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T Cells and Protective Immunity to *Plasmodium berghei* in Rats

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Experiments were carried out in which unfractionated spleen cells, and T lymphocyte subpopulations characterized by certain experimental criteria, were isolated at various times from rats infected with *Plasmodium berghei*. By adoptive transfer it was shown that unfractionated spleen cells, and T cells alone, could transfer protection to syngenic recipients as early as 11 days after infection of the cell donors. The protection conferred by T cells increased with the duration of the infection in the donors, at least up to 100 days. The additional presence of B cells in transferred lymphocyte populations enhanced their protective capacity over that shown by T cells alone. The role of T cells in protective immunity to malaria is discussed.

Many vector-borne host-parasite associations involve integration of parasite and host ecology, biochemistry, and physiology, and also parasite antigenicity with vertebrate host immunity. Survival of the vertebrate host requires that parasite multiplication be reduced to subpathogenic levels. Survival of the parasite, however, requires that the infection persist long enough for effective transmission by a vector, which may be only seasonal in appearance. Even with the most virulent malaria infections, e.g., *Plasmodium falciparum* in humans, there is always a tendency towards chronicity rather than acute disease, and towards commensalism rather than pathogenicity.

When infections are virulent, they are most dangerous during the initial phase of erythrocytic infection, when parasites multiply relatively unchecked by an immune response. Mounting of an effective immune response is characterized by a sudden reduction in parasite numbers to levels that are usually subpathogenic; from then on parasite numbers, although fluctuating, generally remain low. Sometimes occasional recrudescences may produce parasitemias of clinical significance. Parasites frequently remain detectable in the blood for months and sometimes years even in the absence of persistent exoerythrocytic infection, providing a potential source of infection for the vector. Thus, any explanation of malaria immunity has to take into account the fact that not only are many parasites destroyed and their numbers restricted, but also that many survive and erythrocytic infection is chronic.

There is now very good evidence that the chronicity of malaria infections does not derive primarily from an inadequate immune response by the host but from the antigenic lability of the parasite. Intrastrain antigenic variation has been demonstrated in *Plasmodium knowlesi*, *Plasmodium cynomolgi*, and *Plasmodium berghei* (see reviews: 10, 11, 11a), although only in *P. knowlesi* has the phenomenon been studied in any detail. For reasons that are as yet not understood but are probably mainly technical, it has not proved possible to analyze variation serologically in species other than *P. knowlesi*. In the experiments described here, using *P. berghei* in rats, the chronicity of the infection implies that antigenic variation occurs with the same frequency and ease as in *P. knowlesi*, but this has yet to be established with certainty.

Immunity to malaria is thymus dependent (9, 14, 43) and may be transferred to syngenic recipients by passive adoptive transfer of lymphocytes (37). Effective immunity, however, correlates with the presence of protective antibodies in the serum. Consequently, it was postulated (10) that protective immunity, which characterizes chronic subpathogenic infections, results from T cell helper activity. T cells were presumed to be sensitized during the initial parasitemias to antigenic determinants common to all variants and, therefore, able to initiate rapid secondary type B cell responses to later antigenically distinct populations. As a result, these were more effectively controlled and the infection was reduced to subclinical levels. Fluctuation in parasite levels in the chronic phase was considered to be due to differences in the initial level of B cell responsive-

ness to individual variants. Before examining this possibility in detail, it was first necessary to study T cell responses in relation to the development of protective immunity. This paper describes protective T cell activity developing in rats with chronic *P. berghei* infections.

MATERIALS AND METHODS

Animals. Inbred August rats (National Institute of Medical Research [NIMR]) were used. Normally, cell donors and recipients were all males. When recipients were neonates, however, male and female littermates were used.

Donors between 90 and 110 g in weight were infected; recipients were between 80 and 130 g in weight. Weights in one experiment usually did not vary more than 20 g; when the range was greater, rats of different weights were randomized among groups. Each group consisted of five animals.

Normal adult Sandy Lop or Sandy Lop 1 New Zealand white rabbits (NIMR) were used for the preparation of rabbit antiserum to rat immunoglobulin G (RARa-IgG); antilymphocyte serum (ALS) was raised in New Zealand white rabbits. Parkes strain (NIMR) mice, 18 to 22 g, were used in infectivity tests.

Parasites. Two strains of *P. berghei* were used, NK65A (9) for the ALS experiment and KSP11 (46) for the other experiments. Reference populations of these parasites, derived from 6-day infections in inbred August rats, were stored as stabilates (32) at −60°C. Stabilates were thawed and passaged once before intraperitoneal infection of all experimental groups. Both donors and recipients were infected intraperitoneally with 10^6 parasitized erythrocytes. Infections were monitored by Giemsa-stained tail blood smears.

Rats inoculated with *P. berghei* KSP11 at a body weight of 110 g or less remained infected from 60 to 120 days. NK65A infections were sometimes slightly less persistent.

Infectivity tests. A total of 0.2 to 0.3 ml of blood was withdrawn from rats to be tested by cardiac puncture into 1.0 ml of citrate saline (0.85% NaCl, 2% sodium citrate) and immediately injected intraperitoneally into two mice. The mice were examined for infection for at least 2 weeks.

Antilymphocyte serum. ALS was prepared by the method of Levéy and Medawar (30) using August rat thymocytes.

Preparation of spleen cell suspensions. The spleens were removed from suitable donor rats into Krebs saline (29) + 0.2% glucose (KG), pooled, and passed through fine-mesh, stainless-steel sieves to produce cell suspensions. Aggregates were removed by standing and sedimentation, and the cell suspension was centrifuged at 150 × *g* for 5 min, resuspended in KG, and filtered through gauze filters. This procedure gave single cell suspensions. The cells were maintained throughout at +4°C. Samples were taken for total, differential, and viable (trypan blue exclusion) counts and fluorescent antibody tests. In some experiments samples were taken for transmission electron microscopy, kindly performed by D. J. McLaren of this institute.

Cell suspensions were transferred from donors to recipients by intraperitoneal injection in one of three ways: (i) direct cell transfer at donor/recipient ratios of 1:1; (ii) after elution from V26 experimental columns; or (iii) elution from V26 control columns. Cell suspensions inoculated directly into recipients are referred to as spleen cells, cells eluted from V26 experimental columns as T cells, and cells eluted from V26 control columns as T + B cells. The characteristics of the eluted populations are described below. The numbers and types of cells transferred in the various experiments are given in Results.

Normal August rat spleen cells inoculated directly into syngenic recipients at a donor/recipient ratio of 1:1 did not confer any protection on intact recipients (unpublished data).

Except when challenge was delayed, as described in Results, cell recipients and their controls were challenged with 10^6 parasitized cells 2 h after lymphocyte transfer.

Preparation of normal rat Ig. Normal pooled inactivated rat serum was precipitated twice at ambient temperature with 33% saturated ammonium sulfate at pH 7.8. Precipitated protein was redissolved in Krebs saline, pH 7.4, and dialyzed extensively against this buffer at +4°C until sulfate ions were no longer detectable. The Ig solution was then clarified by centrifugation and checked quantitatively by Lowry or $E^{280\,nm}_{1\,cm}$ determinations for total protein and qualitatively by immunoelectrophoresis and polyacrylamide gel electrophoresis. The preparation contained predominantly Ig, with some contamination by other serum proteins. It was divided into suitable samples and stored at −20°C.

Preparation of normal rat IgG. Normal, pooled, heat-inactivated rat serum was precipitated sequentially with 18, 12, and 12% sodium sulfate, pH 8.0, at ambient temperature. The final precipitate containing Ig and other serum proteins was redissolved in 10 mM phosphate buffer, pH 7.4, and dialyzed extensively against the same buffer at +4°C until sulfate ions were no longer detectable. A clarified sample of the Ig solution, concentrated using an ultrafiltration cell (Amicon), was further purified by diethylaminoethyl-ion-exchange chromatography (DE52 Whatman) in 10 mM phosphate buffer, pH 7.4, at +4°C.

The protein obtained under these conditions was found by immunodiffusion and immunoelectrophoresis in gel to contain rat IgG when tested against commercially available antisera monospecific for rat IgG (Nordic) and contained no other rat serum proteins when tested against antisera to rat whole serum. Further polyacrylamide gel electrophoresis analysis demonstrated the presence of only one band when stained gels were scanned at 600 nm. The protein concentration was estimated, and the solution was lyophilized in suitable samples and stored at +4°C.

Preparation of RARa-Ig. Normal rabbits were immunized initially intramuscularly with 3 mg of reconstituted rat IgG in Freund complete adjuvant (Difco) supplemented with 5 mg of heat-killed hu-

man-type tubercle bacilli per ml, mixed C, D, T, and PN strains (kindly supplied by the Central Veterinary Laboratory, New Haw, Weybridge). After 30 days, rabbits were boosted with 3 mg of alum-precipitated rat IgG inoculated intraperitoneally over 6 days; 5 to 7 days later they were bled for serum.

Antiserum prepared in this way was found in immunodiffusion tests to give reciprocal precipitin titers of 16 to 32 against the rat IgG preparation at 1 mg/ml. Immunoelectrophoresis of the antiserum demonstrated activity against normal rat serum components, which were characterized by monospecific anti-rat sera kindly provided by H. Bazin, University of Louvain. This activity appeared to be mainly against IgG subclasses and IgM and was presumably due to antibodies cross-reacting with light-chain determinants and/or heavy-chain determinants in the case of IgG subclasses. No attempt was made to adsorb out this cross-reacting activity.

Preparation of V26 columns. The method used for preparation of V26 columns was based on that described by Goldstein et al. (20) for isolating T cells from mice. Washed, degassed Degalan V26 polymethyl-metacrylic beads (Degussa Wolfgang Ag) in Krebs saline were incubated with 0.5% ammonium sulfate-precipitated rat Ig for 2 h at +45°C and overnight at +4°C. Beads were then packed into jacketed plastic chromatography columns (2 by 30 cm) (Wright Scientific) and maintained at +4°C, and unbound Ig was removed by washing with Krebs saline until the $E^{280\,nm}_{1\,cm}$ was negligible. V26 experimental columns were loaded with heat-inactivated RARa-Ig, and V26 control columns were loaded with inactivated pooled normal rabbit serum. Serum was loaded at predetermined dilutions in KG such that antibody in experimental columns was always in excess. Normal serum was loaded at the same dilution. Thus, the majority of antibody/antigen interactions in the antiserum-loaded columns should presumably have been through monovalent Fab binding, leaving the other antibody-binding site free to combine with surface membrane Ig (SmIg) of passing Ig-bearing lymphocytes. It is possible also that some cells may have been retained through interaction of lymphocyte Fc receptors with column-bound Ig, although Wigzell et al. (45) consider this unlikely.

After 2 h at +4°C, unbound serum proteins were eluted from the columns by washing with KG. Cell suspensions containing up to 10^9 spleen cells were loaded onto the columns and eluted with KG at flow rates of 2 to 3 ml/min. Eluted cells were concentrated by centrifugation at 150 × *g* for 5 min, and a sample was kept for total, differential, and viable counts and for fluorescent antibody tests. In some experiments samples were taken for transmission electron microscopy. No attempts were made to recover or examine retained cells.

Fluorescent antibody tests. A total of 10^7 cells in 500 μl of KG + 5% fetal calf serum (Flow Laboratories) + 4 mM sodium azide (KGFA) was centrifuged, and the cell pellets were gently resuspended in neat fluorescein-conjugated goat antiserum to rat 7S gamma globulins (Hyland-Travenol). After 30 min at +4°C, the fluorescein conjugate was removed by three washes with KGFA, the final cell pellet being gently resuspended in KGFA to 500 μl. In some experiments the conjugate-treated cell pellets were washed three times in azide-free KGF and subsequently incubated at +37°C for 20 to 30 min to demonstrate capping ability.

Samples were mounted in KGFA under cover slips on microscope slides and examined by fluorescence microscopy using (i) an optical system described by Young (47) or (ii) a Laboratory Conference microscope (Gillet & Sibert) fitted with a Halogen quartz light source, dark ground condenser, ×55 0.95 numerical aperture fluorite objective, and a ×10 hi-wide eyepiece. Blue violet illumination was provided by a Turner primary interference filter with 485-nm peak transmission and 515-nm cutoff. Type F or H secondary filters allowed visualization of emission from the fluorescein. Comparison of the two systems showed, within the limitations of the technique, equal sensitivity. Over 100 cells were counted, those fluorescing with anti-Ig fluorescein conjugate being scored as positive. Staining was peripheral and discontinuous due to the metabolic block presented by the 4 mM sodium azide (40).

Possible blocking of fluorescence of SmIg-bearing cells by RARa-Ig eluted from the V26 experimental columns, and/or modulation of SmIg by the RARa-Ig to give apparently SmIg-negative cells, was checked by incubating cells at a concentration of 5 × 10^6 cells/ml in Eagle minimal essential medium + 10% fetal calf serum in tissue culture flasks (Falcon) for 4 h at 37°C. Cultures were held stationary or gently agitated.

Evaluation of results. Two problems complicated presentation of the data: first, the small group size imposed by practical considerations, and, second, the fact that parasites were frequently present in the blood at levels too low to be counted readily by blood smears. Thus, the distribution of counts, and in many cases variation within groups, could not be determined, ruling out parametric tests of significance. Therefore, we have used the Sign Test for median values occurring between day 7, when the effects of transferred immunity became apparent, and day 16. After day 16, the level of immunity in controls developed to a point where comparisons were no longer valid.

Visual examination of the data, however, indicated important differences that the Sign Test could not evaluate. For this reason we show diagrams giving individual daily parasite counts and geometric mean parasitemias for each group. Values of less than log 1.0 indicate that parasite numbers were too low to quantitate satisfactorily. For calculation of geometric means, parasite levels less than 1 in 10^4 erythrocytes were arbitrarily given the value of zero, although low numbers of parasites were undoubtedly present.

RESULTS

Development of protective capacity of the spleen. Spleen cell suspensions were prepared from donor rats 11, 18, 25, 32, and 100 days after infection and transferred directly at a 1:1 donor/

recipient ratio to intact normal syngenic recipients. The results of challenge infections in recipients and controls are shown in Fig. 1.

The experiment established that as early as day 11 of infection spleen cells were, on transfer, able to influence the parasitemia appreciably in the recipients. After 25 to 32 days of infection, their effect reached a maximum detectable by this test. Any subsequent increase in the protective capacity of spleen cells from 32- and 100-day infected donors could not be recorded simply by counting numbers of parasites in blood smears of recipients.

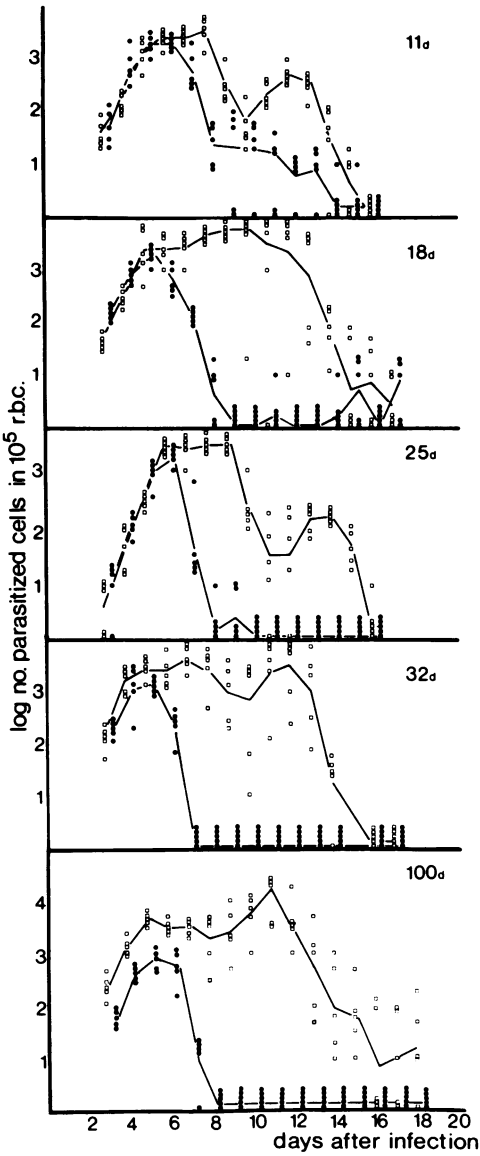

FIG. 1. *Result of transferring spleen cells at a donor/recipient ratio of 1:1 to intact recipients from donors infected 11, 18, 25, 32, and 100 days previously. Recipients were challenged with 10^6 parasitized erythrocytes. Symbols: ●, spleen cell recipients; □, controls; solid line, geometric mean parasitemia. d, Days. Probability for the various groups = 11d versus control, 0.009; 18d versus control, 0.009; 25d versus control, 0.009; 32d versus control, 0.04; 100d versus control, 0.009.*

Effect of lymphocyte depletion on subpatent infections. These experiments were carried out using the NK65A strain. The rationale was as follows. If T cells were essential for continued restriction of parasite numbers to subpathogenic and subpatent levels, then ALS treatment at the time of subpatency should induce a recrudescence of the infection. Likewise, the capacity of spleen cells from such rats to confer protection on intact recipients should be ablated. Figure 2 shows that as a consequence of treatment with ALS on days 27, 29, 30, and 31, both of these expectations were fulfilled.

Infectivity tests on recipient rats on days 39, 60, and 94 showed that recipients of cells from ALS-treated rats were less effective than recipients of cells from normal rabbit serum-treated donors at eradicating the infection, but still better than the controls.

Protection conferred by T cells isolated at various times after infection. (i) Evaluation of isolation technique. Since there is not a well-documented antigenic marker for rat splenic T cells, it was particularly important to satisfy ourselves that the cells eluted from V26 experimental columns were in fact T cells.

(a) **SmIg.** Eluted cells were routinely examined for SmIg typical of B cells by fluorescence, but in addition the possibilities were examined that Ig-negative cells eluted from the columns included B cells that had capped and pinocytosed their SmIg under the influence of RARa-Ig on the column, or shed their SmIg, or bound RARa-Ig to their surface, thus eluting with their SmIg blocked. Table 1 gives the results of two experiments in which cells were eluted from V26 experimental and control columns. Cell populations eluted from experimental columns were virtually devoid of SmIg-positive cells, whereas the percentage of these cells in control column eluates was relatively unchanged (approximately 25.0%). Comparison of cell recoveries and fluorescent antibody tests in Table 1 shows that experimental columns retain some Ig-negative cells in addition to Ig-positive lymphocytes.

Table 2 shows that cells eluted from V26 experimental columns failed to regenerate a

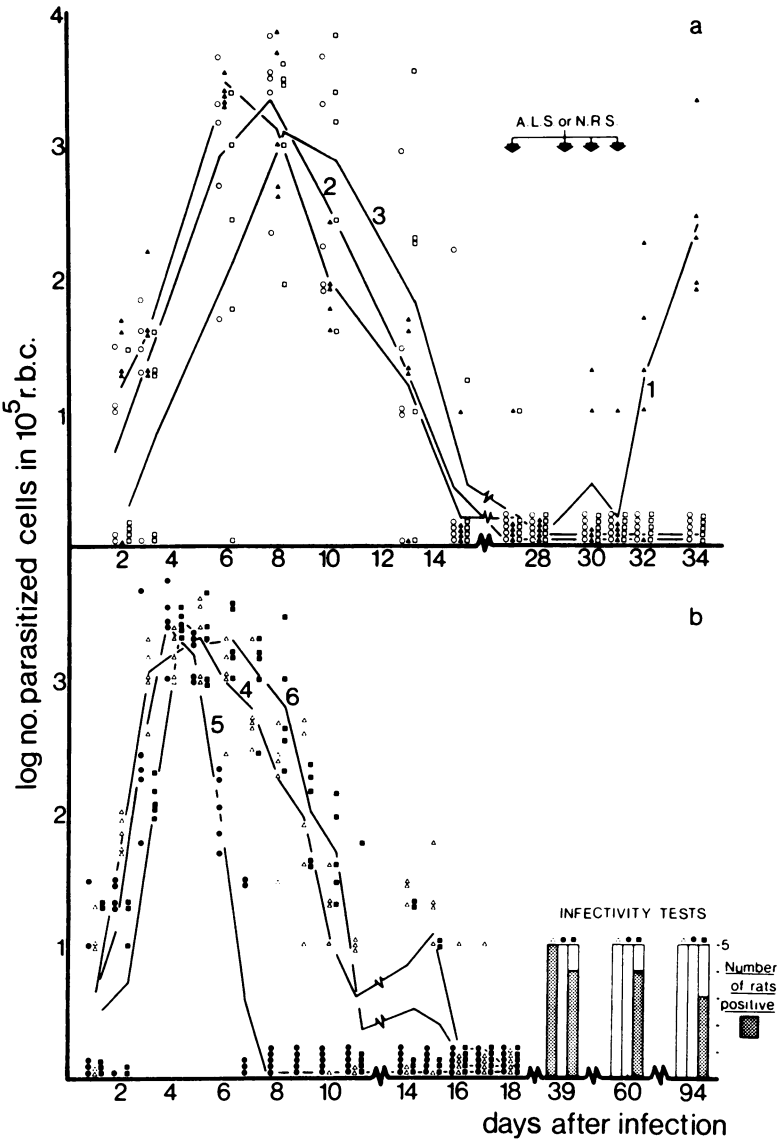

FIG. 2. *Parasitemia (a) in rats treated with ALS or normal rabbit serum (NRS) at the times indicated, and (b) in intact recipients of spleen cells from these rats transferred on day 34. Recipients were challenged with 10^6 parasitized erythrocytes. Symbols: ▲, parasitemia in rats receiving ALS; ○, in rats receiving NRS; □, in controls. Parasitaemia in recipients receiving cells from: △, ALS-treated donors; ●, NRS-treated donors; ■, no cells. Infectivity tests of recipients were on the days indicated. Solid line = Geometric mean parasitemias: 1, 2, and 3, ALS-treated, NRS-treated, and control rats; 4, 5, 6, recipients of cells from ALS- and NRS-treated donors and controls. Probability = group 4 versus 6, >0.1; group 5 versus 6, >0.1; group 4 versus 5, 0.03.*

positive staining reaction with goat-anti-rat Ig fluorescein conjugate even after maintenance at 37°C for 4 h. Cells taken from the control column were, however, shown to be capable of capping their SmIg within 60 min of elution, indicating that lymphocyte cell surface functions were not apparently impaired by the handling procedures used. These results are taken as good evidence that the cells eluted from experimental columns did not include B cells with blocked, removed, or modulated SmIg.

(b) Electron microscopy. Further characterization of eluted cells was carried out by transmission electron microscopy. Samples of cells obtained from rats infected 11, 32, and 105 days previously were examined after elution from

V26 experimental columns. In all samples lymphocytes and erythrocytes predominated. There was substantial contamination with plasma cells in the 11-day samples, and platelet contamination was evident in all samples, especially the 32-day material. Neutrophils were present in 32- and 105-day samples. Macrophages, i.e., cells capable of ingesting 0.8-μm latex beads, were present in all eluates, but in considerably reduced numbers compared with samples taken before treatment.

TABLE 1. *Cell recoveries from V26 experimental and control columns*

Column	No. of cells loaded $\times 10^8$	% SmIg positive loaded	No. of cells recovered $\times 10^8$	% Recovery	% SmIg positive recovered
Experimental	6.25	23.0	1.35	21.0	0
	8.6	25.0	2.43	20.9	0.2
Control	6.25	23.0	4.0	64.0	30.3
	6.35	25.0	3.2	49.2	29.0

TABLE 2. *Effect of incubation in vitro at 37°C on the percentage of SmIg-positive cells in populations eluted from V26 Degalan bead columns*

Column	% of SmIg-positive cells		
	Before loading column	On elution	After 4 h of incubation
Experimental	25.5	0	0.44
Control	25.5	25.7	24.6

(c) **Immunological memory.** Cell populations eluted from V26 experimental columns were tested for their ability to carry immunological memory, specifically, memory for protective immunity to malaria.

Cell donors in this experiment were animals that had suffered chronic infection. On day 213 they were reinfected with homologous parasites, and their blood was found to be negative by subinoculation test on day 225. On day 242, spleen cells were collected, passed through either experimental or control columns, and inoculated into neonatal recipients, which were challenged 56 days later. Details of the experiment and the results are shown in Fig. 3.

Although none of these techniques alone can identify T cells, taken together, these experiments using surface fluorescence, electron microscopy, and delayed parasite challenge established that cell populations isolated from experimental columns were predominantly lymphocytes capable of carrying immunological mem-

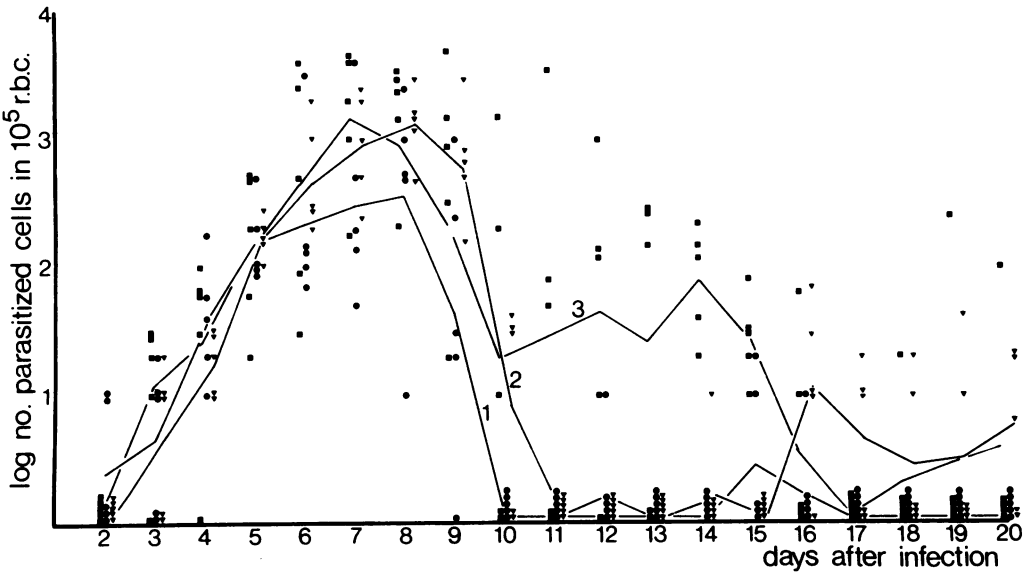

FIG. 3. *Parasitemias in recipients of cells from immune rats initially infected 242 days previously but not harboring a subpatent infection at the time of cell transfer. Challenge with 10^6 homologous parasitized erythrocytes was 56 days after cell transfer. Cell recipients were male and female 5-day-old littermates each receiving: ●, 5×10^6 immune T + B cells (2 males, 3 females); ▼, 4×10^6 immune T cells (3 males, 2 females); ■, 4×10^6 normal spleen cells (3 males, 2 females). Solid line = Geometric mean parasitaemia: 1, immune T + B cell recipients; 2, immune T cell recipients; 3, normal spleen cell recipients. Probability = group 2 versus 3, 0.04; group 1 versus 3, <0.009; group 1 versus 2, >0.11.*

ory and not expressing readily detectable SmIg. These are all T cell characteristics.

(ii) Isolation of T cells at different times after *P. berghei* infection. The experiments described above, involving transfer of unfractionated spleen cells, showed that relatively early in the infection these cells could confer on intact recipients resistance typical of a chronic infection. ALS treatment indicated that T cells were crucial to this protection. It was decided, therefore, to examine how effective T cells, isolated at various stages after infection, were in transferring immunity. Two experiments were performed which gave similar results. In the first experiment, 6×10^7 cells were transferred, and in the second experiment 8×10^7 cells were transferred. The results of the first experiment are given in Fig. 4.

To test whether parasitized erythrocytes present in the eluates, and thus transferred to recipients, added significantly to the number given in the challenge inocula, it was decided in the second experiment to determine how many viable infected erythrocytes were transferred with each T cell inoculum. Samples of each eluate were inoculated intraperitoneally into groups of five mice, and the mean time taken to reach a 2% level of parasitemia was compared with a standard curve prepared from groups of mice inoculated with 10-fold dilutions of parasitized erythrocytes. From this curve an approximate figure for the number of parasites transferred into the recipient rats with the T cell suspensions was determined. They were 10^6, 10^5, and 10^3 for eluates from donor rats harboring 11-, 31-, and 100-day infections, respectively.

Like unfractionated spleen cells, T cells appeared to confer appreciable protection even quite early in the infection. It was conceivable, however, that this effect was due wholly or in part to the transfer of cell-associated or free *Plasmodium* antigen(s) eluting from V26 columns. This possibility was tested in the following experiment.

(iii) Possible antigen transfer in V26 col-

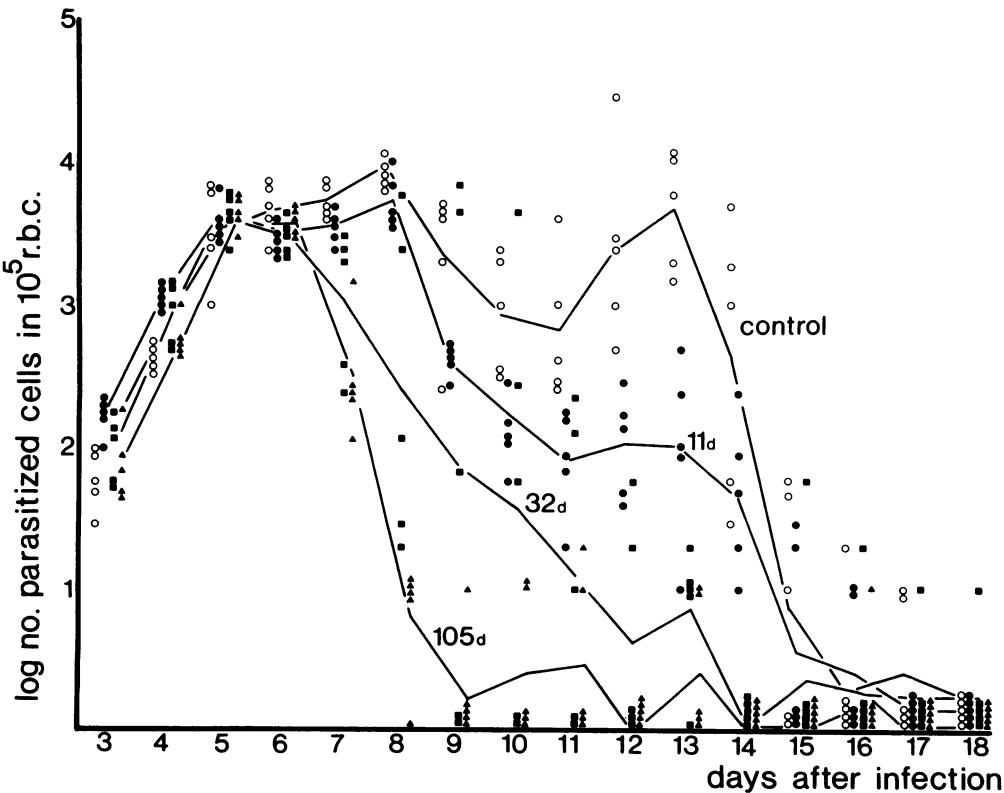

FIG. 4. *Parasitemia in recipients challenged with 10^6 parasitized erythrocytes after receiving T cells from donors infected 11, 32, and 105 days previously. Rats receiving 6×10^7 cells; ●, 11-day; ■, 32-day; ▲, 105-day cells; and ○, no cells. d, Days; solid line, geometric mean parasitemia. Probability for the various groups = 11d versus 32, 0.04; 11d versus 105d, <0.009; 11d versus control, <0.009; 32d versus 105d, >0.11; 32d versus control, 0.009, 105d versus control, 0.009.*

umn eluates. Spleen cell suspensions were prepared from donor rats infected at 11 and 63 days. Each batch of donors was divided into two groups. Spleen cell suspensions were prepared from one group of 11- and 63-day donors and passed through control columns, and the eluates were freeze-thawed four times. While these suspensions were being freeze-thawed, spleen cells were isolated from the remaining 11- and 63-day donors and also passed through control columns. The freeze-thawed eluates and the undisrupted eluates were injected into groups of rats that were then challenged. The number of viable cells injected was adjusted so that it was similar to the number of cells originally in the freeze-thawed inocula. Details of the experiment and the results are given in Fig. 5.

It is clear that transfer of freeze-thawed material did not result in an increase in the rate at which protective immunity appeared in the recipients; in fact, parasitemias were increased in these groups. From the initial parasitemia in the recipients of cells isolated from donors with an 11-day infection, it appears that in this experiment a substantial number of infected erythrocytes were also transferred. In spite of this fact, 11-day as well as 63-day spleen cell suspensions conferred a clear protective advantage on their recipients, and it seems unlikely that transferred antigen is responsible for the protective effect shown by cells eluted from V26 columns.

(iv) **Number of T and T + B cells required to transfer protection.** The results obtained with T cells isolated at different times after infection suggest that T cells in a spleen sensitized to the *P. berghei* antigen(s) concerned with protective immunity increase in number relative to the total cell content of the spleen or in individual effectiveness with duration of the infection. Therefore, an experiment was designed to try to establish the minimum number of splenic T cells capable of conferring protection. The cell donors were rats infected 112 days previously; recipients were inoculated with 10^8, 10^7, or 10^6 T cells. The results are given in Fig. 6.

Under the conditions of these experiments, the number of cells capable of conferring protection was greater than 10^7. A total of 10^6 cells even seemed to have a slight enhancing effect on the infection.

In another experiment, similar numbers of T + B cells were obtained from 113-day donors by elution from control columns. These eluates, which contained 28% Ig-positive cells, were then tested for protective activity. Figure 7 gives the result.

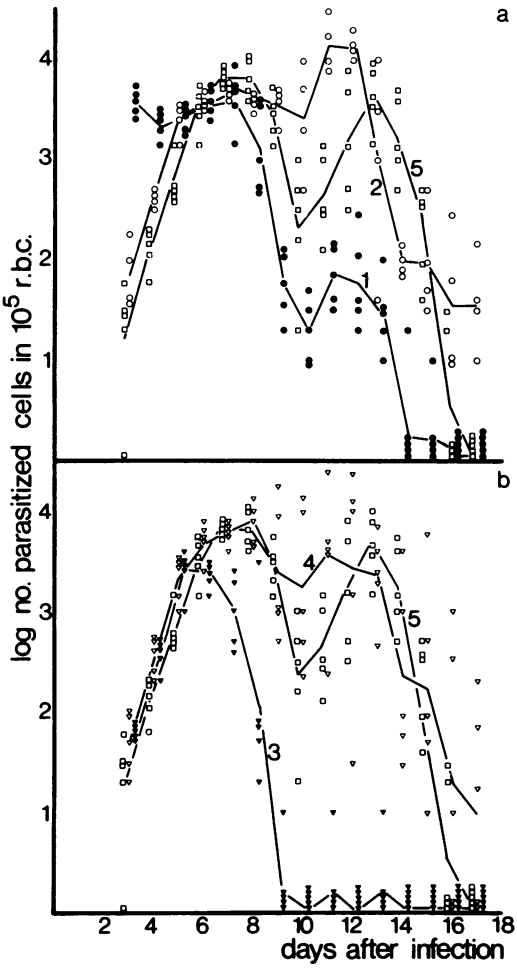

FIG. 5. *Evidence that protective immunity transferred by cell populations eluted from V26 columns was not due to transferred antigen. Before challenge with 10^6 parasitized cells, rats received V26 control column eluates from donors infected 11 and 63 days previously. (a) Recipients of 1.4 × 10^8 cells from 11-day infected donors, either (●) intact or (○) freeze-thawed. (b) Recipients of 10^8 cells from 63-day infected donors, either (▼) intact or (▽) freeze-thawed; (□) controls. Solid line = Geometric mean parasitemia: 1 and 2, recipients of intact and freeze-thawed 11-day cells; 3 and 4, recipients of intact and freeze-thawed 63-day cells; 5, controls. Probability = group 2 versus 5, >0.11; group 1 versus 5, 0.009; group 4 versus 5, >0.11; group 3 versus 5, 0.009.*

Since the cells in this experiment were isolated at the same time after infection, and the number of T cells transferred in both experiments was similar, this result suggests that the additional presence of B cells in a transferred population increases effectiveness substantially.

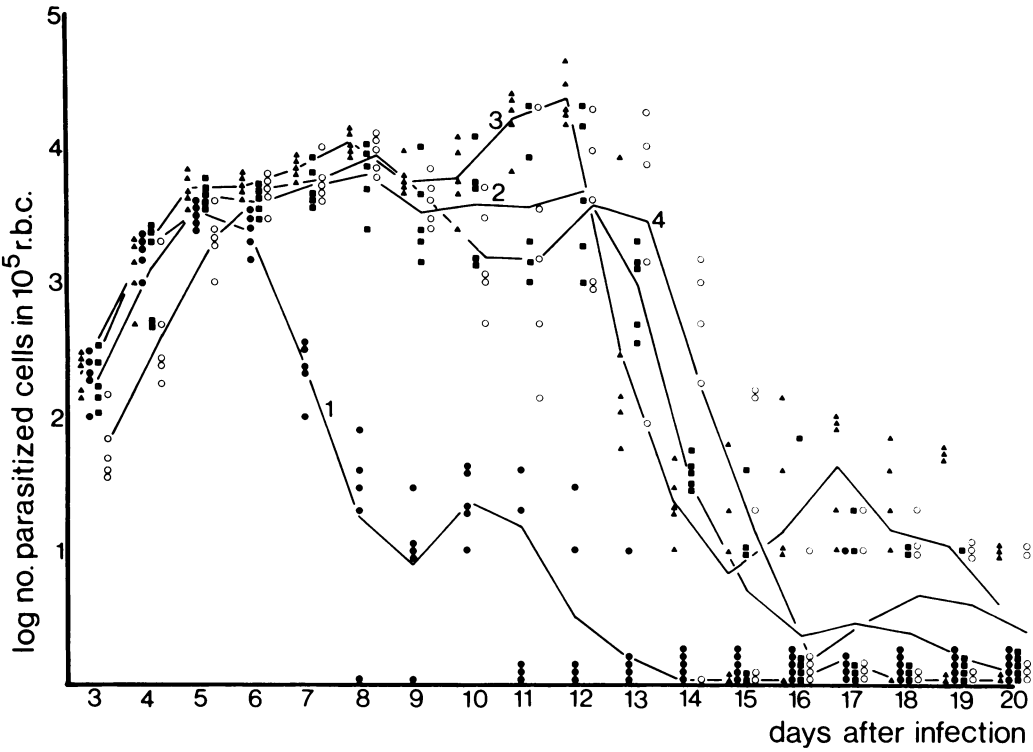

FIG. 6. *Parasitemia in rats receiving (●) 10^8, (■) 10^7, (▲) 10^6 T cells, or (○) no cells and challenged with 10^6 parasitized erythrocytes. Solid line = Geometric mean parasitemia in recipients of: 1, 10^8; 2, 10^7; and 3, 10^6 cells; 4, controls. Probability = group 1 versus 4, 0.009; group 2 versus 4, >0.11; group 3 versus 4, >0.11; group 1 versus 2, 0.009; group 1 versus 3, <0.009.*

DISCUSSION

Erythrocytic malaria infection usually begins with a phase of high parasitemia, and it is at this time that fatalities are most common. Protective immunity to a particular strain can be said to have developed when parasite numbers drop to subclinical levels. Only rarely, however, is the infection eliminated quickly; usually it persists for some time as a low-level parasitemia. Even infections with the most important and virulent human pathogen, *P. falciparum*, can last from a few months to 3 years in the absence of exoerythrocytic schizogony. This chronicity is a primary characteristic of erythrocytic *Plasmodium* infection, and it is our view that any realistic laboratory model for the immunology of malaria must also express such chronicity. Host-parasite associations in which the parasitemia is only of very brief duration are likely to give information of doubtful relevance to human malaria, since they indicate a degree of immune recognition at the T and B cell level not found in normal hosts. It was for this reason that we used the host-parasite model of *P. berghei* in August rats, since this produces a characteristic malarial infection, i.e., high parasitemia, followed by an extended period of low-level continuous erythrocytic infection. One disadvantage of this model is that the parasite develops most readily in reticulocytes. Consequently, use of whole-body irradiation to reduce or ablate immune responsiveness in cell recipients before adoptive transfer is not possible.

Immunity to malaria has been shown to be thymus dependent in rats (9, 10, 43) and in mice (14), and we have published preliminary evidence of the protective activity of sensitized T cells in rats (11). Jayawardena et al. (24) have also provided indirect evidence of T cell involvement in protection.

T cells in an infected or fully immune animal do not have apparently direct or indirect (1, 15, 38) cytotoxic anti-*Plasmodium* effects (but see reference 16). Furthermore, the fact that protective immunity to superinfections in malaria is species and often strain specific (see 7, 8) argues against macrophages activated by sensitized T cells as primary effectors of acquired

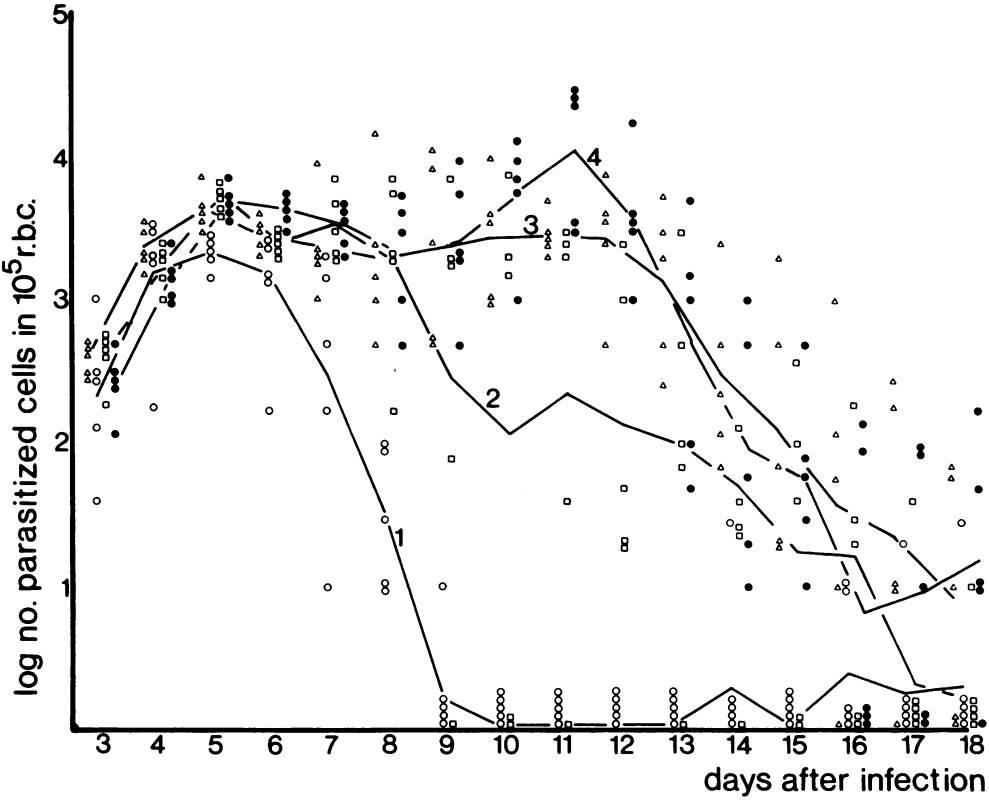

FIG. 7. *Parasitemia in rats receiving* (○) 1.7×10^8, (□) 1.7×10^7, (△) 1.7×10^6 *T + B cells, or* (●) *no cells and challenged with* 10^6 *parasitized erythrocytes. Each inoculum contained 28% SmIg-positive cells. Geometric mean parasitemia in recipients of: 1,* 1.7×10^8*; 2,* 1.7×10^7*; and 3,* 1.7×10^6 *cells; 4, controls. Probability = group 1 versus 4, 0.009; group 2 versus 4, 0.11; group 3 versus 4, >0.11; group 1 versus 2, <0.009; group 1 versus 3, <0.009; group 3 versus 2, 0.04.*

resistance to this parasite. Nonspecific macrophage-mediated protection initiated by specific T cell sensitization has been described for certain bacterial infections (36).

It has been established that protective immunity to malaria correlates with the presence of protective antibodies in the serum (11a). Although antibody is synthesized by B cells, several experimental systems have shown that its production in response to many antigens requires cooperation between nonspecific accessory cells (macrophages), B cells, and thymus-derived lymphocytes (T cells) (5). These cooperative T cell responses are largely antigen specific. There are situations, however, in which specific recognition of antigen by T cells may result in nonspecific enhancement of B cell response. Examples include the "bystander" effect, which may occur as a result of allogenic stimulation (27), and in a more defined system T cells that have been primed to some proteins may produce both specific and nonspecific T and B cell cooperation (34). In malaria, the strain specificity of protection indicates specific T and B cooperation, with a requirement for recognition of strain-specific determinants for effective protection. Nonspecific T cell effects, including macrophage activation and B cell stimulation, may account for the limited degree of cross protection that can occur, particularly in superinfections. These considerations led to the formulation of a hypothesis (10) that took into account the strain specificity of protection, antigenic variation, thymus dependency of immunity, and the protective action of antibody.

It was suggested that the role of T cells in protection is that of helpers in variant-specific antibody synthesis. T cells were said to recognize determinants common to all variants but characteristic of a strain and then act as helpers in variant-specific protective antibody responses to new variants as they developed. These secondary-type responses to later antigenic variants were thought to be associated

with reduction of the parasitemia to subclinical levels. This idea was consistent with the observed dynamics of variant-specific antibody responses to *P. knowlesi* in rhesus monkeys and the emergence of their clinical immunity (12). It also explained the requirement for B cells in recipients of T cells sensitized to *P. chabaudi* (33).

The technique we adapted to obtain rat T cells was based on a method shown to be capable of isolating from mouse spleens a population of lymphocytes over 94% of which were destroyed by a T cell-specific antiserum (45). Our attempts to prepare a specific rabbit anti-rat T cell serum proved unsuccessful, and early hopes that rat T cells could be readily identified using anti-Thy 1.1 (formerly Theta-AKR) (17) antibodies proved groundless. We were therefore obliged to use structural and functional criteria to characterize the putative T lymphocytes. We were able to show that the separation technique using V26 experimental columns isolated a population of cells that were predominantly lymphocytes lacking SmIg detectable by immunofluorescence. Furthermore, incubation at 37°C failed to induce the appearance of Ig on their surface, although the incubation time used should have allowed regeneration of modulated B cell SmIg (3, 31). This result was not unexpected, since during separation the column was maintained at 4°C, a temperature that would tend to preclude capping and endocytosis or shedding of SmIg, a possible reason for misidentification of eluted B cells as Ig-negative T cells. Capping and endocytosis would also require extensive and continuous breakdown of column or cell surface Ig-anti-Ig bonds. The only other known method of removing surface Ig from B cells involves membrane rupture, which has been described only under conditions much more rigorous than encountered on V26 columns (18). Finally, the cell population isolated on experimental columns included specifically sensitized cells, since it was capable of carrying immunological memory. Because of these characteristics, we were satisfied that eluted cells included T cells and that B cells were almost entirely absent. There was a possibility that "null" cells (21) could also be present in eluates. These would not be identified by immunofluorescence, but on theoretical grounds one might anticipate that they could be retained on the column by virtue of their Fc receptors.

The experiments in which freeze-thawed spleen cell eluates were compared with intact controls provide strong evidence that the protective effects observed were not due to transferred antigen. The effectiveness of T cell depletion by the ALS sample used relied on histological studies, which showed depletion in T cell-dependent areas of the spleen and lymph nodes of treated rats (10).

From all of this data we are confident that even in the absence of a positive T cell antigenic marker, we have selectively isolated, and depleted, rat T cells by V26 experimental columns and ALS treatment, respectively.

The first experiment, in which unfractionated spleen cells from infected donors were transferred to intact recipients, established that very early in this infection spleen cells acquired the ability to control the parasitemias. Under the conditions of these experiments, maximal effect was detectable between 25 and 32 days, although further increases in efficiency with duration of infection in the donors might have been detected by (i) measuring the duration of chronic infection in the recipients, (ii) giving graded numbers of spleen cells, or (iii) carrying out infectivity titrations on recipient blood at a given point in time after subpatency had developed, to establish the approximate level of subpatent infection.

In some adoptive transfer systems, ALS treatment is known to reduce helper cells but to leave B cell activity unaffected (23). In the second experiment, T cell depletion by ALS resulted in a recrudescence of the parasitemia within a few days, indicating that T cells are crucial for maintaining subpatency, at least under the conditions used here (see also reference 42). T cell depletion ablated almost entirely the capacity of spleen cells to confer protection, as judged by the patent parasitemia. Subsequent infectivity tests, however, showed that the duration of infection in recipients of cells from ALS-treated donors was shorter than in the controls, although longer than in recipients of cells from normal rabbit serum-treated rats. Thus, T cell numbers appeared to influence the level of parasitemia in donors, the level of parasitemia in recipients, and the duration of infection in recipients.

In the experiments in which T cells were isolated at various times after infection, their ability to influence the challenge infection in recipients was again apparent even by day 11, but appeared to increase with time at least up to 100 days. Evidence is conflicting on whether or not T cells increase in affinity for antigen with time after immunization (25, 35, 41). Our results indicate that either T cell affinity, and presumably effectiveness, increased or that the number of sensitized T cells in the spleen increased with duration of infection.

Subpopulations of T lymphocytes have been described in mice (13, 26), but it is difficult at

this stage to relate these findings to possible T cell subpopulations in malaria-infected rats. Not only is a different host species involved, but the antigenic stimulation provided by chronic malaria is likely to be very different from that used in those studies. Characterization of T lymphocyte subpopulations by conventional techniques, even in chronic malaria, might be difficult, since the continuous antigenic stimulation provided must surely result in some degree of T cell blastogenesis and a much modified pattern of cell traffic. In the experiments described here the immune response that maintains a chronic infection at subpatency appears relatively ALS sensitive, suggesting an involvement of long-lived so-called T_2 cells, but whether these are important as memory cells (2, 4, 26) and as potential helpers (23) of secondary-type B cell responses to new antigenic variants, as experiments in simian malaria suggest (12), has yet to be established.

Relatively large numbers of splenic T cells, approximately 10^8, are needed to transfer protection from donors with prolonged infections. There may be several reasons for this fact. (i) The challenge infection itself multiplies rapidly, and thus the response in the T cell recipient must involve considerable clonal expansion of sensitized cells before host responsiveness overtakes parasite multiplication. (ii) In prolonged chronic infections, when parasites become extremely few or are finally eradicated from the blood, a large proportion of the sensitized T cells may be circulating outside the spleen at any given time as long-lived memory cells. (iii) T cells present in the spleen but carrying absorbed Ig (6, 22) or immune complexes (28) may tend to be retained on V26 bead columns. The data in Table 2 demonstrate that more cells were retained on the column than could be accounted for by selective depletion of B cells alone; some of these retained cells may be sensitized T cells. Since T cells responding to specific antigen may express Fc receptors (19) and thus absorb Ig more readily, the experimental columns may in fact be retaining a number of T cells specifically sensitized and responding to malaria antigen; splenic T cells are continuously exposed to the possibility of antigenic stimulation in chronic malaria infections. There is evidence, however, that Fc receptor-bearing T cells are unlikely to be important in T and B cell cooperation (39, 44).

Experiments with cell populations, which although equivalent in T cell content also contained approximately 25% B cells, showed that these cell mixtures were more effective than T cells alone. Since donor and recipients were infected with parasites derived from the same stabilate, this effect may result from B cell memory to variants experienced by the donor. This memory could enhance variant-specific B cell responses and protective antibody synthesis to those variants as they develop in the recipients. Increased parasite destruction would then result in fewer parasites surviving to initiate later variant populations, thus shifting the dynamic balance of the infection in favor of the host. Alternatively, there may be a B cell response to antigens common to all variants which is protective, reducing but not eliminating the parasitemia. This problem can only be solved with the development of sensitive techniques for identifying variant populations in rodent malarias and using these to determine the specificity of T and B cell responses from donors infected for different periods or immunized in various ways. Such techniques could also make it possible to assess the significance of antigenic differences between parasites in the challenge population and those transferred to recipients in spleen cell suspensions or column eluates. The additional parasitized cells transferred with the lymphocyte populations did not appear to influence unduly the outcome of challenge infections.

In summary, the experiments on *P. berghei* described in this paper demonstrate isolation of splenic T cells effective in the passive adoptive transfer of immunity to intact syngenic recipients. These cells appear in donor spleens early in the infection and apparently increase in number or effectiveness for at least 100 days thereafter. The additional presence of B cells in such lymphocyte populations enhances their protective capacity, on passive transfer, over that produced by T cells alone. It appears most likely that T cells exert their protective action mainly through specific cooperation with B cells.

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