*Proc. Natl. Acad. Sci. USA*
Vol. 82, pp. 6272–6275, September 1985
Immunology

# Monkey-derived monoclonal antibodies against *Plasmodium falciparum*

(malaria)

HAROLD A. STANLEY AND ROBERT T. REESE

Department of Immunology, IMM14, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037

*Communicated by Frank J. Dixon, May 20, 1985*

**ABSTRACT** **A system has been developed that allows efficient production of monkey monoclonal antibodies from owl monkeys. Splenocytes or peripheral blood lymphocytes from monkeys immune to the human malarial parasite, *Plasmodium falciparum*, were fused with P3X63 Ag8.653 mouse myelomas. The resulting hybridomas were screened by an indirect fluorescent antibody test for the production of monkey monoclonal antibodies (mAb) reactive with *P. falciparum*. Most of the mAb reacted with the *P. falciparum* merozoites and immunoprecipitated a parasite-derived glycoprotein having a relative molecular weight of 185,000. These mAb gave a minimum of five different immunoprecipitation patterns, thus demonstrating that a large number of polypeptides obtained when parasitized erythrocytes are solubilized share epitopes with this large glycoprotein. In addition, mAb were obtained that reacted with antigens associated with the infected erythrocyte membrane. One of these mAb bound a $M_r$ 95,000 antigen.**

Particular subspecies of owl monkeys (genus *Aotus*) provide the best model for human malaria. Blood stages of the human malarial parasite *Plasmodium falciparum* produce a life-threatening disease in naive animals that, when cured with drugs, leaves the animals highly immune. Future challenge with very large doses of parasitized erythrocytes produces barely detectable parasitemias (1). Humans respond to the parasite in a similar fashion but may not develop as strong an immunity. However, because of the genetic similarity between primates and humans, it is probable that the immunologic solution devised by the monkey will also be applicable in man. Langreth and Reese (2) have shown that sera from immune owl monkeys contain antibodies that bind to the surface of merozoites and parasitized erythrocytes. These antibodies can inhibit *in vitro* growth of the parasite (1, 3) and provide immunity to other animals when transferred passively (unpublished data).

Here we present a method for producing monoclonal antibodies (mAb) from *Aotus* monkeys. Antibodies that detect a series of different epitopes associated with the surface of merozoites as well as those that bind to parasite antigens found in the membrane or cytoskeleton of the parasitized erythrocyte are given as examples. The fusion efficiency of this monkey–mouse system is at least as high as found with mouse–mouse fusions and the levels of antibody produced by stable hybrids appear to be similar to those from mouse–homo hybrids. This system is likely to have wide application in the study of other infectious agents because the epitopes seen by the primate should be more relevant to producing human immunity than structures seen by a rodent that cannot experience the disease. Similarly, this system may also be useful in elucidating the antigenic composition of plasma membranes from other cells (transformed or non-transformed) that can survive in the monkey and induce a strong immune response.

## MATERIALS AND METHODS

**Cells.** The *Plasmodium falciparum* FVO isolate was grown in human $O^+$ erythrocytes using the candle jar method of Trager and Jensen (4). To obtain material enriched for segmenters and merozoites, the cultures were treated with Physiogel (5) when most of the parasites were early trophozoites. Infected erythrocytes (50–70% parasitemia) were returned to culture (0.5% hematocrit) for 18 hr. Segmenters and free merozoites were harvested, washed twice with RPMI 1640 medium, and used as follows: (*i*) repeatedly frozen and thawed for lymphocyte stimulation, (*ii*) stored at −70°C as antigen for immunoblot analyses, (*iii*) smeared on slides and acetone fixed for indirect fluorescence antibody tests (IFAT), and (*iv*) used unfixed for IFAT on cells in suspension. To obtain metabolically labeled parasites [$^{35}$S]methionine or [$^{3}$H]glucosamine (1 mCi/10 ml; 1 Ci = 37 GBq) was included in the culture medium during the 18-hr incubation of the Physiogel-concentrated parasites.

The P3X63 Ag8.653 myeloma (6) was adapted to and subsequently grown in Dulbecco's high-glucose medium (Irvine Scientific) supplemented with 10% gamma-globulin-free horse serum (GIBCO). The cells were subcultured daily to $4 \times 10^5$ cells per ml for at least 3 days prior to any cell fusion experiments.

**Fusions.** The *Aotus* monkey (karyotype II) used as a source of splenocytes had previously been infected three times with the *P. falciparum* FVO isolate and was judged to be immune to this isolate because the injection of a normally lethal dose of this parasite resulted in little or no detectable parasitemia. One year after the last infection, the monkey was injected intraperitoneally with 0.2 ml of frozen and thawed segmenters and free merozoites. Three days later, the monkey was splenectomized and carefully monitored to ensure complete recovery. The spleen was gently teased apart and the splenocytes were washed with cold phosphate-buffered saline and frozen in 10% dimethyl sulfoxide, 50% RPMI 1640 and 40% fetal bovine serum.

At the time of a fusion one vial of $5–10 \times 10^6$ cells was rapidly thawed at 37°C, washed twice with Dulbecco's high-glucose medium, and mixed with an equal number of similarly washed P3X63 Ag8.653 myelomas. The cells were then pelleted by centrifugation for 10 min at $1300 \times g$. After removing the supernatant, the pellet was gently loosened. One milliliter of 37°C fusion promoter (35% PEG 1000 and 7.5% dimethyl sulfoxide in RPMI 1640) was slowly added over a 1-min time interval. Warm Dulbecco's medium containing 10% gamma-globulin-free horse serum was then slowly added, 2 ml in a 2-min interval followed by 8 ml in 2 min. After centrifugation for 10 min at $1300 \times g$, the fused

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Abbreviations: IFAT, indirect fluorescent antibody test; mAb, monoclonal antibody(ies); PBL, peripheral blood lymphocytes.

cells were diluted to the appropriate cell density in Dulbecco's high-glucose medium containing 10% gamma-globulin-free horse serum, hypoxanthine (14 μg/ml), aminopterin (0.45 μg/ml), and thymidine (7.2 μg/ml) (HAT medium) and dispensed in 96-well plates. BALB/c ByJ mouse thymocytes ($2 \times 10^6$/ml) were used as a feeder layer. The cultures were fed weekly with HAT medium supplemented on days 7 and 15 with fresh thymocytes. Screening of the spent culture supernatants by IFAT using a fluorescein isothiocyanate conjugate of affinity-purified rabbit anti-*Aotus* IgG antibodies was initiated on day 21 and continued for 3 weeks.

A second immune monkey (karyotype VI), used as a source of peripheral blood lymphocytes (PBLs), had previously been infected with the FVO isolate five times within 2 years. A year after the last infection 7 ml of blood was drawn by vena puncture using heparin as an anticoagulant. The mononuclear cells were concentrated using Ficoll/Hypaque (Pharmacia) and washed twice with cold $P_i$/NaCl. Then, $2 \times 10^7$ cells were stimulated *in vitro* for 4 days in 10 ml of Dulbecco's high-glucose medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and 40 μl frozen and thawed segmenters and free merozoites. The surviving PBLs ($1 \times 10^7$) were washed, fused with an equal number of P3X63 Ag8.653 cells, and cultured as described above.

Hybridomas were also prepared using splenocytes from an immunized mouse. In this experiment a BALB/c ByJ mouse was injected three times (days 1, 60, 150) with frozen and thawed parasite material corresponding to $\approx 10^7$ infected erythrocytes. Three days after the final injection, the spleen was removed and the splenocytes were fused with the mouse myelomas as described above. The hybridoma culture supernatants were screened by IFAT using a fluorescein isothiocyanate conjugate of affinity-purified goat anti-mouse IgG and IgM antibodies (Tago, Burlingame, CA).

**Immunological Assays.** Immunoprecipitation assays were done to characterize the molecules with which the monkey mAbs reacted. Metabolically labeled parasites (see above) were lysed in a detergent containing radioimmunoprecipitation assay buffer (RIPA buffer A, ref. 7) and insoluble material was removed by centrifugation for 1 hr at 150,000 × *g*. The lysate was then used to resuspend protein A-Sepharose to which mAbs had been bound previously. Protein A-Sepharose lacking antibody was used as a negative control. After 1 hr in the lysate the beads were washed three times with RIPA buffer, once with RIPA buffer containing 0.5 M NaCl, and once with distilled $H_2O$. The beads were boiled in sample buffer (8) and the eluted antigens were electrophoresed on 8% $NaDodSO_4$/polyacrylamide gels in parallel with $^{14}C$-labeled molecular weight markers (carbonic anhydrase, 30,000; ovalbumin, 43,000; IgG heavy chain, 53,000; and myosin, 200,000).

A previously described (9) immunoblotting procedure was used to test supernatants from some of the hybridomas. The proteins were first resolved by 6% $NaDodSO_4$/PAGE and then blotted onto nitrocellulose sheets. After treating the sheets with the hybridoma supernatants, bound antibodies were detected using $^{125}I$-labeled affinity-purified rabbit anti-*Aotus* IgG antibodies followed by fluorography. In preliminary experiments, $^{14}C$-labeled molecular weight markers (see above) were also run into the gels and blotted. These were used to standardize the reactivity of one mAb in the immunoblots. The antigens recognized by this mAb were subsequently used as molecular weight markers in other immunoblots.

## RESULTS

In preliminary titrations using cells from a fusion of monkey splenocytes and mouse myelomas it was determined that cell concentrations of $2 \times 10^4$ cells per well (in a 96-well plate) resulted in growth in virtually every well. Concentrations of $4 \times 10^3$ cells per well generally gave growth in 40–50% of the wells. At this initial cell density approximately 70% of the wells containing viable hybrids should be clonal (10). Subsequent fusions using monkey splenocytes were therefore plated at $4–5 \times 10^3$ cells per well. The results from one such fusion are given in Table 1. Of the 1380 wells initially plated, 1067 developed viable hybrids. Analyses by IFAT of the culture supernatants from these wells showed the presence of anti-parasite antibodies in at least 97 wells. While most of the culture supernatants gave a bright rim of fluorescence around the acetone-fixed parasites, some gave internal diffuse or internal punctate fluorescence. None of the culture supernatants appeared to react with uninfected erythrocytes. The hybrid cells in the 97 wells containing anti-parasite antibody were then both cloned by limiting dilution and subcultured to 24-well plates. When the supernatants from the 24-well plates were examined by IFAT (11 days after subculturing) 61 wells no longer contained detectable amounts of anti-parasite antibody. However, stable clones were obtained from three of the corresponding wells of the original 96-well plates. Ultimately, 14 hybridomas were obtained that produced antibody for more than 6 weeks after cloning and/or the original fusion. Those of particular interest that have been studied have continued to produce antibody for longer than 3 months. Since a monkey spleen contains enough cells for at least 30 to 40 such fusions, approximately 500 stable anti-parasite hybridomas can be expected from each spleen.

Table 1. Mouse–monkey fusions

| | Monkey fusions | | |
|---|---|---|---|
| | *In vivo* stimulation | *In vitro* stimulation | Mouse fusion |
| Splenocytes | $7 \times 10^6$ | — | $4 \times 10^6$ |
| PBLs | — | $1 \times 10^7$ | — |
| Wells seeded | 1380 | 640 | 480 |
| Myeloma cells per well | $5 \times 10^3$ | $1.5 \times 10^4$ | $8 \times 10^3$ |
| Wells with hybrids | 1067 | 420 | 398 |
| % wells with hybrids | 77 | 66 | 83 |
| Antibody-positive wells* | 97 | 40 | 85 |
| % antibody-positive wells† | 9 | 10 | 21 |
| Stable hybridomas | 14 | 3 | — |
| % stable hybridomas‡ | 1 | <1 | — |
| Stable hybridomas/$10^6$ lymphocytes | 2 | 0.3 | — |

*Wells in the 96-well plates containing antibody against either parasite or erythrocyte antigens.
†(Antibody-positive wells/wells with hybrids) × 100.
‡(Stable hybridomas/wells with hybrids) × 100.

Hybridomas were also obtained from peripheral blood lymphocytes stimulated *in vitro* with frozen and thawed parasitized erythrocytes (Table 1). In a typical experiment a cell density of $1.5 \times 10^4$ cells per well gave viable hybrids in 66% of the wells. Of the 420 wells with viable hybrids, 40 contained antibody against the stimulating antigens (37 against the parasite and 3 against uninfected erythrocyte membrane). Ultimately, only three stable anti-parasite hybridomas were obtained.

The results of a fusion using *in vivo*-stimulated mouse splenocytes are given for comparison (Table 1). Of the 480 wells originally plated at $8 \times 10^3$ cells per well, 83% developed viable hybrids. Of these 398 wells, 21% contained antibody against the stimulating antigen. However, of the 85 hybridomas that produced antibody, 48 contained antibody reactive with uninfected erythrocytes. Of the remaining wells only 15 contained antibody that reacted with antigens specifically associated with merozoites.

All of the 17 stable monkey–mouse hybridomas obtained from the experiments outlined above have produced antibodies reactive by IFAT with *P. falciparum* merozoites. The fluorescence titers for these mAbs were comparable with those obtained with the mouse mAbs. Although two of the monkey–mouse hybridomas have produced antibody that appears by IFAT on acetone-fixed slides to bind to internal merozoite antigens, the rest of the hybridomas produce antibodies that appear to react with antigens associated with the merozoite surface. The binding to merozoite surface polypeptides by some of the mAbs was also observed when intact live merozoites were used as antigen. Of the 15 mAbs that react with surface-associated merozoite antigens, 14 immunoprecipitated a major parasite polypeptide with a relative molecular weight of 185,000 (Fig. 1). This polypeptide can be labeled with both [$^{35}$S]methionine and [$^3$H]glucosamine. Various of these 14 mAbs also immunoprecipitated an array of other polypeptides having lower molecular weights. For instance, mAb 9-6D (lane 2) immunoprecipitated major polypeptides of $M_r$ 185,000, 152,000, 106,000, and 83,000 as well as some minor peptides. mAb 6-6G (lane 1) immunoprecipitated not only these four polypeptides but also at least one additional peptide of $M_r$ 32,000. In contrast, mAb 25-9G (lane 5) immunoprecipitated the $M_r$ 185,000 polypeptide but not the $M_r$ 152,000, 106,000, and 83,000 polypeptides. It did, however, immunoprecipitate at least three other polypeptides ($M_r$ 120,000, 46,000, and 40,000). mAb 24-11H (lane 4) not only immunoprecipitated the $M_r$ 185,000 and 120,000 polypeptides recognized by mAb 25-9G (lane 5) but also the $M_r$ 152,000 polypeptide recognized by mAb 9-6D (lane 2). mAb 24-96 (lane 3) immunoprecipitated these three major polypeptides and the $M_r$ 106,000 polypeptide recognized by mAb 9-6D. The major peptides found in each lane appear to share epitopes rather than being coprecipitated as a complex. This is demonstrated in Fig. 2 (lane 2) since all of the major bands are detectable when the parasite antigens are first separated by $NaDodSO_4$ gel electrophoresis and then subjected to immunoblot analysis with mAb 9-6D.

From a more recent fusion we have obtained several monkey mAbs (e.g., 6-10G) that bind to antigens associated with the plasma membrane of the infected erythrocyte (Fig. 3). mAb 6-10G did not bind to acetone-fixed uninfected erythrocytes, to infected erythrocytes containing live parasites, or to acetone-fixed erythrocytes containing a variant of the FVO isolate that no longer modifies the host membrane by the formation of knobs. The mAb (lane 1 of Fig. 2) reacted strongly with a polypeptide of $M_r$ 95,000 and weakly with a polypeptide of $M_r$ 90,000 when used in immunoblot analyses.

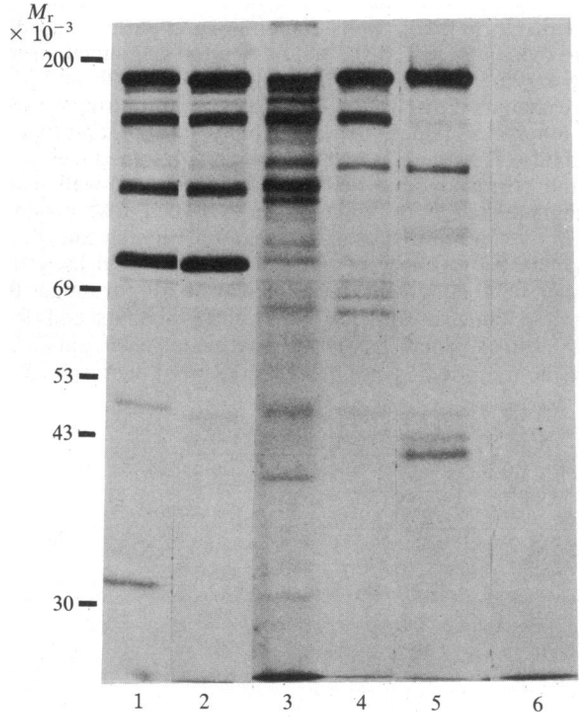


FIG. 1. $NaDodSO_4$/PAGE of *P. falciparum* polypeptides immunoprecipitated by monkey-derived mAbs. The [$^{35}$S]methionine-labeled polypeptides were immunoprecipitated with protein A-Sepharose coated with mAbs 6-6G (lane 1), 9-6D (lane 2), 24-9C (lane 3), 24-11H (lane 4), or 25-9G (lane 5). Polypeptides nonspecifically precipitated by protein A-Sepharose lacking antibody are shown in lane 6.

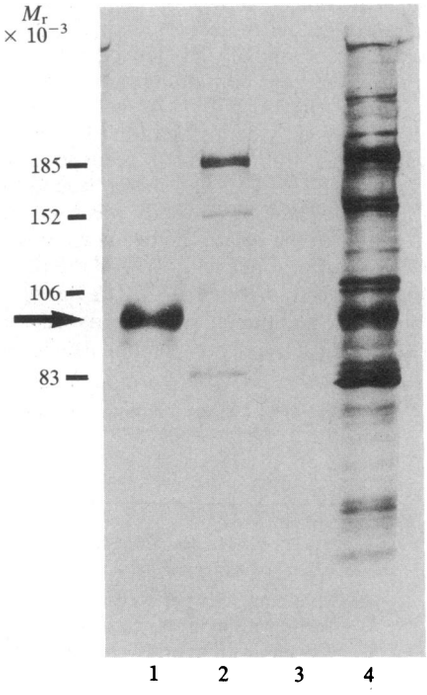


FIG. 2. Immunoblot analysis of mAbs reactive with the antigen(s) associated with the infected erythrocyte. Lanes: 1, the $M_r$ 95,000 antigen (arrow) recognized by mAb 6-10G; 2, the four antigens recognized by mAb 9-6D (see Fig. 1, lane 2); 3, antigens recognized by culture medium lacking any mAb; 4, antigens recognized by immune *Aotus* serum. Affinity-purified $^{125}$I-labeled rabbit anti-*Aotus* IgG was used to visualize the bound antibodies.

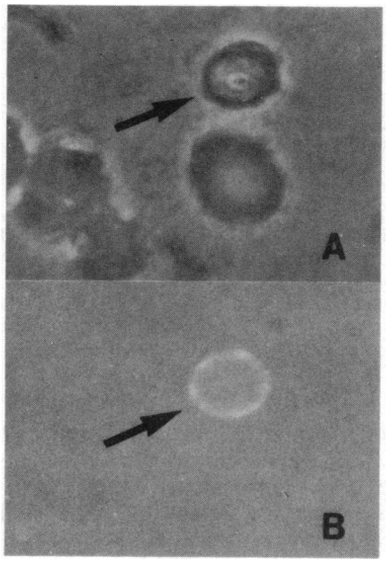


FIG. 3. mAb binding to the antigen(s) associated with the infected erythrocyte membrane. (*A*) Phase-contrast photograph showing an infected erythrocyte (arrow) and a noninfected erythrocyte. (*B*) Fluorescence photograph showing that mAb 6-10G binds only to the infected erythrocyte (arrow).

## DISCUSSION

Monoclonal antibodies produced from a primate that can experience an infectious disease have a series of theoretical advantages over mAbs produced from a mouse (refractory to the disease) immunized with the infectious agent. For example, with human malarial parasites, the antigen can be presented to the animal in its own erythrocytes. This eliminates a large amount of the background antierythrocytic response that is typically found using the mouse system. More than 50% of our mouse mAbs bind to uninfected erythrocyte antigens while virtually every monkey mAb reacts with the parasite or with antigens specifically associated with the parasitized erythrocyte membrane.

Immunization by infection provides the animal with the organism and its components in an authentic replicating form in contrast to the possibly altered forms found in a culture. In the case of *P. falciparum*, ample evidence suggests that dramatic changes can occur within a relatively short time in culture (3, 11). Immunization with such material is likely to provide limited stimulation against some antigens that may be important in immunity. Evidence that the monkey system has helped this situation comes from the observation that from one fusion using $10^7$ monkey splenocytes, we have obtained at least three stable mAbs that react with parasite-induced antigens associated with the erythrocyte membrane. In contrast, antibodies with such specifications have not been obtained from approximately 20 mouse–mouse fusions in our laboratory and, so far as we know, there are no published accounts of such monoclonal antibodies from any other laboratory.

One of our mAbs that reacted with an antigen associated with the infected erythrocyte membrane bound a $M_r$ 95,000 antigen by immunoblot analysis. This antigen was not detectable by IFAT using acetone-fixed infected erythrocytes lacking the membrane modifications called knobs. Whether this antigen is the histidine-rich protein thought to comprise the electron-dense material forming part of the knob (12) remains to be determined.

A further advantage of the monkey mAb system should be its potential for recognizing antigens that are likely to be relevant in stimulating immunity. None of the mouse mAb reacted with the parasitized erythrocyte membrane and only 18% bound merozoite antigens. In contrast, most of the monkey mAb reacted with antigens associated with the merozoite surface. In particular, 14 monkey mAbs immunoprecipitated a $M_r$ 185,000 glycoprotein that recently had been shown to induce a protective immune response in monkeys (13). Previous work has shown that this glycoprotein itself is not on the merozoite surface but that at least one processing product of $M_r \approx 80{,}000$ (14) is on the surface. In addition, it is known that the $M_r$ 185,000 glycoprotein shares epitopes with a glycoprotein of $M_r$ 45,000–50,000. Since the monkey mAbs appear to specifically immunoprecipitate numerous polypeptides in addition to the $M_r$ 185,000 glycoprotein, they may be recognizing epitopes on widely different regions of the $M_r$ 185,000 molecule. This should make them useful in elucidating the method whereby the $M_r$ 185,000 glycoprotein is processed into lower molecular weight peptides. The mAb should also serve as valuable reagents to distinguish those regions of this molecule that may be most useful in stimulating immunity.

We are grateful to Joanne Martinis and Karen Burnett for helpful suggestions during the course of our experiments, to Cynthia Somers and John'L Reed for technical assistance, and to Cathy Amundsen for labeling the antibodies with $^{125}I$. This research was supported by Agency for International Development Contract DPE-0453-C-00-1017-00. Donor blood samples were obtained from the General Clinical Research Center, Grant RR00833 from the Division of Research Resources. This is publication no. 3663-IMM from the Research Institute of Scripps Clinic.

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