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Role of T cells in preventing transmission of rodent malaria

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Summary. Transmission blocking immunity induced by microgamete vaccination is fully effective for at least 12 months. Passive transfer of immune T cells reduced transmission of a subsequent infection by 95%, the effect being partly due to a significant reduction in numbers of circulating gametocytes during the infection. This immunity was apparently independent of specific antibody, though these were produced within a few days after challenge infection and was mediated by a T cell of the GK1.5$^+$, Ly 2.2 phenotype. Immune serum and immune T cells, administered together, showed a strong additive effect and blocked transmission completely.

INTRODUCTION

We have previously shown that vaccination of mice with microgametes of *Plasmodium yoelii nigeriensis* induces a stage-specific transmission blocking immunity which persists at least 6 months (Mendis & Targett, 1982). Here, we have extended studies on the long-term immunity and the mechanisms involved, and show that the strong immunity persists at least 12 months, correlates with the induction of immune T-cells, and may involve the T-cell mediated release of cytotoxic macrophage products.

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MATERIALS AND METHODS

Parasite

The isolation of *Plasmodium yoelii nigeriensis* has been described previously (Killick-Kendrick, 1973); the parasite has since been maintained by cryopreservation (LUMP 1766) and by syringe passage in mice with regular cyclical transmission. Throughout these experiments, parasites from the first animal passage were used to initiate infections.

Mice

BALB/c mice used in this study were supplied by Olac (1976) Ltd, Bicester, Oxon. Tuck outbred mice were supplied by A. Tuck & Son Ltd, Battlesbridge, Essex.

Vector

Anopheles stephensi (Beech strain) mosquitoes, bred in our laboratory, served as the vector in all experiments.

Preparation of gametes

Preparation of gametes has been described previously (Mendis & Targett, 1982). Briefly, infected mouse blood with a gametocytaemia of 0·5–1·5% (parasitaemia 40–60%) was washed in suspended animation (SA) medium (0·21% Tris, 0·96% NaCl, 0·2% glucose, pH 7·4) in which gametogenesis is reversibly suppressed (Carter & Nijhout, 1977). The blood was resuspended in SA solution and layered onto a discontinuous Percoll (Sigma Chemical Co., Poole, Dorset) gradient consisting of equal volumes of 35%, 50% 56% and 80% Percoll equilibriated with SA

solution (Knight & Sinden, 1982). After centrifugation at 3200 *g* for 20 min, the gametocyte-enriched fraction was removed from the 30%:50% interface, washed and resuspended 1:5 v/v in gamete-releasing medium (2·5 vols 5% NaCl, 2·5 vols 10% glucose, 20 vols 1·46% $NaHCO_3$ and 100 vols heat-inactivated fetal calf serum adjusted to pH 8·0) (Carter & Chen, 1976). After exflagellation, the male microgametes were separated by differential centrifugation pelleted at 13,000 *g* and counted prior to use.

Indirect immunofluorescent assay

Anti-gamete antibody was measured by a slide fluorescence method (Voller & O'Neill, 1971) using air-dried acetone-fixed microgametes. Two-fold dilutions of immune sera were applied to the gamete antigen slides at 20° for 30 min after which the slides were washed and treated with fluorescein-conjugated (FITC) polyvalent rabbit anti-mouse immunoglobulin (Ig) for a further 30 min; the fluorescence was read in an ultraviolet incident light microscope. The end point was taken as the last dilution showing clear gamete-specific fluorescence.

Determination of infectivity

Mice were inoculated i.v. with 10^6 *P.y. nigeriensis*-infected erythrocytes. On Day 3 after infection, batches of mosquitoes were fed for 30 min on each group of mice, after which the mosquitoes were maintained at a temperature of 24–27°. One week after feeding, all mosquitoes were dissected and their midguts examined microscopically for oocytes. Impairment of transmission was reflected in reduced oocyst numbers. Each batch consisted of at least 50 mosquitoes.

Spleen cell preparation

Spleens were removed from donor mice and single cell suspensions made by teasing and aspirating in serum-free RPMI-1640 medium. The red cells were lysed by treating the whole spleen cell suspension with 0·17 M Tris-ammonium chloride buffer (pH 7·2) for 4 min at room temperature. The white cells were then washed twice in RPMI-1640 prior to use.

T-cell enrichment of spleen cells

Splenic white cells were prepared as above and fractionated on nylon wool columns as described previously (Julius, Simpson & Herzenberg, 1973). The resulting nylon wool non-adherent cells, which were contaminated with less than 2% surface Ig-positive cells, were further depleted by two successive incubations at 4° on petri-dishes coated with affinity-purified sheep anit-mouse Fab (Wellcome Research Laboratories, Beckenham, Kent) (Mage, McHugh & Rothstein, 1977). The non-adherent cell population was washed twice in serum-free RPMI-1640 medium prior to injection.

B-cell enrichment of spleen cells

Splenic white cells were prepared as above and incubated with 1 ml of monoclonal anti-Thy 1.2 (final concentration 1:50) per 10^8 cells for 30 min at 37° with diluted (1:4) guinea-pig complement which had been absorbed with mouse spleen cells. The cells were then washed and the dead cells removed by filtration over glass wool. The resulting B-cell enriched population was shown by fluorescence to be at least 93% surface Ig-positive.

Anti-Thy 1.2 and complement treatment of T cells

T cells, 5×10^7, were purified as above and resuspended in 1 ml of monoclonal anti-Thy 1.2 (diluted 1:50 v/v in RPMI) for 30 min at 37°. Cells were washed and resuspended in guinea-pig complement (diluted 1:4 v/v with PBS) pre-absorbed with mouse spleen cells. The resulting cell population was washed three times in serum-free RPMI-1640 medium prior to i.v. injection.

Anti-GK 1.5 and anti-Ly 2.2 depletion of T cells

Splenic T cells, 10^8, were prepared as above, resuspended in 1 ml of anti-GK 1·5 or anti-Ly 2·2 (diluted 1:50 v/v in serum-free Hanks' balanced salt solution) and incubated at room temperature for 30 min. The cells were washed twice, resuspended in 2 ml of rabbit complement diluted (1:19 v/v), pre-absorbed with mouse spleen cells and incubated for 45 min at 37°. The cells were washed, resuspended in 1 ml and the depletion procedure repeated. After the second depletion step, the cells were washed three times in serum-free RPMI-1640 medium prior to i.v. injection. The antisera were kindly provided by Dr F. Y. Liew, Wellcome Laboratories).

Assessment of cell viability

Cell viability was assessed by fluorescence under blue incident light using the Ethidium Bromde/Acridine orange method of Lee, Singh & Taylor (1975).

RESULTS

Duration of transmission-locking immunity induced by microgamete vaccination

Twenty-five BALB/c mice were vaccinated intravenously (i.v.) with 10^7 formalin-fixed *P.y. nigeriensis* microgametes. Various times later (0·5, 3, 6, 9 or 12 months), five mice were randomly selected and infected i.v. with 10^6 *P.y. nigeriensis*-infected erythrocytes, and infectivity to mosquitoes was tested 3 days later. All mosquitoes fed on control mice were found to be heavily infected; each midgut had a minimum of 350 oocyts (Table 1). However, with mice immunized with formalin-fixed microgametes, transmission-blocking immunity was still fully effective when the interval between vaccination and challenge was 12 months.

Specific antibody responses to microgamete vaccination

Transmission-blocking immunity induced by microgamete vaccination is thought to be largely serum-mediated (Mendis & Targett, 1982). We were interested in determining whether the long-term transmission blocking immunity observed (Table 1) was due to residual gamete-specific serum antibodies

Table 1. Duration of transmission-blocking immunity induced by microgamete vaccination

Vaccine	Interval between vaccination and challenge (months)	Mean no. oocysts/mosquito (range)
+	0·5	0
+	3	0
+	6	0
+	9	0
+	12	0·05 (0–1)
Controls		350 (350)

Female BALB/c mice were vaccinated i.v. with 10^7 formalin-fixed *P.y. nigeriensis* microgametes and, at various times thereafter, infected i.v. with 10^6 *P.y. nigeriensis*-infected erythrocytes. Three days later, batches of 50 mosquitoes were fed for 30 min on each group of infected mice. Mosquitoes were maintained at 25–27°C for 1 week prior to dissection and microscopic examination of midguts for oocysts. Results are quoted as the mean number of oocysts per mosquito for each batch; the figures in parentheses denote the range of oocysts found in that group. Mosquitoes fed on control mice gave almost indentical results and the data were therefore pooled.

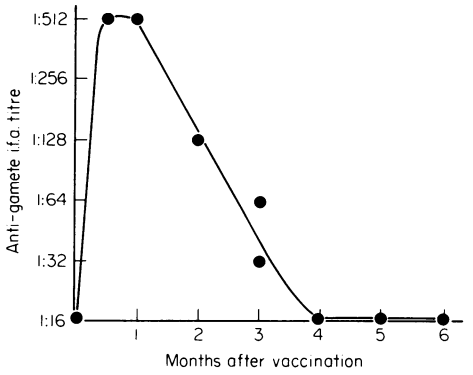

Figure 1. Specific antibody response to microgamete vaccination. Female BALB/c mice were immunized intravenously (i.v.) with 10^7 formalin-fixed microgametes, and at various times thereafter groups of three mice were randomly selected and their sera pooled. Gamete-specific antibody titres were measured by immunofluorescence (Voller & O'Neill, 1971). The minimum titre considered to be gamete-specific was 1:16.

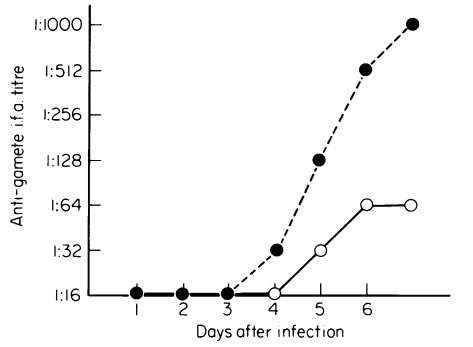

Figure 2. Antibody responses to infection. Female BALB/c mice were immunized i.v. with 10^7 formalin-fixed microgametes (●——●) or a control vaccine made from uninfected mouse red cells (○——○). Six months later, all mice were infected with 10^6 *P.y. nigeriensis*-infected erythrocytes. Sera from each group of mice were collected at various times after infection and the gamete-specific antibody titres measured as described in Fig. 1.

persisting in vaccinated mice. Accordingly, mice were vaccinated as before and the anti-microgamete serum antibody titres measured at various times thereafter. The serum IFA titres are shown in Fig. 1.

Anti-gamete antibody titres rose from background levels on Day 4 to a peak titre of 1:256–1:512 2 weeks after vaccination. Residual microgamete-specific antibody was still detectable by immunofluorescence after

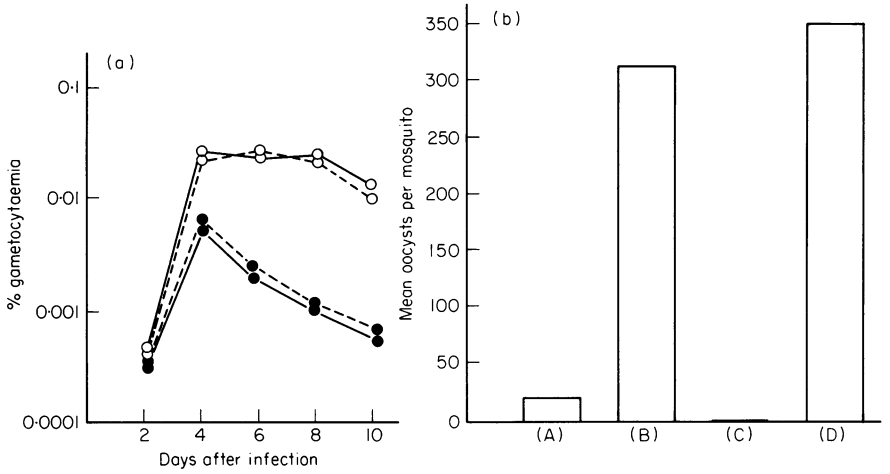

Figure 3. Passive transfer of transmission-blocking immunity. Female BALB/c mice were vaccinated as described in Table 1, and 6 months later inoculated with 10^6 *P.y. nigeriensis*-infected erythrocytes; the sera collected 3 days after served throughout these experiments as a source of 'immune sera'. Female BALB/c mice vaccinated 6 months previously served as a source of 'immune' spleen cells. (a) Age-matched syngeneic mice were injected i.v. with 5×10^7 immune (●) or control (○) spleen cell and/or serum from immune (——) or control mice (– – –) 2 hr prior to i.v. infection with 10^6 *P.y. nigeriensis* parasites. The mean gametocytaemia in each group is shown; each group consisted of at least five mice. (b) Batches of 50 mosquitoes were fed for 30 min on each group of mice, after which they were maintained at a temperature of 24–27° for 1 week. The results are quoted as the mean number of oocysts per mosquito (± 1 SD) for each batch of mosquitoes. (A) Mice given immune spleen cells plus normal mouse serum. (B) Mice given serum from vaccinated mice that had been infected for 3 days, plus normal spleen cells. (C) Mice given immune spleen cells plus immune serum. (D) Controls given normal spleen cells plus normal mouse serum.

3 months at a titre of 1:64, but was no longer detectable after 6 or 12 months.

Antibody responses to infection in immune and non-immune mice

Mice were vaccinated and, 6 months later, when gamete-specific antibody was no longer demonstrable (Fig. 1), were challenged with 10^6 *P.y. nigeriensis*. The anti-gamete antibody titres measured at various times after this infection are shown in Fig. 2. Three days after challenge, at a time when a complete block on transmission could be shown, the gamete-vaccinated animals still had no detectable gamete-specific serum antibodies. By Day 6 after infections, the antibody titres had risen to a titre of 1:512. In control mice, anti-gamete antibody titres rose from background levels on Day 4 of infection to a titre of 1:64 6 days after inoculation.

Passive transfer of transmission-blocking immunity

In order to determine mechanisms involved in the

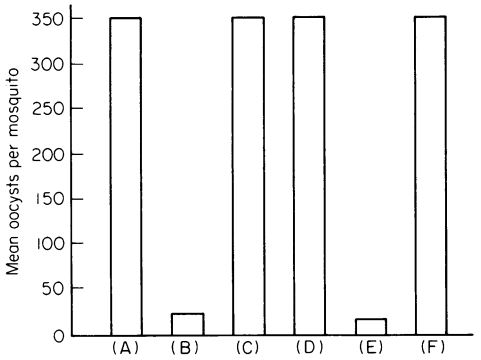

Figure 4. Transmission-blocking immunity is transferrable by T cells. Spleen cells from mice vaccinated 6 months previously were isolated as described in Fig. 3. Naive syngeneic recipient mice were injected i.v. with (A) 5×10^7 B cells, (B) T cells, (C) T cells treated with anti-Thy 1.2 plus complement, (D) anti-GK 1.5 plus complement, or (E) anti-Ly 2.2 plus complement. Two hours later, all mice were infected with 10^6 *P.y. nigeriensis* and mosquito-fed 3 days thereafter. Transmission was measured as the mean number of oocysts per mosquito in each group. Mosquitoes fed on control mice gave almost indentical results and the data were therefore pooled (F).

long-term transmission-blocking immunity described above, naive BALB/c mice were first injected i.v. with immune spleen cells taken from mice vaccinated 6 months previously. The mice were then infected and, 3 days later, tested for their ability to transmit infection to mosquitoes. The results are shown in Fig. 3.

Spleen cells, 5×10^7, from the mice vaccinated 6 months previously with microgametes caused a 95% reduction in transmission after a subsequent infection relative to controls. Furthermore, the gametocytaemias of these mice following infection were significantly lower than those that occurred in controls.

Mice vaccinated 6 months previously with microgametes were infected with *P.y. nigeriensis* and their sera collected 3 days later when it was still anti-gamete antibody-negative (see Fig. 2), but when transmission from the donors was not possible (see Table 1). On passive transfer, this serum alone mediated only a 5% reduction in transmission relative to controls and induced no change in the numbers of circulating gametocytes found in infected recipient mice. However, if the immune serum and immune spleen cells used previously were passively transferred, a strong additive effect and block on transmission were demonstrable (Fig. 3). Fractionation of lymphoid spleen cells prior to transfer indicated that the transmission-blocking immunity mediated was T-cell dependent (Fig. 4). Pretreatment of the T-cell population with anti-Thy 1.2 or anti-GK 1.5 and complement prior to transfer completely abrogated their ability to transfer immunity passively. Conversely, pretreatment of T cells with anti-Ly 2.2 and complement did not impair transmission.

Viability of gametocytes

Microgametocytes produced after infection of mice that had been pretreated with immune T cells or serum, as described above, were tested for their ability to exflagellate. They were concentrated on Percoll and suspended in gamete-releasing medium. The results are shown in Table 2. Gametocytes isolated form mice pretreated with immune T cells from vaccinated animals showed significantly fewer exflagellations (Groups A and C) than controls (D) or mice pretreated with serum collected 3 days after infection of vaccinated mice. In no case was agglutination of free microgametes demonstrable.

DISCUSSION

Transmission-blocking immunity induced by microgamete vaccination is fully effective for at least 12 months (Table 1). The passive transfer studies indicated that T-cell mediated mechanisms which operate independently of specific antibody cause a reduction in the numbers of circulating gametocytes in recipient animals upon subsequent infection and reduced transmission by 95%. On the other hand, serum taken from animals that had been vaccinated 6 months previously and infected for 3 days passively conferred

Table 2. The viability if microgametocytes of *P.yoelii nigeriensis* from mice inoculated with immune cells or serum prior to infection

Pretreatment of infected mice	% gametocytes*	No. exflagellating gametocytes/20 fields	Agglutination of microgametes
(A) Immune T cells	39	10	–
(B) Immune serum	42	131	–
(C) Immune T cells + immune serum	40	4	–
(D) Controls	37	123	–

Mice were inoculated with (A) T cells from vaccinated mice plus normal mouse serum, (B) T cells from normal mice plus a serum collected 3 days after infection of vaccinated mice, (C) immune T cells plus immune serum, and (D) normal mouse spleen cells and serum. Each group of mice was then infected, the gametocytes collected 3 days later, concentrated on a Percoll gradient and tested for viability by suspending in a gamete-releasing medium and counting the numbers of exflagellating microgametocytes.

* Gametocyte percentages obtained after concentration on Percoll gradients.

only limited (5%) protection, even though the donors of the serum were incapable of transmitting the infection. The same serum combined with lymphoid cells from vaccinated mice blocked transmission completely, indicating a strong additive effect. The transmission-blocking immunity was shown to be T-cell mediated, being abrogated by treatment with anti-Thy 1.2, serum plus complement, and due to cells of the GK 1.5^+, Ly 2.2^- phenotype, which includes delayed-type hypersensitivity (DH) and helper T (T_H) cells.

The tests of viability were particularly interesting, since it appears that infection of mice previously inoculated with T cells from vaccinated donors leads to production of apparently normal gametocytes; however, few of these were able to exflagellate by comparison with controls. The effects seem unlikely to be antibody-mediated; certainly antibody which could agglutinate gametes could not be shown at the time this failure to exflagellate was demonstrable.

Effective immunity to asexual forms of malaria correlates with increased activity and accumulation of macrophages in the spleen (Shear, Nussenzweig & Bianco, 1979) and liver (Dockrell, de Souza & Playfair, 1980) of infected hosts apparently under the influence of soluble lymhocyte-derived chemotactic factor(s) (Wyler & Gallin, 1977). Such a correlation could be equally important for effective immunity to the sexual stages of the disease. Gamete-specific DH or T_H cells could, after transfer to recipient mice, be triggered by gametocytes of a subsequent infection to release lymphokines capable of inducing macrophages to secrete soluble cytotoxic factors (Clark *et al.*, 1975; 1977b; Clark, Cox & Allison, 1977a) with the potential to kill gametocytes within the red cell, similar to that reported with *P. gallinaceum* (Cantrell & Jordan, 1946; Eyles, 1952). Such a mechanism would certainly explain the significantly reduced gametocytaemias in those mice which were passively protected by immune T cells. We are presently examining the sera of such passively protected mice for cytotoxic macrophage products such as tumour necrosis serum (Taverne, Dockrell & Playfair, 1981) or reactive oxygen intermediates (Allison & Eugui, 1982; Clark, Cowden & Butcher, 1983; Wozencraft *et al.*, 1984) and examining their effects on gametocytes present in infected animals.

It is well documented that specific anti-gamete antibodies will block transmission of rodent (Mendis & Targett, 1979), avian (Carter & Chen, 1976) and simian (Gwadz & Green, 1978) malarias. However, it seems that an alternative mechanism also operates following vaccination with microgametes of *P.y. nigeriensis*; transmission-blocking immunity was still fully effective when the interval between vaccination and challenge was 6 or 12 months (Table 1), despite the apparent absence by then of specific anti-gamete antibodies in the serum of these mice at the time of mosquito feeding (Fig. 2). This serum, on passive transfer prior to infection, mediated only a 5% reduction in transmission after mosquito feeding 3 days later. As IgM antibodies in the mouse have a half-life of only a few hours (Bazin & Malet, 1969), it is possible that protective IgM antibodies introduced passively would no longer be present at the time of mosquito feeding. However, a similar reduction in transmission was noted when passive transfer of the serum was delayed until 2 hr prior to mosquito feeding (data not shown). This indicates that, in the early stages of infection, transmission-blocking immunity is predominantly an antibody-independent process.

The gamete-specific T-cell mediated component of immunity appeared to act early after a subsequent infection to limit the numbers of circulating gametocytes and block transmission; it was only later that antibodies appeared and were effective by agglutinating exflagellating microgametes in the mosquito midgut.

We do not yet know the mediators of the proposed cell-mediated response, though macrophage secretory products are most likely. However, the crucial observations is the apparent requirment of triggering by T cells responding specifically to sexual-stage antigens.

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