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Proliferative Responses of T Subsets from Malaria-naive Human Donors [$CD4^+$, $CD8^+$, and Naive T Cell ($CD45RA^+$)-enriched and Memory T Cell ($CD45RO^+$)-enriched Subpopulations], to *Plasmodium falciparum* Polypeptide Antigens Isolated from the Merozoite Stage

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ABSTRACT

Mononuclear cells, $CD2^+$ T cells and T subsets ($CD4^+$, $CD8^+$) from HLA-typed malaria-naive donors were stimulated with isolated surface antigens (Ags) from *Plasmodium falciparum* merozoites ($MSP1_{83}$, $MSP1_{36}$, MSP2). Cell responses in lymphocyte proliferation assays were MHC-class II and mainly DR restricted. Donors could be grouped into high, non or low responders according to the proliferative response. The high responders possessed the combination DR7/DRw53 as a common HLA-restriction element. Non and low responders did not have this Ag combination, but some were characterized by DQwl.

Individual donors did not show any Ag dependent response. $CD45RA^+$ (naive, non-stimulated) enriched T cells from high-responders were strongly stimulated by the merozoite Ags, whereas stimulation of $CD45RO^+$ (memory) enriched T cells was much lower.

INTRODUCTION

Antigens from extracellular blood stages of *Plasmodium* such as sporozoites, merozoites and gametocytes are considered to be targets for protective B and T cell responses. The natural Ags represent a complex mixture; numerous strains and stages of the parasite exist, all of which express specific Ags. In addition each Ag carries an unknown number of different epitopes, and for most of these very little or nothing is known about the type of immune response which they can trigger.

Malaria research on the molecular basis is restricted at present to a relatively small number of plasmodial Ags. Recent immunological investigations into the circumsporozoite (CSP) Ag have identified T cell epitopes in this molecule (Good et al. 1987; Guttinger et al. 1988; Sinigaglia et al. 1988a; Kilgus et al. 1989; Nardin et al. 1989; de Groot et al. 1989; Romero et al. 1989), as well as MHC-restriction elements which are necessary for T cell stimulation of the various HLA-haplotypes in the population (Guttinger et

al. 1988; Kilgus et al. 1989; Sinigaglia et al. 1988b). As yet, however, no applicable vaccine has been developed on the basis of this research (Ballou et al. 1987; Herrington et al. 1987; Etlinger et al. 1988; Sherwood et al. 1991). The various possibilities proposed to explain the poor immunogenicity of the vaccine candidates used in man include: (i) that people living in endemic areas develop only partial immunity against *P. falciparum* sporozoites (Hoffmann et al. 1987a; Good et al. 1988), (ii) that the original native Ags are not always recognized by CSP-peptide specific T cell clones (Kilgus et al. 1989) and (iii) the possible existence of a genetic HLA-restriction of CSP epitopes (Good et al. 1987; Sinigaglia et al. 1988a; del Giudice et al. 1986).

In view of this unsatisfactory situation it is clearly necessary to isolate, and immunologically characterize, vaccine candidates from other plasmodial stages such as merozoites and gametocytes, and to identify their ability to induce cell-mediated immune responses and protective immunity. Some Ags from asexual blood stages, such as the MSP1 merozoite surface Ag (Crisanti et al. 1988; Sinigaglia et al. 1988c) and the Pf155/RESA (Kabilan et al. 1988; Rzepczyk et al. 1988), have been shown to possess T cell epitopes. These (Hall et al. 1984; Siddigui et al. 1987; Collins et al. 1986), and mixtures of peptides derived from erythrocytic blood stages of *P. falciparum* (Patarroyo et al. 1987; Patarroyo et al. 1988; Collins et al. 1988), have also been shown to induce protection in animals and in man.

Several investigators have shown that native, synthetic or recombinant malaria Ags can induce specific proliferation responses in T lymphocytes, even when from naive donors (Guttinger et al. 1989; Sinigaglia et al. 1988; Kilgus et al. 1989; Sinigaglia et al 1988; Chizzolini and Perrin 1986; Good et al. 1987; Riley et al. 1988; Jaureguiberry et al. 1988; Roussilhon et al. 1989; Perlmann et al. 1989). The possible mechanisms of such stimulation, priming of naive cells by malaria Ags or cross-reactive recall of memory T cells stimulated by non-plasmodial Ags, are still discussion subjects.

Here we demonstrate in vitro the antigenic potential of various isolated merozoite surface polypeptides, such as the fragments $MSP1_{83}$ and $MSP1_{36}$, both derived from the 185-195 kDa MSP1, and the 46-55 kDa MSP2 glycoprotein. For these experiments we used mononuclear cells and/or enriched $CD2^+$, $CD4^+$ and $CD8^+$ T cell subsets isolated from peripheral blood from malaria-naive donors. Donors were HLA-typed in order to recognize a possible specific correlation between the strenghth of immune responses and certain HLA-restriction elements. In order to discover whether naive or memory T cells are responsible for the proliferation, monoclonal antibodies (mAbs) specific for the CD45RO molecule of activated (Smith et al. 1986), or the CD45RA molecule of non-activated T cells (Ledbetter et al. 1985) were used to isolate these T subsets then tested with merozoite Ags in lymphocyte proliferation assays.

MATERIALS AND METHODS

Preparation of Ags: *Plasmodium falciparum* parasites (FCB1 strain from Colombia) were cultured at a haematocrit of 3% in human A^+ RBCs (Trager and Jensen 1976). Cultures were synchronized by alternating Plasmagel (Pasvol et al. 1978; Jensen 1978) and sorbitol (Lambros and Vanderberg 1979) treatment. Merozoites were isolated from synchronous cultures using the filter procedure (Heidrich et al. 1983).

Extraction of merozoite polypeptides was performed on ice for 60 min in Medium A (11 mM triethanolamine, 11 mM HC1, 0.05 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 0.15 mM NaCl, 2% aprotinin, pH 7.4 adjusted with 1 N KOH) supplemented with 2 mM phenylmethylsulphony1 fluoride (PMSF), the protease inhibitors antipain, bestatin and pepstatin all at a final concentration of 1 mg/ml, and 5 mM Zwittergent 3-12 (McBride and Heidrich 1987).

Ags were isolated chromatographically using Sephacryl S-300 and Mono-Q ion exchange resin (Heidrich 1988).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinous 5-20% acrylamide system (Heidrich et al. 1983) and stained with Coomassie Brillant Blue. Separated polypeptide bands were electro-eluted (Hunkapiller et al. 1983) as described for *P. falciparum* Ags (Heidrich et al. 1989).

Prior to use in the lymphocyte stimulation test, all Ag extracts were dialyzed for 12 h in PBS, pH 7.4, followed by a 12 h dialysis against complete RPMI 1640 medium (see below). Protein concentrations in the dialysates were too low for precise quantitative protein determination. Therefore the various Ag solutions were adjusted to about the same concentration after an immune dot blot with a rabbit anti-merozoite antiserum (Herbrink et al. 1982). The intensity of the blot spots was compared with known concentrations of unpurified merozoite Ag as standards.

Preparation of lymphocyte subsets and antigen-presenting cells (APCs): Mononuclear leucocytes were isolated from warm peripheral blood of naive (non-exposed to *P. falciparum* donors (3 male, 7 female aged between 24 and 45) by centrifugation at 400 x g for 30 min at RT on a discontinuous Ficoll-Hypaque gradient (Boyum 1968) and then washed and resuspended in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (h.i) and 2 mM glutamine (complete RPMI medium).

$CD2^+$ T lymphocytes were enriched by the resetting technique. SRBCs were treated with 2-aminoethylisothiouronium bromide hydrobromide (AET), rosettes were separated on a Ficoll-Hypaque gradient and the RBCs were lysed (Saxon et al. 1976). Non-T lymphocytes (B lymphocytes, monocytes, etc.) were found in the interface of the gradient. The isolated cells were washed in complete RPMI medium.

$CD2^+$ subpopulations ($CD4^+$ and $CD8^+$) were enriched by negative selection using the panning method (Tsoi et al. 1982; Countre and Engleman 1987). Briefly, plastic petri dishes were coated with goat anti-mouse IgG (10 μg/ml in 0.05 M Tris buffer, pH 9.5) followed by either anti-CD4 or anti-CD8 mAb (10 μg/ml in PBS). 2 x 10^7 $CD2^+$ cells in 3 ml 5% FCS-PBS were added to the dishes. Non-adherent lymphocytes of the $CD8^+$ or the $CD4^+$ phenotype were removed after incubation for 70 min at 4^OC (negatively selected populations). The purity of these cells was controlled by immunofluorescence and was routinely greater than 95%.

Naive T cells were enriched by the same method using a mAb (UCHL1) specific for the CD45RO molecule and other mAbs specific for HLA-DR and the CD25 anti-IL2 receptor.

Memory T cells were enriched by negative selection using a mAb (G1-15) specific for the CD45RA

molecule (MAbs were a generous gifts from Dr. E. P. Rieber, Institute for Immunology, University Munich).

Monocytes/macrophages, as APCs, were enriched by incubation of 5 x 10^6 mononuclear cells per ml in complete RPMI medium (with only 5% PCS) for 1 h at 37^OC in polystyrene plastic petri dishes. The adherent cells (monocytes/macrophages) were washed and then removed from the plate with ice cold PBS after a 20 min incubation at 4^OC (Lehner 1983).

Lymphocyte proliferation assay: 1 x 10^6 monocytes in 1.5 ml complete RPMI-medium as presenting cells were incubated together with electro-eluted Ag fractions (1.5 - 3 µg per 1 x 10^6 monocytes in 1.5 ml, i.e. 1 - 2 µg/ml) in complete medium at 37^OC. After 15 h incubation they were washed at 100 x g for 10 min at RT. For stimulation, autologous APC (2 x 10^4 cells per well) were added to mononuclear cells or enriched T subsets (2 x 10^5 cells per well in a 96 flat bottom plate) and incubated for 6 days. The proliferation, as correlated with [^{3}H]-thymidine uptake by the cells (1 µCi per well), was measured 18 h later in counts per min (cpm). The number of naive and memory T cells obtained by the negative selection method was low so that it was not possible to test all three Ags in all experiments with these cells. Mitogenic stimulations were done with 6 µg/ml PHA. Blank control stimulations were done with complete medium and without addition of Ags.

HLA-typing: The standard lymphocyte microcytotoxicity technique with Terasaki Typing Trays (One Lambda, Los Angeles, USA) was used for HLA-typing (Mittal 1978) following the manufacturer's instructions.

Blocking of T subset proliferation by monoclonal antibodies specific for MHC products: MAb w6/32 (anti-class I heavy chain anti-αβ2m) and mAb L206 (class II anti-DQ, DP, DR) were used in blocking studies (Mellins et al. 1987; Barnstable et al. 1978). Monoclonal antibodies used in class II blocking experiments were L243 (anti-HLA-DR), mAb antiαβ1/αβ3, mAb Tü22 (anti-HLA-DQ) which reacts with a subset of DQ molecules, and mAb B7/21 (anti-DP monomorphic) (all generous gifts from Dr. D. Schendel, Institute of Immunology, University Munich).

Blocking studies were performed using 1: 50 dilutions of ascites as described in the proliferation assay except that mAb were added during the start of cultures. Blocking experiments were performed in triplicate.

Statistical analysis: Comparisons were made based on merozoite polypeptides , lymphocyte proliferation and HLA-phenotype and comparing proportions with the Mann-Whitney-U-test. A p-value of less than 0.05 was considered significant.

RESULTS

HLA typing: The results of MHC class I (A, B, C) and class II (DR, DQ) typing are summarized in Table 1.

T cell responses to individual electro-eluted merozoite polypeptides 1. Merozoite Ags: Merozoite surface polypeptide fractions ($MSP1_{83}$ and $MSP1_{36}$, both derived from MSP1 through protein processing, and 46 kDa MSP2) were separated on SDS-PAGE and electro-eluted. Isolated Ags have been characterized

thoroughly as described elsewhere (for a summary see Heidrich 1988). The characterization of the antigens used here included identification with polyvalent and monoclonal antibodies in immunoprecipitation and/or immunoblotting experiments, and determination of the N-terminal sequence of isolated antigens.

Table 1 : HLA phenotypes of the 10 Caucasian donors used in the present expedients. The 7 female and 3 male donors were aged 24-45. All were clinically healthy. None had ever been in contact with *P. falciparum* (malaria-naive donors), confined by negative serological analyses using Ags from *P. falciparum* in imunoblots (not shown).

donor	HLA-pattern
RG	A3, A28, B35, Bw55, Cw4; DR1, DR2, DQw1, DQw3
TP	A2, A25, B13, B44, Cw6; DR3, DR5, DRw8, DRw52, DQw2, DQw3
MM	A2, Aw33, Bw4, B13, B14, B57, Cw4; DR1, DR3, DRw52, DQw6
AH	A2, A9, B5, Bw6, B51, Cw5; DR5, DR8, DRw52, DQw3
CG	A1, A2, Aw36, Bw6, B8, Bw41, Cw7; DR3, DRw11, DRw52, DQw2, DQw3
AP	A2, A25, Bw6, B7, B18, Cw7; DR5, DRw6, DRw13, DRw52, DQw1, DQw6
AS	A2, Bw4, Bw6, B13, Cw3; DR2, DRw8, DRw52, DQw1, DQw6
AL	A1, A3, Bw6, B8, B40; DR3, DRw6, DRw13, DRw52, DQw1, DQw2
MÜ	A2, A29, Bw4, Bw6, B7, B13, Cw6; DR5, DR7, DRw52, DRw53, DQw2, DQw3
RR	A2, B12, Bw40, Cw3; DR2, DR7, DRw9, DRw53, DQw2, DQw3

2. Electro-transferred versus electro-elated merozoite Ags for proliferation assays: In the first proliferation experiments the polypeptide Ags used were electro-transferred from SDS-PAGE onto nitrocellulose and further processed to an Ag-bearing nitrocellulose particle suspension. No significant differences were observed in T cell proliferation using nitrocellulose particles with or without polypeptides, but the blank controls had a high background (data not shown). It was not possible to show a reproducible Ag-specific T cell response using this technique.

The high background in blank controls was eliminated when electro-eluted Ag fractions were used in the lymphocyte stimulation assays. In order to reduce any influence of the detergents (present in small amounts even in thoroughly dialyzed Ag extracts) on T cells, APC (1 x 10^6 monocytes) were incubated with 1.5 - 3 µg Ag overnight. After incubation monocytes were washed and their vitality checked using the trypan blue exclusion test. The morphological (ultrastructural) integrity of cells was assessed by viewing thins sections of embedded cells in a transmission electron microscope. Cells appeared vital and morphologically intact.

3. Optimal Ag concentration for lymphocyte proliferation assays: Ags $MSP1_{83}$, $MSP1_{36}$ and MSP2 were used in lymphocyte proliferation assays in various concentrations (between about 0.1 µg and 15 µg per 10^6 APC in 1.5 ml) in order to test the dose-response on the proliferative responses of mononuclear cells, and $CD2^+$, $CD4^+$ and $CD8^+$ T cells in the presence of autologous APCs. The optimal range for suitable T cell activation was quite wide, i.e. between 1 and 10 µg per 10^6 APC. Good proliferations were obtained for all three Ags within about the same concentration range, thus 1.5 - 3 µg Ag was routinely used to treat 10^6 APC prior to the proliferation test.

4. Stimulation of mononuclear cells and T subsets from HLA-typed malaria-naive donors: One of the important questions to be answered in this study was whether or not lymphocytes and/or lymphocyte

subsets from donors differing in HLA respond differently and individually to merozoite Ags. Proliferation experiments using mononuclear cells or unseparated $CD2^+$ T cells (data available but not shown) showed some, but not consistent, differences in the response to the various Ags depending on the donor. However, T cells from the same donor showed no significant differences in their response to the three different Ags.

These effects were recognized much better when enriched $CD4^+$ T subpopulations were used (Table 2). Only the $CD4^+$ cells from donors MÜ and RR responded well to all three Ags, in addition cells from donors MM and CG responded well to MSP2. With the exception of donors MM and CG, the three polypeptides tested induced very similar $CD4^+$ proliferation responses ($p = 0.906$). Four of the 8 non/low responders to the Ags possessed the common restriction element DQwl ($p = 0.055$). This value is at the border of significance. The two high-responders were characterized by the restriction element combination DR7 and DRw53 ($p = 0.0367$).

Table 2: Proliferation responses of $CD4^+$ subsets from 10 malaria-naive Caucasian donors, to the *P. falciparum* merozoite surface Ags $HSP1_{83}$ = 83 kDa, HSP2 = 46 kDa and $HSP1_{36}$ = 36 kDa. Donors are grouped according to T cell proliferation, the first group are non/low responders with the common restriction elenent DQwl. The second group are non/low responders without a characteristic common HLA-pattern. (The p-value in both of these groups for DQwl is at the border of significance ($p = 0.055$).) The third group are high responders and were characterized by the HLA restriction element conbination DR7 and DRw53 ($p = 0.0367$). All values in triplicate. PHA-P: mitogenic control stimulation with PHA; Medium: blank control values. Proliferation is expressed in counts per minute (cpm).

donor	HLA phenotype	proliferative response (cpm) to 83 kDa	46 kDa	36 kDa	PHA	medium
RG	DR1,DR2, DQw1, DQw3	1243 ± 91	1042 ± 330	759 ± 233	6600 ± 200*	359 ± 36
AS	DR2,DRw8,DRw52, DQw1, DQw6	1432 ± 157	1407 ± 507	1871 ± 299	169250 ± 9798	94 ± 78
AP	DR5,DRw6,DRw13,DRw52, DQw1, DQw6	636 ± 156	657 ± 411	361 ± 273	53500 ± 3535	184 ± 253
AL	DR3,DRw6,DRw13,DRw52, DQw1, DQw2	2346 ± 900	610 ± 184	553 ± 156	43599 ± 18240	491 ± 32
AH	DR5,DR8,DRw52,DQw3	852 ± 286	1734 ± 144	797 ± 264	138524 ± 4045	772 ± 472
TP	DR3,DR5,DR8,DRw52,DQw2,DQw3	1124 ± 350	1372 ± 352	1950 ± 395	14947 ± 1460	488 ± 189
CG	DR3,DRw11,DRw52,DQw2,DQw3	2669 ± 363	5106 ± 809	3268 ± 263	71092 ± 4250	1524 ± 507
MM	DR1,DR3,DRw52,DQw6	2618 ± 533	4404 ± 1267	2658 ± 520	224420 ± 11842	313 ± 76
RR	DR2, DR7, DRw9, DRw53, DQw2,DQw3	14922 ± 1811	8149 ± 1331	14251 ± 1391	57294 ± 10218	23 ± 312
MÜ	DR5, DR7, DRw52, DRw53, DQw2,DQw3	33685 ± 1415	40460 ± 6280	33367 ± 2105	56564 ± 6477	567 ± 91

* The low proliferative response of cells from this donor to PHA was shown reproducibly in repeated experiments over a number of years.

With the exception of cells from donors MÜ and AS, $CD8^+$ T cells showed only a low response to the three Ags (Table 3). The proliferative responses to the three Ags by cells from the same donor were the same ($p = 0.9172$), except for a much higher proliferation response with $MSP1_{36}$. This suggests the possibility that the HLA-Bw4 molecule might be a dominant restriction element in the CD8-proliferation responses ($p = 0.472$).

Table 3: Proliferation responses of $CD8^+$ subsets from 10 naive Caucasian donors to the *P. falciparum* merozoite surface Ags $MSP1_{83}$ = 83 kDa, MSP2 = 46 kDa and $MSP1_{36}$ = 36 kDa. See text. All values in triplicate. PHA-P: mitogenic control stimulation with PHA; Medium: blank control values. Proliferation in counts per ninute (cpm).

donor	HLA phenotype	proliferative response (cpm) to 83 kDa	46 kDa	36 kDa	PHA	medium
RG	A3,A28,B35,Bw55,Cw4	191 ± 32	268 ± 50	278 ± 75	5653 ± 70*	197 ± 54
AS	A2,Bw4,Bw6,B13,Cw3	6002 ± 1251	4014 ± 493	4750 ± 393	139146 ± 4138	1263 ± 207
AP	A2,A25,Bw6,B7,B18,Cw7	177 ± 94	112 ± 6	142 ± 5	43824 ± 2733	234 ± 67
AL	A1,A3,Bw6,B8,B40	418 ± 212	280 ± 114	595 ± 251	39240 ± 5074	48 ± 21
AH	A2,A9,B5,Bw6,B51,Cw5	2927 ± 1737	921 ± 117	999 ± 176	43726 ± 5499	632 ± 257
TP	A2,A25,B13,B44,Cw6	274 ± 117	431 ± 78	510 ± 104	18586 ± 7903	56 ± 29
CG	A1,A2,Aw36,Bw6,B8,Bw41,Cw7	1739 ± 335	1963 ± 210	1756 ± 330	38193 ± 6909	502 ± 123
MM	A2,Aw33,Bw4,B13,B14,B57,Cw4	1578 ± 386	2570 ± 393	2707 ± 445	122992 ± 66	189 ± 111
RR	A2,B12,Bw40,Cw3	2897 ± 385	1301 ± 339	2447 ± 445	91515 ± 3857	403 ± 43
MÜ	A2,A29,Bw4,Bw6,B7,B13,Cw6	5663 ± 442	7902 ± 706	31895 ± 777	71425 ± 9940	363 ± 187

* The low proliferative response of cells from this donor to PHA was shown reproducibly in repeated experiments over a number of years.

HLA-blocking by monoclonal antibodies specific for MHC products: Monoclonal antibodies recognizing framework determinants of class I (w 6/32) or class II (L206) MHC-restriction elements were used in blocking experiments. Proliferation was MHC-class II restricted (results not shown). The MHC-class II elements involved were further specified using anti-DR, anti-DP and anti-DQ mAbs and Ags $MSP1_{83}$, $MSP1_{36}$ and MSP2. The results were very similar for all three antigens tested. Inhibition of proliferation was mainly DR-specific. Fig. 1 shows as an example proliferation responses obtained with Ag $MSP1_{36}$

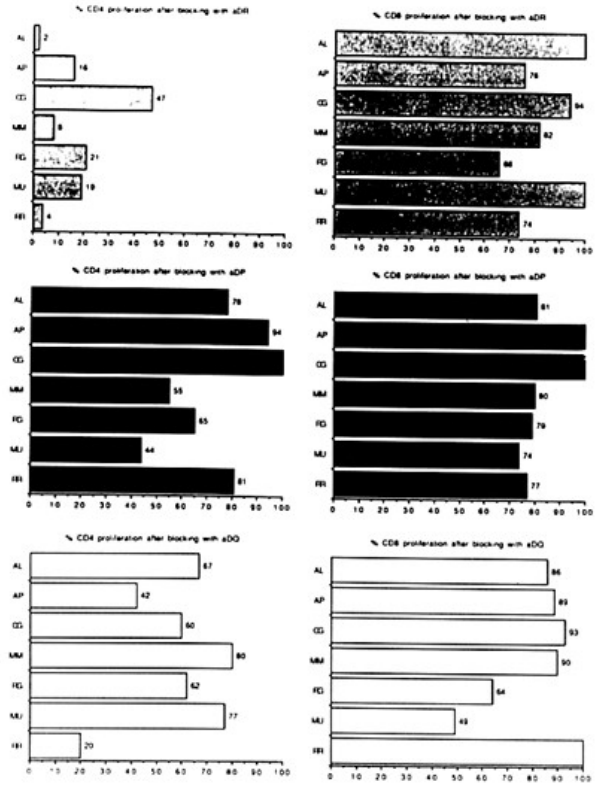

Fig.1: It Proliferative responses of CD4+ and $CD8^+$ T cells to merozoite surface $MSP1_{36}$ = 36 kDa after blockage with anti-DR (upper panel), anti-DP (middle panel) and anti-DQ (lower panel) monoclonal antibodies. Control proliferation without addition of mAbs was 100%. Similar proliferation responses were obtained with Ags $MSP1_{83}$ = 83 kDa and MSP2 = 46 kDa (not shown).

Stimulation of $CD45RA^+$ naive and $CD45RO^+$ memory T lymphocytes: The origin of T cell proliferation, i.e. primary response of naive T cells or restimulation of cross-reacting memory T cells, was investigated

in enriched (negatively selected) populations of $CD2^+$, $CD4^+$ or $CD8^+$ T subsets from 5 donors, among them the high-responding donor MÜ. T memory cells and activated T cells were removed by panning with mAbs directed against $CD45RO^+$, $CD25^+$ and anti-MHC-class II. Naive cells were removed by panning with an anti-CD45RA mAb. The enriched cell populations were tested for lymphocyte proliferation using all three merozoite Ags. Cells from the high-responder MÜ showed the most obvious blastogenesis upon stimulation, and thus differences between the subsets were seen best with this donor.

35-50% of the cells remaining in the $CD2^+$ cell pool after removal of T memory and activated T cells were $CD45RA^+$ positive, i.e. naive cells. 45-72% of cells remaining after removal of naive cells were $CD45RO^+$ positive, i.e. memory cells. The enriched naive ($CD45RA^+$ $CD2^+$) T lymphocyte population showed higher proliferation rates than the enriched memory cells ($CD45RO^+$ $CD2^+$) after stimulation with the merozoite Ags $MSP1_{83}$, MSP2 and $MSP1_{36}$. This was seen particularly clearly in cells from the high-responders. The data of the high-responding donor MÜ are shown in Fig. 2.

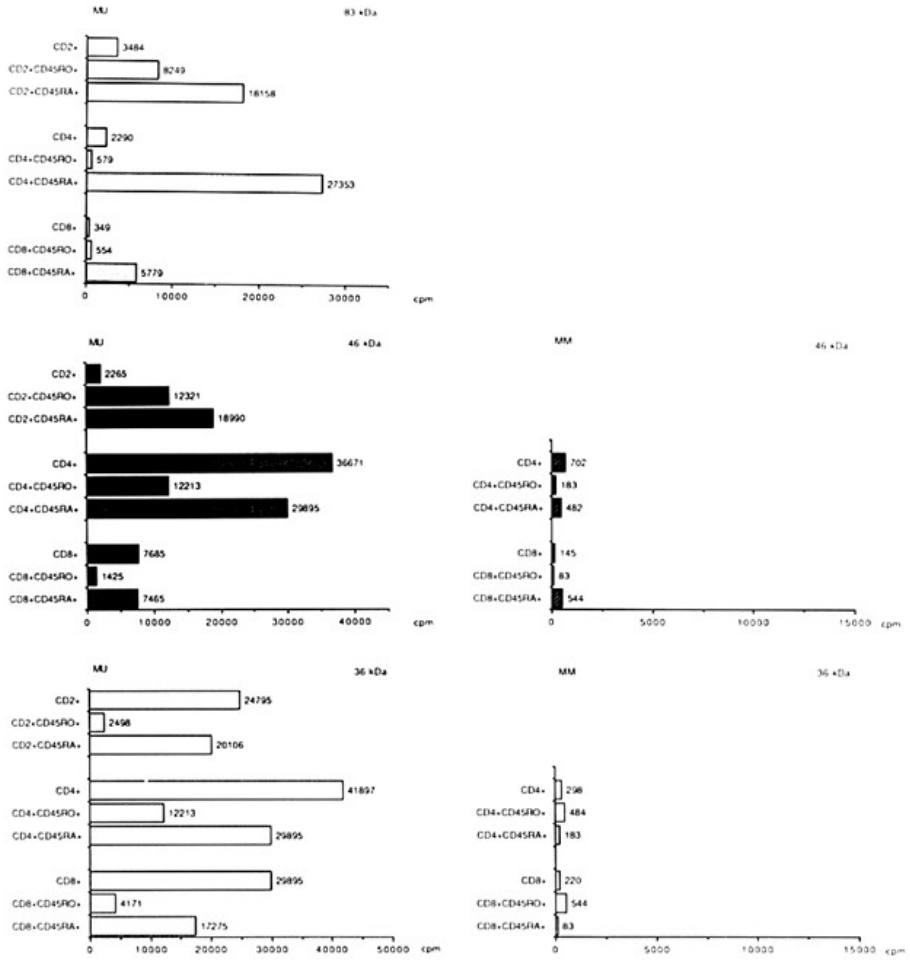

Fig. 2: Proliferative responses of $CD45RO^+$ (memory) and $CMSRA^+$ (naive) T cells in the $CD2^+$, $CD4^+$ and $CD8^+$ subpopulations from a high (MÜ as example, left panels) and in the $CD4^+$ and $CD8^+$ subpopulations from a low/medium responder (MM as example, right panels) to $MSP1_{83}$ = 83 kDa (only donor MÜ), MSP2 = 46 kDa and $MSP1_{36}$ = 36 kDa. Proliferation is expressed in counts per minute (cpm).

Differences in blastogenesis following stimulation were more obvious in the enriched $CD4^+$ populations than in the $CD2^+$ cells. In most of the donors, $CD45RA^+$ (naive) cells were clearly more strongly stimulated with all the Ags than were $CD45RO^+$ (memory) cells. This was seen best in the experiments with cells from the high-responder MÜ (Fig. 2).

Naive $CD8^+$ cells from the high responder donor MÜ clearly showed a stronger proliferative response than did the memory $CD8^+$ cells (Fig. 2). The same tendency was seen in the $CD8^+$ cells from low responding donors but the proliferation rates were much lower and thus the differences less marked. There seemed to be some differences in the proliferation rates between the Ags used.

DISCUSSION

The results show that it is possible to use isolated polypeptide Ags from P. *falciparum* merozoites to analyze the role of such Ags in the polyclonal T cell proliferative response. Enriched $CD2^+$, $CD4^+$ and $CD8^+$ T cells from naive, HLA-typed Caucasians were stimulated in the presence of autologous APCs which had been incubated with merozoite surface Ags prior to the lymphocyte proliferation assays, and the nature of responses and of MHC restriction were analyzed. The primary response appeared to result from proliferation of $CD45RA^+$ (naive) cells, as shown by tests using populations enriched in naive or memory cells.

We screened selected surface polypeptides of P. *falciparum* merozoites, two fragments of the 185-195 kDa MSP1 (the 83 kDa MSP183 and the 36 kDa MSP136) and MSP2 (the 46 kDa glycoprotein), for T cell stimulation. These Ags were selected for several reasons. Firstly, they are located on the surface of merozoites and antibodies against the molecules have been shown to inhibit invasion of RBCs by merozoites (Strych et al. 1987; Clark et al. 1989). Secondly their amino acid sequences are known. This means that the degree of polymorphism in different P. *falciparum* strains is known (Heidrich et al. 1989; Tanabe et al. 1987; Smythe et al. 1990) and that the presence, numbers and nature of T cell epitope regions within the molecules can be analyzed. This is an important issue in vaccine-design, i.e. in the selection of epitopes to be used as components of a vaccine, since conserved epitopes are considered to be better candidates.

The other prerequisite for analysing the immunogenic potential of polypeptides or their epitopes in cell-mediated immune responses is the availability of T cells subsets with defined functions. Here we used enriched cell subsets from the polyclonal T-cell system. These have been shown by others to be suitable for characterizing plasmodial Ags (for a review see Good and Miller 1989). Naive donors who had never had contact with P. *falciparum* were studied because one of the goals in malaria research is to develop a vaccine suitable for the vaccination of naive individuals. Experiments with cells from acutely-infected and immune donors are under way.

Stimulation of mononuclear cells and T subsets from HLA typed malaria-naive donors

The present experiments show clearly that there are striking donor-dependent differences in T cell proliferation, and thus T cell recognition, of the merozoite Ags. The donors could be grouped into three basic groups, non, non/low and high responders (Table 2), on the basis of the response of their $CD4^+$ T

cells to stimulation. The strong immune responses of MÜ and RR appeared to be associated with HLA-DR7/DRW53 alleles ($p < 0.05$). The restriction element DR7 has also been shown to be associated with a good immune response (Sinigaglia et al. 1988b) when using synthetic *P. falciparum* peptides (CSP peptides), in particular CS-T3 which is derived from the conserved region of the circum-sporozoite Ag. This is the only *P. falciparum*-like molecule recognized by HLA-molecules DR1, DR2, DR4, DR5, DRw6, DR7 and DR9 (Guttinger et al. 1988; Sinigaglia et al. 1988b; Kilgus et al. 1989).

The largest group of our donors either did not respond, or responded poorly, to the three Ags used. The possibility that low-responders exist in the human population, and that this low response to malaria Ag molecules is MHC-linked, has been considered by others (Hoffman et al. 1987). Four non-responders in our experiments (RG, AS, AP, and AL in Table 2) had the HLA-element DQwl as a common Ag, although the association of low response with DQwl was below the level of statistical significance. This MHC-product has been studied in *Schistosoma japonicum* infections (Hirayama et al. 1987; Sasazuki et al. 1989). There appears to be a correlation between DQwl, a product of the genes responsible for immunosuppression, and DR2, a product of immunoresponse genes, the DR2 being epistatically suppressed by DQwl. The DQwl molecule has also been shown to be associated with absence of T cell immune responses to *M. leprae* (Vries et al. 1989).

The different merozoite surface Ags did not induce any significant Ag-dependent differences in T subset blastogenesis ($p = 0.9066$ and 0.9172). Such an effect, if seen, would have indicated the presence of genetically restricted recognition of the different Ags by the HLA-restriction elements of the various donors. Although this possibility has been considered by several authors (Hoffman et al. 1987b; Carter et al. 1989; Quakyi et al. 1989) a number of observations suggest that it is unlikely to occur. Genetic HLA-restrictions, at least those from sporozoites and gametocyte Ags, were not seen in natural malaria infections in immune Papua New Guineans (Graves et al. 1990) or patients in an endemic area of West Africa (Riley et al. 1990). We have also screened sera from infected individuals from The Gambia, an endemic area (McBride, Lamb and Heidrich, unpublished observations). The three merozoite surface Ags $MSP1_{83}$, MSP2 and $MSP1_{36}$ were all recognized by all the individuals tested (acutely-infected and asymptomatic) although the strength of the reaction varied. Unfortunately the donors were not HLA-typed so no correlation could be made between HLA-haplotype and immune response. The three merozoite Ags used here have been shown by algorithm analysis (Rothbard and Taylor 1988, not shown) to possess a large number of T cell epitopes, which might account for their recognition by all the different HLA-haplotypes tested.

Blocking of T subset proliferation by monoclonal antibodies specific for MHC products

The blocking experiments using MHC-class I specific (w 6/32) or MHC-class II specific mAbs (L 206) showed the expected class II dependence when $CD2^+$ and $CD4^+$ T cells were used (not shown). As expected proliferation of $CD8^+$ cells could not be blocked with mAb L 206.

The blocking experiments also showed DR to be the main restriction determinant for the Ag-specific T cells. Much lower, variable and donor-dependent inhibition was observed when anti-DQ and anti-DP antibodies were used to block proliferative responses. This phenomenon has also been observed by other

authors. Mellins et al. (1987) showed that the T cell response to complex Ags was 60% or more a DR response. The non-DR restricted response was derived from DQ- and DP-encoded determinants as shown by mutational analysis and blocking with class II-specific mAbs. This phenomenon is explained by a tight linkage disequilibrium between DR and DQ genes and the frequent sharing of epitopes among products of different class II loci (Eckels and Zeewi 1986).

Stimulation of T subsets depleted of $CD45RO^+$ or $CD45RA^+$cells

The $CD45RO^+$ memory T cells comprise 40 - 50% of mononuclear cells in peripheral blood, thus cross-reaction of $CD45RO^+$ cells activated with non-plasmodial Ags cannot be excluded when using *P. falciparum* Ags in proliferation assays. A number of observations, however, suggest that the proliferative response is at least in part a primary reaction. Pink and Sinigaglia (1989), who originally favoured the idea of cross-reaction, now postulate the presence of a primary response because (i) the number of memory cells in naive donors is low, and (ii) cord blood lymphocytes contain no or very few memory cells (Hayward et al. 1989) but do respond to malaria Ags (Jones et al. 1990). Strong proliferative responses to plasmodial schizont antigens have also been found in donors naive to malaria (Roussilhon et al. 1989). These authors postulated that at the time of first contact with *P. falciparum* all the requirements for a T cell stimulation are met, but they did not differentiate between subsets of activated and non-activated T lymphocytes. Malaria Ag-specific T cell clones can be induced in vitro from malaria-naive donors (Chizzolini and Perrin 1986; Good et al. 1987) further evidence that naive T lymphocytes can be stimulated by soluble plasmodial Ags to proliferate. The possibility that stimulation of naive T cells and re-stimulation of memory T-cells can both take place has also been discussed (Beverly 1990). The latter author suggested that immune reaction of unexposed T cell populations to Ags requires an interaction of high-affinity between the T cell receptor and an Ag/MHC complex, whereas low-affinity (cross-reactive) binding is sufficient for stimulation of memory T cell proliferation because of higher expression of adhesion molecules in this population.

We isolated $CD45RO^+$ memory T cells and naive T cells (Akbar et al. 1988: Sanders et al. 1988; Merkenschlager et al. 1988) from five HLA-typed donors by negative selection using panning with mAbs anti-CD45RA and anti-CD45RO, respectively. The loss of the 200/220 kDa CD45RA determinant of the leukocyte common Ag, and the gain of the 180 kDa CD45RO determinant, is a unidirectional switch which characterizes the phenotypic change from a naive to a memory T cell population.

The T cell responses in the naive $CD45RA^+$ subset were considerably higher than those in the memory ($CD45RO^+$) T cells for all the three Ags in almost all of the assays. This was particularly clear in cell subsets from the high-responder MÜ (Fig. 2). The $CD45RO^+$ T cells showed a donor-dependent proliferation which could have resulted from cross-reaction. The stimulation was low, however, indicating that the memory T cells played only a minor role in the proliferation response. This finding contrasts with the observation of others that only memory T cells are stimulated by soluble Ags in vitro (Smith et al. 1986; Akbar et al. 1988; Damle et al. 1987), and that polyclonal in vitro proliferation response from naive

donors to *P. falciparum* Ags requires memory T cells (Jones et al. 1990). This discrepancy in findings might result from experimental differences such as (i) using isolated soluble Ags rather than Ag-bearing particles in experiments, (ii) using cells from high-responders which show the differences in proliferation much more clearly than do these from non/low-responders, and (iii) the use of highly enriched $CD45RA^+$ and $CD45RO^+$ T cells to selectively test the reaction. The observations in our experiments that autologous APCs were necessary for Ag processing, that the proliferation responses were at a maximum on day 6 (and not earlier) and that the responses obtained were MHC restricted, provide further support to the finding that the proliferation obtained did not simply result from cross-reactions of the recall-type or from mitogenic effects.

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