1,2,3,4-tetrahydrochrysene, but with 5-, 3-, and 20-fold less sensitivity (respectively) than with BPDE-I-modified DNA.
10. M. C. Poirier *et al.*, *Cancer Res.* **40**, 412 (1980).
11. Statistical analysis was conducted by nonparametric procedures including Fisher's exact test, Kendall τ_B rank correlations, and Wilcoxon rank sum tests with programs provided by SAS Institute, Cary, NC.
12. In all positive samples, the levels of adduct 1 were close to the analytical limit of the ^{32}P-postlabeling assay, and prolonged autoradiography for 3 to 6 days at −80°C with intensifying screens was required for its detection. An intensity scale for adduct 1 was derived by visual comparison of spot darkness on autoradiograms for different specimens assayed under identical conditions. Three or four replicate analyses of DNA were performed for each specimen. For intensities greater than 4 these estimates were equivalent to data (count/min) obtained by direct measurement of radioactivity, which provided estimates of absolute levels of adducts. For these measurements adduct and normal nucleotide spots and adjacent blank areas were cut from separate polyethyleneimine-cellulose chromatograms and assayed by Cerenkov counting (*6*). The strongly positive sample D (Fig. 1) exhibited 14 count/min after subtraction of the blank (counting efficiency 42 percent), the corresponding count rates for samples C and B (Fig. 1) were 9 and 6 count/min, respectively. Although low, these values for ^{32}P were replicable, with standard deviations for triplicate analyses averaging 30 to 40 percent. Count rates of spots <4 count/min could not be reproducibly determined by counting (relative standard deviations >50 percent). Since the assay was performed under conditions that resulted in a 10- to 20-fold greater labeling of adducts relative to normal nucleotides [E. Randerath *et al.*, *Carcinogenesis* **6**, 1117 (1985)], the DNA sample presented in Fig. 1, sample D, had 60 to 120 adducts in 10^{10} normal nucleotides, the approximate number of bases per mammalian genome. The estimated range of adduct 1 for the DNA specimens analyzed was 2 to 140 adducts per 10^{10} normal nucleotides.
13. E. Randerath *et al.*, *Proc. Am. Assoc. Cancer Res.* **26**, 84 (1985).
14. A. K. M. Shamsuddin *et al.*, *Cancer Res.* **45**, 66 (1985).
15. L. C. Green *et al.*, *ibid.* **44**, 4254 (1984).
16. F. P. Perera *et al.*, *Carcinogenesis* **3**, 1405 (1982).
17. L.-J. W. Lu *et al.*, *Proc. Am. Assoc. Cancer Res.* **26**, 89 (1985).
18. S. J. Stowers and M. W. Anderson, *Chem. Biol. Interact.* **51**, 151 (1984); R. B. Everson, *Lancet* **1980-II**, 123 (1980); D. P. Sandler *et al.*, *Am. J. Public Health* **75**, 487 (1985).
19. We thank I. B. Weinstein of Columbia University for encouraging and facilitating our collaboration and providing useful advice at several stages of this study; S. Hartnett, K. St. Claire, B. B. McPherson, and T. M. Price and other personnel at Survey Research Associates, Inc., for collecting placenta specimens and clinical information; S. Viet, N. Dharmaraja, and B. Silber for conducting ELISA assays; N. J. Haley of the American Health Foundation for conducting the thiocyanate and cotinine analyses; and D. L. Shore for statistical analyses. Supported by PHS grants CA 32157 and CA 10893 and by a DuPont Occupational Environmental Health Grant.

3 June 1985; accepted 15 October 1985

Human Monoclonal Antibodies to Pf 155, a Major Antigen of Malaria Parasite *Plasmodium falciparum*

RACHANEE UDOMSANGPETCH, KATARINA LUNDGREN, KLAVS BERZINS, BIRGITTA WÅHLIN, HEDVIG PERLMANN, MARITA TROYE-BLOMBERG, JAN CARLSSON, MATS WAHLGREN, PETER PERLMANN, ANDERS BJÖRKMAN

Pf 155, a protein of the human malaria parasite *Plasmodium falciparum*, is strongly immunogenic in humans and is believed to be a prime candidate for the preparation of a vaccine. Human monoclonal antibodies to Pf 155 were obtained by cloning B cells that had been prepared from an immune donor and transformed with Epstein-Barr virus. When examined by indirect immunofluorescence, these antibodies stained the surface of infected erythrocytes, free merozoites, segmented schizonts, and gametocytes. They bound to a major polypeptide with a relative molecular weight of 155K and to two minor ones (135K and 120K), all having high affinity for human glycophorin. The antibodies strongly inhibited merozoite reinvasion in vitro, suggesting that they might be appropriate reagents for therapeutic administration in vivo.

DUE TO THE RAPID RESURGENCE of malaria in many parts of the world and its enduring prevalence in tropical Africa, major efforts are being made to develop vaccines against the disease. Since the asexual erythrocytic stages of the parasite are the cause of the morbidity and mortality of malaria, interest has been focused on the identification and characterization of antigens expected to be instrumental in inducing protective immunity against these stages. Among the major candidates for a vaccine are antigens present on the merozoites or on the surface of infected erythrocytes. Antibodies to these antigens have been shown to mediate (or amplify) killing of the parasites, to block merozoite-erythrocyte interactions, or to block or reverse sequestration of parasitized erythrocytes, thereby preventing the parasites from escaping destruction in the spleen (*1–4*).

Of the four species of *Plasmodium* that cause malaria in humans, *P. falciparum* is the most malignant. Recently, we identified a *P. falciparum* antigen, Pf 155, which is deposited in the erythrocyte membrane at merozoite invasion. Pf 155 is a heat-stable polypeptide (molecular weight, 155K) associated with merozoites and present in the supernatants of *P. falciparum* cultures (*5*). By means of a modified immunofluorescence assay (IFA), Pf 155 is easily detected on erythrocytes infected with ring-stage parasites (*3, 5, 6*). Antibodies to Pf 155 efficiently inhibit merozoite reinvasion in *P. falciparum* cultures in vitro (*3, 6*). They are present in patients with acute *P. falciparum* infection as well as in clinically immune individuals (*3, 5, 6*). In residents of a holoendemic area of Africa (*7*), a good correlation was found between serum titers of antibodies to Pf 155 and acquisition of clinical *P. falciparum* immunity (*6*). Although the existence of an antigenic variation within or between *P. falciparum* strains has not been ruled out, some of the major antigenic structures of Pf 155 are present in all strains and clones investigated thus far (*5, 8*). Hence, Pf 155 appears to be a suitable candidate for a *P. falciparum* vaccine.

The use of rodent monoclonal antibodies (MAb's) has contributed to much of the progress in the characterization of *P. falciparum* antigens achieved thus far (*1*). However, in human disease, MAb's from B cells of malaria-immune donors should offer several advantages over mouse MAb's, including the possibility of assessing the antibody-forming potential of patients' B cells and a more direct identification of antigenic epitopes relevant for protection. In this report we describe the production of human MAb's specific for Pf 155. These antibodies, which efficiently inhibited merozoite reinvasion in vitro, were further used to establish the relation of Pf 155 to similar *P. falciparum* antigens (*9–11*).

Cell cultures producing human monoclonal antibodies were obtained by repeated cloning by limiting dilution early after immortalization of B lymphocytes transformed with Epstein-Barr virus (*12*). The B lymphocytes were from a donor hyperimmune to *P. falciparum*, who had a very high serum titer of antibodies to Pf 155 (1:15,000) as detected by IFA on glutaraldehyde-fixed and air-dried erythrocytes infected with *P. falciparum* (*3, 5*). This IFA procedure was also used to screen the cultures for production of antibodies to Pf 155. Twelve immu-

R. Udomsangpetch, K. Lundgren, K. Berzins, B. Wåhlin, H. Perlmann, M. Troye-Blomberg, J. Carlsson, M. Wahlgren, and P. Perlmann, Department of Immunology, University of Stockholm, S-106 91 Stockholm, Sweden.
A. Björkman, Department of Infectious Diseases, Karolinska Institute, Roslagstull Hospital, S-114 89 Stockholm, Sweden.

noglobulin M (IgM)–and three immunoglobulin G (IgG)–producing cultures were positive in IFA and these immunoglobulins were shown to be monoclonal by isoelectric focusing. Two of the clones, the IgM clone G2 and the IgG clone B6, were selected for further propagation and characterization. The IgM clone showed a high growth rate and has now been stable for over 1 year with regard to both growth and antibody production (10 to 15 μg/ml in 72 hours). In contrast, the IgG-producing clone grew slowly and produced small amounts of IgG (0.2 μg/ml in 72 hours).

Figure 1a shows the typical staining pattern of the two MAb's in IFA on glutaraldehyde-fixed and air-dried monolayers of *P. falciparum*–infected erythrocytes. Supernatants from cultures of the G2 clone specifically stained the membrane of infected erythrocytes at a 1:125 dilution and sometimes weakly also at 1:625, while the B6 supernatants gave a weak staining at 1:5.

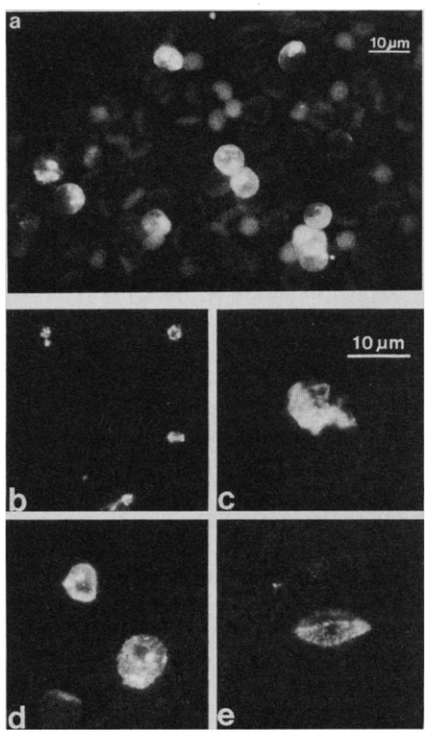

Fig. 1. Indirect immunofluorescence of monolayers of *P. falciparum*–infected erythrocytes, either glutaraldehyde-fixed and air-dried (a) or unfixed and air-dried (b to e). The infected erythrocytes in (a) to (d) were from in vitro cultures of the F32 (Tanzania) strain at parasitemias of 4 to 8 percent. Cultures of the HB3 clone of the Honduras 1/CDC strain (*20*) were used (e) to obtain gametocyte-containing monolayers. Erythrocyte suspensions (1 percent) in tris-buffered Hanks solution (TH) were applied to eight-well multitest slides treated with bicarbonate buffer (*p*H 9.6). Immediately after being washed in TH, the monolayers were either fixed briefly in 1 percent glutaraldehyde in phosphate-buffered saline (*p*H 7.4) and then air-dried extensively under a fan or air-dried directly without fixation. The monolayers were kept frozen until being used. For immunofluorescence the dried monolayers were incubated sequentially for 30 minutes at room temperature with monoclonal antibody (G2), biotinylated antihuman Ig, and fluorescein-conjugated avidin. In (a) the monolayer was counterstained with ethidium bromide to reveal parasites inside the erythrocytes (*5*).

To locate in the parasites the antigenic epitopes recognized by MAb's G2 and B6, the antibodies were tested by IFA on monolayers of unfixed but air-dried infected erythrocytes. The reactivity patterns of the two MAb's were identical (Fig. 1). The antibodies stained free merozoites (Fig. 1b) and segmented schizonts (Fig. 1c). Both antibodies also stained trophozoites, giving a speckled pattern of immunofluorescence over the parasite. High-intensity staining of two small ringlike structures was frequently seen (Fig. 1d). A similar staining pattern has been reported by others using antibodies to a 120K *P. falciparum* polypeptide (*13*). The antibody also gave strong but diffuse staining of gametocytes (Fig. 1e). Masuda *et al.* (*11*) recently found a mouse MAb that stained both sexual stages and the surface of ring-infected erythrocytes from several *P. falciparum* isolates. Like Pf 155, this antigen was released during schizogony and had a relative molecular weight of 150K.

The G2 and B6 MAb's were used for probing in immunoblotting of *P. falciparum* merozoite extracts separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*5, 14*). Figure 2a shows the reactivity of the G2 antibodies. The B6 antibodies gave an identical pattern. Both MAb's reacted mainly with a polypeptide of 155K, corresponding to Pf 155 (arrow), but also weakly with two polypeptides of 135K and 120K, respectively. The heavy staining in the 50K region and below is due to contaminating human IgG in the merozoite extracts and appears also in conjugate (antihuman Ig) controls in which no antibodies to *P. falciparum* are added (*5*). Figure 2a also shows that the MAb's recognize an antigenic epitope that is present in Pf 155 from three different *P. falciparum* strains: the Tanzanian strain F32, the K1 strain from Thailand, and the 7G8 clone of the Brazilian isolate IMTM22 (*15*).

Perkins (*10*) recently described the high-affinity binding of two *P. falciparum* merozoite surface proteins (155K and 130K) to human erythrocyte glycophorin, and suggested that these proteins constitute parasite recognition molecules in the merozoite-erythrocyte interaction. We passed supernatants from *P. falciparum* cultures through columns charged with glycophorin-polyacrylamide conjugates. Eluates obtained by boiling for 5 minutes in 4 percent SDS were separated by SDS-PAGE and analyzed by immunoblotting with both MAb G2 and serum antibodies from the same donor. As seen in Fig. 2b, Pf 155 as well as the 135K and 120K polypeptides were glycophorin-binding, together with an additional polypeptide of 65K. This last component was seen only when culture supernatants were the antigen source, and it probably represents a proteolytic breakdown product derived from one of the components of higher molecular weight. In any event, these results suggest that the proteins recognized by MAb G2 are either the same as those described by Perkins (*10*) or are antigenically related to them.

Coppel *et al.* (*9*) recently reported a *P. falciparum* antigen (RESA) present in the membrane of ring-infected erythrocytes and having an apparent molecular weight similar

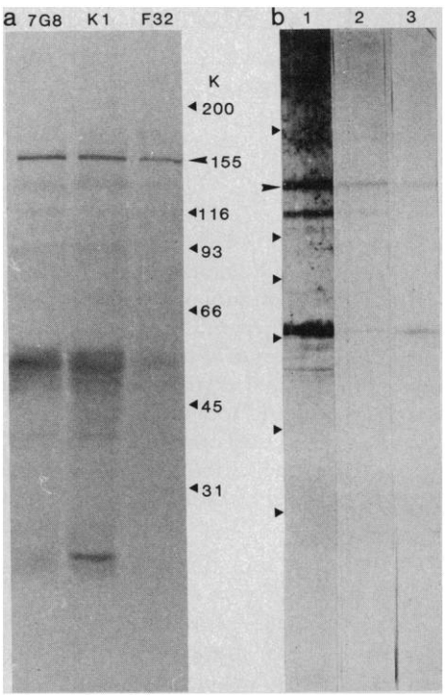

Fig. 2. Immunoblotting of *P. falciparum* polypeptides. (a) Merozoite extracts from in vitro cultures of the *P. falciparum* strains K1 (Thailand) and F32 (Tanzania) or the clone 7G8 (Brazil) were prepared (*5*). The parasite polypeptides were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to nitrocellulose (*14*). The blots were probed with the human monoclonal antibody G2 diluted 1:25. (b) Spent *P. falciparum* (F32) culture medium was adsorbed to human glycophorin–polyacrylamide beads (*10*). The binding material was eluted with 4 percent SDS and boiling. After SDS-PAGE and blotting as above, the blots were probed with a human *P. falciparum* immune serum diluted 1:1000 (lane 1), human MAb G2 diluted 1:25 (lane 2), or rabbit antiserum to the octapeptide diluted 1:100 (lane 3). The rabbit antiserum was prepared by conjugating the octapeptide (*16*) with keyhole limpet hemocyanin (*21*).

to that of Pf 155. From the nucleotide sequence of cloned complementary DNA coding for a major fragment of RESA, it was deduced that the antigen in its carboxyl-terminal end had a region of tandemly repeated sequences, including the octapeptide Glu-Glu-Asn-Val-Glu-His-Asp-Ala, repeated five times (*9*). This octapeptide appears to be conserved in different *P. falciparum* strains (*8, 16*). Approximately 20 percent of the human antibodies reacting with the surface of infected erythrocytes in the modified IFA bind to affinity columns containing the octapeptide as a ligand (*17*). However, although they were derived from a donor whose serum antibodies were strongly octapeptide-reactive, the MAb's did not react with the synthetic peptide when assayed by binding to affinity columns or by dot-immunoblotting with an octapeptide–bovine serum albumin conjugate used as a ligand. In competitive binding experiments, antibodies to the carboxyl-terminal amino acid repeats of RESA inhibited the immunofluorescence staining of the surface of infected erythrocytes by the human MAb's (*18*), suggesting that they also recognized an epitope in the carboxyl-terminal region. When the heat-stable *P. falciparum* antigens were eluted from the glycophorin-polyacrylamide column described above and were probed in parallel in immunoblotting with the human MAb's and a rabbit antibody specific for the octapeptide (*17*), identical banding patterns were obtained (Fig. 2b). These results suggest that the glycophorin-binding molecules recognized by MAb G2 also contain antigenic structures related to some of the repeated amino acid epitopes characteristic of RESA.

Because human polyclonal serum antibodies to Pf 155 are potent inhibitors of merozoite reinvasion into erythrocytes (*3, 6*), it was of interest to establish whether the human monoclonal antibodies to this antigen would also mediate inhibition. Crude supernatants from both the IgM- and IgG-producing clones efficiently inhibit parasite reinvasion in vitro (Fig. 3). Control B-cell supernatants containing similar amounts of IgM antibodies to human tumor cells (*19*) were inactive. To ascertain that inhibition was antibody-mediated, the IgM antibody

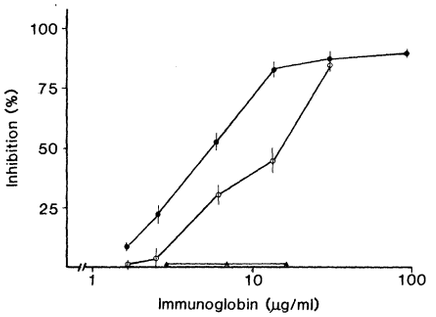

Fig. 3. Inhibition of *P. falciparum* reinvasion in vitro by the human monoclonal antibody G2. The experiments were performed as described by Wåhlin *et al.* (*3*). The parasite cultures were diluted with normal O+ erythrocytes to a parasitemia of 0.5 percent and a hematocrit of 2 percent. Aliquots of the parasite suspension (100 μl) were seeded in quadruplicate in 96-well flat-bottomed microculture plates. MAb G2 was added, either as culture supernatant (○) or after purification on concanavalin A–Sepharose of ammonium sulfate–precipitated culture supernatants (●) (*22*). Control B-cell supernatants contained IgM antibodies to human tumor cells (▲) (*19*). After 20 hours of incubation at 37°C in a candle jar (*23*), the erythrocytes from each well were washed and monolayers were prepared on eight-well multitest slides as described in the legend to Fig. 1. The parasites were stained with acridine orange. The number of parasitized erythrocytes was obtained by counting 4 × 10^4 erythrocytes per sample. Vertical bars represent standard deviations from quadruplicate counts.

was purified on a concanavalin A–Sepharose column (*22*). The 50 percent inhibition titer of the purified IgM antibody was 5.5 μg/ml, whereas that of the crude supernatant was 14 μg/ml. The ratio between these titers correlated well with that in the modified IFA, where the minimal amount of IgM needed for positive erythrocyte surface immunofluorescence was 0.1 μg/ml for the crude supernatant and 0.03 μg/ml for the purified antibodies. The 50 percent inhibition titer of the crude IgG MAb was ~20 μg/ml.

Polyclonal human serum antibodies specific for one or several epitopes in the repeated octapeptide region efficiently inhibit merozoite reinvasion (*17*). Although the fine specificity of the epitopes recognized by the monoclonal human antibodies remains to be defined, the finding that these antibodies also are very strongly inhibitory further strengthens the supposition that the immune response to Pf 155 is of protective significance. Our results indicate that MAb's derived from the natural host of the parasite constitute exquisite tools for the structural analysis and delineation of antigenic epitopes relevant for the disease. Moreover, selected human MAb's with a high capacity for inhibiting parasite reinvasion might be appropriate reagents for therapeutic administration.

REFERENCES AND NOTES

1. L. H. Perrin and R. Dayal, *Immunol. Rev.* **61**, 245 (1982).
2. G. V. Brown, R. F. Anders, G. F. Mitchell, P. F. Heywood, *Nature (London)* **297**, 591 (1982).
3. B. Wåhlin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7912 (1984).
4. I. J. Udeinya, L. H. Miller, I. A. McGregor, J. B. Jensen, *Nature (London)* **303**, 249 (1983).
5. H. Perlmann *et al.*, *J. Exp. Med.* **159**, 1686 (1984).
6. M. Wahlgren *et al.*, in *Vaccines* **85**, R. A. Lerner, R. M. Chanock, T. Brown, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1985), p. 51; M. Wahlgren *et al.*, *Am. J. Trop. Med. Hyg.* **35**, 22 (1986).
7. A. Björkman *et al.*, *Ann. Trop. Med. Parasitol.* **79**, 239 (1985).
8. H. Perlmann *et al.*, paper presented at the Seventh European Immunology Meeting, Jerusalem (1985).
9. R. L. Coppel *et al.*, *Nature (London)* **310**, 789 (1984).
10. M. E. Perkins, *J. Exp. Med.* **160**, 788 (1984).
11. A. Masuda, F. Zavala, V. Nussenzweig, R. S. Nussenzweig, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 982 (1985).
12. K. Lundgren *et al.*, *J. Immunol.* **131**, 2000 (1983).
13. R. L. Coppel, G. V. Brown, G. F. Mitchell, R. F. Anders, D. J. Kemp, *EMBO J.* **3**, 403 (1984).
14. K. Berzins, M. Wahlgren, P. Perlmann, *Clin. Exp. Immunol.* **54**, 313 (1983).
15. T. R. Burkot, J. L. Williams, I. Schneider, *Trans. R. Soc. Trop. Med. Hyg.*, in press.
16. A. F. Cowman *et al.*, *Mol. Biol. Med.* **2**, 207 (1985).
17. K. Berzins *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
18. R. Udomsangpetch *et al.*, in preparation.
19. S. Paulie *et al.*, *Scand. J. Immunol.* **20**, 461 (1984).
20. V. K. Bhasin, W. Trager, *Am. J. Trop. Med. Hyg.* **33**, 534 (1984).
21. G. Rougon, B. Ceard, J. Van Rietschoten, B. Jordan, J. Barbet, *Mol. Immunol.* **21**, 461 (1984).
22. R. Klein, M. Kapperstück, A. I. Chukhrova, V. A. Lapur, *ibid.* **16**, 421 (1979).
23. W. Trager and J. B. Jensen, *Science* **193**, 673 (1976).

24. We thank I. Andersson and M. Hagstedt for excellent technical assistance and P. Nguyen-Dinh for providing gametocyte-containing cultures. Supported by grants from the Rockefeller Foundation Great Neglected Diseases Network, the Swedish Medical Research Council, the Swedish Agency for Research Cooperation with Developing Countries, the Epidemiology Component of the United Nations Development Programme/World Bank/World Health Organization, Special Program for Research and Training in Tropical Diseases, and the National Board for Laboratory Animals.

28 June 1985; accepted 23 October 1985