CD4+ Cytolytic T Cell Clone Confers Protection against Murine Malaria

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Summary

A CD4+ T cell clone (A1.6) was derived from spleen cells of mice immunized with irradiated sporozoites. This T cell clone recognizes an antigen that is shared by sporozoites and blood forms of *Plasmodium berghei* and differs from the circumsporozoite protein. Clone A1.6 displays cytotoxic activity, produces IFN-γ and IL-2 in vitro, and recognizes the plasmodial antigen in the context of the class II I-E^d molecule. Passive transfer of this CD4+ clone into naive mice resulted in a high degree of protection against sporozoite challenge.

Protective immunity against malaria sporozoites is mediated, in part, by neutralizing antibodies that recognize the repeat domain of the circumsporozoite (CS)[1] protein. The protective role of these antibodies has been demonstrated by the resistance to sporozoite challenge upon passive transfer of anti-CS mAbs and also by the antibody-mediated protection induced by immunization with synthetic peptides (1).

T cell effector mechanisms, mediated by CD8+ cytotoxic T cells, also play a major role in antisporozoite immunity, as shown by the reversal of immunity obtained after in vivo depletion of CD8+ T cells (2, 3). Furthermore, it has recently been shown that passive transfer into naive mice of CD8+ T cell clones, which recognize a defined epitope of the CS protein, confers protection against sporozoite challenge (4).

While CD4+ T cells are known to play an important role in the induction of protective antisporozoite antibodies, there has up to now been no evidence indicating that these T cells may also inhibit the infectivity of sporozoites and/or the development of liver stages.

Here we report some of the basic characteristics of a CD4+ T cell clone we obtained from mice immunized with irradiated sporozoites. This T cell clone recognizes an antigen that is shared by sporozoites and blood forms of *Plasmodium berghei* and differs from the CS protein. Passive transfer of this CD4+ clone into naive mice protected most of these animals against sporozoite challenge.

[1] *Abbreviations used in this paper:* CS, circumsporozoite; FPLC, fine performance liquid chromatography; NMS, normal mouse serum; NRBC, normal red blood cells; NSG, noninfected mosquito salivary glands; PRBC, parasitized red blood cells; SI, stimulation index.

Materials and Methods

Parasites, Mice, and Schedule of Immunization. Sporozoites and blood stages of *P. berghei* (NK65 strain) were obtained as previously described (5). 6-wk-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were immunized intravenously with 10^5 irradiated sporozoites, twice a week, for 1 mo.

Antigens. Sporozoite extracts were prepared by sonicating these parasites at a concentration of 10^7/ml, in RPMI-1640 culture medium, containing 10% FCS. The lysate was then centrifuged at 10,000 *g* for 3 min, and the supernatant frozen at -20°C, until used. *P. berghei* parasitized red blood cells (PRBC) were obtained from infected BALB/c mice and the erythrocytes lysed by saponin treatment. After extensive washing, the blood stages were disrupted by sonication and parasite extracts were prepared as described above. Control extracts, prepared from an equivalent number of sonicated noninfected mosquito salivary glands (NSG) and normal red blood cells (NRBC), were processed similarly.

Fractionation of the PRBC Extract. The blood stage extract was fractionated on a DEAE-5DW column (LKB Instruments Inc., Bromma, Sweden), using a fine performance liquid chromatography (FPLC) apparatus (Pharmacia Fine Chemicals, Uppsala, Sweden), according to a method previously described (6). All fractions were dialyzed twice against PBS, aliquoted, and frozen at −20°C until used.

Antibodies. Affinity-purified anti-L3T4 mAb (GK1.5), and anti–class II mAbs of different specificities—namely, anti-I-A^s (MK-S4), anti-I-A^d (MK-D6), and anti-I-E^d (14-4-4S)—were kindly provided by Dr. Jeanette Thorbecke, of New York University.

Proliferative Response of Splenic T Cells. Immune spleen cells were depleted of cell surface Ig+ cells, by adherence to anti-mouse Ig-coated petri dishes, as described (7). An aliquot of 3×10^5 splenocytes of this T cell–enriched fraction, was stimulated with different amounts of PRBC extract, in RPMI-1640 medium, containing 0.5% normal mouse serum (NMS), and in the presence of irradiated syngeneic APC (5×10^5). The proliferative response was measured on day 5 by ^{3}H-TdR incorporation.

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Establishment of a T Cell Growth Factor–dependent, Antigen-Specific T Cell Clone. Splenic T cells from sporozoite-immunized mice were stimulated in vitro with a blood stage extract (2.5×10^6 PRBC/ml), in the presence of irradiated unprimed spleen cells, and maintained for 1 wk in RPMI 1640 culture medium containing 0.5% NMS. They were then restimulated for 10 d with a PRBC extract, in RPMI 1640 medium containing 10% FCS and 10% T cell growth factor (7), in the presence of irradiated syngeneic APC. T cell clones were derived from this bulk culture by limiting dilution (7). One of these clones, A1.6, was selected and expanded by weekly stimulation with a PRBC extract. The generation and maintenance of an OVA-specific, BALB/c-derived, cytolytic $CD4^+$ clone (5.9), which was kindly provided to us by Dr. Charles Janeway (Yale University, New Haven, CT), has been earlier described (8).

Proliferative Response, IFN-γ and IL-2 Production by the Cloned T Cells. Two wk after antigen stimulation, 10^4 cloned T cells were restimulated by adding irradiated, antigen-pulsed, stimulator cells, obtained by incubation of unprimed syngeneic spleen cells with a blood stage extract. The supernatant fluids were removed, after 48 h of cultivation, and their concentrations of IFN-γ and IL-2 determined. While the IFN-γ titer in the supernatants of stimulated T cell clones was measured by an RIA (4), the IL-2–dependent cell line, CTLL-2, was used to detect this lymphokine in the T cell supernatants (9). The proliferative response was measured on day 3 by the ^{3}H-TdR incorporation and expressed as stimulation index (SI).

Cytolytic Assays. This assay was performed as described elsewhere (10). Briefly, ^{51}Cr-labeled, BALB/c-derived, A20.2J cells, kindly provided by Dr. Ralph Kubo (National Jewish Center, Denver, CO), were pulsed with a PRBC extract (5×10^6 PRBC/ml) and then incubated with splenic T cells or the A1.6 T cells, at different E/T ratios, as indicated. After 12 h, the ^{51}Cr release from target cells was measured and expressed as the percent of specific ^{51}Cr release.

Effect of Selected mAbs on the Proliferative Response of Clone A1.6. The inhibitory effect of anti–class II and anti–L3T4 antibodies on the antigen-induced proliferation of T cell clone A1.6 was determined by adding the corresponding mAbs to the culture, at a final concentration of 0.5 μg/ml (11).

Adoptive Transfer Experiments. Various numbers of cloned T cells or comparable numbers of normal splenocytes were injected intravenously into BALB/c mice. Immediately before transfer, the cells were resuspended in serum-free culture medium (0.5 ml) containing 10^3 U of human rIL-2. The mice were challenged, 4 h after the cell transfer, by i.v. injection of sporozoites. A second dose of 10^3 U of rIL-2 was administered intravenously, 20 h after sporozoite challenge. Thin blood smears were prepared daily, between days 3 and 12 after challenge, stained with Giemsa, and examined by light microscopy to detect erythrocytic parasites. Protection was defined as the absence of parasitemia.

Results and Discussion

Initial experiments showed that splenic T cells from sporozoite-immunized BALB/c mice were able to proliferate and produce IFN-γ, upon in vitro stimulation with an extract of blood stages of *P. berghei* (Fig. 1). Spleen cells of naive mice were not stimulated by this blood stage extract. These findings suggested that the observed activation of T cells of sporozoite immunized mice was antigen specific, rather than the result of mitogenic activity of the PRBC extract.

To further analyze this phenomenon, a T cell clone, A1.6, was derived from the spleen cells of sporozoite-immunized BALB/c mice. This clone (A1.6), which expresses the CD 4^+8^- phenotype, proliferates and produces IFN-γ as well as IL-2 upon incubation with antigen.

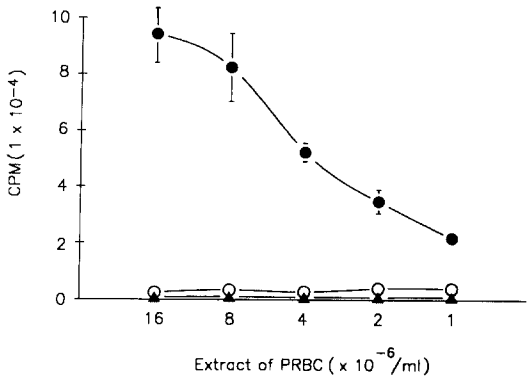

Figure 1. Proliferative response of splenic T cells to extracts of *P. berghei* PRBC. Splenic T cells (3×10^5) from irradiated sporozoite-immunized mice were stimulated with an extract from different amounts of PRBC (●), or equivalent amounts of NRBC (○), in the presence of syngeneic APC (5×10^5). Splenic T cells from naive mice were also stimulated with the PRBC extract under identical conditions (▲). The proliferative response was measured on day 5 by the incorporation of ^{3}H-TdR.

The pattern of reactivity of clone A1.6, cultured in the presence of various antigen preparations, is summarized in Table 1. We found that clone A1.6 displayed a strong prolifer-

Table 1. *Specificity of the Proliferative Response and IFN-γ Production of $CD4^+$ T Cell Clones to Antigen Stimulation*

Clone	Antigen*	SI‡	IFN-γ§
			ng/ml
A1.6	PRBC	70.4	102 ± 12
	Sporozoites	3.3	2 ± 0.6
	OVA	1.4	Neg
	NRBC	1.1	Neg
	NSG	0.8	Neg
	–	1.0	Neg
5.9	PRBC	0.9	Neg
	Sporozoites	1.0	Neg
	OVA	37.2	64 ± 6
	–	1.0	Neg

* 10^4 cloned T cells were cultured in the presence of 5×10^5 antigen-pulsed APC. Extracts of PRBC and sporozoites were obtained from 5×10^6 parasites/ml. The NSG extract was derived from the same number of noninfected mosquitoes as the sporozoites. The NRBC extract was prepared from the same number of RBC as the PRBC. OVA was used at a concentration of 100 μg/ml.

‡ The stimulation index (SI) was calculated as the cpm of cultures in the presence of antigen, divided by the cpm in the absence of antigen.

§ The concentration of IFN-γ was determined in triplicate samples.

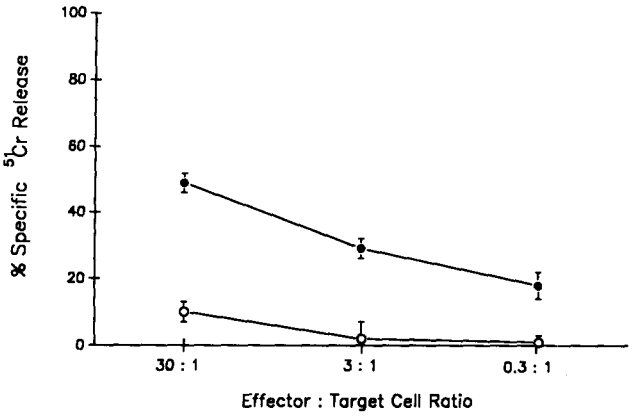

Figure 2. Cytolytic assay of clone A1.6. Clone A1.6 was incubated with ^{51}Cr-labeled A20.2J cells, at the indicated E/T ratios, and pulsed with a PRBC extract (●) or just incubated in medium (○). Supernatants were removed 12 h later to assess the specific ^{51}Cr release.

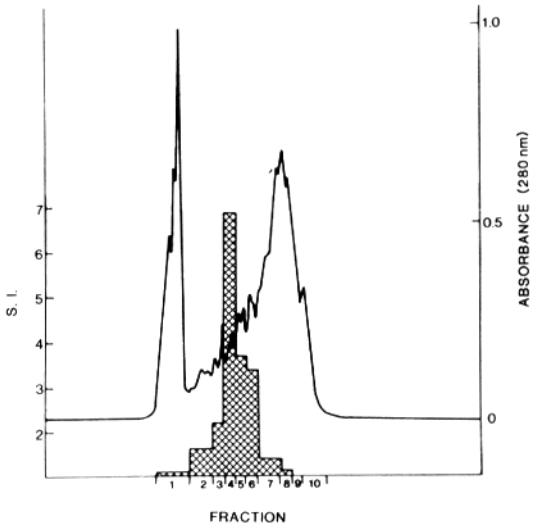

Figure 4. Fractionation of PRBC extract by DEAE FPLC. An antigen extract, corresponding to 2 × 10^9 PRBC, resuspended in 10 mM Tris containing 10 mM NaCl at pH 8.4, was loaded on a DEAE-5DW column. After extensive washing with this buffer, the bound molecules were eluted by a 30-ml linear gradient, from 10 mM to 1 M NaCl, at a flow rate of 0.5 ml/min. The protein content of each fraction was monitored at 280 nm, and is represented by the solid line. The height of the bars corresponds to the SI of clone A1.6 induced by the different fractions.

ative response and high levels of IFN-γ production when incubated with an extract of *P. berghei* PRBC, and a more modest, but clearly positive, response upon stimulation with an extract prepared from a comparable number of sporozoites. No activation was observed when NRBC, NSG, or OVA was used as antigen.

In contrast, an OVA-specific, highly reactive CD4$^+$ T cell clone (5.9) did not proliferate nor produce IFN-γ when incubated with a PRBC or sporozoite extract. These results corroborated our earlier findings obtained with immune spleen cells, indicating that the activation of A1.6 is plasmodial specific.

The response of clone A1.6 to the sporozoite extract was consistently much lower than that obtained with a PRBC extract, suggesting that the corresponding antigen or epitope is more abundant in blood stages. However, the corresponding antigen (or epitope) is undoubtedly also present in sporozoites. Besides, the T cell clone (A1.6) must have been induced by a sporozoite and/or liver stage antigen, since it was derived from mice immunized with irradiated sporozoites, which invade hepatocytes, but are unable to transform into blood stages (12).

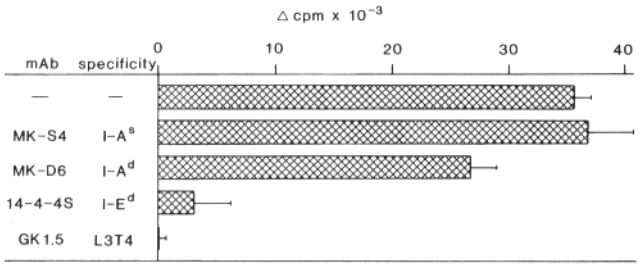

Figure 3. Incubation of antigen-induced A1.6 proliferation by anti-class II mAbs and anti-L3T4 mAb. Clone A1.6 (10^4 cells) was stimulated by syngeneic APC (5 × 10^6), pulsed with 5 × 10^6 PRBC in the presence of 0.5 μg/ml of anti-class II mAbs or anti-L3T4 mAb, and added to the culture medium. The proliferative response was measured on day 3 of culture by the incorporation of ^{3}H-TdR. The data are expressed as cpm minus background values.

An interesting finding was that clone A1.6 displayed cytolytic activity upon stimulation with antigen. As shown in Fig. 2, by a ^{51}Cr release assay, this T cell clone lysed antigen-pulsed A20.2J target cells, while, in the absence of antigen, only minimal lytic activity was detected.

To identify the restrictive element involved in the recognition of this antigen, we analyzed the proliferative response of clone A1.6 in the presence of several anti-class II mAbs (Fig. 3). A mAb directed against I-E^d (14-4-4S) strongly inhibited this proliferative response, while antibodies directed against I-A^d (MK-D6) and I-A^s (MK-S4) had no significant effect. As expected, GK1.5, a mAb reactive with the CD4 molecule, completely inhibited the proliferative response of A1.6.

With the purpose of characterizing the antigen recognized by clone A1.6, the PRBC extract was fractionated by FPLC, using a DEAE-5DW column. Fig. 4 shows the corresponding elution profile, obtained by using a linear NaCl gradient, and the proliferative response induced by the corresponding FPLC fractions, upon incubation with clone A1.6. Significant proliferation of clone A1.6 was only induced by fractions 3–6, fraction 4 inducing the strongest proliferative response.

We found that fraction 4 does not contain CS protein, as determined by a two-site RIA, and also by Western blot analysis. Furthermore, clone A1.6 failed to be stimulated by a recombinant *P. berghei* CS protein, and by overlapping synthetic peptides, representing the repeat domain and the

Table 2. *Effect of Passively Transferred T Cell Clones on the Outcome of the Challenge of Mice with Different Developmental Stages of P. berghei*

Cells transferred*	Challenge	Mice infected/mice challenged	Mice infected
		n/n	*%*
Exp. 1			
A1.6	Sporozoites‡	1/5	20
Normal splenocytes	Sporozoites‡	5/5	100
None	Sporozoites‡	5/5	100
Exp. 2			
A1.6	Sporozoites§	0/7	0
5.9	Sporozoites§	7/7	100
None	Sporozoites§	6/7	86
Exp. 3			
A1.6	Blood forms‖	5/5	100
Normal splenocytes	Blood forms‖	5/5	100
None	Blood forms‖	5/5	100

* BALB/c mice were injected intravenously with cloned T cells, normal spleen cells, or just RPMI medium; 10^7 cells were transferred in Exp. 1, and 5×10^6 cells in Exps. 2 and 3.
‡ Mice challenged by the intravenous injection of 5×10^3 sporozoites.
§ Challenged by the intravenous injection of 3×10^3 sporozoites.
‖ Challenged with 10^3 blood forms of *P. berghei*.

flanking regions of this antigen (data not shown). Taken together, these results indicate that an antigen which differs from the CS protein is being recognized by this clone. We are currently attempting to purify and further characterize this antigen, using additional chromatographic techniques.

To determine whether clone A1.6 could protect against sporozoite-induced malaria, these T cells were adoptively transferred to naive BALB/c mice, which were challenged 4 h later, by the intravenous injection of *P. berghei* sporozoites. We found that clone A1.6 consistently conferred a high degree of protection to the recipient mice (Table 2). No protection was observed in control groups receiving the same number of normal spleen cells or of clone 5.9, an OVA-specific $CD4^+$ T cell clone.

The protection conferred by A1.6 is stage specific since mice that received an identical number of these T cells were fully susceptible to challenge with blood stages (10^3 PRBC). Furthermore, the prepatent period of these PRBC-challenged mice, their level of parasitemia, and their course of infection was identical to that of controls.

This lack of protection against blood stage challenge may be due to a number of different causes, including differences of susceptibility of the different developmental stages to certain lymphokines. In fact, while minute amounts of IFN-γ have a strong inhibitory effect on the liver stages of Plasmodia (13), large amounts of this mediator inhibit only partially the development of blood stages (13–15). The cell type(s) involved in the presentation of this plasmodial antigen as well as the nature of the protective effect induced by the transfer of these $CD4^+$ T cells remains to be determined. It is conceivable that these T cells recognize the processed antigen on the surface of Kupffer cells, and secrete lymphokines which, in turn, may have an inhibitory effect on the plasmodial infected hepatocytes. It is also possible that these $CD4^+$ cells have a direct inhibitory effect on liver stages, after recognizing the processed antigen on the surface of infected hepatocytes. Since parenchymal liver cells have been shown to express class II MHC antigens after some viral infections (16), this appears to be a plausible working hypothesis.

In conclusion, the present results indicate that the effector mechanisms of antisporozoite immunity may include not only neutralizing antibodies (1) and cytotoxic $CD8^+$ T cells (4) but also "protective" $CD4^+$ T cells. Also important, from the point of view of vaccine development, is the finding that an antigen that differs from the CS protein and is shared by sporozoites and blood stages may contribute to protection against sporozoite challenge.

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