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Testosterone-Induced Abrogation of Self-Healing of *Plasmodium chabaudi* Malaria in B10 Mice: Mediation by Spleen Cells†

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This study investigates the suppressive effect of testosterone (Te) on the self-healing of *Plasmodium chabaudi* malaria in female mice of the strain C57BL/10, and, in particular, the possible role of spleen cells in mediating this Te effect. Our data show the following. (i) About 80% of B10 mice infected with 10^6 *P. chabaudi*-infected erythrocytes are capable of self-healing the infections. This capability is progressively impaired and finally abrogated after pretreating the B10 mice with Te for 3 weeks. (ii) The spleen is Te responsive. This becomes evident in a reduction of total spleen cells from 1.05×10^8 to 0.54×10^8 on average after Te treatment for 3 weeks. Moreover, Te treatment causes an increase in the relative proportion of $CD8^+$ cells by about 4% and a decrease of Ig^+ cells by about 4.5%, as revealed by flow cytometry. (iii) Spleen cells mediate the suppressive Te effect as revealed by adoptive transfer experiments. The percentage of self-healing mice dramatically decreases to about 8% when they receive, just prior to infection, nucleated spleen cells isolated from mice treated with Te for 3 weeks. This suppressive effect can be transferred by T cells in particular but also by non-T cells, though to a lesser extent. (iv) The adoptively transferred cells mediate their suppressive effect on self-healing only if the recipient mice receive Te during infection. Our data suggest that spleen cells become functionally changed by the Te treatment for 3 weeks. Particularly T cells, but also non-T cells, gain *P. chabaudi*-specific suppressive activities, and the cells require a Te-induced factor(s) to mediate these activities.

Epidemiological studies have revealed that protective immunity to malaria can be acquired after repeated infections and that it is predominantly directed against the blood stages of malaria parasites of the genus *Plasmodium* (for reviews, see references 5, 6, and 21). However, the mechanisms mediating protective immunity have remained largely unknown to date.

The murine malaria parasite *Plasmodium chabaudi* is a suitable model to study protective immunity against malarial blood stages. Recent immunogenetic investigations with inbred and recombinant mouse strains have shown that the development of protective immunity against *P. chabaudi* is under polygenic control involving both genes of the mouse major histocompatibility complex, i.e., the *H-2* complex, and genes of non-*H-2* background (4, 14, 25, 27, 28, 36). Intriguingly, however, the male sex hormone testosterone (Te) imposes restrictions on this polygenic control, i.e., Te is able to suppress those genes normally controlling the development of malaria-specific immunity (35, 36). This becomes evident, for instance, in mice of the strain C57BL/10. Females are capable of self-healing of *P. chabaudi* infections, whereas these infections take a fatal course in males. After castration, however, males can self-heal the infections too. Te, in turn, converts castrated males as well as females from healers to nonhealers. Such Te effects on host resistance appear to be of more general importance. Indeed, Te is known to increase host susceptibility to numerous other parasitic diseases (for a review, see reference 1) and even to promote several forms of cancer (13, 17, 18).

At present, however, the mechanisms by which Te prevents self-healing of *P. chabaudi* malaria are totally unknown. It would be a major experimental step forward to define those tissues and/or cells which mediate the effect of Te-induced susceptibility or nonhealing. A priori, a candidate target organ may be the spleen, which is one of the major lymphoid organs involved in the defence against blood stage malaria (7, 33, 38). Some information that Te may directly and/or indirectly affect the spleen is available. For instance, the spleen exhibits a relatively high binding capacity for Te (29), and the number of cells in the spleen has been reported to decrease with Te treatment (3). The present study shows that Te induces changes in spleen cells of B10 mice and that Te-changed spleen cells, in particular T cells, mediate the Te-induced suppression of self-healing of *P. chabaudi* malaria.

MATERIALS AND METHODS

Antibodies. The following antibodies were used: Anti-Thy1, clone 59AD 2.2 (20); anti-Thy1.2 (biotin-conjugated anti-mouse Thy1.2; Becton-Dickinson); anti-CD3, clone 500 A2 (2); anti-CD4, clone GK 1.5 (8); anti-CD8, clone 53-6.72 (20); anti-Ia, clone ER-TR3 (30); anti-kIg-fluorescein isothiocyanate (anti-kIg-FITC)-conjugated rabbit anti-mouse immunoglobulins (Dako, Copenhagen, Denmark); FITC-conjugated swine anti-hamster immunoglobulins (Nordic, Tilburg, The Netherlands); and FITC-conjugated rabbit anti-rat immunoglobulins (Cedarlane, Toronto, Canada).

Mice. Specific-pathogen-free mice of strains C57BL/10 and NMRI were obtained from the animal facilities of the Max-Planck Institute for Immunobiology and from the Heinrich-Heine University. They received a standard diet and water ad libitum.

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Te treatment. Females of strain C57BL/10, 8 to 14 weeks old, were subcutaneously injected twice a week with 0.9 mg of Te (Testoviron-Depot-50; Schering, Berlin, Germany) suspended in 200 μl of sesame oil (Roth, Karlsruhe, Germany). Controls were treated with sesame oil alone.

Parasite infections. Blood infections of *Plasmodium chabaudi* subsp. *chabaudi* were maintained in NMRI mice by weekly passages of infected blood (37). The B10 mice were infected with 10^6 *P. chabaudi*-infected erythrocytes. Te-pretreated mice were always challenged 2 days after Te pretreatment. The Te treatment was continued during infection if not otherwise stated. Parasitemia was evaluated in Giemsa-stained blood smears. Erythrocytes were counted in a Neubauer chamber.

Isolation of spleen cells. Spleens were aseptically removed from mice and gently dissociated through a stainless steel sieve into RPMI medium (GIBCO-BRL, Karlsruhe, Germany) supplemented with 5% fetal calf serum (Boehringer GmbH, Mannheim, Germany) and collected by pelleting at 1,200 rpm in a Beckman GPKR centrifuge. Erythrocytes were lysed for 1 min in 155 mM NH_4Cl–17 mM Tris-HCl (pH 7.2). The suspension was then diluted 10-fold with RPMI medium containing 5% fetal calf serum and centrifuged, and the lysis was repeated once. The cell number was determined in a Neubauer chamber.

Flow cytometry. Flow cytometry was performed as described previously (31). In brief, spleen cells prefixed with 0.5% paraformaldehyde (pH 7.2) were diluted to 10^7 cells per ml. Aliquots of 150 μl were centrifuged, and the cell pellets were incubated with 50 μl of monoclonal antibodies at 4°C overnight. After washing, the cells, except those labeled with anti-Ig, were incubated with the respective FITC-labeled secondary antibodies at room temperature for 45 min, washed, and finally suspended in phosphate-buffered saline (PBS) containing 0.1% paraformaldehyde. All antibodies were used at optimal concentrations, with FITC-labeled ones diluted in 1% normal mouse serum. The cells were analyzed in a FACScan (Becton-Dickinson, Sunnyvale, Calif.), the sample size being 10,000 cells gated on the basis of forward and side scatter. The acquired data were stored in list mode, and the fluorescence parameters were recorded after logarithmic amplification and processed with the FACScan software.

Isolation of T cells. T cells were isolated from total spleen cells according to the nylon-wool procedure (16).

Isolation of T-cell-depleted spleen cells. The method of magnetic cell sorting was used as described by Miltenyi et al. (22). In brief, total spleen cells were labeled with biotin-conjugated anti-Thy1.2 monoclonal antibody, streptavidin-FITC, and biotinylated magnetic microparticles. The labeled cells were then separated by passage through a magnetized column. The effluent cells were pelleted and suspended in PBS.

Adoptive transfer experiments. The cells were suspended in 200 μl of PBS at the desired cell concentration and injected intravenously into the tails of the recipient syngeneic B10 females, which were 10 to 12 weeks old.

Spleen morphology. Spleens were measured with respect to weight and size immediately after their removal from mice. The distribution of cells in spleens was examined on 5- to 6-μm-thick cryosections by using the immunoperoxidase technique with the avidin-biotin-peroxidase complex described by Hsu et al. (15).

Statistical analysis. A statistical analysis was performed with Wilcoxon's rank sum test.

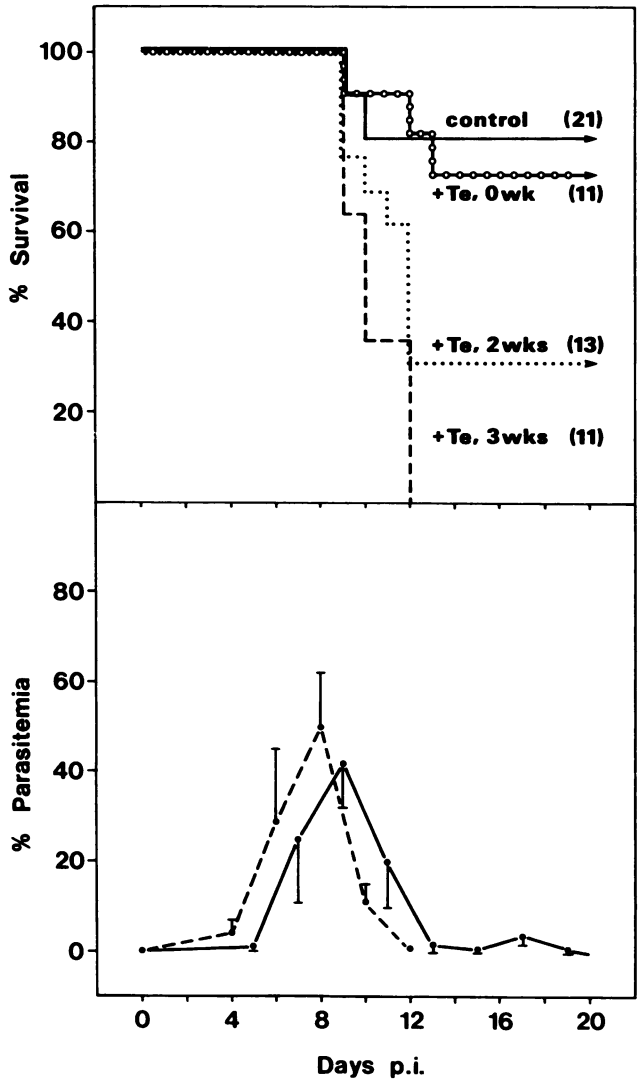

FIG. 1. Effect of Te on *P. chabaudi* infections in female C57BL/10 mice. Mice were challenged with 10^6 *P. chabaudi*-infected erythrocytes and received Te during infection. Two groups of mice were pretreated with Te for 2 or 3 weeks. Parentheses indicate the number of mice per group. Parasitemia was evaluated only in control mice (●——●) and in those pretreated with Te for 3 weeks (●----●). p.i., postinfection.

RESULTS

Te-suppressed self-healing. Female mice of the inbred strain C57BL/10 possess the capability of self-healing of blood stage infections with the malaria parasite *P. chabaudi*. About 80% of mice challenged with 10^6 *P. chabaudi*-infected erythrocytes survived the infection (Fig. 1). The surviving mice cleared a fulminant parasitemia during the first 2 weeks postinfection. Parasitized erythrocytes disappeared from the peripheral blood after 3 weeks postinfection (Fig. 1). The percentage of such self-healing mice was not significantly changed when the mice received Te during the infection (Fig. 1; see Table 3). However, when mice were pretreated, prior to infection, with Te for 2 weeks, the percentage of self-healers was decreased to 31% (Fig. 1). Self-healing was abrogated in all mice after pretreatment with Te for 3 weeks (Fig. 1). Incidentally, this abrogation also occurred even if

TABLE 1. Sizes and weights of spleens from Te-treated and control B10 mice

Te treatment[a]	No. of mice	Mean ± SD body wt (g)	Mean ± SD wet wt of spleen (g)	Spleen wt/ body wt (%)	Mean ± SD spleen size (mm)	
					Length	Width
−	4	22.05 ± 2.5	0.1015 ± 0.01	0.46	14.25 ± 1.25	4.00 ± 0.00
+	8	26.80 ± 1.8	0.0980 ± 0.01	0.36	14.87 ± 1.12	4.25 ± 0.46

[a] B10 females were treated with Te for 3 weeks as described in Materials and Methods.

the Te treatment was not continued during infection (data not shown). The Te pretreatment for 3 weeks affected only slightly, if at all, the course of parasitemia (Fig. 1).

Te-induced changes in spleen cells. Te pretreatment of mice for 3 weeks appeared to result in a slight increase in the total weight of mice (Table 1). However, the spleens of Te-treated mice retained the same weight and size (Table 1). Also, the gross organization of the spleen was not changed during the Te treatment, as examined with hematoxylin-stained cryosections. Immunocytochemistry of cryosections revealed that the $Thy1^+$, $CD4^+$, and $CD8^+$ cells remained localized in the center and the Ia^+ cells remained at the periphery of the white pulpa regions (data not shown). However, Te pretreatment of mice for 3 weeks induced a significant decrease ($P = 0.0005$) in the total number of spleen cells (Table 2). About 1.05×10^8 cells were isolated from the spleens of untreated mice, whereas only 0.54×10^8 cells, on the average, were recovered from the spleens of the Te-treated mice. Moreover, Te treatment had a significant effect on the relative proportions of distinct cell populations as revealed by flow cytometry. Thus, the relative proportion of Ig^+ cells significantly decreased from about 57 to 52% ($P = 0.0005$) and that of the $CD8^+$ cells increased from 11 to 15% ($P = 0.0005$) after Te treatment for 3 weeks (Table 2). Incidentally, the increase in $CD8^+$ cells entailed a decrease in the $CD4^+/CD8^+$ ratio from about 1.6 to 1.2 after Te treatment for 3 weeks.

Adoptive transfer of Te-suppressed self-healing. In order to analyze a possible role of spleen cells in mediating the suppressive Te effect, several sets of adoptive transfer experiments were performed. In one type of experiment, 5×10^7 total spleen cells were transferred from B10 females pretreated with Te for 3 weeks into syngeneic, untreated B10 females. Immediately thereafter, recipient mice were infected with 10^6 *P. chabaudi*-parasitized erythrocytes, and they did not receive any Te during infection. These recipient mice obviously retained their capability of self-healing of *P. chabaudi*, since their survival rate was about the same as that of the corresponding Te-untreated control mice (73 versus 80%; Table 3). By contrast, when the recipient mice were treated with Te during infection, the adoptively transferred spleen cells caused a dramatic decline in the survival rate of the recipient mice to 8% (Table 3). Obviously, total spleen cells can transfer the suppressive Te effect, but only in the presence of Te in the recipient mice.

In the next set of experiments, spleen cells of Te-pretreated mice were depleted of $Thy1.2^+$ cells by magnetic cell sorting. This method removed more than 99% $Thy1.2^+$ cells from the total spleen cells, as revealed by flow cytometry (Fig. 2). When 3.5×10^7 of these non-T cells were adoptively transferred to syngeneic B10 females immediately prior to infection with *P. chabaudi*, about 46% of the recipient mice survived infection in the presence of Te (Table 3). This decrease was not as pronounced as that observed after the transfer of total spleen cells (8%; Table 3). In another experimental set, T cells were adoptively transferred from Te-treated mice. Total T cells were isolated from spleens of mice pretreated with Te for 3 weeks. The T cells were more than 90% $Thy1^+$ cells, as revealed by flow cytometry (Fig. 3). Adoptive transfer of 2×10^7 $Thy1^+$ cells into recipient B10 females before infection induced a fatal outcome of the infection in 82% of the recipient mice in the presence of Te (Table 3).

DISCUSSION

The present data show that Te treatment progressively impairs and eventually abolishes the capability of B10 mice to be self-healed of *P. chabaudi* malaria. Self-healing obviously reflects the manifestation of the successful mounting of protective immune mechanisms, since mice after self-healing of *P. chabaudi* infections have acquired long-lasting immunity against homologous rechallenge (34). Thus, it is reasonable to assume that Te prevents self-healing by suppressing the development of protective immunity. Consistently, a series of other studies have revealed a suppressive capacity of Te of diverse cellular and humoral immune reactions (for reviews, see references 11, 12, 26, and 29).

Moreover, our data show Te responsiveness of the spleen, which is widely considered to be an important organ of the immune system involved in self-healing of malarial infections (for a review, see reference 38). This Te responsiveness becomes evident from the decline of total spleen cells as well as from the increase of cytotoxic/suppressive $CD8^+$ cells and the decrease of Ig^+ cells after Te treatment for 3 weeks. In accordance, other authors also found a Te-induced decrease in total spleen cells (3, 19, 24), as well as an increase in suppressor T cells (3, 9) and a decrease in plaque-forming cells in the spleens of Te-treated mice (10, 19, 23, 24). In B10 mice, the Te-induced changes in spleens

TABLE 2. Flow cytometric analysis of spleen cells from Te-treated and control B10 mice

Te treatment[a]	Total no. of spleen cells (10^8)	% Spleen cell populations[b]					
		$Thy\text{-}1^+$	$CD3^+$	$CD4^+$	$CD8^+$	Ia^+	Ig^+
−	1.05	30.2 ± 2.1	28.0 ± 1.4	18.1 ± 1.3	11.0 ± 1.3	52.8 ± 3.3	56.7 ± 1.3
+	0.54	31.7 ± 3.2	32.7 ± 2.7	18.4 ± 2.7	15.1 ± 1.2	52.1 ± 5.5	52.2 ± 1.8

[a] B10 females were treated with Te for 3 weeks.
[b] Values represent means ± standard deviations of duplicate determinations from at least six mice.

TABLE 3. Adoptive transfer of spleen cells isolated from B10 females pretreated with Te for 3 weeks: outcome of *P. chabaudi* infections in the recipient syngeneic mice

Te treatment of donor mice (*n*)[a]	Transferred spleen cells pooled from donor mice (*n*)[b]	Te treatment during infection	No. of recipient mice/no. of surviving mice in expt:				Avg survival rate (%)
			1	2	3	4	
−	PBS	−	5/3	6/5	10/9	4/3	80
−	PBS	+		6/4	5/4	9/9	85
−	PBS[c]	−	10/0	5/0	5/0	3/0	0
+ (10)	Total spleen cells (5×10^7)	−	8/7	7/4			73
+ (8)	Total spleen cells (5×10^7)	+		8/1		5/0	8
+ (34)	Non-T cells (3.5×10^7)	+			5/2	8/4	46
+ (23)	T cells (2×10^7)	+			6/1	5/1	18

[a] *n*, number of mice.
[b] *n*, number of transferred spleen cells pooled from donor mice.
[c] PBS was injected intravenously in mice pretreated with Te for 3 weeks.

were observed after Te treatment for 3 weeks. This, in turn, was found to be the minimal period of Te treatment to achieve complete abrogation of self-healing of *P. chabaudi* infections. This may signal that Te-induced changes in the spleen are somehow associated with the suppression of self-healing of *P. chabaudi* malaria.

Our adoptive transfer experiments provide the first evidence that spleen cells are involved in mediating the suppressive Te effect on self-healing of *P. chabaudi* infections. Indeed, the adoptive transfer of total spleen cells from Te-pretreated mice on syngeneic recipient mice just prior to infection prevents self-healing in most of the recipient B10 mice. This, however, is achieved only if the recipient mice receive Te during infection. Nonetheless, the immunosuppressive effect is derived from the transferred cells and not from the injected Te, since Te applied without transferred cells does not severely affect, if at all, the survival of normal B10 mice (Table 3). Thus, it is reasonable to assume (i) that Te treatment for 3 weeks induces spleen cells to change functionally, i.e., spleen cells gain suppressive activities, and (ii) that these changed cells after adoptively transferred into recipient mice require the presence of Te and/or a Te-induced factor(s) for maintaining and mediating their suppressive activities.

It has been suggested that Te-induced suppressive activities in the immune system are mainly propagated by cytotoxic/suppressive $CD8^+$ cells, for example, by an increased cell number and/or by enhanced cellular activities (3, 24, 32, 37). According to this suggestion, it is possible that the Te-induced increase of $CD8^+$ cells we have found in B10 mice causes secondarily the decrease of Ig^+ cells and even the decrease of total nucleated cells in the spleen after 3 weeks of Te treatment. This view, however, cannot explain the Te-induced suppression of self-healing of *P. chabaudi* malaria. This phenomenon appears to be more complex. Our adoptive transfer experiments with selected spleen cells indicate that T cells, in particular, from Te-treated mice mediate the suppressive activities. However, non-T cells can also mediate the suppressive Te effect, though to a lesser extent than T cells. Thus, both T cells and other nucleated spleen cells have gained immunosuppressive activities during the Te treatment, and the interactions between them may be important for suppressing self-healing of *P. chabaudi* malaria.

Some authors previously suggested that spleen cells are Te targets (3). Our data that spleen cells are Te responsive and even mediate Te-induced suppressive activities are in line with this suggestion. However, it is known that spleen lymphocytes (24) and T cells (1, 29) do not possess any detectable amounts of Te receptors. Thus, if spleen cells are primary Te targets at all, then nonlymphoid cells such as macrophages or epithelial cells are presumably the Te targets. Indeed, such cells are known to contain Te receptors (for reviews, see references 1 and 20). Thus, it is attractive to speculate that the 3-week Te treatment of B10 mice induces nonlymphoid spleen cells to produce a Te-specific gene product(s) which mediates its effect via paracrine mechanisms to splenic T cells and B cells. These, in turn, then develop their own immunosuppressive activities which ultimately prevent self-healing of *P. chabaudi* malaria. Currently, we are trying to identify and characterize such Te-induced mediators in spleen cells from Te-treated B10 mice. The specific neutralization of such immunosuppres-

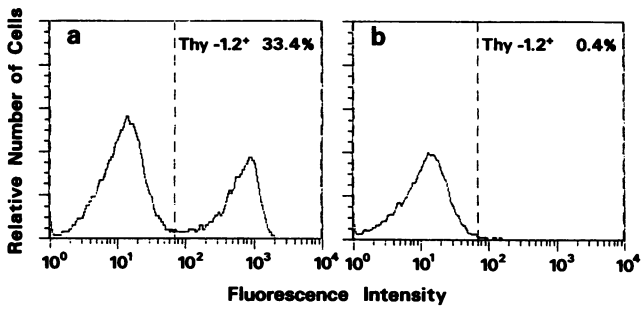

FIG. 2. FACScan analysis of splenic Thy1.2$^+$ cells before (a) and after (b) magnetic cell sorting.

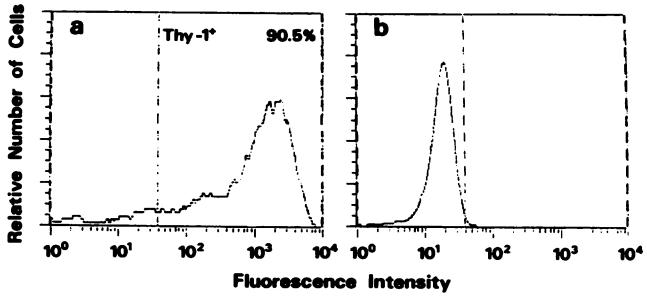

FIG. 3. FACScan analysis of splenic Thy1$^+$ cells after separation on a nylon-wool column (a) and the corresponding negative control (b).

sive mediators might open new prespectives for the treatment and prophylaxis of malaria.

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