Malaria toxins: effects on murine spleen and bone marrow cell proliferation and cytokine production *in vitro*

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SUMMARY

The ability of deproteinated malaria exoantigens from *Plasmodium falciparum* (Pf-MT) and *P. berghei* ANKA (PbA-MT) to activate murine haematopoietic cells was analysed *in vitro*. Malaria toxins (MT) of both plasmodium species induced cell proliferation and the production of IFN-γ in overnight and long-term (5 days) spleen and bone marrow cultures and a reduction of the number of TNF-α spot forming cells (SFC). When added to cells of malaria-experienced animals, MT decreased the number of IL-4 SPC and increased the number of IL-5 SPC. However, the same proliferative and IFN-γ induction properties as in naive cells were observed. Simultaneous addition of IL-2 and PbA-MT to spleen cells inhibited the proliferation but increased the IFN-γ production usually induced by IL-2. Flow cytometric analysis revealed that the addition of MT triggered an expansion of $CD3^+$ and $GR1^+$ cell populations. Our results suggest that malaria toxins of different species can induce an immediate and strong proliferation and a TH1-type cytokine release by murine cells, independently of previous *in vivo* priming.

Key words: malaria toxins, *Plasmodium falciparum*, *Plasmodium berghei* ANKA, murine haematopoiesis, immunity.

INTRODUCTION

Haematopoietic disturbance during malarial episodes is an element in the pathophysiological events causing severe complications during infection. Several malaria antigens have been described and their possible roles in the development of pathogenesis have been analysed. Miller and colleagues (1989) showed a role of toxic antigens released by rupturing schizonts in the malarian pathogenesis. Exoantigens are thought to induce the release of tumour necrosis factor (Bate, Taverne & Playfair, 1989) leading to pathologies in mouse models (Clark, 1987; Taylor *et al*. 1992; Kwiatkowski, 1993, 1995; Jakobsen *et al*. 1995). Taverne and colleagues (1995) described a common TNF-inducing determinant in exoantigen preparations from various *Plasmodium* species. Originally classified as a phospholipid moiety, the main active part of this antigen was later characterized as a glycosylated phosphatidylinositol-like molecule (Bate *et al*. 1992*a*; Schofield *et al*. 1993; Schofield & Hackett, 1993; Gerold, Dieckmann-Schuppert & Schwarz, 1994; Bate & Kwiatkowski, 1994) and similar toxins have been isolated from *Plasmodium vivax* schizonts (Bate *et al*. 1992*b*; Wijesekera *et al*. 1996). Exoantigens that have been isolated as protein-free molecules possess the ability to stimulate macrophages, and to induce, in a T-cell independent fashion, specific but transient antibodies when coupled to a carrier (Bate *et al*. 1990).

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It is thought that the development of protective immunity or pathology during malaria infections depends on the activation of all compartments of the immune system associated to a great variety of produced cytokines (Mshana *et al*. 1991).

The present experiments aimed to analyse the influence *in vitro* of ubiquitous malaria toxins on the proliferation and cytokine production of spleen and bone marrow murine cells.

MATERIALS AND METHODS

Animals

Female Balb/c, 8–10 weeks old, and female ICR mice, older than 8 weeks, were purchased from BRL (Füllinsdorf, Switzerland). Mice were maintained on standard mouse chow and water *ad libitum*.

Parasites

Plasmodium berghei ANKA strain was a kind gift from Professor D. Walliker, University of Edinburgh, UK. *P. falciparum* K1 strain was kindly provided by Dr H. Matile, Hoffmann-La-Roche, Basel, Switzerland.

Chloroquine treatment

Balb/c mice were infected by intraperitoneal injection of *P. berghei* ANKA-parasitized red blood cells (PRBC) from a donor animal of the same strain. After development of a parasitaemia over 30%, the animals were injected i.p. twice daily with chloroquine (0·2 mg chloroquine sulfate in 0·2 ml of NaCl

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0·9 %/10 g body weight/injection) consecutively during 4 days. The animals were considered as cured if the thick blood smears were free of parasites on days 10, 15 and 17 after treatment.

Culture medium

Iscove's modified Dulbecco's medium (IMDM) with L-glutamine supplemented with $NaHCO_3$, 2-mercaptoethanol, penicillin, streptomycin, MEM non-essential amino acids and 10 % heat-inactivated foetal calf serum (IMDM/FCS; all reagents from Life Technologies, Basel, Switzerland).

Production of malaria toxins

Plasmodium berghei ANKA malaria toxin (PbA-MT) was prepared according to the modified protocol of Bate *et al*. (1993) and Taverne *et al*. (1995). Briefly, ICR mice were infected by i.p. injection of *P. berghei* ANKA-parasitized red blood cells (PRBC) from a donor animal of the same strain. After development of a parasitaemia over 30 %, the animals were sacrificed and bled by cardiac puncture. Blood cells were washed twice with HBSS (Hanks' BSS without Ca^{2+} and Mg^{2+}; Life Technologies, Basel, Switzerland). Leucocytes were removed by CF 11 cellulose (Whatman Biochemicals, Springfield Mill, Maidstone, Kent) filtration. RBC were eluted with IMDM, washed and resuspended in IMDM supplemented with 10 mM glucose. Leucocyte depletion was controlled using a blood cell counter (Sysmex F-500, Digitana, Switzerland). Leucocyte contamination was lower than 1 %. Then 25 ml of suspension (4×10^8 PRBC/ml IMDM-glucose) were incubated in a 75 cm^2 culture flask for 24 h at 37 °C in an atmosphere of 10 % O_2, 5 % CO_2 and 85 % N_2. After centrifugation at 10000 ***g*** at 4 °C, supernatants were boiled for 5 min, centrifuged for 10 min at 20000 ***g*** at 4 °C and incubated overnight at 37 °C with shaking with 2 U/ml of beaded pronase E (Sigma Chemicals, Buchs, Switzerland). After removal of the pronase beads by centrifugation, supernatants were treated with 5 μg/ml of polymyxin B agarose beads for 2 h at room temperature with shaking. The beads were removed and supernatants were filtered through a 0·22 μm membrane. Protein was not detectable with the Bio-Rad assay. RBC from non-infected animals were similarly prepared as a control. The solutions obtained were stored at 4 °C until use.

Plasmodium falciparum malaria toxin (Pf-MT) was produced using the same procedure. *P. falciparum* K1 strain was cultivated *in vitro* by standard techniques as published by Trager & Jensen (1976). Non-infected human RBC were used as control.

Single-cell suspensions

Spleen excision from mice was performed after sacrifice by cervical dislocation. Single-cell suspensions were obtained by gently squeezing the organ through a sterile steel mesh. After washing with HBSS, the viability was determined by ethidium bromide/acridine orange dye exclusion and the cells were resuspended in IMDM 10 % FCS (5×10^5 viable cells/ml for thymidine incorporation and 5×10^6/ml for ELISPOT).

For bone marrow, single-cell suspensions were made by flushing tibiae with HBSS. Washing and resuspension were as for spleen cells.

Proliferation assays

The cell suspension (200 μl) was placed into wells of flat-bottomed 96-well culture plates (Costar, Integra Biosciences, Wallisellen, Switzerland). After addition of 20 μl of MT or mitogen, plates were incubated at 37 °C in a 5 % CO_2 water-saturated atmosphere for 18 h or 5 days and 1 μCi [^{3}H]methyl thymidine (TRA 120, Amersham, Zürich, Switzerland) was added/well for the last 18 h of incubation. Supernatants were removed, aliquoted and stored frozen until cytokine quantification. Cells were harvested on filter mats, beta-counted and the proliferative responses were calculated as mean counts per min (cpm) of quadruplicate samples. The magnitude of stimulation from a sample is expressed as stimulation index (SI) and calculated as follows: cpm of sample divided by cpm of the corresponding control.

TNF-α, IFN-γ, IL-4 and IL-5 ELISA

ELISA was performed as previously described (Favre, Bordmann & Rudin, 1997). Briefly, Immulon 4 plates (Dynatech, Embrach, Switzerland) were coated with rat monoclonal anti-mouse TNF-α (clone MP6-XT22; Endogen, Bioreba, Reinach, Switzerland), rat monoclonal anti-mouse IFN-γ (clone R4-6A2), rat monoclonal anti-mouse IL-4 (clone BVD4-1D11) or rat monoclonal anti-mouse IL-5 (clone TRFK-5; All three: Minikits from Endogen). The plates were blocked with PBS + 1 % BSA and incubated overnight after addition of test samples and standards. Affinity-purified rabbit IgG anti-mouse TNF-α (Endogen) or biotinylated anti-mouse IFN-γ, IL-4, or IL-5 (Endogen) were used as secondary antibodies. Alkaline phosphatase (ALPH)-labelled goat anti-rabbit $F(ab')_2$ fragment (Jackson Immuno-Research, Milian, La Roche, Switzerland) for TNF or horse-radish peroxidase (HRPO)-conjugated streptavidin (Zymed, Bioreba, Reinach, Switzerland) for IFN-γ, IL-4 and IL-5 were used for detection. *P*-nitrophenylphosphate (*p*-NPP, Bio-Rad, Glattbrugg, Switzerland) for TNF or 1,1′-trimethylene-*bis*-(4-formylpyridinium bromide) (TMB; KPL, Bioreba, Reinach, Switzerland) for IFN-γ, IL-4, and IL-5 were added and colour reactions were recorded at 405 nm for ALPH and 620 nm for HRPO.

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Table 1. Influence of MT on [^{3}H]TdR incorporation and number of TNF-α, IFN-γ, IL-4 and IL-5 SCF of spleen and bone marrow cells from naive mice after 18 h of stimulation *in vitro*

	[^{3}H]TdR (cpm† $\times 10^{-3}$)	TNF-α spots‡	IFN-γ spots‡	IL-4 spots‡	IL-5 spots‡
Spleen					
Pf-MT§	8·1 ± 0·3*	203 ± 18*	397 ± 19**	46 ± 8	132 ± 17
Hu RBC control§	2·2 ± 0·4	947 ± 76	33 ± 4	55 ± 8	111 ± 14
PbA-MT§	5·7 ± 0·9*	443 ± 51*	166 ± 14**	60 ± 4	108 ± 12
Mu RBC control§	2·0 ± 0·1	831 ± 39	19 ± 5	48 ± 9	96 ± 14
Protein A (10 μg/ml)	8·9 ± 0·4	1744 ± 96	431 ± 48	82 ± 7	122 ± 19
Medium control§	2·1 ± 0·5	872 ± 56	19 ± 8	56 ± 7	136 ± 15
Bone marrow					
Pf-MT§	11·6 ± 0·6*	82 ± 13**	623 ± 19**	52 ± 11	89 ± 11
Hu RBC control§	3·0 ± 0·1	262 ± 27	55 ± 8	63 ± 4	117 ± 16
PbA-MT§	8·5 ± 0·4*	91 ± 21*	311 ± 9**	62 ± 2	101 ± 19
Mu RBC control§	2·8 ± 0·1	211 ± 12	48 ± 5	50 ± 10	137 ± 21
Protein A (10 μg/ml)	13·2 ± 0·6	437 ± 51	788 ± 67	72 ± 5	107 ± 15
Medium control§	3·0 ± 0·4	244 ± 18	51 ± 8	48 ± 5	92 ± 14

† Mean ± S.D. of 4 cultures from 2 different animals containing 10^5 viable cells at cultivation start.
‡ Mean ± S.D. spots/10^6 cells of 4 cultures from 2 different animals.
§ 10% volume/volume in the cultures.
* Statistical difference to corresponding control; $P < 0{\cdot}05$.
** Statistical difference to corresponding control; $P < 0{\cdot}001$.

TNF-α, IFN-γ, IL-4 and IL-5 ELISPOT assays

ELISPOT assays were performed as previously described (Favre *et al*. 1997). Briefly, 96-well Maxisorb plates (Nunc, Life Technologies, Basel, Switzerland) were coated with rat monoclonal anti-mouse TNF-α (clone XT3, Endogen), rat monoclonal anti-mouse IFN-γ (clone R4-6A2, Endogen), rat monoclonal anti-mouse IL-4 (clone 1D11, Endogen), or rat monoclonal anti-mouse IL-5 (clone TRFK4, Endogen). Plates were blocked with PBS + 1% BSA and serial dilutions of spleen or bone marrow cells were added. After incubation, cells were removed and rabbit IgG anti-mouse TNF-α (Endogen), biotinylated rat anti-mouse IFN-γ (clone XMG1·2, Endogen), biotinylated anti-mouse IL-4 (clone 24G2, Endogen), or biotinylated anti-mouse IL-5 (clone TRFK-5, Endogen) were added. Alkaline phosphatase-conjugated goat anti-rabbit F(ab′)$_2$ fragment (Jackson Immuno-Research, Milian, La Roche, Switzerland) for TNF, or ALPH-conjugated avidin (Sigma, Buchs, Switzerland) for IFN-γ, IL-4 and IL-5 were added as conjugates. After incubation, 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Bio-Rad, Glattbrugg, Switzerland) was added as substrate. The colour reaction was stopped by rinsing the plates with distilled water and spots were counted under ×16 magnification. Protein-A (Pharmacia Biotech AG, Dübendorf, Switzerland) served as an internal control for proliferation, SFC and cytokine production assays.

Flow cytometry analysis

Spleen leucocytes were isolated from single-cell suspensions of spleen cells by lymphocyte separation medium (LSM; Organon-Tecknika, Pfäffikon, Switzerland) gradient centrifugation and adjusted to 10^6 cells/ml in PBS$^+$ 0·1% BSA and 0·1% NaN_3. Cells were incubated for 1 h on ice with phycoerythrin or fluorescein isothiocyanate (FITC)-labelled monoclonal antibodies; rat anti-murine erythroid cells, TER-119, rat anti-murine myeloid differentiation antigen, GR-1, rat anti-murine CD45R (Pharmingen, San Diego, CA, USA) or with rat anti-murine CD3, CD4 and CD8 (Serotec, Oxford, UK). All antibodies were used at a predetermined optimal dilution of 1:100 in PBS. Cells incubated with normal rat serum were included as negative controls. Labelled cells were analysed on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA).

Statistical analysis

Results were analysed for statistical significance by using Student's *t*-test. $P < 0{\cdot}05$ was considered as significant.

RESULTS

Influence of Pf-MT and PbA-MT in vitro *on proliferation of spleen and bone marrow cells and production of TNF-α, IFN-γ, IL-4 and IL-5*

Short-term cultures (*see Table 1*). To determine whether cell proliferation could be induced by MT from different parasite species, *in vitro* [^{3}H]thymidine uptake was measured in spleen and bone marrow cells from naive mice after overnight

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Table 2. Influence of MT on [^{3}H]TdR incorporation and TNF-α, IFN-γ, IL-4 and IL-5 secretion of spleen and bone marrow cells from naive mice after 5 days of stimulation *in vitro*

	[^{3}H]TdR (cpm† $\times 10^{-3}$)	TNF-α (pg‡/ml)	IFN-γ (pg‡/ml)	IL-4 (pg‡/ml)	IL-5 (pg‡/ml)
Spleen					
Pf-MT§	19·3 ± 1·1**	< 5·0	22250**	5·2	8·9
Hu RBC control§	1·8 ± 0·1	< 5·0	37	8·3	10·6
PbA-MT§	12·7 ± 1·3**	< 5·0	13400**	6·6	12·1
Mu RBC control§	2·2 ± 0·2	< 5·0	47	6·0	8·5
Protein A (10 μg/ml)	56·3 ± 4·1	25·0	29310	12·0	10·2
Medium control§	2·0 ± 0·3	< 5·0	55	5·0	10·6
Bone marrow					
Pf-MT§	9·1 ± 1·2**	< 5·0	6320**	3·5*	10·2
Hu RBC control§	1·8 ± 0·1	< 5·0	50	12·2	8·0
PbA-MT§	6·7 ± 0·5*	< 5·0	4250**	2·5*	4·2
Mu RBC control§	1·4 ± 0·3	< 5·0	60	5·5	8·0
Protein A (10 μg/ml)	31·2 ± 2·7	10·2	16310	8·0	10·2
Medium control§	1·9 ± 0·2	< 5·0	65	2·4	12·0

† Mean ± S.D. of 4 cultures from 2 different animals containing 10^5 viable cells at cultivation start.
‡ Mean of 4 culture supernatants from 2 different animals.
§ 10 % volume/volume in the cultures.
* Statistical difference to corresponding control; $P < 0{\cdot}05$.
** Statistical difference to corresponding control; $P < 0{\cdot}001$.

Table 3. Influence of MT on [^{3}H]TdR incorporation and number of TNF-α, IFN-γ, IL-4 and IL-5 SCF of spleen cells from PbA experienced mice cultivated *in vitro* for 18 h

Spleen	[^{3}]TdR (cpm† $\times 10^{-3}$)	TNF-α spots‡	IFN-γ spots‡	IL-4 spots‡	IL-5 spots‡
Pf-MT§	9·3 ± 0·7*	97 ± 12	447 ± 39**	19 ± 8*	176 ± 17*
Hu RBC control§	1·8 ± 0·3	412 ± 55	43 ± 28	99 ± 8	21 ± 8
PbA-MT§	6·1 ± 0·8*	146 ± 19*	120 ± 24*	36 ± 4*	121 ± 17*
Mu RBC control§	2·1 ± 0·3	432 ± 39	48 ± 11	117 ± 10	9 ± 9
Protein A (10 μg/ml)	10·6 ± 0·9	834 ± 72	411 ± 24	112 ± 7	16 ± 17
Medium control§	2·2 ± 0·3	438 ± 56	44 ± 12	96 ± 7	17 ± 15

† Mean ± S.D. of 4 cultures from 2 different animals containing 10^5 viable cells at cultivation start.
‡ Mean ± S.D. spots/10^6 cells of 4 cultures from 2 different animals.
§ 10 % volume/volume in the cultures.
* Statistical difference to corresponding control; $P < 0{\cdot}05$.
** Statistical difference to corresponding control; $P < 0{\cdot}001$.

cultures (18 h) in the presence of Pf- or PbA-MT. Addition of Pf-MT to cell cultures induced a significant stimulation (SI = 3·7, $P < 0{\cdot}05$, for spleen and SI = 3·9, $P < 0{\cdot}05$, for bone marrow) as compared to corresponding RBC controls. Slightly lower stimulation indices were found in cultures treated with PbA-MT (SI = 2·9, $P < 0{\cdot}05$, for spleen and SI = 3·0, $P < 0{\cdot}05$, for bone marrow cells). No stimulation was found after treatments with either human or murine RBC extracts. TNF-α, IFN-γ, IL-4 and IL-5 spot-forming cells (SFC) were enumerated by ELISPOT assays. The number of TNF-α SFC spleen cells was reduced to about 20 % by Pf-MT and to about 50 % by PbA-MT of the corresponding RBC controls. In bone marrow cultures with Pf-MT and PbA-MT, the number of TNF-α SFC represented 33 % and 50 % respectively of the corresponding controls. Incubation with human or murine RBC controls did not influence the number of TNF-α SFC of either organ. In contrast to TNF-α, the number of IFN-γ spots was increased 12-fold in spleen ($P < 0{\cdot}001$) and 11-fold in bone marrow ($P < 0{\cdot}001$) cultures after addition of Pf-MT, as compared to RBC controls. PbA-MT increased 8·7-fold the number of IFN-γ spleen SFC ($P < 0{\cdot}001$) and 6·5-fold the bone marrow IFN-γ SFC ($P < 0{\cdot}001$). MT from both parasite species did not influence the number of IL-4 and IL-5 SFC in both organs.

Long-term cultures (*see Table 2*). As in short-term cultures, Pf-MT and PbA-MT led to a strong proliferation of spleen cells in long-term cultures (5 days; SI = 10·7 for Pf-MT, $P < 0{\cdot}001$, and SI = 5·8 for PbA-MT, $P < 0{\cdot}001$). In bone marrow cultures, the proliferation induced by both MT were slightly weaker (SI = 5·0 for Pf-MT, $P < 0{\cdot}05$, and SI = 4·8,

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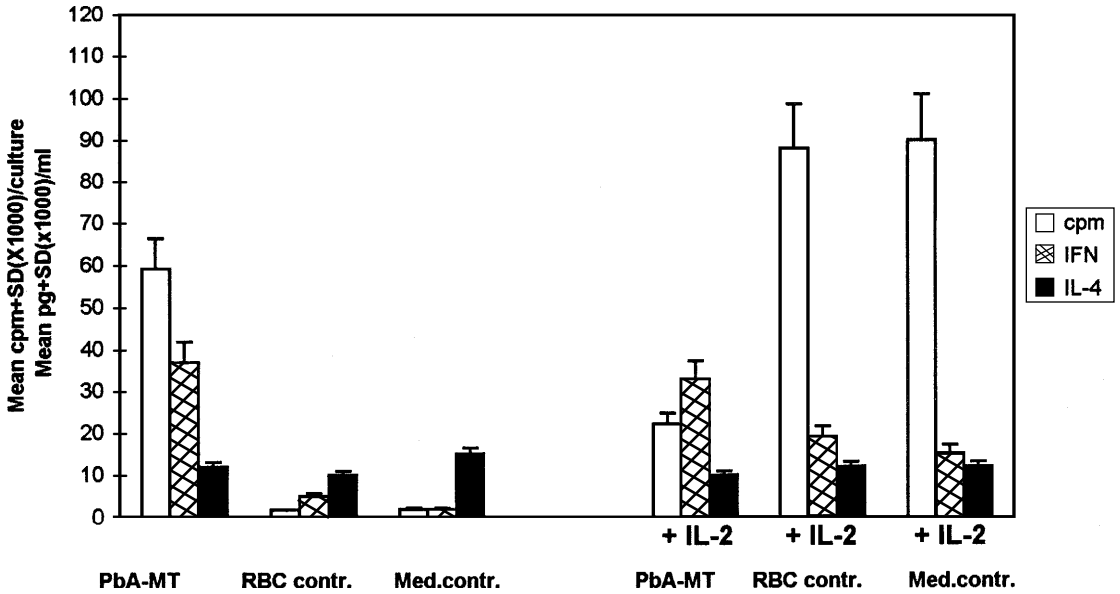

Fig. 1. Influence of PbA-MT on the action of IL-2. Spleen cells from naive mice were incubated (2×10^5 viable cells/well), with or without IL-2, in the presence of PbA-MT or control preparations. After 5 days of culture, [^{3}H]thymidine uptake and secretions of IFN-γ and IL-4 were measured, as described in the Materials and Methods section. Results are given as means of triplicates $\pm$ S.D.

$P < 0{\cdot}05$, for PbA-MT). At the end of the cultivation period, the viability of MT-treated cells remained above 85 % in all cultures in comparison to less than 60 % for the controls.

In all culture supernatants, the measured TNF-α levels were below 5 pg/ml, except for the included protein A internal controls. A 600-fold increase ($P < 0{\cdot}001$) of the IFN-γ concentrations in Pf-MT-treated spleen cell cultures, and a 285-fold increase ($P < 0{\cdot}001$) with PbA-MT were measured when related to the corresponding controls. In bone marrow cultures, IFN-γ secretion was also significantly increased but, to a lesser extent by the addition of MT (126-fold, $P < 0{\cdot}001$, for Pf-MT and 70-fold, $P < 0{\cdot}001$, for PbA-MT) when compared to the corresponding controls. IL-4 and IL-5 concentrations in supernatants of cell cultures treated with MT showed no significant differences as compared to corresponding RBC controls.

Effect of Pf-MT and PbA-MT on spleen cells from primed animals (*see Table 3*)

In order to determine whether MT influence Th1 or Th2-type immune responses, cells previously primed *in vivo* by a *P. berghei* ANKA infection were used. [^{3}H]thymidine uptake was similar in spleen cells from *P. berghei* ANKA-experienced mice (6 weeks after last detection of blood-stage parasites) as compared to spleen cells from naive animals.

The numbers of TNF-α SFC in spleen cell cultures from primed mice were reduced in all samples to 50–60 % of those from naive animals. As in short-term spleen cultures from naive animals, a decrease of 76·5 % for TNF-α SFC was registered after incubation with Pf-MT ($P < 0{\cdot}001$), and of 66·2 % ($P < 0{\cdot}05$), for PbA-MT of the corresponding controls. In contrast, the number of IFN-γ SFC increased 10-fold ($P < 0{\cdot}001$) with Pf-MT, and 2·5-fold ($P < 0{\cdot}05$) with PbA-MT, as compared to the corresponding controls. Pf-MT reduced the number of IL-4 SFC to 54·5 % ($P < 0{\cdot}05$), and PbA-MT to 74·2 % ($P < 0{\cdot}05$), of the corresponding controls. No significant difference was observed in the numbers of IFN-γ and IL-4 SFC between naive and primed animals. However, priming augmented the number of IL-5 spleen SFC in cultures supplemented with Pf-MT (2·6-fold, $P < 0{\cdot}05$). This was not significant for PbA-MT (1·9-fold).

Influence of MT on the proliferative and the IFN-γ inducing properties of IL-2 (*see Fig. 1*)

IL-2, a potent T cell stimulator, enhances the production of a variety of Th1-type cytokines, including IFN-γ. To determine whether MT and IL-2 act in a synergistic way, spleen cells from naive mice were stimulated for 5 days simultaneously with murine IL-2 and PbA-MT. As compared to the culture stimulated with IL-2 only (87600 $\pm$ 6744 cpm), a reduction of 74·7 % (22124 $\pm$ 1913 cpm, $P < 0{\cdot}001$) of the [^{3}H]thymidine incorporation was measured in spleen cell culture stimulated simultaneously with both IL-2 and PbA-MT. Compared to PbA-MT cultures (59316 cpm $\pm$ 4174), thymidine uptake was lowered to 37·2 % in PbA-MT/IL-2 cultures (22124 $\pm$ 1913 cpm, $P < 0{\cdot}05$).

Surprisingly, the measured IFN-γ levels were similar in IL-2/Pba-MT cultures (35411 $\pm$ 1932 pg/ml) to those without IL-2 (37124 $\pm$ 2115 pg/ml). Pba-MT in IL-2 cultures increased 1·8-fold the IFN-γ levels ($P < 0{\cdot}05$), as compared to

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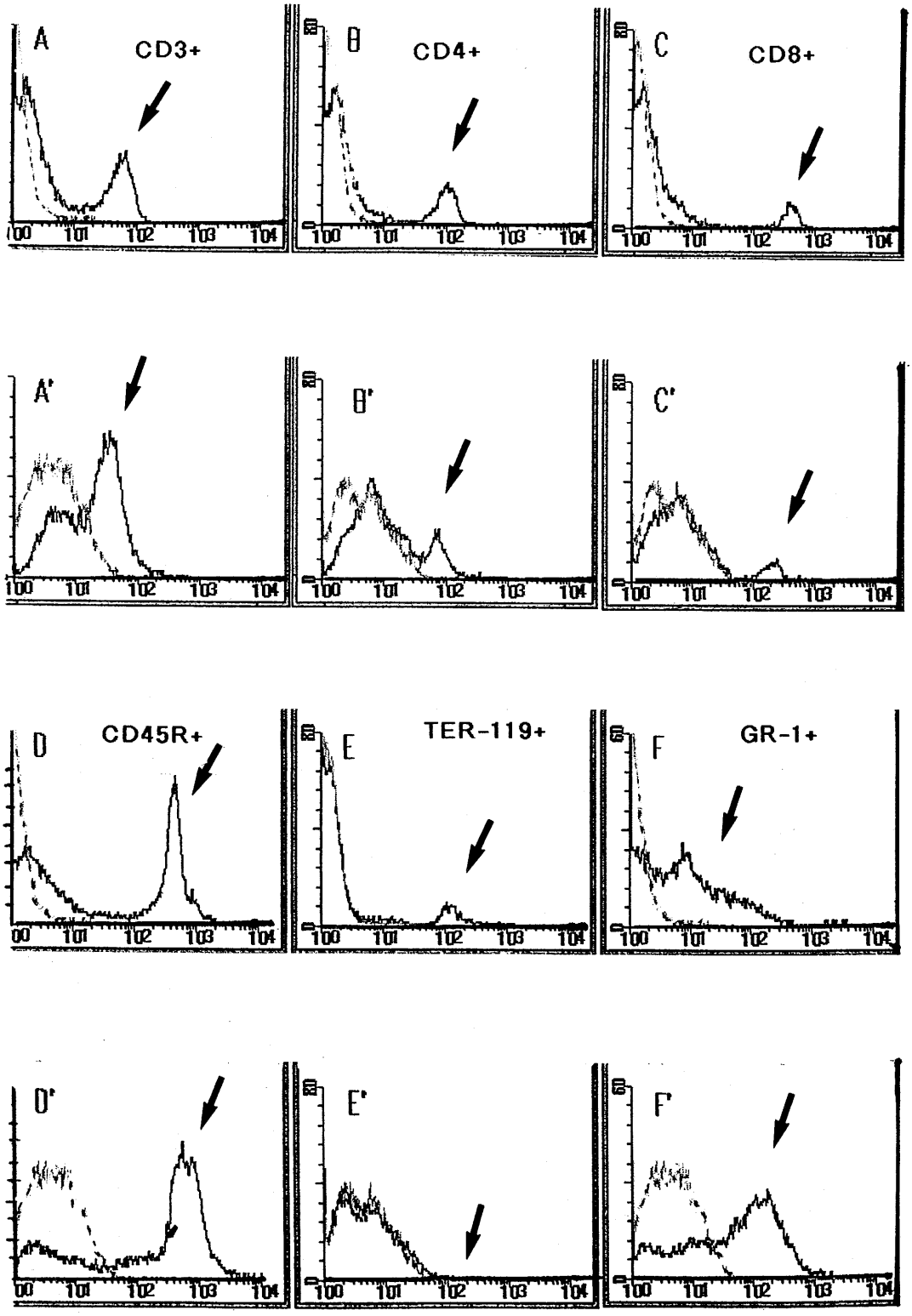

Fig. 2. Influence of Pf-MT on the populations of spleen cells from naive animals cultivated *in vitro* during 5 days. (A) Distribution of $CD3^+$ cells in RBC control cultures; (A′) in cultures containing Pf-MT; (B) distribution of $CD4^+$ cells in RBC control cultures; (B′) in cultures containing Pf-MT; (C) distribution of $CD8^+$ cells in RBC control cultures; (C′) in cultures containing Pf-MT; (D) distribution of $CD45R^+$ cells in RBC control cultures; (D′) in cultures containing Pf-MT; (E) distribution of TER-119^+ cells in RBC control cultures; (E′) in cultures containing Pf-MT; (F) distribution of GR-1^+ cells in RBC control cultures; (F′) in cultures containing Pf-MT. X-axis: fluorescence intensity; Y-axis: number of cells. Cells were stained as described in the Materials and Methods section. In the histograms, reactive cell populations are designated by an arrow, and unstained controls are represented by dotted lines.

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RBC/IL-2 cultures (20304 ± 1882 pg/ml). In all culture supernatants, with and without IL-2, the measured IL-4 levels were not significantly modified by the addition of PbA-MT.

Flow cytometry (*see Fig. 2*)

The above results led us to analyse the cell population subsets by flow cytometry. The addition of Pf-MT to the spleen cell cultures from naive mice during 5 days increased the number of T cells. However, the $CD4^+/CD8^+$ ratio remained unchanged. No difference was observed in the cell population expressing the $\gamma\delta$TCR between MT-treated and control populations (data not shown). The expression of the $CD45R^+$ antigen on B-cells was strongly stimulated by the addition of MT. Surprisingly, the number of cells expressing the murine myeloid differentiation antigen GR-1 was higher after addition of Pf-MT to the cultures. In contrast, the cell population carrying the murine erythroid T-119 antigen was reduced. Identical results were obtained with PbA-MT (data not shown).

DISCUSSION

Deproteinated *P. falciparum* and *P. berghei* malaria toxins, when added to cultures of spleen or bone marrow cells from naive mice, rapidly induce a strong proliferative response and a high IFN-γ production, whereas the number of TNF-α spot forming cells is not affected. In addition, MT was unable to induce a Th2-like response even after *in vivo* priming of the cells by malaria infections. MT significantly triggered the proliferation of $GR1^+$ granuloid cell population and, to a lesser extent, the proliferation of B- and T-cells, without influencing the $CD4^+/CD8^+$ ratio. It is now well established that single cytokines in the extremely complex network of the immune system may have different or contrary effects depending on the targeted cells and/or on the modulating activities of other cytokines (Romagnani, 1994). The importance of cytokines in protective and pathological responses to malaria infection is reflected by the encountered multiple aspects of the disease (Grau, 1990; Clark & Rockett, 1994*a*, *b*; Eling & Kremsner, 1994). Experimental models of infections with a variety of pathogens suggest that IFN-γ and TNF-α significantly contribute to host resistance to infection (Grau *et al*. 1987; Shear *et al*. 1989; Grau, 1992; Stevenson, Tam & Nowotarski, 1990; Kremsner *et al*. 1992; Tsuji *et al*. 1995). In our experimental model, the immediate and strong reactions of naive murine spleen and bone marrow cells to Pf-MT and PbA-MT suggest that an active host defence could be induced at the cellular level by products deriving from the parasites without the contribution of living organisms. The high proliferation and strong stimulation of IFN-γ production observed in short-term cultures indicates that MT of different parasite strains can induce an immediate cell activation.

The number of TNF-α SFC was considerably reduced in spleen and bone marrow after overnight cultivation with MT and no TNF-α production was measured in any of the culture supernatants. This correlates with the reported inconsistencies in TNF-α release obtained by different MT preparations (Taverne *et al*. 1995). It could also be due to co-extraction of a TNF-α inhibitor (Sheik *et al*. 1996). An overproduction of interleukin-10 induced by MT could be another explanation for the reduction of TNF-α spot forming cells (Ho *et al*. 1995). In addition, MT-Pf and MT-PbA failed in all experiments to induce the production of NO (data not shown). This finding is in accordance with the results of Rockett *et al*. (1994), and Ahvazi, Jacobs & Stevenson (1995), who reported that an overproduction of NO suppresses lymphoproliferation.

High concentrations of IFN-γ were measured at day 5 in supernatants of stimulated spleen and bone marrow cultures. In contrast, two Th2-type cytokines, IL-4 and IL-5, in long-term cultures, and the numbers of IL-4 producing cells, in short-term cultures, were not significantly affected by the addition of MT, even after *in vivo* priming by PbA infection. Our results indicate that soluble malaria antigen can trigger naive cells of both haematopoietic compartments to mount a strong Th1-type response. During a malaria infection, many of the mouse models show a switch from Th1 to Th2 pathway which may be important for clearance of the parasites (Phillips, Mathers & Taylor-Robinson, 1994; Grau & Behr, 1994; Troye-Blomberg, Berzins & Perlmann, 1994; Yap, Jacobs & Stevenson, 1994; Cruz Cubas, Gentilini & Monjour, 1994). This switch leads to the production of anti-plasmodium antibodies, produced by a non-selective expansion of the Ig-secreting B-cells (von der Weid *et al*. 1994, Vanham & Bisalinkumi, 1995). In our model, the failure to induce a Th2-like pathway by PbA-MT stimulation of spleen cells from *P. berghei* ANKA malaria-experienced animals might be explained by the fact that we used deproteinated antigens (Bate *et al*. 1993). In addition, we observed in our model that MT of human or murine parasite species are able to upregulate rapidly the production of IFN-γ without affecting the TNF-α secretion even from cells which have been exposed previously to malarial antigens.

The IL-2 proliferative effect on spleen cells (Cantrel & Smith, 1984; von der Weid & Langhorne, 1993) was significantly inhibited by MT without affecting the IFN-γ production. This phenomenon suggests that independent mechanisms not yet elucidated are involved in the regulation of activation and proliferation. This sustains the observations that during a malarial infection cellular responses which are related to a large extent to non-specific activation

(Allison & Clark, 1977) can lead to exaggerated production of cytokines and eventually to immunopathology.

In our experiments, MT failed to influence the differentiation of spleen cells cultivated in medium containing haematopoietic stem cell growth factors (data not shown). However, we found that MT induced an expansion of the granuloid GR-1$^+$ cell compartment. It seems that our preparations of MT possess inflammatory properties independent of the ability to induce the production of TNF-α. *In vivo* experiments failed to demonstrate in mice any influence of repeated MT injections on cells of eosinophilic lineage (data not shown). This finding correlates with the inability of MT to induce the production of IL-4 (de Kossodo & Grau, 1993; Elghazali, Esposito & Troye-Blomberg, 1995), even if the cells were previously sensitized *in vivo* through malarial infection. The function of this granulocyte activation during a malaria infection has to be investigated. Furthermore, MT could be involved in the recruitment of immune-competent cells capable of determining the cellular and/or the humoral immune responses during a malaria infection (Golding, Zaitseva & Golding, 1994). Our observations suggest that MT might act indirectly as a mediator in haematopoiesis.

In conclusion, our results suggest that malaria toxins of different *Plasmodium* species can induce a Th1-type immune response, independently of previous *in vivo* priming, and thus contribute to pathogenesis during malaria infection. Furthermore, our experiments showed that the activation of granuloid cells (GR1$^+$) might be due to malaria toxins. Further experiments will have to determine the exact role of this cell population, and whether the observed properties of MT also affect cells from lymphoid organs other than spleen or bone marrow. Whether the observed phenomena are restricted only to *in vitro* situations has also to be investigated. Such experiments might help to understand and to control the development of pathogenesis during malaria infection.

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