CULTIVATION OF THE EXOERYTHROCYTIC STAGE OF *PLASMODIUM BERGHEI* IN PRIMARY CULTURES OF MOUSE HEPATOCYTES AND CONTINUOUS MOUSE CELL LINES

GARY W. LONG, SIMON LEATH, RICHARD SCHUMAN, MICHAEL R. HOLLINGDALE,
W. RIPLEY BALLOU¹, BETTY KIM LEE SIM, AND STEPHEN L. HOFFMAN

*Malaria Program, Infectious Diseases Department, Naval Medical Research Institute, Bethesda, Maryland
20814 (G. W. L., S. L. H.); Biomedical Research Institute, 12111 Parklawn Drive, Rockville,
Maryland 20852 (S. L., M. R. H.); Antibody Resources, 649 D Los Strand Lane, Rockville,
Maryland 20850 (R. S.); and Department of Immunology, Division of Communicable
Diseases and Immunology, Walter Reed Army Institute of Research,
Washington, DC 20307 (W. R. B., B. K. L. S.)*

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SUMMARY

Plasmodium berghei exoerythrocytic (EE) stages have been cultured in vitro in human continuous cell lines and primary cultures of both human and rat hepatocytes. Although the predominant experimental model of irradiated sporozoite-induced protective immunity is the mouse, *P. berghei* has not been cultivated in primary mouse hepatocytes or in continuous mouse lines. Because of this, target cells are not available for determining if these immunized mice produce cytotoxic T lymphocytes (CTLs) that recognize *P. berghei* antigens expressed on hepatocytes in the context of class I major histocompatibility (MHC) antigens. We report the development of methods for cultivating the (EE) stage of *P. berghei* in murine hepatocytes and in two cell lines derived from the livers of BALB/c mice; one line produced from a primary hepatocyte culture and the other produced by fusion of mouse hepatocytes with a continuous rat liver line. Mature parasites were detected by microscopy and by DNA probe in both cell lines, each of which supported complete development of *P. berghei* liver stages and production of infectious merozoites. Since class I MHC antigens are present on the surface of primary hepatocytes and the mouse × rat hybrid line, these cells can be used to detect cytotoxic T cells against liver stage parasites.

Key words: malaria; *Plasmodium berghei*; exoerythrocytic; hepatocyte; primary culture; continuous culture.

Mice immunized with irradiated *Plasmodium berghei* sporozoites are solidly protected against subsequent sporozoite challenge (4,12). Immunized mice produce antibodies to the circumsporozoite (CS) protein of *P. berghei* and such antibodies may protect against moderate sporozoite challenge (2,8). Sporozoite-induced immunity does not protect against blood-stage infection (11) and may be directed against either sporozoites or exoerythrocytic (EE) stages of the parasites in the liver. Protection against large sporozoite challenge can be achieved independent of antibodies (1) and is probably dependent on a T-cell-mediated response (2,15,18).

Sporozoites rapidly invade hepatocytes after entry into the host and develop within these cells. A possible mechanism for cell-mediated protective immunity is the recognition of malaria antigens by cytotoxic T lymphocytes (CTLs) on the hepatocyte surface. CTLs recognize

foreign antigens only when expressed in the context of class I major histocompatibility (MHC) antigens. Cells expressing MHC antigens and capable of maintaining parasite growth are therefore required for demonstration of antigen specific, class I restricted destruction of EE stages of malaria. Research in this area has been hindered by the lack of a suitable in vitro model. Exoerythrocytic stages of *P. berghei* have been cultured in a human embryonic lung line (W138) and a human hepatoma cell line (HepG2) (5,7), in primary cultures of rat hepatocytes infected in vivo (3), and in rat hepatocytes (14). Sporozoites invade and develop in these cells to mature EE stages. However, due to the class I restriction, these systems cannot be used to study mouse CTL responses against EE-stage antigens. Research on the cultivation of rodent hepatocytes has concentrated on primary cultures of rat cells. Little work has been carried out on mouse liver cells, and their maintenance in culture has proven to be more difficult. We report the development of a system for culturing mouse hepatocytes, and the production of two continuous mouse liver

¹ Present address: Univax Corporation, 12111 Parklawn Drive, Rockville, MD 20852.

cell lines, that support the invasion of *P. berghei* sporozoites and their development to the mature liver stage.

MATERIALS AND METHODS

Culture media. Primary hepatocyte cultures were grown in Williams' medium E (WME) with 10% fetal bovine serum (FBS), 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 5 µg/ml Fungizone (Flow Laboratories Inc., McLean, VA), supplemented with 10 µg/ml insulin, 10 µg/ml transferrin, 10 ng/ml selenous acid (ITS Solution; Collaborative Research Inc., Lexington, MA), 1 µM dexamethasone (Collaborative Research), 5 ng/ml epidermal growth factor, 0.1 µM glucagon, 10 µU/ml somatotropin, and 20 mU/ml prolactin (Sigma Chemical Co., St. Louis, MO).

Hepatocyte isolation and culture. Hepatocytes were isolated from C57Bl/6 or BALB/c mice by the method of Klaunig et al. (9). The livers were perfused in situ by cannulating the thoracic inferior vena cava. The hepatic portal vein was severed and the perfusate allowed to run to waste. Fifty milliliter of calcium free Hank's balanced salt solution (HBSS; Flow Laboratories) plus 0.5 mM

FIG. 1. *Plasmodium berghei* exoerythrocytic schizont in primary mouse hepatocyte 48 h postinfection. $\times 952$. Immunoperoxidase staining with a monoclonal antibody [NBS1 (3:28:1)] raised against irradiated *P. berghei* sporozoites which recognizes DPAPPNAN, the major octapeptide repeat on the *P. berghei* CS protein.

FIG. 2. Giemsa-stained EE stage schizont 48 h after in vitro infection of primary hepatocyte culture. $\times 1050$.

EGTA followed by 100 ml 0.05% collagenase (Sigma Chemical Co.) in HBSS containing 5 mM calcium chloride were perfused at a rate of 10 ml/min. All solutions were maintained at 37° C and continuously gassed with a mixture of 95% O₂:5% CO₂. The liver was removed and the cells were released into cold, 2.5% (wt/vol) fatty acid-free bovine serum albumin (BSA) (Sigma Chemical Co.) in HBSS by teasing, and further separated by pipetting several times with a large-tip pipette. The suspension was filtered through nylon mesh and centrifuged for 5 min at 50 $\times g$. The cells were resuspended in culture media and plated at a density of 1×10^5 cells/cm² on 12-mm glass cover slips coated with mouse-tail collagen. The culture media was changed 3 h after plating to remove nonattached cells and every 24 h thereafter. Approximately 60 000 cells remained per cover slip 48 h after plating.

Production of Cell Lines

Mouse hepatocyte line. Hepatocytes were collected from the livers of BALB/c mice as above. Cells were cultured in WME (Flow Laboratories) supplemented with 10% FBS, 10^{-7} M dexamethasone, and 10 µg/ml insulin

TABLE 1

NUMBER OF EXOERYTHROCYTIC STAGE SCHIZONTS
IN PRIMARY MURINE HEPATOCYTES 48 H AFTER
INFECTION WITH 40 000 SPOROZOITES^a

Experiment Number	Number of 48-H Schizonts ^a	Number of Sporozoites Added, $\times 10^3$	Percent Sporozoites that Invade and Develop	Percent Hepatocytes Infected ^b
1	145	37.5	0.39	0.24
2	176	40	0.44	0.29
3	143	40	0.36	0.24
4	162	35	0.53	0.27
Mean	157		0.41	0.26

^aAverage of two cover slips. Parasites quantitated by direct counting of immunoperoxidase stained liver stages.

^bBased on an estimated 60 000 hepatocytes per cover slip.

(Collaborative Research) for 2 wk. After 2 wk, several colonies of cells were observed on the plates. These were subcultured and passaged in WME with 10% FBS.

Mouse/rat hybrid line. Production of the BALB/c hepatocyte $\times$ rat hybrid cell line (designated E2) was previously described (16). Briefly, the BALB/c hepatocytes were fused to a malignant rat liver cell line (FRL; provided by Dr. M. K. Wilson, FCRF, Frederick, MD) and hybrids selected by addition of HAT (hypoxanthine, 1×10^{-4} M, aminopterin, 4×10^{-7} M; thymidine, 1.6×10^{-7} M; Flow Laboratories).

Infection of cells. The NK 65 strain of *P. berghei* was grown in *Anopheles stephensi* mosquitoes. Salivary glands were dissected into Medium 199 containing 10% heat inactivated mouse serum, disrupted in a tissue grinder, and counted. Sporozoites suspended in 200 μ l of media were added to cover slips in shell vials and incubated for 2 h. Fresh media containing 200 U/ml penicillin and 200 μ g/ml streptomycin was added to the cultures and changed twice daily thereafter. In some cases, monolayers were infected by centrifugation. Sporozoites were added to cells growing on cover slips in shell vials. These were capped and centrifuged at room temperature (1000 $\times g$ for 5 min). The cultures were incubated for 1 h at 37° C, at which time the cultures were washed and the media replaced as above.

Immune Staining

Infected cell monolayers were fixed with methanol, and parasites were visualized by immunoperoxidase (6) with a monoclonal antibody against the sporozoite CS protein [NBS1 (previously designated 3:28.1) Charoenovit, Y. personal communication]. Immune staining for flow cytometry was performed following standard methods. Class I MHC antigens were detected by incubation with monoclonal antibody to H-2 D^d (ATCC HB-75, HB-102) (13), and a rat monoclonal to H-2 monotypic antigen (M1/42, Hybritech, Inc. San Diego, CA) (17) followed by treatment with fluorescein-labeled goat antimouse Ig (Becton Dickinson and Co., Mountain View, CA) or goat

antirat Ig (Boehringer Mannheim Biochemicals, Indianapolis, IN). Samples were measured on a FACS II (Becton Dickinson and Co.) coupled to a PDP 11/40 computer (Digital Equipment Corp., Maynard, CA). Control samples were treated with second antibody alone. Intracellular albumin was detected by methanol fixation and permeabilization of the suspended cells (10) before staining with rabbit antimouse albumin (Cooper Biomedical, Malvern, PA).

Detection of Parasite DNA

A highly repeated fragment of *P. berghei* DNA (pPB3; Sim, unpublished) was used as a probe to detect parasite DNA. *P. berghei* infected, and uninfected cell monolayers growing on cover slips, were incubated for 24 h at 37° C inverted onto nitrocellulose filters, backed by Whatman 3MM paper soaked with 0.5 M EDTA, pH 9.5, 1.0% sarcosyl and 2 mg/ml proteinase K (Boehringer Mannheim). The cover slips were removed and the nitrocellulose filters were air dried. Retained DNA was denatured by placing filters onto puddles of 0.5 M NaOH (twice for 2 min), and then neutralized with 1 M tris (pH 7.4) (twice for 5 min), and 1.5 M NaCl, 0.5 M tris (pH 7.4) (twice for 5 min). The specimens were air dried and baked for 2 h at 80° C in vacuo and then prehybridized for 2 h at 42° C in $10 \times$ Denhardt's (50 $\times$ Denhardt's: 1.0% Ficoll, 1.0% BSA, 1.0% polyvinylpyrrolidone), 2.5 $\times$ SSC (sodium chloride, sodium citrate) (20 $\times$ SSC: 3.0 M NaCl, 0.3 M NaCitrate, pH 7.0), 100 μ g/ml salmon sperm DNA, 50% formamide, and 0.1% sodium dodecyl sulfoxide (SDS). The filters were then hybridized for 12 h with ³²P-labeled pPB3 and washed 3 times 30 min at 50° C (0.1 $\times$ SSC, 0.5% SDS) before autoradiography.

RESULTS

Exoerythrocytic stage development in primary hepatocytes. Primary hepatocyte suspensions of approximately

TABLE 2

PLASMODIUM BERGHEI SCHIZONT DEVELOPMENT
AND LIVER MEROZOITE INFECTIVITY IN
CONTINUOUS BALB/c CELL LINES

Cell Line	Hours Postinfection	Number of Schizonts ^a	Size, μ M $\pm$ SE	Merozoite Infectivity ^b
BALB/c Line	28	199.0	10.3 $\pm$ 0.3	ND
	42	149.5		0/3
	45	142.0	12.9 $\pm$ 0.6	0/3
	47	114.5	12.8 $\pm$ 0.6	2/3
	49	50.5		ND
Mouse $\times$ Rat Hybrid	28	31.5	10.9 $\pm$ 1.1	ND
	42	25.5		0/3
	45	16.5	15.1 $\pm$ 1.0	0/3
	47	10.5	15.1 $\pm$ 0.8	1/3
	49	6.0		ND

^aCells grown on 12-mm cover slips and infected by centrifugation of 50 000 sporozoites. Number is the average of two cover slips determined by immunoperoxidase staining.

^bMice each received i.v. injection of supernatants from four cultures.

80% viability (trypan blue exclusion) were produced using the described procedure. Exposure of the monolayers to *P. berghei* sporozoites resulted in the formation at 48 h of schizonts averaging 30 μm in diameter. Liver stages were positive by immunoperoxidase using a monoclonal antibody to the CS protein (Fig. 1) and with antisera produced against blood-stage parasites by repeated inoculation of mice with infected blood. Giemsa-stained parasites (Fig. 2) seemed to be mature by 48 h and contained numerous merozoites. Injection of supernatants from these cultures into mice failed to produce blood-stage infections, suggesting that complete development of schizonts with rupture and release of infectious merozoites did not occur.

The results of four experiments using hepatocytes cultured from C57Bl/6 mice are shown in Table 1. We added 40 000 sporozoites per cover slip 3 h after plating of the cells, and consistently observed by immunoperoxidase staining approximately 150 liver schizonts per cover slip representing 0.4 to 0.5% of the sporozoites added; an infection rate of 0.25% of the 60 000 hepatocytes per cover slip. Similar results were obtained using hepatocytes cultured from BALB/c mice. Attempts to increase the number of schizonts by addition of larger numbers of sporozoites have thus far been unsuccessful.

Infection of cell lines. One of the spontaneously transformed BALB/c cell lines, and the mouse $\times$ rat hybrid line, supported invasion and development of *P. berghei* sporozoites to late liver schizonts in vitro (Table 2). Consistent results with these lines were best obtained by centrifuging the sporozoites onto the cell layers. This was not the case for primary hepatocytes, which did not tolerate centrifugation. The diameter of schizonts produced in the cell lines seemed smaller on fixed slides than those produced in the hepatocyte cultures (approximately 13 to 15 μm in diameter compared to 30 μm in primary cultures). Rupture of the schizonts and release of infectious merozoites occurred between 45 and 47 h after infection of the cultures. Two of three BALB/c mice that received intravenous injections of 47-h culture supernatants from the spontaneously transformed line, and one of three from the mouse/rat hybrid line, developed blood stage infections (Table 2).

Characterization of cell lines. We tested both lines for murine MHC antigens by flow cytometry to determine if either cell line could be used in an assay to assess class I MHC restricted killing of infected cells by immune lymphocytes. Both lines were tested for murine class I antigens using monoclonal antibodies to H-2 D^d (Fig. 3 A, B) and to H-2 monotypic antigen. The mouse $\times$ rat hybrid

FIG. 3. Flow cytometry analysis of surface class I MHC antigens of continuous cell lines derived from livers of BALB/c mice. A, the spontaneously transformed BALB/c line has been treated with the monoclonal (ATCC HB-75) against H-2D^d; B, is the mouse $\times$ rat hybrid treated with the same monoclonal. Graphs show results plotted along with negative control (fluorescein conjugate only). Continuous BALB/c line (C) and mouse-rat hybrid line (D) were also tested for intracellular albumin. Graphs plotted with negative control.

line was positive for surface H-2 D^d (Fig. 3 B) and also for monotypic antigen (not shown). The parent rat line was negative. The BALB/c hepatocyte line was negative with antibody to H-2D^d (Fig. 3 A) and with H-2 monotypic antibody (not shown). We also tested the lines for the production of albumin, which is a hepatocyte-specific function. Both lines were positive for intracellular mouse albumin by flow cytometry (Fig. 3 C, D).

Assessment of infection by DNA probe. Measuring rates of infection by light microscopy is laborious and often difficult. We therefore attempted to assess the development of parasite-specific DNA by hybridization. Using the *P. berghei* specific DNA probe (pPb3) we were

able to demonstrate the development of parasite-specific DNA during the cycle of infection and development (Fig. 4).

DISCUSSION

We have developed a method to culture murine hepatocytes and have infected them with *P. berghei* sporozoites. The hepatocytes support growth of the parasites and the production of EE schizonts. Schizonts average 30 μ m in diameter, which is equivalent to development in the human hepatoma line HepG2. These schizonts seem to develop normally in vitro; however, they fail to reach the stage at which rupture and release of merozoites occurs. The fragile nature of these cells and their failure to support complete development of liver merozoites led us to attempt to develop continuous cell lines that could be used in these experiments, and to produce a means by which to measure development of the parasites in cultivation.

Two cell lines were produced that supported growth and complete development of *P. berghei* liver stages in vitro. One of these lines (mouse $\times$ rat hybrid; E2) expresses surface murine class I MHC antigens, which were detected by flow cytometry (Fig. 3). Both cell lines were positive for albumin, indicating they had retained this hepatocyte-specific function. The ability of these two lines to support invasion and development of *P. berghei* sporozoites differs considerably. Approximately 0.3% of the sporozoites develop to form schizonts in the BALB/c line, whereas approximately 0.05% do so in the mouse $\times$ rat line. The schizonts achieved approximately 75% of their final size by 28 h after infection. The DNA probe indicated that the parasite produced little or no new DNA during the first 24 h; however, considerable new DNA was detected 40 h after infection. This is approximately 7 h before merozoite release occurs in this system (47 h postinfection). Merozoite release occurs at 42 to 44 h postinfection in vivo.

These studies demonstrate that *P. berghei* EE stages can be cultured successfully in primary murine hepatocytes and in continuous mouse cell lines. The EE stages develop morphologically to a mature schizont stage and, in the case of the continuous lines, proceed further to rupture and release infectious merozoites. Such systems should prove to be of great importance for studying the interaction between immune T-lymphocytes and the liver stages of *P. berghei*.

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FIG. 4. Autoradiograph of infected monolayers of BALB/c cell line following reaction with a *P. berghei* DNA probe. Cultures were analyzed 40 h after infection with *P. berghei* sporozoites (A); and 20 hours after infection (B). Uninfected cultures are shown in C.

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Book Review

Benchmarks: Alternative Methods in Toxicology

M. A. Mehlman, Editor

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Workers in the area of Toxicology have for some years recognized two basic conditions; descriptive Toxicology, as embodied by the LD₅₀ and the Draize test, is no longer a scientifically viable area or vehicle for study, and the cost of statutorily mandated toxicity and carcinogenicity testing has become extraordinary (estimated to be about \$1,000,000/test chemical). Compound these factors with the public demand for humane treatment of laboratory animals, reduction of the number of animals used, and the need to reduce the expense and time delay involved with standard animal testing protocols, then the need for development of alternative methods in Toxicology is clear. This volume, *Benchmarks: Alternative Methods in Toxicology*, the first in a series, presents review articles on technical aspects of the topic of alternatives to animal testing in Toxicology. The editor, Dr. Myron A. Mehlman, has assembled contributions from recognized experts in the major topics in alternative testing. The nine technical chapters in the book address the areas of statistical and computer analysis and modeling of test results; pharmacokinetic modeling; structure activity relationships; and *in vitro* testing methodologies for genotoxicity, teratogenesis and carcinogenesis including bacterial and tissue culture methods. Methods and potential uses are evaluated realistically and practically, without suggestion that in the foreseeable future animal testing can be completely abandoned. The chapters are not intended to be methodological summaries in the sense of an application manuals, although the references are complete and provide an excellent compendium of the current literature in these topics. The greatest value of this book is that all of the newest alternatives in the testing area are described in a single location.

The tenth chapter is contributed by representatives of the animal right movement. Animal use in science has not been historically characterized by rational discussion of balancing the public health necessity of research and testing with the need to treat animals humanely and with respect for their status as sentient individuals. Although there is no clear moral high ground in this issue, participation by these representatives recognizes that the scientific community is trying to address question of humane and efficient use of animals to promote public safety.

Benchmarks: Alternative Methods in Toxicology provides a worthwhile summary of the current efforts to develop accurate, dependable and predictive method for toxicity testing with minimal use of animals.

Michael J. Meredith, Ph.D. Department of Biochemistry School of Dentistry, Oregon Health Sciences University, 611 S.W. Campus Drive, Portland, Oregon 97201.