV in cells from HIV-infected adults

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SUMMARY

We have tested the hypothesis that activation of T cells by exposure to malaria antigens facilitates both de novo *HIV infection and viral reactivation and replication. PBMC from malaria-naïve HIV-uninfected European donors could be productively infected with HIV following* in vitro *stimulation with a lysate of* Plasmodium falciparum *schizonts and PBMC from malaria-naïve and malaria-exposed (semi-immune) HIV-positive adults were induced to produce higher levels of virus after stimulation with the same malaria extract. These findings suggest that effective malaria control measures might contribute to reducing the spread of HIV and extending the life span of HIV-infected individuals living in malaria endemic areas.*

Keywords *HIV, malaria,* Plasmodium falciparum

INTRODUCTION

HIV replication in $CD4^+$ T lymphocytes requires that the cells express an activated phenotype and intercurrent infections that induce immune activation have been found to increase HIV disease progression (1). In large areas of the tropics, HIV and malaria coexist with considerable potential for co-infection. In non-immune individuals, malaria infection leads to a fulminant pro-inflammatory (T helper 1-type) immune response with activation of $CD4^+$, $CD45RO^+$ T cells to produce IFN-γ; T cell-derived signals synergise with parasite-derived stimuli to activate monocyte macrophages to produce IL-1β and TNF-α (2). As the $CD4^+$ $CD45RO^+$ T cell subset is a preferred target for HIV replication (3), these would seem to be ideal conditions for rendering individuals susceptible to HIV infection and for re-activation of viral replication in infected individuals, raising the possibility that malaria infection may lead to more rapid progression to AIDS and death.

Until recently it was believed that there was little interaction between HIV and malaria (4). However, numerous studies have now shown that HIV-infected individuals are more susceptible to infection or clinical episodes of malaria and are less able to clear infections after anti-malarial chemotherapy, and that susceptibility to malaria is positively correlated with HIV viral load and inversely correlated with $CD4^+$ count (5).

Xiao *et al*. (6) have shown that primary infection of $CD8^+$ T cell-depleted PBMC by HIV *in vitro* is enhanced in the presence of *P. falciparum* malaria antigens, that this effect is TNF-α-dependent and that malaria antigen stimulation activates long-terminal repeat (LTR)-directed viral transcription. However, the significance of these findings for malaria-endemic populations is unclear, because experiments were conducted only with PBMC from malaria-unexposed and HIV-uninfected donors. The ability of malaria antigen to promote reactivation of latent HIV infection in

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donors with some acquired immunity to both HIV and malaria is not known. Thus, we have compared the effects of activation by malaria antigens on HIV replication in PBMC from HIV-infected and uninfected, malaria-unexposed and malaria-immune donors from the UK and from Uganda.

MATERIALS AND METHODS

Subjects

Malaria-unexposed, HIV-infected adults were recruited from the Lothian Regional Infectious Diseases Unit, Edinburgh City Hospital and the Department of Genitourinary Medicine at the Edinburgh Royal Infirmary prior to initiation of anti-retroviral therapy. Malaria-unexposed, HIV-negative adults were recruited from the Scottish National Blood Transfusion Service or were laboratory volunteers. Malaria-exposed HIV-infected and uninfected adults were recruited from attendees at the Uganda Virus Research Institute (UVRI) HIV testing clinic and the AIDS Support Organization (TASO) clinic in Entebbe, Uganda. The study was approved by ethical review committees in Edinburgh and Uganda. Written, informed consent was obtained from all donors. Subjects attending for HIV testing in Uganda received pre- and post-test counselling according to guidelines laid down by the Ugandan Ministry of Health. HIV status was determined by serology and viral loads were determined using a commercial PCR detection kit (Nuclisens HIV-1 QT, Organon Teknika, Basingstoke, UK). Ugandan donors were screened for current malaria infection and for anti-malarial antibodies as described previously (7). CD4/CD8 counts were performed and $CD4^+$ and $CD8^+$ T cells were analysed for activation and differentiation markers (CD69, CD38, CD25 and HLA-DR) by flow cytometry; all antibodies were from Becton Dickinson (Oxford, UK). Samples for PCR analysis and cytokine detection were shipped from Uganda to the UK on dry ice.

Plasmodium falciparum schizont antigen

Plasmodium falciparum schizont extract (PfSE) was prepared as described previously (7). Cultures were screened to confirm absence of mycoplasma infection using a commercial PCR kit (BioWhittaker, Wokingham, UK). Preparations of uninfected erythrocytes (uRBC) were used as controls.

T cell activation assays

PBMCs were separated from heparinized blood by density centrifugation and immediately put into short-term culture as described (7). Purified protein derivative (PPD) of *Mycobacterium tuberculosis* (Statens Serum Institute, Copenhagen, Denmark) (100 iu/mL) was used as a control for recall antigen-induced T cell activation. PfSE and uRBC were used at an equivalent of 10^4 red blood cells/mL. Negative control cultures were incubated with growth medium alone. Lymphocyte proliferation was determined at day 7 by incorporation of tritiated thymidine (7) and culture supernatants were saved for cytokine and viral p24 determination. IFN-γ in supernatants was measured by ELISA, as described (7).

HIV infection

HIV (strain C-98, isolated from an Edinburgh haemophiliac patient), at a multiplicity of infection of 0·005, was added to 6-day cultures of PBMC from HIV^- donors and supernatants were harvested for viral detection after periods of up to 21 days.

HIV detection

To increase the sensitivity of virus detection in cultures from HIV^+ donors, supernatants were co-cultured with 3-day PHA-activated lymphoblasts (from an HIV^- donor) for up to 28 days. Fresh PHA blasts were added to the cultures every 7 days. Supernatant was collected at intervals, stored at $-80^{\circ}C$ and tested for p24 by ELISA. HIV production was assessed by ELISA, using an anti-HIV p24 capture antibody (Aalto Bioreagents, Dublin, Eire) and a biotinylated anti-HIV p24 detecting antibody (ARP454; MRC AIDS reagent programme, National Institute for Biological Standards and Control, South Mimms, UK). Viral p24 concentrations were determined by comparison to a standard curve generated with recombinant baculovirus-derived p24 (ARP620; MRC AIDS reagent programme). The detection threshold for p24 was 0·05 ng/mL.

RESULTS

Characteristics of blood donors

To assess the extent of baseline differences in HIV and malaria-related infection parameters and immunological function between the different donor populations, blood samples were collected from 18 malaria-naïve, HIV-uninfected, 26 malaria-naïve HIV-infected, 44 malaria-exposed HIV-uninfected and 23 malaria-exposed HIV-infected donors from the UK and Uganda, respectively. Donors ranged in age from 19 to 57 years and there was no significant difference in ages between the donor groups.

Peripheral CD4 percentages were lower in HIV^+ than HIV^- Ugandan donors (27 ± 6 vs. 38 ± 3) but did not differ significantly between Ugandan and UK HIV^+ donors (27 ± 6 vs. 21 ± 9). Similarly, viral loads were not significantly

different between HIV$^+$ Ugandan and UK donors (4·9 ± 0·2 vs. 4·2 ± 1·0/mL $\log^{10}$). All but one of the Ugandan donors was seropositive for anti-malarial antibodies and there was no significant difference between HIV$^+$ and HIV$^-$ donors in mean antibody levels (OD at 1 : 1000, 1·4 ± 0·5 vs. 1·6 ± 0·5). The only significant difference between the groups in *ex vivo* activation status of lymphocytes was a higher percentage of HLA-DR$^+$ CD4$^+$ (36 ± 11 vs. 24 ± 4) and CD8$^+$ (69 ± 8 vs. 52 ± 4) cells in the HIV$^+$ than in the HIV$^-$ Ugandan donors ($P < 0·05$ in each case); this was unrelated to asymptomatic malaria infection. Cells from all donors proliferated strongly in response to malaria antigen stimulation and although proliferation did not differ significantly between UK and Ugandan donors, it was higher in HIV$^-$ than HIV$^+$ donors (mean cpm 35 496 and 10 895 in HIV$^-$ and HIV$^+$ Ugandan donors and 29 395 and 16 450 in HIV$^-$ and HIV$^+$ UK donors, respectively, $P < 0·001$ in both cases); cpm for uRBC was < 2700 in all cases. Cells from 39/44 (89%) UK donors but only 46/67 (69%) of Ugandan donors produced IFN-γ after stimulation with PfSE ($\chi^2 = 5·9$, d.f. = 1, $P < 0·025$).

Susceptibility of PBMC to HIV infection

HIV infection assays were carried in all HIV-negative donors from whom sufficiently large blood volumes could be collected (i.e. five UK donors and 16 Ugandan donors). PBMC from five HIV-uninfected, malaria-naïve UK donors were infected with HIV isolate C-98 after 7 days incubation with PfSE, uRBC and (where cell numbers were sufficient) PPD. Cells from all five donors became productively infected after co-culture with PfSE, but not uRBC, with p24 levels of up to 40 ng/mL detectable within 8 days (Figure 1a). In the two donors whose cells were also stimulated with PPD, p24 was also detected. We observed a strong correlation between p24 concentration and IFN-γ concentration in cell supernatants (Figure 1b).

We made several separate attempts to infect PBMC of 16 different HIV$^-$, malaria-exposed Ugandan donors with exogenous HIV. Although viral p24 could be detected in most cultures stimulated with PHA (data not shown), no evidence of viral replication was seen in PBMC co-cultured with malaria antigen.

Reactivation of HIV

HIV reactivation assays were carried out in all HIV-positive donors from whom sufficient cell numbers were available (i.e. seven UK donors and six Ugandan donors).

Malaria-unexposed donors

PBMC from seven malaria-unexposed, HIV$^+$ UK donors were cultured for up to 9 days with PfSE or uRBC. For five

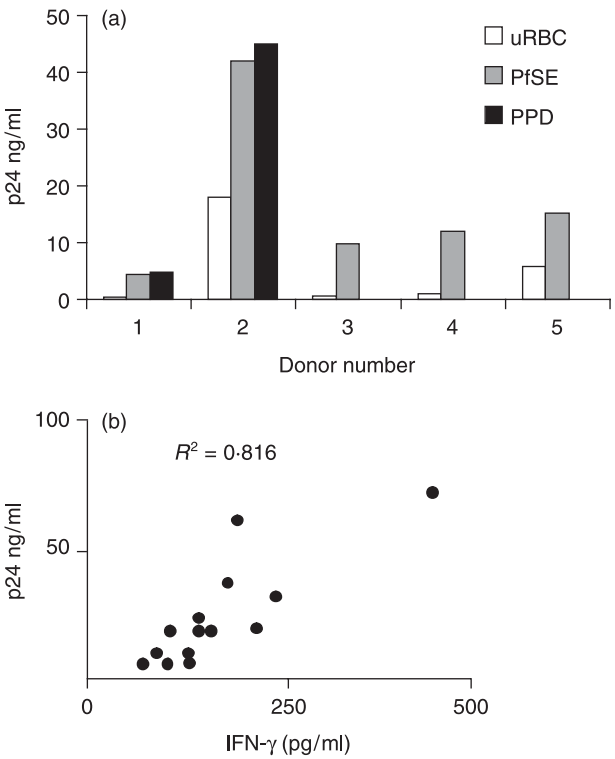

Figure 1 *De novo* infection of PBMCs after *in vitro* restimulation with *P. falciparum* antigens. PBMCs from five HIV-uninfected, malaria-naïve, European donors were cultured *in vitro* for 7 days with *P. falciparum* schizont extract (PfSE), extract of uninfected red blood cells (uRBC) or PPD (two donors only) and then infected with HIV. (a) HIV p24 in culture supernatants was measured by ELISA; maximal p24 concentration after either 4 or 8 days is shown. (b) Correlation between maximal HIV p24 concentrations and IFN-γ concentration in the cell supernatant at 7 days (prior to HIV infection).

of the donors, no viral p24 was detected in any of the cultures at any time point, but p24 was detected in day 9 supernatants from PfSE-stimulated (but not uRBC-stimulated) cultures of two donors with p24 concentrations of 0·12 ng/mL and 0·06 ng/mL, respectively. The donor whose cells produced the higher levels of p24 when stimulated with PfSE also showed a very strong proliferative and IFN-γ response to PfSE (data not shown). These results indicate that exposure to malaria antigens in HIV$^+$ malaria non-immune individuals can lead to HIV reactivation and replication.

Malaria-exposed donors

PBMC from six malaria-exposed, HIV$^+$ Ugandan donors were cultured with PHA for 3 days and with PfSE or uRBC for 7 days; supernatants were incubated with HIV$^-$ PHA blasts for up to 28 days and tested for HIV p24 by ELISA.

Supernatants from two donors were consistently negative for HIV p24 after activation with either PfSE or PHA

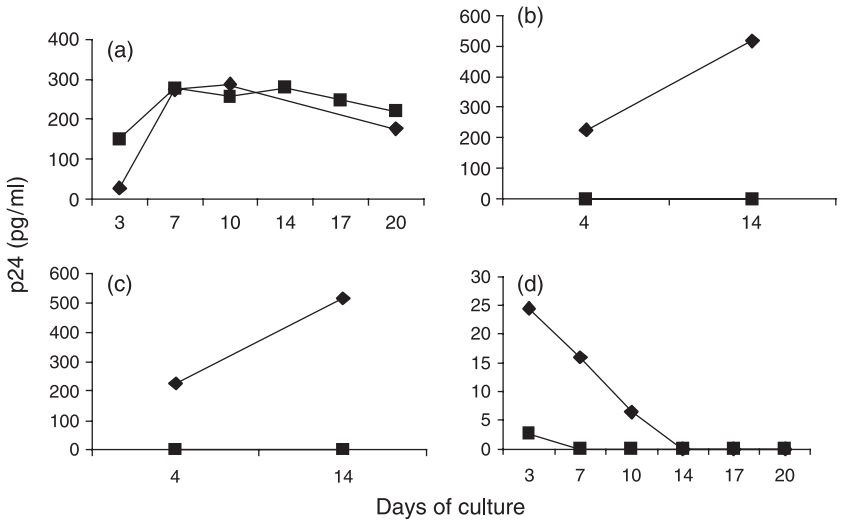

Figure 2 Reactivation of HIV replication in PBMC from HIV$^+$ malaria-exposed donors following *in vitro* stimulation with *P. falciparum* antigens. PBMC from four HIV-infected, malaria-exposed, African donors (a–d) were cultured *in vitro* with *P. falciparum* schizont extract (PfSE; ◆) or an extract of uninfected red blood cells (uRBC; ■). Supernatants were collected after varying periods of time, co-cultured with HIV-uninfected PHA blasts for 28 days and viral p24 measured by ELISA.

(data not shown). Cells from two other donors were spontaneously shedding HIV as high levels of p24 were detected in all supernatants, with or without reactivation with malaria antigen (Figure 2a,b). However, for the other two donors, spontaneous HIV shedding was undetectable or low and p24 concentration was markedly enhanced by activation of the cells with malaria antigens (Figure 2c,d). For one donor (Figure 2d), viral replication was transient (already maximal at 3 days and undetectable by 14 days) whilst for the other (Figure 2c) viral replication persisted for at least 14 days. Transient HIV replication may have been due to extensive cell death within the culture.

DISCUSSION

This study shows – for the first time – that, in HIV-infected donors, stimulation of PBMC with malaria antigens can lead to HIV reactivation, replication and release of viral proteins; this reactivation of natural infection occurs in both malaria-naïve (European) and malaria-exposed (African) donors. Although the number of donors tested was small, and the donor populations differed in a number of ways in addition to prior malaria exposure, we believe that the observation that concomitant exposure of cells to malaria antigens and HIV can exacerbate HIV replication is an important finding that justifies further (larger) studies to determine the interaction between HIV and malaria at the cellular level.

The reactivated viruses in the African donors were not typed, but the patients came from a well-characterized Ugandan study cohort where the virus population comprises subtypes A, D and a low frequency of subtype C (8,9). This suggests that more than one viral subtype can be reactivated by exposure to malaria. Clinical data indicating that malaria infection in HIV-infected individuals leads to a transient increase in HIV viral load (5) suggest that periodic reinfection with malaria may play an important role in the pathogenesis and/or spread of HIV in sub-Saharan Africa. Information on the untreated natural history of HIV suggests that the median survival in Africa may be up to 2 years shorter than in developed countries (10,11). Concomitant infections, such as malaria, may, in part, account for this shorter survival.

We have also shown that PBMC from malaria-naïve, HIV$^-$ donors can be productively infected with HIV after activation of the T cells with malaria extracts, confirming the previous report of Xiao *et al.* (6). Importantly, we were able to infect PBMCs without prior depletion of CD8$^+$ T cells (suggesting that infection may occur within the context of an intact immune system *in vivo*) and with *P. falciparum* antigen that was confirmed to be mycoplasma free. Cells from all malaria-naïve donors showed strong lymphoproliferative responses to malaria, and the majority also produced IFN-γ. Responses to malaria antigens in naïve donors are attributed to reactivation of T cell clones primed by cross-reacting antigens from environmental organisms, common pathogens and commensals; the responding cells are typically CD4$^+$ memory cells and produce copious IFN-γ (2). The correlation between HIV replication and antigen-induced IFN-γ production in UK donors suggests either that IFN-γ is causally associated with HIV replication or that it is a marker for T cell activation.

Despite many attempts, we were unable to infect cells from malaria-exposed HIV$^-$ donors with exogenous antigen using PfSE as the T cell activator. Neutralization of malaria antigens by anti-malarial antibodies is unlikely, because PBMC were extensively washed to remove immune serum and resuspended in non-immune European serum. It is

possible that our assays were not sensitive enough to detect low level virus production (viral p24 was analysed directly rather than following amplification in PHA blasts) but p24 was detected in supernatants of cells from HIV⁻ European donors using an identical procedure. The most plausible explanation for the failure of malaria antigens to facilitate infection of cells from malaria-immune donors is that the PBMCs were not appropriately activated by malaria antigens and were thus not susceptible to HIV infection. Anti-malarial immunity is associated with a switch from a predominantly pro-inflammatory (IFN-γ and TNF-α) to an anti-inflammatory (TGF-β and IL-10) immune response (2); TGF-β has been shown to suppress HIV replication (12). In support of this, IFN-γ responses to PfSE were lower in HIV⁻ Ugandans than in HIV⁻ UK donors, indicating down regulation of Th1-like responses.

In conclusion, we have demonstrated that activation of PBMCs with malaria antigens facilitates both *de novo* HIV infection in malaria-unexposed donors and HIV replication in cells from naturally infected malaria-naïve and malaria-exposed donors. Our data strengthen the case for implementation of interventions aimed at reducing exposure to malaria infection in HIV-infected individuals.

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