Epstein-Barr Virus Transformation of Saimiri sciureus (Squirrel Monkey) B Cells and Generation of a Plasmodium brasilianum-Specific Monoclonal Antibody in P. brasilianum-Infected Monkeys

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The new-world monkeys Saimiri sciureus (squirrel monkeys) are currently used as a model to test the efficacy of vaccines against human malaria. To improve our knowledge on this model, we tested the susceptibility of S. sciureus B cells to Epstein-Barr virus (EBV) infection. B-lymphoblastoid cell lines were obtained from six of six healthy animals after infection with the B95-8 source of EBV. The frequency distributions of spleen B cells clonally committed to the production of immunoglobulins M and G, as measured by limiting dilution analysis, were from 1 in 179 to 1 in 1,085 and from 1 in 45 to 1 in 60, respectively, in three monkeys naturally infected with Plasmodium brasilianum. In the same three animals, the frequency of spleen B cells committed to the production of P. brasilianum-specific antibody ranged from 1 in 2,211 to 1 in 9,099. One B-lymphoblastoid cell line producing anti-P. brasilianum-specific antibody was cloned twice, and the immunoglobulin G produced was purified. This monoclonal antibody recognized a parasite component of 197 kDa and was specific for Plasmodium malariae and P. brasilianum parasites. These data document that squirrel monkey B cells naturally primed by an infectious agent can be efficiently used to produce monospecific antibodies against the infectious agent.

Epstein-Barr virus (EBV) is a herpesvirus that infects human B cells (reviewed in reference 27). EBV binds specifically to the complement receptor type 2 (CD21) for the C3d fragment of the third component of the complement expressed on the surface of mature B cells (11). Although most B cells are infected by EBV, only a fraction of them are induced to express viral antigens such as EBNA-1 and then to proliferate and produce immunoglobulins in a T-cell-independent fashion (1, 32). EBV-infected B cells can thus give rise to long-term proliferating lymphoblastoid cell lines (LCLs). Through appropriate selection of antibodies produced by B-LCLs, human monoclonal antibodies have been obtained (3, 4).

Beside human B cells, EBV has been shown to transform primate B cells. However, inefficient or no EBV transformation of B cells from new-world monkeys, in particular from Saimiri sciureus (squirrel monkeys), has been reported (10, 15, 19). Squirrel monkeys are naturally infected by Plasmodium brasilianum, which is thought to be a Plasmodium malariae strain that has recently become adapted in new-world monkeys (7). In addition, squirrel monkeys are permissive hosts for the human malaria species Plasmodium falciparum and P. vivax (7). As such they have been used in recent years to evaluate the efficacy of vaccines designed to induce protective immunity against sporozoites and blood stages of these two species (9, 21). The possibility of obtaining monoclonal antibodies (MAbs) efficiently from infected squirrel monkeys could allow the identification of conformationally intact antigenic determinants expressed by living parasites. In addition, passive transfer of homologous antibodies specific for defined parasite antigens could prove useful in evaluating their protective effect.

In this study we reassessed the capacity of EBV to infect squirrel monkey B cells. We addressed this issue by quantitating in limiting-dilution culture assays (17) the B lymphocytes clonally committed to the production of immunoglobulin M (IgM), IgG, and P. brasilianum-specific immunoglobulin. In addition, the ability of EBV to transform squirrel monkey B cells was used to generate a monoclonal B-LCL-producing antibody specific for a P. brasilianum component of 197 kDa.

MATERIALS AND METHODS

EBV. The B95-8 marmoset lymphoma cell line (provided by A. A. Ansari, Emory University, Atlanta, Ga.) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, nonessential amino acids (1% of a 100× stock solution), 1 mM sodium pyruvate, and 5 × 10⁻⁵ 2-mercaptoethanol (all from Gibco, Grand Island, N.Y.). This medium is hereafter referred to as complete medium (CM). The B95-8 culture supernatant containing living virus was obtained as previously described (14), was stored at −80°C until use.

B cell preparation. Blood (3 ml) was drawn from the femoral veins of six healthy adult S. sciureus boliviensis monkeys (three males and three females) by using heparinized Vacutainers. Peripheral blood mononuclear cells (PBMC) were purified by using Ficoll Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation. After plastic adherence, PBMC were reacted with sheep erythrocytes (RBC) previously treated with 2-aminoethylisothiouronium bromide hydrobromide (Sigma Chemical Co., St. Louis, Mo.). Rosetting cells were depleted by gradient centrifugation as described previously (6). Nonrosetting cells (T cell-depleted PBMC) were used as the source of B cells. Because of the small number of cells available (0.2

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× 10⁶ to 1.3 × 10⁹), no attempt was made to quantify the actual B cells contained in these cell preparations.

Spleens were surgically removed from three other adult S. sciureus boliviensis naturally infected with P. brasili anum. Spleen tissue was minced with scissors, and a single-cell suspension was obtained by using a homogenizer. Spleen cells were incubated in petri dishes (Falcon; Becton Dickinson, Oxnard, Calif.) for 1 h at 37°C in CM to allow the cells to shed cytophilic immunoglobulin and allow monocytes and macrophages to adhere to plastic. Nonadherent cells were then submitted to two cycles of rosetting with sheep RBC. The nonrossetting cells contained less than 8% sheep RBC rosette-forming cells and were 24.1, 34.6, and 41.5% positive for membrane immunoglobulin in three monkeys as determined by cytofluorometric analysis (FACSscan cytoflurometer; Becton Dickinson) with fluorescein isothiocyanate (FITC)-labeled goat anti-S. sciureus IgG (provided by V. Tsang, Centers for Disease Control, Atlanta, Ga.) and FITC-labeled goat anti-mouse immunoglobulin as negative controls.

**ELISA titration of IgM and IgG.** The Falcon assay screening test–enzyme-linked immunosorbent assay (ELISA) system was adopted to determine immunoglobulin titers in culture supernatants by a modification of the procedure of Hancock and Tsang (13). In brief, knobs were coated for 150 min with 5 μg of affinity-purified goat anti-human IgM (gamma chain specific) or anti-human IgG (mu chain specific) (Cappel Laboratories, Organon Teknika Co., West Chester, Pa.) per ml of phosphate-buffered saline (PBS) (pH 7.2). Knobs were incubated with appropriate dilutions of culture supernatants or reference standards for 60 min and then with anti-S. sciureus IgG (H and L chain specific) conjugated with horseradish peroxidase for 30 min. The knob-bound enzyme was revealed with the substrate 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.), and the A₄₅₀ was measured with an automated ELISA reader (Titertek Multiscan; Flow Laboratories, McLean, Va.). Reference binding curves were obtained by using purified human IgM or IgG (Cappel). The assays for IgM and IgG had a sensitivity of 20 ng/ml without evident cross-reactivity up to 20 μg/ml. The specificity of this assay for S. sciureus immunoglobulin was verified and confirmed a posteriori when supernatants from B-LCLs producing IgM or IgG were available.

**Immunofluorescence detection of P. brasili anum-specific antibodies.** Antigens for the immunofluorescence assay (IFA) were smears of parasite-infected RBC prepared as described previously (25). P. brasili anum, P. vivax, and Plasmodium inui were from infected S. sciureus. Plasmodium cynomolgi was from infected Macaca mulatta. P. malariae was from infected Pan troglodytes. P. falciparum was in vitro culture (29). Culture supernatants were incubated on slides for 30 min and then treated sequentially with FITC-conjugated goat anti-S. sciureus immunoglobulin and with FITC-conjugated swine anti-goat IgG. Slides were examined on a fluorescence microscope (Olympus; Southern Micro Instruments, Inc., Atlanta, Ga.).

**EBV infection of S. sciureus B cells.** T cell-depleted PBMC or enriched spleen B cells (0.2 × 10⁶ to 3 × 10⁶) were incubated overnight at 37°C in 1 ml of freshly thawed EBV. T cell-depleted PBMC were then cultured in 24-well plates (Costar) with 1 million spleen cells and culture medium every 6 days until vigorous proliferation was evident. Mass cultures were then maintained in flasks by weekly dilution. Control cultures not incubated with EBV were similarly maintained.

**Determination of frequencies of antibody-producing precursors.** After incubation with EBV, enriched spleen B cells were diluted and plated at 2,500, 500, 100, 20, and 4 cells per well in round-bottom 96-well plates in the presence of 0.2 × 10⁶ irradiated (2,500 rads) normal human PBMC per well as feeder cells. Forty-eight replicates for each cell dose, including 48 negative controls (feeder cells alone), were seeded. Twenty-one days later, 150 μl of each culture supernatant was harvested, diluted 1:4 in CM, and tested for the presence of IgM, IgG, or P. brasili anum-specific antibodies. Positivity for IgM or IgG presence in supernatants was defined as an optical density equal to or greater than the optical density plus 3 standard deviations of the 48 replicates of feeder cells (0.05 to 0.1 optical density unit in different experiments) as determined in the Falcon assay screening test-ELISA. The fraction of negative wells was then recorded and utilized to compute the frequency of precursors of immunoglobulin-producing cells as described previously (22, 26).

**Monoclonal P. brasili anum-specific IgG-producing S. sciureus cell line.** Cells from a culture producing P. brasili anum-specific antibody were cloned by limiting dilution. The cloned line H12 was then expanded, and the antibody in the culture supernatant was purified by precipitation in 50% saturated ammonium sulfate. The antibody contained in culture supernatants from the parental cell line or other mass cultures was also purified.

**P. brasili anum antigen preparation.** Heparinized blood from two S. sciureus monkeys infected with P. brasili anum (3% parasitemia) was centrifuged. The pellet was suspended in 2 ml of PBS and applied to a column of CF 11 cellulose (Whatman Biosystem Ltd., Maidstone, United Kingdom) and glass beads (Sigma) previously equilibrated with PBS to remove the leukocytes and platelets (12, 24). The recovered RBC were fractionated by centrifugation (800 × g for 15 min) over a discontinuous multistep isotonic Percoll gradient (2). More than 50% of the RBC in the layer recovered at the interface between 40% and 60% Percoll were infected. The parasites, from young trophozoites to mature schizonts, were used as antigen for Western immunoblotting.

**Western blotting.** After two washes in PBS, enriched P. brasili anum-infected RBC were extracted in 10 volumes of lysis buffer (25 mM Tris-HCl [pH 7.6], 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1.5 mM phenylmethylsulfonyl fluoride, leupeptin [1 μg/ml], pepstatin A [1 μg/ml]; all from Sigma). Noninfected RBC used as a control were submitted to the same procedure. Lysed samples were fractionated under reducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (0.45-μm pore size; Schleicher and Schuell Inc., Keene, N.H.) by using standard procedures (28). After transfer, nitrocellulose strips were incubated with purified B-LCL antibody (50 μg/ml) overnight at 4°C. After the strips were washed, they were incubated with horseradish peroxidase-conjugated anti-S. sciureus immunoglobulin for 1 h. To visualize the recognized bands, enzyme activity was revealed by using the substrate 3,3-diamino benzidine. Pre-stained molecular markers (Bethesda Research Laboratories, Gaithersburg, Md.) were used to determine the relative molecular masses.

**RESULTS**

Squirrel monkey B cell susceptibility to EBV infection. T cell-depleted PBMC from six healthy squirrel monkeys were infected with EBV. From 10 to 15 days later, vigorous cell
proliferation was observed. By day 18, the growing cells required subculturing, and continuously growing B-LCLs were successfully established in all cases. Both IgG and IgM were detected in the supernatants of B-LCLs. No growth was recorded when T cell-depleted PBMC from four of these squirrel monkeys were maintained in culture for 8 weeks without previous infection with EBV (Table 1).

**Frequency analysis of Saimiri B cell precursors committed to the production of IgM and IgG.** The results described above demonstrate that EBV is able to transform *Saimiri* B cells. We were then interested in measuring the frequency of B cell precursors activated by EBV to produce IgM or IgG. To this aim, enriched spleen B cells from three squirrel monkeys naturally infected with *P. brasilianum* were used in a limiting dilution analysis. A representative experiment is shown in Fig. 1. In mass cultures of spleen B cells from the three infected animals, the time to transformation was in the same range of that observed with T cell-depleted PBMC from normal animals. For three monkeys the frequency of B cells committed to the production of IgG, ranging from 1 in 45 to 1 in 66, did not overlap and was higher than the frequency of B cells committed to the production of IgM (ranging from 1 in 179 to 1 in 1,085) (Table 2). Because these monkeys were infected with *P. brasilianum*, we were also

![Fig. 1. Limiting dilution analysis to determine the precursor frequencies of IgM- and IgG-secreting cells from squirrel monkeys in EBV-stimulated cultures. Forty-eight replicate cultures were started for each cell dose. IgM and IgG concentrations in 21-day-old supernatants were determined by using an ELISA. The dashed line at 0.37 corresponds to the zero term of the equation according to the Poisson distribution when one B cell precursor is seeded in each well. In this experiment, 1 in 51 added B cells produced IgG and 1 in 179 produced IgM. The dotted lines represent the 95% confidence intervals.](image-url)
TABLE 3. Determination of the frequency of *P. brasilianum*-infected squirrel monkey spleen B cell precursors induced by EBV to *P. brasilianum*-specific antibody production

<table>
<thead>
<tr>
<th>Animal</th>
<th>Precursor frequency*</th>
<th>n</th>
<th>Chi square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1930</td>
<td>1/0.099 (1/5.501 to 1/15.038)</td>
<td>2</td>
<td>0.14</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>S1938</td>
<td>1/2.211 (1/1.642 to 1/2.978)</td>
<td>3</td>
<td>3.59</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>S1939</td>
<td>1/4.959 (1/5.336 to 1/7.373)</td>
<td>3</td>
<td>1.71</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* The supernatant from each microculture was tested for the presence of *P. brasilianum*-specific antibody by the IFA. For further explanation see the footnotes to Table 2.

interested in evaluating the progenitor frequency of B cells producing *P. brasilianum*-specific antibodies. Parasite-specific antibodies were detected by IFA in cultures from the three monkeys, with frequencies ranging from 1 in 2,211 to 1 in 9,099 (Table 3).

**Generation and characterization of a *P. brasilianum*-specific MAb.** Cells from a culture seeded at 2,500 cells and positive for the presence of IgG and parasite-specific antibodies were submitted to sequential cloning by limiting dilution first at 0.4 cell per well and then at 10 cells per well because less than 10% of the wells were growing. The quantitation of IgG in culture fluid of the cloned H12 B-LCL tested at the end of 7 days of subculture was consistently above 5 mg/ml in four distinct determinations. The H12 IgG recognized *P. brasilianum* and *P. malariae* asexual blood stages at similar concentration by the IFA but did not react when tested against *P. falciparum*, *P. vivax*, *P. cynomolgi*, and *P. inui*.

The immunofluorescence reaction pattern was diffuse or reticulated with increased intensity from late trophozoites to mature schizonts. In the segmenting cells, the pattern appeared to delineate individual merozoites (Fig. 2). By Western blot the H12 MAb recognized a major band of 197 kDa specifically on parasite-infected RBC but not on noninfected RBC. Minor bands were also recognized corresponding to 183, 160, 136, 95, 82, and 43 kDa. No bands were recognized by purified immunoglobulin from an uncloned B-LCL obtained from a healthy animal (Fig. 3).

**DISCUSSION**

The present study was undertaken to determine whether EBV is able to infect and transform B cells from squirrel monkeys, a new-world primate species. The results show that (i) B cells from healthy or malaria-infected squirrel monkeys were efficiently transformed by EBV; (ii) the frequency of spleen B cell precursors transformed by EBV and committed to IgM production; (iii) through the selection and subcloning of a B-LCL secreting an antibody of interesting specificity, relatively large amounts of squirrel monkey monoclonal IgG were easily obtained; and (iv) partial biochemical and structural characterization of a *P. brasilianum* antigenic component was performed with the H12 squirrel monkey MAb.

Beside human B cells, EBV and EBV-like viruses have been shown to infect and transform B cells from apes and old-world monkeys (10, 15, 19, 23). In one study on 16 animals of seven new-world species, including squirrel monkeys, negative results were reported (15). However, in agreement with Falk et al. (10) and with Miller et al. (19), we found that squirrel monkey B cells are transformed by the B95-8 source of EBV. That exogenous EBV but not endogenous virus transformed *Saimiri* B cells in our assays is supported by the lack of spontaneous growth observed in cultures of B cells not incubated with B95-8 supernatants. The 100% rate of successful B cell transformation with squirrel monkeys in this study is higher than the 20 to 30% success rates previously reported (10, 19). However, technical factors such as EBV preparation and T-cell depletion before the EBV infection of B cells may explain this difference.

Of interest is the high frequency of EBV-transformed squirrel monkey B cells. Indeed, the measured frequencies for IgG B cell precursors (1 in 45 to 1 in 66) or IgM B cell precursors (1 in 179 to 1 in 1,085) are possibly underestimates. In fact, the starting population was composed of enriched but not pure B cells, and no attempts were made to correct the results for the number of actual B cells plated. Although the frequency of EBV-transformed B cells reported in the literature for humans differs according to the particular protocol followed, the number of B cells committed to IgG production in humans as detected by the methodology applied here (30) was from 1 in 145 to 1 in 450, i.e., 2 to 10 times lower than that measured for squirrel monkeys.

**FIG. 2.** Immunofluorescence staining pattern of MAb H12 with *P. brasilianum*-infected squirrel monkey RBC.
Moreover, in humans, B cell precursors for IgM production have been reported to have higher frequencies than have IgG B cell precursors. This possibly reflects the higher proportion of B cells bearing mu immunoglobulin heavy chains in PBMC. In fact, EBV has been shown to activate and transform human B cells bearing mu, gamma, or alpha immunoglobulin heavy chains to produce IgM, IgG, or IgA, respectively, with equal efficacy (20). However, a direct comparison with results obtained with human PBMC may be inappropriate. In this study, spleen cells and not PBMC were used to perform the limiting dilution analysis. Thus, our results might reflect a preferential homing in the spleen of B cells bearing gamma immunoglobulin heavy chains. In addition, the spleens were obtained from *P. brasilianum*-infected animals. Malaria parasites are potent T-cell-dependent B mitogens (5). Thus, it is possible that B cells with various degrees of activation were populating the spleen and were more susceptible to EBV transformation. Indeed, *P. falciparum* products have been shown to enhance human lymphocyte transformation by EBV (16).

The quantification of B cells committed to the production of malaria-specific immunoglobulin has been attempted with a different methodology (31). In that work, human B cells were activated by mutant EL4 thymoma, leading to activation and differentiation of about 90% of the B cells. The frequencies of *P. falciparum*-specific B cells in infected humans reported by these authors (from 0.1 to 1%) are higher than those against *P. brasilianum* found in infected squirrel monkeys. Besides differences in the experimental system utilized, it is also possible that the two different malaria species had different interactions with the immune systems of the reciprocal vertebrate hosts, thus explaining discrepancies between the results.

Finally, we describe here the *P. brasilianum* antigenic component recognized by a cloned squirrel monkey B-LCL, H12. Mouse MAbs have been produced in the past to characterize *P. brasilianum* components (8), but none of these recognized a 197-kDa antigen. Because of its molecular mass, including the presence of minor bands of lower molecular mass in the Western blot, and its IFA pattern, the antigen described here is highly reminiscent of the *P. falciparum* gp 195, the precursor of the major merozoite surface antigen (18). The epitope recognized by H12 MAb appears to be species specific. This MAb did not cross-react with any other malaria species, with the exception of the closely related species *P. malariae* (7).

MAbs produced in mice by the hybridoma technology have been extensively used to characterize several cellular antigens. They have the greatest chance of success, because repeated immunizations are possible and spleen cells are easily obtained. However, when the immunogen used is denatured, as it is with parasites that do not grow in rodents, mouse MAb could fail to detect conformational epitopes that might have important biological significance. In this instance, transformation of B lymphocytes from the parasite-susceptible host might be useful in generating MAbs from cells involved in the ongoing in vivo immune process.

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REFERENCES


