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Differential Effect of Immunoglobulin on the In Vitro Growth of Several Isolates of *Plasmodium falciparum*

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Immunoglobulin isolated from the sera of individuals living in a malarious area of Papua New Guinea was tested for an effect on the growth in vitro of four isolates of *Plasmodium falciparum*, three from Papua New Guinea and one from Thailand. The Papua New Guinea isolates were inhibited to the same degree by individual immunoglobulin preparations, and inhibition varied from 0 to 98% (assessed by a radioisotopic readout). Immunoglobulin preparations which inhibited the Papua New Guinea isolates caused less inhibition of the Thai parasites. Biosynthetically labeled parasite proteins were analyzed by two-dimensional gel electrophoresis, and differences were detected in the protein and antigenic composition of isolates which differed in their sensitivity to inhibitory immunoglobulin. Three acidic proteins (M_r 200,000, 150,000, and 65,000) were found only in the Papua New Guinea isolates. All Papua New Guinea isolates contained a high-molecular-weight basic protein with an M_r of 220,000 (Pf220), but the corresponding protein of the same molecular weight in the Thai isolate had a more acidic isoelectric point. Another isolate (from Africa) initially showed a degree of resistance to inhibition by Papua New Guinea immunoglobulin (although not to the same extent as the Thai parasite), but in later experiments, this isolate was susceptible to inhibition. During the course of this series of experiments, the antigenic composition of this (uncloned) isolate changed so that it became similar (but not identical) to the Papua New Guinea isolates.

Total antimalarial antibody is a poor index of protective immunity against malaria, even though large amounts of antimalarial antibody are produced in response to infection in humans. That a component of serum antibody is protective is inferred from the observation that passive transfer of immunoglobulin from immune adults to infected children (9) effectively reduced parasitemias. At present, no tests are available to distinguish protective from nonprotective antibody, but it has been shown that some human sera (2, 8, 19, 21, 28) and immunoglobulin G from immune individuals (1, 15) inhibit the growth of *Plasmodium falciparum* in vitro.

Strain specificity of protective immunity against *P. falciparum* was demonstrated in chimpanzees (22), in which a primary infection protected animals against challenge with the homologous but not a heterologous strain of the parasite. In human volunteers (10, 20), modification of the course of a second infection with heterologous strains was noted but was not as marked as the effect observed on the course of a second infection with the homologous strain. Different isolates of African *P. falciparum* showed differing susceptibility to inhibition by serum in vitro (28), suggesting the importance of isolate-specific immunity to the parasites affecting humans.

In this paper, antigenic diversity of asexual stages of *P. falciparum* is demonstrated by the varying effect of immune human immunoglobulin on the in vitro growth of three isolates of *P. falciparum* from the same region of Papua New Guinea and a different effect on an isolate from Thailand. In addition, we compare protein composition of isolates differing in susceptibility to inhibition by immunoglobulin.

MATERIALS AND METHODS

Parasites. Isolates of Papua New Guinean *P. falciparum* established in this and other laboratories have been maintained in culture for more than 2 years (7). In this paper, FCQ2, FCQ27, and FCQ46 are abbreviated to FC2, FC27, and FC46, respectively. Isolate K1 (from Thailand) and NF7 (from Ghana) were gifts to G.K. in Papua New Guinea from D. Walliker. Cultures were maintained by using HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered RPMI 1640 medium (culture medium) with 10% human serum by the candle jar method of Trager and Jensen (11,

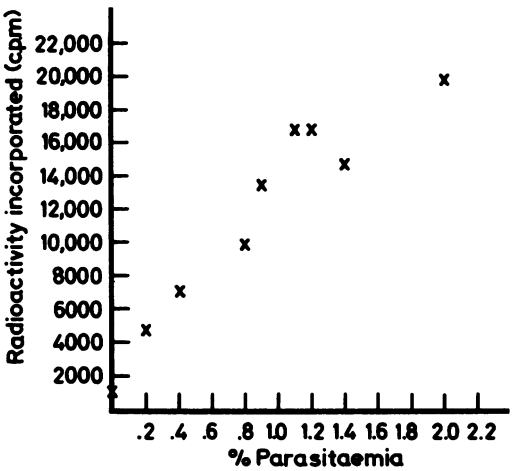

FIG. 1. Correlation of percentage parasitemia determined microscopically with incorporation of [^{3}H]leucine into TCA-precipitable material.

26). All parasites were cultured in group O erythrocytes.

Immunoglobulin preparations. Sera were obtained from adult blood donors in the Madang region of Papua New Guinea, where malaria is endemic and seasonally variable. Sera were stored for up to 1 week at −20°C, transported to Melbourne, Australia, on dry ice, and stored at −70°C. Control sera were obtained from adults living in Melbourne, where there is no malaria transmission. Immunoglobulin was isolated by affinity chromatography on protein A-Sepharose (protein A-Sepharose CL-4B; Pharmacia, Uppsala, Sweden) which had been equilibrated in human tonicity phosphate-buffered saline (HTPBS). Immunoglobulin (predominantly immunoglobulin G) was eluted from the protein A-Sepharose in 0.1 M acetate (pH 3) and immediately dialyzed against HTPBS for 6 to 8 h. Individual immunoglobulin preparations were then dialyzed against culture medium for 18 to 24 h with a change of dialysis fluid after 12 h. Protein concentration was estimated from the absorbance at 280 nm (assuming an absorbance of 1.4 for 1 mg/ml solution), and samples were diluted to 5 mg/ml in medium before sterilization by filtration. After the addition of control human serum to a final concentration of 10%, this "immunoglobulin-supplemented medium" was used for parasite culture in the inhibition assay.

Inhibition assay. Infected cells (approximately 0.5% parasitized) in culture medium (8% [vol/vol]) containing 10% normal serum were incubated in 200-μl volumes in flat-bottomed wells of a microtiter tray (Linbro; Flow Laboratories, Inc., Hamden, Conn.). After 4 to 12 h, 180 μl of medium was replaced with the same volume of immunoglobulin-supplemented medium. Daily medium changes were made for 4 days, and then immunoglobulin-supplemented medium was replaced with leucine-free medium (RPMI 1640; Select-Amine Kit; GIBCO, Grand Island, N.Y.) supplemented with L-[4,5-^{3}H]leucine (50 μCi/ml). After a period of biosynthetic labeling (5 h), incorporation of label into parasites was estimated by adding 20 μl of resuspended cells to 200 μl of 10% fetal calf serum in HTPBS and precipitating with 10% trichloroacetic acid (TCA). Precipitates were washed three times in TCA, dissolved in 0.4 M NaOH, mixed with 300 μl of Soluene-350 (Packard Co., Downers Grove, Ill.), and then added to 4 ml of toluene-based scintillant (4 g of PPO [2,5-diphenyloxazole] per liter and 0.1 g of dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)benzene] per liter). Radioactivity was measured in a Packard liquid scintillation counter.

In control experiments, smears of infected cells were made from each well, stained, and coded, and the percentage of parasitized cells was estimated from about 30 high-power fields. Incorporation of radioactivity into TCA-precipitable material correlated with the percentage of parasitized cells (Fig. 1).

Labeled antigen preparation. Medium without methionine (RPMI 1640; Select-Amine Kit; GIBCO) supplemented with [^{35}S]methionine (200 μCi/ml) (Radiochemical Centre, Amersham, England), 10% normal human serum, and gentamicin (40 μg/ml) was used for biosynthetic labeling of asynchronous parasite cultures (2 to 4% parasitemia). After a 20-h labeling period, the cell suspension was centrifuged for 7 min at 350 × *g*, and the supernatant was removed and recentrifuged at 25,000 × *g* for 30 min. The pellets from both centrifugations were combined and solubilized in 2 ml of 0.5% Triton NET (12) containing methionine (2 mg/ml). After 30 min on ice, the sample was centrifuged at 25,000 × *g* for 30 min, and the supernatant was loaded onto a Sephadex G-25M column (PD10 column; Pharmacia) which was equilibrated and eluted with 0.5% Triton NET containing methionine (2 mg/ml). To prepare samples for isoelectric focusing, samples were supplemented with 1 mg of urea per μl of sample and added to an equal volume of buffer containing 2% Triton X-100, 2% ampholines (LKB) (4:1 [vol/vol] pH 5 to 8:pH 3.5 to 10 ampholines), 9.5 M urea, and 50 mM dithiothreitol.

Immunoprecipitation. Immunoprecipitation with purified immunoglobulin (100 μg per test) and heat-killed and Formalin-fixed *Staphylococcus aureus*, Cowen strain (100 μl of 10% *S. aureus*) was performed as described previously (2), essentially according to the method of Kessler (12). To prepare samples for isoelectric focusing, the final pellets of antigen-antibody complexed with *S. aureus* were washed three times in Triton NET and frozen at −70°C or solubilized in 2% (wt/vol) sodium dodecyl sulfate lysis buffer containing 9.5 M urea, 2% total ampholines (LKB) (4:1 [vol/vol] pH 5 to 8:pH 3.5 to 10 ampholines), and 50 mM dithiothreitol. After 20 min at room temperature, five times the volume of lysis buffer containing 2% Triton X-100, 2% ampholines, 9.5 M urea, and 50 mM dithiothreitol was added. Incubation continued for 15 min at room temperature before samples were centrifuged prior to analysis and, if necessary, stored at −70°C.

Polyacrylamide gel electrophoresis. Two-dimensional analysis was performed according to the method described by O'Farrell (14, 16), with 10% acrylamide in the second dimension. Molecular weight standards were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ampholines of pH range 3.5 to 10 and 5 to 8 were obtained from LKB (Bromma, Sweden). Gels were prepared for fluorography with En^{3}Hance (New England Nuclear Corp., Boston, Mass.) before drying down and exposure to Kodak X-omat S film.

TABLE 1. Effect of purified immunoglobulin on growth in vitro of a Papua New Guinea isolate of *P. falciparum* (FC27)

Sample	Radioactivity incorporated (cpm)[a]	% Inhibition[b]
5	20,786 ± 1,325	0
9	20,178 ± 2,413	3
8	16,265 ± 1,309	26
7	14,937 ± 61	37
6	12,787 ± 127	46
4	11,368 ± 479	55
3	10,654 ± 419	59
1	8,896 ± 2,469	69
2	4,687 ± 274	94
10	3,907 ± 129	98
Control A[c]	21,585 ± 1,728	
Control B[c]	20,511 ± 1,183	
No immunoglobulin added	20,716 ± 1,088	
Uninfected cells[d]	3,650 ± 114	

[a] Arithmetic mean of three cultures ± standard error of the mean.

[b] $\frac{\text{[Control (no immunoglobulin) − uninfected] − [test-uninfected]}}{\text{Control (no immunoglobulin) − uninfected}} \times 100.$

[c] Two different samples from individuals living in a nonendemic area.

[d] Cells used for parasite culture.

RESULTS

Immunoglobulin preparations inhibited growth of Papua New Guinea isolates of *P. falciparum* by up to 98% after 4 days in culture. The range of inhibitory activity in 10 immunoglobulin preparations from sera taken at a time of high malaria transmission (April 1981) is shown in Table 1. Seasonal variation in the prevalence of inhibitory antibody, clinical features of donors, serological and biochemical properties of serum, and analysis of immunoprecipitates using these sera will be the subjects of a separate publication.

Four immunoglobulin samples varying in their inhibitory activity for Papua New Guinea isolates were tested for inhibitory activity against the Thai isolate. Sample 10 was equally inhibitory for all three Papua New Guinea isolates (FC2, FC27, and FC46), and three other sera had the same inhibitory effect on two Papua New Guinea isolates tested. These sera were less effective in inhibiting the other geographical isolate, K1 (Table 2). The effect was apparent for sera which caused marked inhibition, e.g., serum 10, or moderate inhibition, e.g., serum 1. The same trend was observed with a sample causing mild inhibition, serum 12, but the difference was not significant.

The protein composition of the different isolates was examined by two-dimensional gel electrophoresis of ^{35}S-labeled infected cell lysates. No differences were apparent among the three Papua New Guinea isolates, FC2, FC27, and FC46, but there were some differences apparent when the Papua New Guinea and non-Papua New Guinea isolates were compared (Fig. 2). As shown in Fig. 2, two acidic high-molecular-weight proteins labeled p are present in the Papua New Guinea isolates but are absent from the Thai isolate, K1. There is no evidence for the presence in K1 of proteins of similar size but of different isoelectric point. All isolates have a major high-molecular-weight basic protein, with a molecular weight of approximately 220,000 (Pf220). The corresponding protein has a more basic pI in all Papua New Guinea isolates (Fig. 2).

The antigenicity of proteins p, p65, and Pf220 has been demonstrated by analysis of immunoprecipitates generated by using a pool of Papua New Guinea immune serum (Fig. 3). Proteins not found in K1 are shown to be antigenic and represented in all other immunoprecipitates (arrows in Fig. 3). There were also differences between K1 and all other isolates in the antigens precipitated from the supernatants of [^{35}S]methionine-labeled parasite cultures (data not shown).

Another isolate (NF7, from Africa) initially showed resistance to inhibition by Papua New Guinea immunoglobulin but not to the same extent as K1 (Table 3, a and b). When tested 4 months later with different immunoglobulin preparations, this isolate showed the same susceptibility to inhibition as Papua New Guinea isolates (Table 3, c and d).

Consistent with this change in susceptibility to inhibition of growth by immunoglobulin is the change over this period in the protein pattern on two-dimensional gels. A comparison of the two-dimensional gels analyzing the proteins in this (uncloned) isolate at the beginning and end of this series of experiments indicates that the high-molecular-weight acidic proteins (p) characterizing all Papua New Guinea isolates were not present initially but were present in the later analysis (Fig. 4). Furthermore, the protein designated Pf220 had a more basic pI when the isolate was reexamined at the completion of the growth inhibition experiments (Fig. 4). These results suggest that a minor or contaminant population of parasites (of Papua New Guinea origin) had overgrown the dominant starting population.

DISCUSSION

Data presented in this paper demonstrate that many adults living in an area of Papua New Guinea in which malaria is endemic develop antibodies which can inhibit the growth of *P. falciparum* in vitro. These antibodies are equally

TABLE 2. Inhibition of different geographical isolates of *P. falciparum* by immunoglobulin from Papua New Guinea

Sample	Isolate	Radioactivity incorporated (cpm)[a]		% Inhibition[b]
		Control immunoglobulin	Papua New Guinea immunoglobulin	
10	FC2	47,061 ± 861	6,997 ± 372	94
	FC27	31,729 ± 1,381	6,295 ± 320	93
	FC46	26,387 ± 1,293	5,669 ± 219	95
	K1	33,891 ± 916	17,558 ± 235	56[c]
4	FC2	15,244 ± 797	6,144 ± 65	63
	FC27	19,326 ± 371	8,139 ± 432	60
	K1	13,418 ± 289	11,698 ± 277	14[c]
1	FC2	15,244 ± 797	5,678 ± 192	66
	FC27	19,326 ± 371	7,257 ± 231	65
	K1	13,418 ± 289	7,201 ± 289	49[d]
12	FC27	27,871 ± 1,285	23,955 ± 2,513	15
	FC46	53,523 ± 665	44,823 ± 1,392	17
	K1	39,368 ± 3,405	36,018 ± 834	9[e]

[a] Mean of three samples ± standard error of the mean.

[b] % Inhibition = {[counts per minute (control immunoglobulin) − counts per minute (Papua New Guinea immunoglobulin)]/[counts per minute (control immunoglobulin) − counts per minute (uninfected cells)]} × 100. Statistical evaluation: comparison of FC27 and K1 by using three pairs of observations to calculate a paired *t* statistic (2 degrees of freedom). The *P* value was assessed from the area in two tails of the *t* distribution.

[c] $P < 0.01$.

[d] $P < 0.05$.

[e] $P > 0.1$.

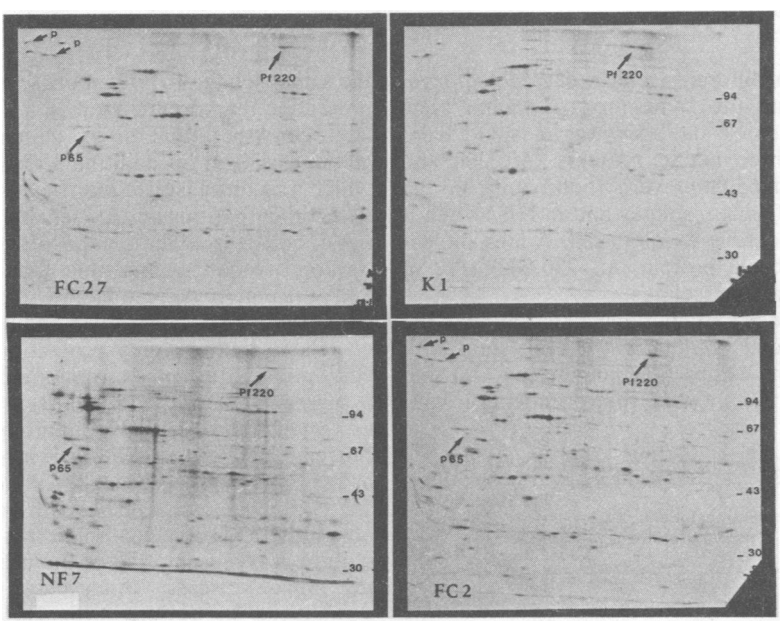

FIG. 2. Autoradiograph following two-dimensional gel electrophoresis of [^{35}S]methionine biosynthetically labeled parasite proteins. First-dimension isoelectric focusing (basic region to the right) was followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions in the second dimension. Two isolates from Papua New Guinea (FC27 and FC2), one from Africa (NF7), and one from Thailand (K1) were used. Molecular weight markers are at the right (kilodaltons). p65 and p, Proteins seen in all isolates except K1; Pf220, high-molecular-weight protein (molecular weight ≅220,000) of K1 with different pI from a protein of approximately the same size in NF7 and the Papua New Guinea isolates.

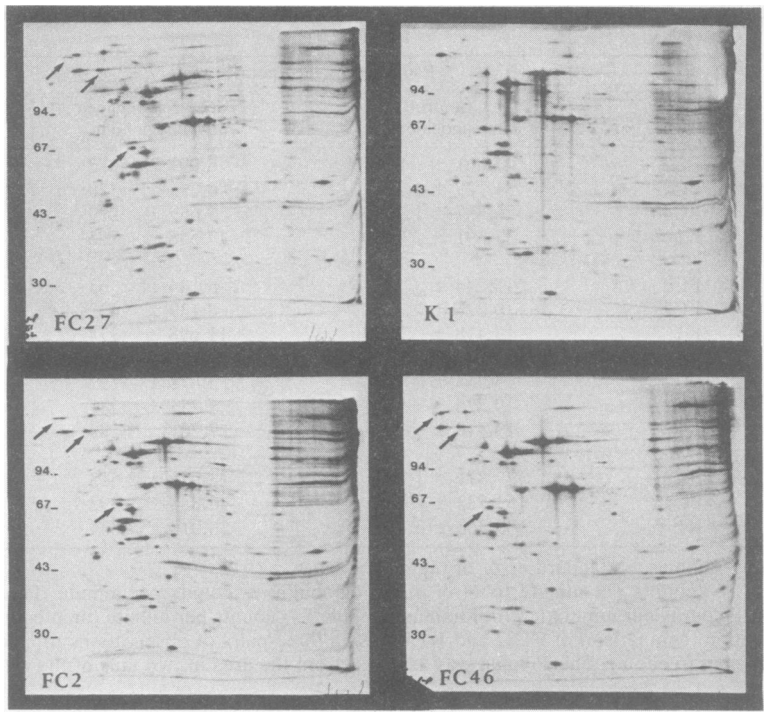

FIG. 3. Autoradiograph following two-dimensional gel electrophoresis of immunoprecipitates of [^{35}S]methionine-labeled parasite proteins. Immunoprecipitates were generated by using a pool of immune serum. Arrows indicate proteins p and p65, which are present in all Papua New Guinea isolates (FC27, FC2, and FC46) but not the isolate from Thailand (K1).

inhibitory for different isolates of *P. falciparum* obtained from the same area of Papua New Guinea but are less inhibitory for an isolate from Thailand. Three acidic proteins (M_r 200,000, 150,000, and 65,000) were found only in the Papua New Guinea isolates and in NF7 when it became susceptible to inhibition. A high-molecular-weight basic protein (M_r 220,000) (Pf220) also varied among isolates.

This evidence for geographical variation in the antigenicity of *P. falciparum* is consistent with immunization studies in chimpanzees (22) and humans (10, 20) showing that preinfection with the same isolate of *P. falciparum* provides more protection than preinfection with a different isolate. Growth inhibition by standard immunoglobulin is then an additional characteristic by which a cultured isolate may be described to add to its other features of drug sensitivity, morphology (knobs), isozyme composition, protein pattern on two-dimensional gels, S antigen pattern, and antigenicity defined by the binding of monoclonal antibodies. If growth inhibition in vitro correlates with in vivo protection, population susceptibility to a new strain or isolate could be assessed in this way, particularly if the sample to be tested were initiated from a cloned parasite. Since many or most isolates are mixed infections as assessed by S antigens (27) or isozymes (5, 6, 23, 25), more precise results will only be obtainable if there is some guarantee that the isolate in culture is representative of the organisms causing human disease. If all parasites could be established in culture with 100% efficiency, studies such as those described using whole sera and fresh isolates (28) would provide further valuable information.

TABLE 3. Changing pattern of growth inhibition

Date	Immunoglobulin preparation	% Inhibition	
		Papua New Guinea	NF7
a 13/8/81	10	94	68[a]
b 27/8/81	12	16	7[b]
c 17/12/81	1	66	63[c]
d 17/12/81	4	62	54[c]

[a] $P < 0.01$.

[b] $P < 0.1$ for comparison with one Papua New Guinea isolate; $P > 0.1$ for a second isolate.

[c] $P > 0.1$. For description of statistical evaluation, see Table 2, footnote *b*.

The three isolates from Papua New Guinea appeared identical in protein composition and were equally susceptible to inhibition by immunoglobulin. It is possible that culture con-

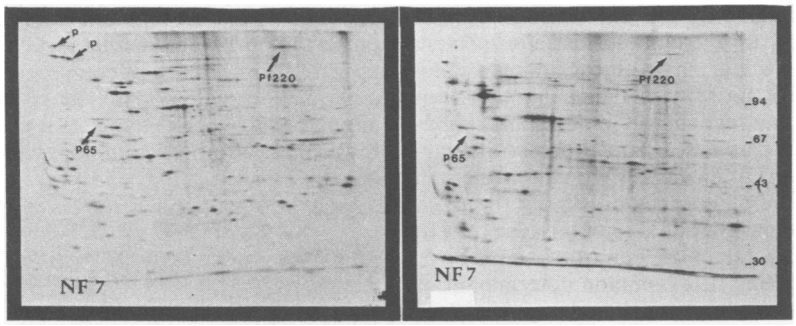

FIG. 4. Change in protein composition of isolate NF7. Left, January 1982; right, April 1981.

ditions select for a subset of parasite antigenic types; thus, our results are not necessarily inconsistent with the existence of significant antigenic diversity within Papua New Guinea. We have recently shown by cloning that one of the isolates (FC27) consists of at least two S antigen types (unpublished observations). Further study is required to determine whether the African (NF7) or Thai (K1) antigenic types are present in Papua New Guinea, and if so, whether their growth can be inhibited by purified immunoglobulin.

There are several previous studies of the reaction of human serum with antigens of *P. falciparum* (1–3, 13, 17, 21), three of which have confirmed that the immune serum reacts predominantly with antigens of the mature parasite, particularly schizonts (3, 13, 17). However, large amounts of antibody may be present in serum which does not inhibit parasite growth (2, 21), and at the time of acute infection, individual sera (analyzed by two-dimensional gel electrophoresis of immunoprecipitates) may have large amounts of antibody to most of the antigens recognized by a pool of immune serum, yet the antibody is inadequate in quality or quantity to prevent infection (Brown et al., submitted for publication). Two-dimensional gel electrophoresis would be necessary to determine whether antigens recognized by sera described in other papers (17, 21) are common to all isolates used in our study.

The results of our inhibition studies are consistent with analysis of the antigens. No differences were detected in [^{35}S]methionine-labeled products of the three Papua New Guinea isolates analyzed by two-dimensional electrophoresis, but several obvious differences were seen between K1 and the other parasites (mainly in high-molecular-weight proteins). NF7 was also different from Papua New Guinea isolates (and similar to K1) when it was less susceptible to inhibition. There may well be other differences in protein composition not detected by our technique (minor proteins, proteins outside the pI and molecular weight range we have examined, proteins containing little or no methionine, or proteins poorly soluble in the detergents we have used).

The change in pattern of inhibition of NF7 was associated with a change in its protein composition during continuous long-term culture. In addition to the changes evident in the two-dimensional gels presented here, we have recently established that there was a change in the S antigen serotype of NF7 during this same period of continuous culture. There are several possibilities for these changes, including overgrowth of the major population of parasites with a minor population having survival advantage in vitro, change to another antigenic type, or contamination from another isolate growing in the laboratory at the same time. The last possibility appears unlikely, since no other isolate so far examined has Pf220 with the same isoelectric point as the final form of NF7.

The high-molecular-weight proteins which differ among isolates are obvious candidates for the molecules involved in antigenic variation. The techniques used in this study are very similar to those used by Tait (24) to describe protein variations in different isolates of *P. falciparum*. It is possible that the two larger "p" proteins we have identified as differing among isolates are parts of the complex designated protein no. 1 by Tait, although K1, described but not shown in his paper, apparently had only a single component (B) for protein no. 1. We have noted variability in the degree of labeling of the high-molecular-weight parasite proteins which vary among isolates, presumably related to the efficiency of labeling and the relative abundance of different parasite forms in asynchronous cultures (3, 13). We note that Tait did not include a description of Pf220 or its equivalent in his classification of proteins.

Small differences in amino acid composition may cause only minor changes in the molecular weight or isoelectric point of a total protein but considerable change in binding or function of

antibodies directed against the original protein. Changes such as those described for protein Pf220 may be important both for immunity and for detection of antigenic variants. Another consideration is that recognition (binding and immunoprecipitation) is not synonymous with function, so the appearance of a particular protein in an immunoprecipitate does not imply the presence of relevant functional antibody directed to it (let alone to unlabeled nonprotein moieties). Antibody reacting to a common determinant of several molecules binds to all and coprecipitates the variable portions whether or not there is functional antibody directed against them.

Parasite growth in vitro may be affected by antibodies of various unique specificities (18), and immune serum is likely to contain variable quantities of different inhibitory antibodies. The heterogeneity of this component of the protective immune response is demonstrated by the wide range of inhibition (0 to >95%), despite the large amounts of antibody to *P. falciparum* in all samples (measured by enzyme-linked immunosorbent assay), even in those causing no inhibition (R. F. Anders, unpublished observation). Many antigens from K1 were precipitated by heterologous (Papua New Guinea) immunoglobulin, and most Papua New Guinea immunoglobulin samples caused a degree of inhibition of the growth of K1. It is likely that protective antibody develops against some determinants common to all isolates, with specific antibody giving additional protection. Blocking or enhancing antibody could play a role, and we have noted that, in another series of immunoglobulin samples tested against one Papua New Guinea isolate, those samples with large amounts of antibody which had no effect on, or actually enhanced, parasite growth in vitro were most efficient in precipitation of antigen Pf220. It is possible that heterologous challenge could stimulate such an inappropriate cross-reacting antibody.

Differences in protein composition and susceptibility to growth inhibition by immunglobulin are important characteristics of individual isolates which influence the strategy for detection of candidate host-protective antigens. We have previously identified two candidate antigens (M_r 96,000, termed Pf96) (1) and note that these proteins are present in all 15 isolates so far examined from Papua New Guinea and in the 2 non-Papua New Guinea isolates used for these experiments. Another approach is to use the variant molecules to find a common carrier determinant which would induce host protection against all variants (4). In this assay, we have studied only one aspect of the total immune response to malaria and demonstrated some function in heterologous antibody, but it is quite possible that other immune mechanisms, e.g., opsonization or cell-mediated killing, could be triggered in vivo by effector cells recognizing determinants common to all species. Specific antibodies to variant antigens are the next step toward isolation of common carrier determinants.

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