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Human T lymphocyte clones specific for malaria *(Plasmodium falciparum)* antigens

Francesco Sinigaglia and J.Richard L.Pink

Central Research Units, F. Hoffmann-La Roche & Co. Ltd., CH-4002 Basel, Switzerland

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Peripheral blood mononuclear cells (PBM) from a patient who had lived in a malarial-endemic area were cultured in the presence of malarial antigens (a lysate of *Plasmodium*-infected erythrocytes). Responding cells were grown in IL-2-containing medium and then cloned, and subsequently subcloned, in the presence of phytohemagglutinin and allogeneic irradiated PBM. Ten clones were specific for malarial antigens. They proliferated in response to *P. falciparum* extract, but not to a lysate of uninfected erythrocytes. The response was HLA-restricted. All the clones tested responded to lysates of cells infected with parasites of either African or Asian origin. Six clones had the T4+/T8− phenotype and four the T4−/T8+ phenotype. Two of the T4+ clones recognised a parasite antigen of apparent mol. wt. ~50 000. All of the clones tested produced γ-interferon following antigen stimulation.

Key words: malaria/*Plasmodium falciparum*/T lymphocyte clones

Introduction

In attempts to identify antigens which might stimulate immunity to malarial infection, most effort has so far been concentrated on the antibody response to the parasite. However, there is good evidence that T cell-mediated mechanisms, as well as antibodies, are important in anti-malarial immunity. In some animal malaria models, immunity can be induced in the absence of antibodies (Weidanz and Grun, 1983), and in others, immunity can be transferred to naive animals by purified T cells (Jayawardena *et al.*, 1982). The role of T cells in resistance of humans to *Plasmodium falciparum* or other malarial species is not clear, but may be important, since malaria-infected patients and people living in malaria-endemic areas develop T cell responses to parasite antigens (Wyler and Oppenheim, 1974; Wyler and Brown, 1977; Troye-Blomberg *et al.*, 1983; Bygbjerg *et al.*, 1985), which so far have not been characterized.

In order to obtain more information about the mechanisms and specificity of T cell-mediated immunity to *P. falciparum*, we have started to isolate and characterize anti-malarial T cell clones from previously infected individuals. In this paper we report results on 10 such clones.

Results and Discussion

A soluble extract of parasitized red blood cells induced a strong proliferation of peripheral blood mononuclear cells (PBM) from a previously infected donor (SB) [stimulation index (S.I.) = 13] but not from several normal individuals (S.I. <1.5). Proliferation of PBM from immunized individuals induced by similar extracts of *P. falciparum* has been reported previously (Wyler and Oppenheim, 1974; Wyler and Brown, 1977; Troye-Blomberg *et al.*, 1983; Bygbjerg *et al.*, 1985), but the reported mitogenic activity of some extracts (Wyler and Brown, 1977; Troye-Blomberg *et al.*, 1983) was not observed in our experiments on cells from three normal individuals. The antigen-stimulated cells from donor SB were expanded in IL-2-containing medium and cloned in the presence of phytohemagglutinin (PHA) and allogeneic antigen-presenting cells (APC) as described (Sinigaglia *et al.*, 1985a). After the cloning procedure, cells from 44 wells were assayed for their proliferative response to the parasite extract. Cells from eight wells proliferated (S.I. >3) in the presence of the extract and autologous APC [autologous irradiated PBM and Epstein-Barr virus (EBV)-transformed B cells were equally efficient in stimulating T lymphocyte clones (TLC) to proliferate to antigen]; the remaining cultures did not respond (S.I. <1.5). Cells from the eight wells were recloned and 10 distinct antigen-specific clones were established from the initial cultures. Each clone responded to the extract of parasite-infected cells but not to an extract of uninfected red blood cells (Table I). Six of the clones were typed as OKT4+/T8− and four as OKT4−/T8+. The T4+ clones responded significantly better (as measured by thymidine uptake) than the T8+ clones in the presence of antigen and APC. All of the clones produced γ-interferon (γ-IFN) following antigen stimulation, in amounts ranging from 30 to 3600 U/ml (Table I).

The clones only responded to parasite antigens in the presence of appropriate APC. Table II shows that the responses were major histocompatibility complex-restricted. Clone SB33, for example, responded to antigen only in the presence of HLA-DR4 APC (either autologous, or from other donors), whereas clones SB3 and SB22 responded only in the presence of autologous APC. Similar results were obtained with the one T8+ clone tested (data not shown). The responses of clones SB24 and SB37 to different APC deserve further mention. These clones gave the expected antigen-specific proliferative response in the presence of autologous or DR4-positive APC. However, they also respond-

Table I. Antigen-specific proliferation, phenotype and γ-interferon (IFN-γ) production of *P. falciparum*-specific T lymphocyte clones

TLC	[^{3}H-]Tdr uptake (c.p.m.)[a] *P. falciparum*	RBC	Medium	Phenotype	IFN-γ (U/ml)
SB3	26825	841	1026	T4	n.d.[b]
SB22	44603	497	367	T4	3600
SB24	12 7839	1571	1347	T4	3000
SB25	11757	1150	1531	T8	30
SB31	20571	1840	1822	T8	1800
SB33	13 3315	1918	1660	T4	1800
SB34	20916	1447	1717	T8	n.d.
SB35	27690	1713	2162	T4	3600
SB36	18291	1574	1775	T8	90
SB37	16 5244	1476	1312	T4	100

TLC cells were stimulated with *P. falciparum* antigen, or with a lysate of uninfected red blood cells (RBC), in the presence of autologous iEBV-B cells.

[a]Values expressed as mean c.p.m. of triplicate cultures.

[b]n.d. = not done.

Table II. HLA-DR restriction of *P. falciparum*-specific T lymphocyte clones

EBV-B cells	DR	Ag	Clones				
			SB3	SB22	SB24	SB33	SB37
Autologous	4,–	–	1241	1084	1487	2421	1083
		+	50 186	65 060	23 420	105 051	22 089
Allogeneic	1,4	–	2270	1366	1188	2722	1055
		+	2725	1525	12 326	120 624	12 080
Allogeneic	3,4	–	3183	542	8805	2134	7547
		+	2961	501	15 222	62 652	11 726
Allogeneic	1,3	–	1262	984	23 966	1277	24 985
		+	1145	1556	24 449	1950	24 691
Allogeneic	3,–	–	2714	1357	21 991	1234	21 736
		+	2305	1842	22 975	1149	23 311
Allogeneic	1,2	–	2623	1408	1235	968	1212
		+	2132	1287	1568	1112	1216

TLC cells were stimulated with *P. falciparum* antigen (Ag) in the presence of iEBV-B cells from a panel of HLA-DR typed donors. Values expressed as mean c.p.m. of triplicate cultures.

ed strongly to DR3-positive APC, even in the absence of malarial antigen (Table II). This behavior is most easily interpreted as a cross-reaction between DR3 antigens and a putative DR4-parasite antigen complex. Cross-reactions of this type (for example of an H-2^b-restricted H-Y-specific clone to H-2^d; or a DR3-restricted tetanus toxoid-specific clone to DR4) have previously been reported for both mouse and human clones (Von Boehmer *et al.*, 1979; Umetsu *et al.*, 1985). Of four clones tested (SB24, SB25, SB33, SB37), all responded (S.I. >4) to extracts of *P. falciparum* isolates from different geographical regions (Thailand, East and West Africa) and at least three of the clones also responded strongly to an extract of mouse red blood cells infected with *P. berghei* (data not shown). The epitopes detected by the clones are therefore not isolate-or species-specific, in contrast to some of the epitopes reacting with antibodies in the sera of infected individuals (Wilson and Phillips, 1976). However, since we selected for widely cross-reactive clones by using an extract of an Asian (Thailand) parasite isolate (Kl) to stimulate cells from a donor infected in West Africa, we do not know whether the wide cross-reactivity is a general property of malaria-specific T cell responses.

We also studied the reactivity of four clones to different stages of the red blood cell (asexual) cycle of the parasite. Figure 1 shows the responses of the clones to intact parasitized red blood cells and to lysates prepared from synchronized cultures containing either immature (ring) or mature (trophozoite and schizont) parasite forms in the presence of autologous APC. All clones tested reacted to intact parasitized cells. Clone SB33 proliferated most strongly in the presence of intact cells. Clones SB24 and SB37 proliferated most strongly to lysates of infected cells (irrespective of stage), suggesting that these clones do not recognize antigens present on the surface of infected erythrocytes. Clone SB22 responded most strongly to a lysate of erythrocytes infected with mature parasites, suggesting that it may recognise a stage-specific antigen of the parasite's blood cell cycle.

To identify the antigens to which the clones reacted, we fractionated an extract of parasitized cells by gel electrophoresis under reducing conditions. The fractions were tested for their ability

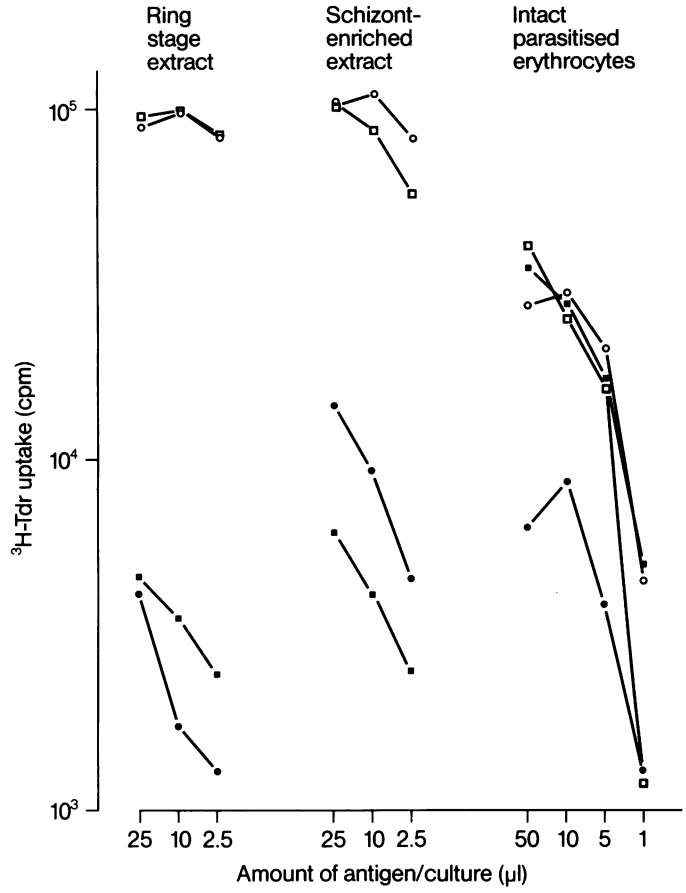

Fig. 1. Responses of T cell clones to different *P. falciparum* antigen preparations. The proliferative responses (c.p.m.) of clones SB22 (●—●), SB24 (○—○), SB33 (■—■) and SB37 (□—□) to different amounts (µl) of extracts of ring stage (left) or schizont-enriched (center) infected erythrocytes, or to intact infected erythrocytes (right), are shown. Intact erythrocytes, containing viable unsynchronized parasites (4% parasitemia), were added as the indicated volumes of a 2% suspension. All responses to uninfected intact red blood cells (50 µl) were <2000 c.p.m.

to stimulate proliferation of the clones. Figure 2 shows that two clones (SB24, SB37) out of five T4+ and one T8+ tested responded to fractions containing material of apparent mol. wt. ~50 000. This material may correspond to a major parasite protein, of similar mol. wt., detected in lysates of infected cells (Perrin and Dayal, 1982). No material capable of stimulating clones SB22, SB31, SB33 or SB35 was recovered from the gel.

Our results indicate that both T4+ and T8+ lymphocytes respond to malarial antigens. The roles of these cell types in resistance to infection are not known. T4+ lymphocytes could act as helper cells for antibody production; it is known that helper cells are required for induction of anti-malarial immunity in mice (Brown, 1971). The T8 antigen is typically present on cytotoxic T cells, but there is so far no evidence for a direct cytotoxic effect of T lymphocytes on malaria-infected cells (Deans and Cohen, 1983; Allison and Eugui, 1983). However, a possible role for both T4+ and T8+ cells in resistance to malaria is suggested by the ability of both cell types to release γ-IFN following antigen stimulation. Interferon can activate monocyte-derived macrophages to kill malaria parasites *in vitro* (Ockenhouse *et al.*, 1984), and the probable importance of this mechanism *in vivo* has been emphasized by Allison and Eugui (1983). We found that all malaria-specific clones tested released γ-IFN after antigen

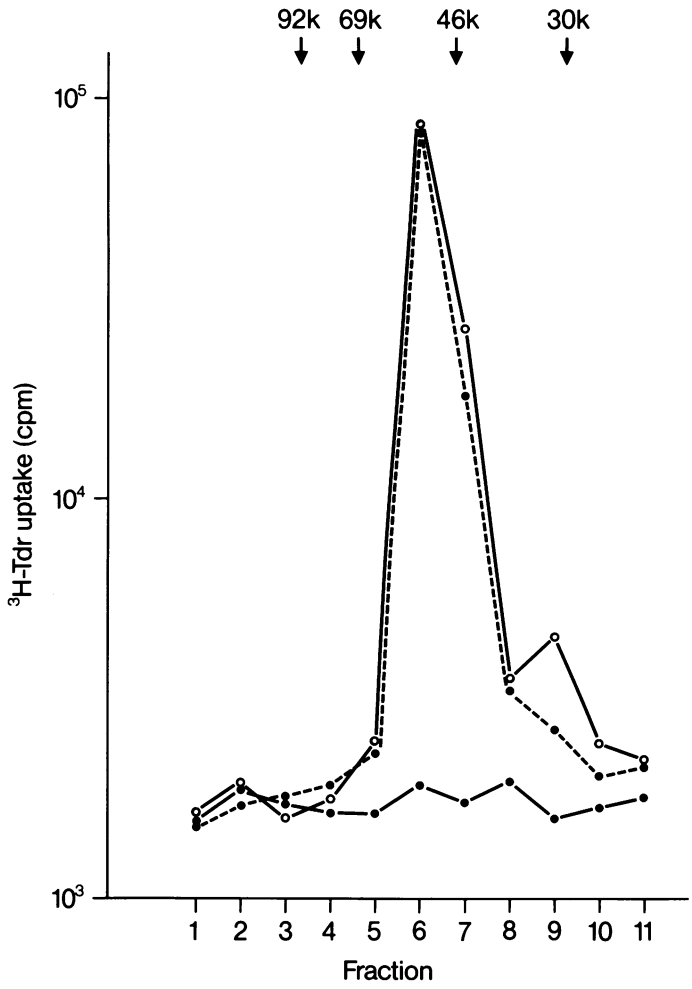

Fig. 2. Responses (c.p.m.) of T cell clones SB22 (●—●), SB24 (○—○) and SB37 (●--●) to *P. falciparum* extract (20 μl) fractionated by SDS-polyacrylamide gel electrophoresis in reducing conditions. Positions of mol. wt. standards (phosphorylase b, 92 K; bovine serum albumin, 69 K; ovalbumin, 46 K; carbonic anhydrase, 30 K) are indicated by arrows.

stimulation. Whether the T4+ and T8+ clones have other distinct functions in protection against malaria remains to be seen.

Materials and methods

Parasite antigens

The Kl strain of *P. falciparum* was maintained in culture in 5% O_2/5% CO_2 as described (Trager and Jensen, 1976), except that the culture medium contained hypoxanthine (50 μg/ml). For preparation of antigen extracts, 3 ml of packed erythrocytes from an unsynchronized culture with parasitemia of ~10% were washed in Dulbecco's calcium- and magnesium-free phosphate-buffered saline (PBS) and lysed by the addition of 1 ml 0.15% saponin in PBS to the packed cells. The detergent-insoluble material released following a 20-min. incubation at 37°C was washed in PBS by two centrifugations at 3500 *g* (10 min each). The washed pellet was suspended in 1 ml PBS, frozen and thawed twice, supplemented with 1 ml of RPMI-1640 medium containing 10% pooled AB serum and dialysed against PBS. After dialysis, insoluble material was removed by centrifugation (2 min, 5000 *g*) and filtration through a 0.45 μm Millipore filter. The soluble antigen extract was added to cultures at the stated concentrations. Similar extracts were made from other *P. falciparum* isolates, from uninfected red blood cells or from cultures of the FCH-5 *P. falciparum* isolate which had been synchronized using sorbitol (Lambros and Vanderberg, 1979) and contained >90% ring forms or >50% schizonts, respectively.

Cells

Peripheral blood mononuclear cells (PBM) were prepared by Ficoll-Hypaque centrifugation from a 20 ml blood donation from a Swiss donor (SB) who had resided for 17 months in Gambia in an area in which malaria was endemic. The donor had experienced one attack of acute malaria 1 year before the blood sample was taken.

Media for cell stimulation and growth

The culture medium was RPMI-1640 supplemented with L-glutamine (2 mM), 50 U/ml penicillin, 50 μg/ml streptomycin and 10% pooled AB serum (RPMI-HS) or 10% fetal calf serum (RPMI-FCS) (Gibco Laboratories, Paisley, UK). To support the antigen-independent growth of TLC the medium was supplemented with 100 U/ml recombinant IL-2 (Roche, Nutley, NJ).

Cloning of P. falciparum-specific T cells

PBM were isolated from heparinized whole blood by Ficoll-Hypaque centrifugation. PBM were adjusted to a concentration of 2×10^6 cells/ml in RPMI-HS and incubated with antigen for 6 days at 37°C in 5% CO_2/air. The cells were then washed twice and transferred to IL-2-containing medium, and, after another 7 days, cloned by limiting dilution. For cloning, T blasts were seeded at 0.3 cell/well in Terasaki trays (Falcon Division, Becton Dickinson & Co., Cockeysville, MD) in the presence of phytohemagglutinin (PHA-L) (Polysciences, Inc., Warrington, PA) (2 μg/ml) and 10^4 allogeneic irradiated (2500 Rad) PBM (iPBM) and IL-2. After 1–2 weeks cell growth was detected microscopically and the growing wells were expanded further in medium with PHA, allogeneic iPBM and IL-2. The subcloning procedure was identical to that described above.

Establishment of EBV-transformed cell lines

PBM from the patient or from a panel of HLA-typed volunteer donors were transformed with EBV as described (Rosen *et al.*, 1983). Briefly, 10^7 PBM were resuspended in 10 ml RPMI-FCS containing 30% supernatant of the EBV-producing marmoset cell line B95.8 and 600 ng/ml cyclosporin A (Sandoz, Basle, Switzerland), and distributed in the wells of a flat-bottom 96-well microculture plate at 5×10^4/well. All EBV-transformed B cells (EBV-B cells) were expanded for at least 2 months before being tested for their ability to serve as antigen-presenting cells (APC). All the lines were screened for mycoplasma contaminations once a month (Sinigaglia *et al.*, 1985b).

Proliferation assay

2×10^4 TLC were cultured in 0.2 ml RPMI-HS in flat-bottom microplates with 1×10^5 iPBM or 1×10^4 irradiated (5000 Rad) EBV-B cells (iEBV-B) in the presence or absence of *P. falciparum* extract. The antigen was used in stated amounts or at the optimal concentration for inducing maximum proliferation of the donor whose cells were cloned (5%). After 48 h the cultures were pulsed with 1 μCi of [^{3}H-]Tdr (2 Ci/mmol, Radiochemical Center, Amersham, UK) and incorporation was determined after another 16 h.

Cell surface analysis

TLC were analyzed for cell surface phenotype by indirect immunofluorescence on a fluorescence-activated cell sorter (FACS II) using monoclonal antibodies of the OKT (Ortho Diagnostic, Raritan, NJ) and Leu (Becton/Dickinson, Sunnyvale, CA) series.

γ-IFN production

4×10^5 TLC were cultured with 1×10^5 autologous irradiated EBV-B cells in Costar 24-well plates in the presence or absence of *P. falciparum* extract. Culture supernatants were harvested after 48 h for IFN determination. IFN activity was measured as protection of human Wish fibroblasts from the cytopathic effect of encephalomyocarditis virus (Stewart, 1981). Titers of IFN preparations are reported as the reciprocal of the highest dilution inhibiting the cytopathic effect by 50%. In addition, enzyme immunoassays specific for IFNα, β (Gallati, 1982) and a radioimmunoassay specific for IFNγ (Centocor Inc., Malvern, PA) were used to characterize the anti-viral activity.

Antigen fractionation by gel electrophoresis

Infected erythrocytes (K1 isolate, 10% parasitemia) were lysed in saponin as described above. The washed pellet obtained from lysis of 3 ml erythrocytes was suspended in 1 ml of PBS containing 10^{-3} M phenylmethylsulfonyl fluoride, frozen and thawed twice and dialyzed against water. To 0.2 ml of the dialyzed preparation were added 40 μl of SDS (10% in H_2O), 20 μl of Tris-HCl (1M, pH 6.8), 25μl of glycerol and 15 μl of β-mercaptoethanol. The suspension was heated for 1 min in a boiling water bath and applied in a 5 cm band to a 12.5% SDS-polyacrylamide gel. Following electrophoresis, the gel was cut into 1 cm strips, from which antigen was extracted in 5 ml H_2O containing 100 μg/ml cytochrome *c* as previously described (Huser *et al.*, 1978). Fractions were concentrated 10-fold in a Minicon concentrator (Amicon Co., Danvers, MA) before use.

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References

Allison,A.C. and Eugui,E.M. (1983) *Annu. Rev. Immunol.*, **1**, 361-392.
Brown,K.N. (1971) *Nature*, **230**, 163-167.
Bygbjerg,I.C., Jepsen,S., Theander,T.G. and Odum,N. (1985) *Clin. Exp. Immunol.*, **59**, 421-426.
Deans,J.A. and Cohen,S. (1983) *Annu. Rev. Microbiol.*, **37**, 25-49.
Gallati,V.H. (1982) *J. Clin. Chem. Clin. Biochem,*, **20**, 907-914.
Huser,H., Ziegler,A., Knecht,R. and Pink,J.R.L. (1978) *Immunogenetics*, **6**, 301-307.
Jayawardena,A.N., Murphy,D.B., Janeway,C.A. and Gershon,R.K. (1982) *J. Immunol.*, **129**, 377-381.
Lambros,C. and Vanderberg,J.P. (1979) *J. Parasitol.*, **65**, 418-420.
Ockenhouse,C.F., Schulman,S. and Shear,H.L. (1984) *J. Immunol.*, **133**, 1601-1608.
Perrin,L.H. and Dayal,R. (1982) *Immunol. Rev.*, **61**, 245-269.
Rosen,A., Persson,K. and Klein,G. (1983) *J. Immunol.*, **130**, 2899-2902.
Sinigaglia,F., Scheidegger,D., Garotta,G., Scheper,R., Pletscher,M. and Lanzavecchia,A. (1985a) *J. Immunol.*, in press.
Sinigaglia,F., Scheidegger,D., Talmadge,K. and Garotta,G. (1985b) *J. Immunol. Methods*, **76**, 85-92.
Stewart,W.E.,II (1981) *The Interferon System,* 2nd edn., Springer-Verlag, NY pp.17-18.
Trager,W. and Jensen,J.B. (1976) *Science*, **193**, 673-675.
Troye-Blomberg,M., Perlmann,H., Patarroyo,M.E. and Perlmann,P. (1983) *Clin. Exp. Immunol.*, **53**, 345-353.
Umetsu,D.T., Yunis,E.J., Matsui,Y., Jabara,H.H. and Geha,R.S. (1985) *Eur. J. Immunol.*, **15**, 356-361.
Von Boehmer,H., Hengartner,H., Nabholz,M., Lernhardt,W., Schreier,M.H. and Haas,W. (1979) *Eur. J. Immunol.*, **9**, 592-597.
Weidanz,W.P. and Grun,J.L. (1983) *Adv. Exp. Med. Biol.*, **162**, 409-439.
Wilson,R.J.M. and Phillips,R.S. (1976) *Nature*, **263**, 132-134.
Wyler,D.J. and Oppenheim,J.J. (1974) *J. Immunol.*, **113**, 449-454.
Wyler,D.J. and Brown,J. (1977) *Clin. Exp. Immunol.*, **29**, 401-407.

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