A NEW HUMAN DUFFY BLOOD GROUP SPECIFICITY DEFINED BY A MURINE MONOCLONAL ANTIBODY

Immunogenetics and Association with Susceptibility to *Plasmodium vivax*

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The Duffy blood group system of human erythrocytes comprises a membrane glycoprotein recently characterized by immunoprecipitation techniques (1). It is a polymorphic system that includes determinants for five alloantigens, two of which are Mendelian alleles (Fy^a and Fy^b) (2, 3). A null genotype, designated $Fy(a^-b^-)$ (4), lacks the expression of both Fy^a and Fy^b, and is inherited as a third allele of the series. Homozygous $Fy(a^-b^-)$ red cells, however, also lack expression of the Fy3 (5) antigen, which is present on all other Duffy types. Fy4 (6) occurs on almost all samples of $Fy(a^-b^-)$, but the expression is weak on some. In addition Fy4 is occasionally found on red cells of $Fy(a^+b^+)$, so that its exact Duffy status is not in perfect agreement with the absence of both Fy^a and Fy^b (7). Fy5 (8) resembles Fy3 but is also absent from Rh-null red cells regardless of their other Duffy types.

These five specificities are inherited *en bloc* but they differ in their susceptibility to proteolytic enzyme treatment; the high frequency antigens Fy3, Fy4, and Fy5 being resistant to the effects of chymotrypsin, ficin, and papain, while Fy^a and Fy^b are completely destroyed by these enzymes (7). Duffy maps to chromosome 1 (9) (i.e., is syntenic to Rh), which may be related to the peculiar absence of Fy5 on Rh-null cells.

Interest in the Duffy system has been considerable since the demonstration by Miller et al. (10) that $Fy(a^-b^-)$ erythrocytes are resistant to the penetration of *Plasmodium knowlesi* merozoites in vitro (10), and that the red cells of individuals of that type are not infected by *Plasmodium vivax* (11) even in regions of very high endemism (12, 13). Confirmation of the role of Duffy molecules in the penetration of merozoites has been obtained by specific though partial blocking of the *P. knowlesi* receptor function by alloantibody to Fy^a (10).

We have prepared a murine monoclonal antibody, anti-Fy6, that reacts with all human red cells except for those of the $Fy(a^-b^-)$ phenotype. The specificity defined by anti-Fy6 differs significantly from others previously identified in the Duffy system. In this report we show that Fy6 is not only characteristic of Duffy, but is related to the penetration of red cells by *P. vivax* merozoites.

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Materials and Methods

Monoclonal Anti-Duffy (Anti-Fy6). Originally designated NYBC-BG6, anti-Fy6 was obtained essentially according to Kohler and Milstein (14) using a protocol described previously (15). Briefly, pooled human red cells, used as immunogen, were injected intraperitoneally into BALB/c mice, and the spleen cells from an immunized mouse were fused with NSO/1 mouse myeloma cells. Primary fusion well culture supernatants were tested for antibody activity using conventional saline, enzyme, and indirect antiglobulin techniques (16). The anti-Fy6-producing cell line resulted from a colony, cloned three times, that has remained stable for well over a year. Fresh, fully typed human red cells, and liquid nitrogen–stored reconstituted human and nonhuman primate red cells were used to define specificity. Indirect antiglobulin tests of monoclonal anti-Fy6-coated red cells were performed with a locally prepared rabbit anti–mouse IgG reagent exhaustively preadsorbed with human red cells. Similar tests with human alloantibody coated cells were performed with a locally prepared monoclonal mouse anti–human IgG reagent.

Immunoglobulin Class Determination. The immunoglobulin class and subclass were identified by Ouchterlony immunodiffusion (17) using rabbit anti–mouse κ and goat anti–mouse λ (Research Products International Corp., Mt. Prospect, IL), anti–mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA (Miles Scientific, Naperville, IL).

Alloantisera. Anti-Fy^a and anti-Fy^b reagents were purchased from Gamma Biologicals, Inc. (Houston, TX). Anti-Fy3 was kindly provided by Dr. W. L. Marsh of the New York Blood Center.

Antibody Elution. For tests of red cells from nonhuman species, human alloantibodies were isolated by adsorption onto positively reacting human cells and elution using ether and heat.[1]

Enzyme Treatment of Red Cells. 1% (vol/vol) red cell suspensions (10^8 cells/ml) were incubated in each of the different enzyme solutions at 37°C, as described by Mason et al. (18). Neuraminidase (*Clostridium perfringens*, type X, purified, 160 U/mg protein), trypsin (bovine pancreas, type XI, DPCC-treated), chymotrypsin (bovine pancreas, type VII, TLCK-treated) crude ficin, and trypsin inhibitor (soybean type 1-S) were all obtained from Sigma Chemical Co., St. Louis, MO, while papain (African papaya) was from Calbiochem Behring Corp., San Diego, CA. All enzymes were used in phosphate (0.015 M, pH 7.3)-buffered physiological saline solution (PBS) neuraminidase at a concentration of 100 mU/ml, and the others at 1 mg/ml. Neuraminidase-treated red cells were agglutinated by *Arachis hypogoea* lectin indicating that they had been T activated (19). Freshly chymotrypsin-treated red cells were incubated for 10 min at room temperature in PBS containing 10^{-3} M phenylmethylsulfonyl fluoride (PMSF)[2] and 5% 2-propanol. Freshly trypsin-treated red cells were incubated for 10 min at room temperature in soybean trypsin inhibitor solution. All enzyme-treated cells were washed three times in PBS and suspended in PBS for use in serological tests.

Purification and Measurement of Monoclonal Antibody Protein Concentration. The monoclonal antibody was precipitated from ascites fluid with an equal volume of 45% saturated ammonium sulfate. The precipitate was dissolved in PBS, dialyzed against 20 mM Tris, 10 mM NaCl (pH 8.6) and passed through a DEAE Sephacel column (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 20 mM Tris, 10 mM NaCl buffer (pH 8.6). Adsorbed proteins were eluted with a 20–200 mM NaCl gradient. Fractions were tested for anti-Duffy activity, and the antibody was found to elute as a single, narrow peak. The IgG content of this peak was measured both by absorbance at 280 nm, and by ELISA blocking using a highly purified mouse IgG1 myeloma protein as standard (20).

Radiolabeling. 100 μl of purified monoclonal antibody (215 μg) was labeled by solid-phase radioiodination using Iodo-Gen (Pierce Chemical Co., Rockford, IL), and freed of unbound iodine by filtration through Sephadex G-10, followed by dialysis against PBS at 4°C overnight. The specific activity of the labeled antibody preparation was determined by measuring both TCA-precipitable radioactivity in a gamma counter, and IgG concen-

[1] Marsh, W. L., cited in Widmann (16).

[2] *Abbreviations used in this paper:* HRP, horseradish peroxidase; PMSF, phenylmethylsulfonyl fluoride.

tration was determined by ELISA blocking assay (20). Specifically binding antibody in the labeled protein as a fraction of the total radioactivity was measured by adsorption with an excess of cells of the Fy(a^+b^+), Fy(a^+b^-), Fy(a^-b^+), and Fy(a^-b^-) types in the presence and in the absence of 2,500–10,000-fold excess of unlabeled anti-Fy6.

Assay of Antibody Binding by Red Cells of Different Duffy Types. Fresh red blood cells were washed four times in PBS containing 10% (vol/vol) indifferent murine Freund's adjuvant–induced ascites fluid, and resuspended in the same solution to a cell count of exactly 10^8 cells/ml, determined electronically (Coulter S + IV, Coulter Electronics, Miami, FL). This suspension was distributed in 0.5 ml volumes, centrifuged, and 0.4 ml of supernatant was removed without disturbing the pellets. The tubes containing red cell pellets were assembled in sets of duplicates for each cell type and each antibody concentration. The labeled antibody displayed 4.2×10^6 dpm/μg IgG which, assuming 150 kD molecular mass, corresponded to 6.3×10^5 dpm/pM. 1-ml aliquots of labeled antibody solutions, containing respectively 2.4, 1.8, 1.2, and 0.6 pM in PBS with 10% murine ascites, were added. Reaction mixtures were incubated at 37°C for 1 h, and then overnight at 4°C with occasional mixing. Free antibody was determined by counting the radioactivity in 0.1 ml of supernatant fluid and correcting for the total reaction volume and mean corpuscular volume of the reacted red cells. Bound antibody was computed as the difference between the total antibody applied and the free antibody measured. This value was ~10% greater than the counts on the respective adsorbing red cells separated by passage through a 4:1 mixture of di-*n*-butyl and di-nonyl phthalate (Eastman Kodak Company, Rochester, NY). By either method, Fy(a^-b^-) red cells were found to bind <3% of applied antibody. Nevertheless, the uptake of Fy6 to Fy(a^-b^-) cells was subtracted from the uptake of $Fy6^+$ phenotypes, after which the reciprocal of the moles of bound antibody was plotted against the reciprocal of the moles of free antibody (21). For each of the four $Fy6^+$ phenotypes, the data plotted linearly, and least squares point-estimates of slope and intercept were calculated directly. The reciprocal of the *y*-intercept thus corresponds to maximal antibody bound in the presence of infinite free antibody, while intercept divided by slope (correcting for volume) corresponds to K_a, the constant of binding of the antigen-antibody reaction equilibrium. A one-to-one correspondence of bound antibody to antigen was assumed to calculate antigen sites from *p*M, the point of maximum bound antibody.

Detection of Duffy Molecules in Western Blots. Red cell ghosts were prepared by hypotonic lysis with six volumes of 10 mM Tris HCl (pH 7.4) containing 1 mM PMSF for 30 min at 4°C. After several washes in the hypotonic buffer to remove all residual hemoglobin, the membranes were extracted in 1% deoxycholate (DOC) for 30 min at 4°C and insoluble materials were removed by centrifugation at 12,000 *g*. Equal volumes of supernatant were mixed with 0.1 M Tris (pH 6.8) containing 10% SDS, 10% glycerol, and 0.01% bromophenol blue, and boiled for 3 min. 100 μl was loaded on a 4–12% gradient SDS–polyacrylamide gel using a 3% stacking gel, and electrophoresed with the discontinuous buffer system of Laemmli (22). Molecular weight standards (Pharmacia Fine Chemicals, Piscataway, NJ) were run in parallel. The separated molecular species were electroblotted onto nitrocellulose membranes using a trans-blot cell (Bio-Rad Laboratories, Richmond, CA) for 7–9 h at 4°C and 250 mA with Tris glycine buffer (20 mM Tris, 192 mM glycine) containing 20% (by volume) methanol. The membranes were blocked against nonspecific adsorption of labeled antibody by overnight incubation at 4°C in 50 mM Tris (pH 8.0) containing 100 mM sodium chloride, 20% FCS, and 0.05% sodium azide. The blots were then incubated overnight in anti-Fy6-containing culture supernatant diluted 1:2 in 50 mM Tris buffer (pH 8.0) containing 100 mM sodium chloride and 0.05% Tween 20. This antibody solution (~2.5 μg of antibody immunoglobulin per milliliter) had an indirect antiglobulin titer value of 1:1,000. Following three 10-min washes in Tris buffer containing 1% BSA, the blots were incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated rabbit anti–mouse IgG and diluted 1:1,000 in Tris/BSA buffer. Blots were washed three times in Tris/BSA, and HRP color development was obtained by the addition of 4-chloro-1-napthol substrate.

Erythrocyte Invasion by P. vivax. Squirrel monkeys undergoing synchronous infection with *P. vivax* (Belem) provided red cells infected with trophozoite or early schizont

TABLE I
Reactions of NYBC-BG6 with Various Human Red Cell Samples

Blood type	Anti-Fy^a	Anti-Fy^b	Anti-Fy3	Anti-Fy5*	NYBC-BG6
Fy(A$^+$b$^-$)	+	−	+	+	+
Fy(a$^-$b$^+$)	−	+	+	+	+
Fy(a$^+$b$^+$)	+	+	+	+	+
Fy^{x}Fy^{x‡}	+	+	+	+	+
Fy(a$^-$b$^-$)	−	−	−	−	−
Fy(a$^-$b$^+$) Rh null§	−	+	+	−	+
Fy(a$^+$b$^+$) Rh null‖	+	+	+	−	+

Human and murine antisera were tested with 4% red cell suspensions by the indirect antiglobulin method.
* Data from Colledge et al. (8).
‡ These red cells carry a weak Fy^b antigen and support only one-fourth the titration endpoint obtained with other Fy(b$^+$) samples.
§ Amorphic type; homozygous for amorphic alleles at the Rh locus.
‖ Regulator type; homozygous for inhibitor genes unlinked to the Rh locus.

parasites (31). Platelets and leucocytes were separated by passage through glass bead and CF11-cellulose columns after admixture with ADP to induce platelet aggregation. After the red cell suspension was centrifuged against a 54% percoll cushion, the parasitized cells were recovered at the interface with a purity of 85–90%. The target human erythrocytes, obtained from the interface of a 65% percoll cushion, were then added to the parasitized cells in a ratio of 5:1 or 10:1, depending on the degree of parasitemia. Invasion was allowed to proceed for 8–10 h before harvesting, preparing Giemsa-stained films, and counting 2,000 cells under light microscopy to determine the proportion of cells containing ring-stage parasites.

Results and Discussion

Fy6 Duffy Specificity. The presence of anti-Duffy antibody in an initial supernatant was confirmed by testing it with a panel of selected red cells, all of which were agglutinated, except for those of type Fy(a$^-$b$^-$). After cloning, the IgG1κ monoclonal antibody, designated NYBC-BG6, was tested against a second panel of selected erythrocytic blood groups and against the red cells of 500 random, consecutive donors. Again, it only failed to react with all 22 examples of Fy(a$^-$b$^-$) individuals in these tests. Other tests to confirm specificity included cells that lacked high frequency antigens; e.g., Vel:-1,-2, Lu(a$^-$b$^-$), Jk(a$^-$b$^-$), Oh, Jr(a$^-$), Ge$^-$, Tj(a$^-$), Hy$^-$, Jo(a$^-$), Wr(b$^-$), U$^-$, Ko, McLeod, and especially, cells of both types of Rh null (23). These tests are particularly important because of the Duffy-Rh relationship disclosed by anti-Fy5, which fails to react not only with Fy(a$^-$b$^-$) red cells but also with Fy^{a+} or Fy^{b+} cells if they are Rh null (8). Fy5 is thus an interactive product distinct from all other Duffy blood group specificities. The results, shown in Table I, indicate that the epitope recognized by the NYBC-BG6 antibody is indeed within the Duffy system, with a distribution similar to that of Fy3.

As shown in Table II, the reactivity of Fy^a, Fy^b, and Fy6 are destroyed by ficin, papain, and chymotrypsin, all of which actually enhance the reactivity of the Fy3 antigen. Trypsin, which does not affect Fy^a, slightly reduces Fy^b while enhancing both Fy3 and Fy6.

The Number of Fy6 Sites on Human Red Cells. A previous study (24) using

TABLE II

Effect of Enzyme Treatment of Red Blood Cells on Titer Values of Human Polyclonal Anti-Fy^a, -Fy^b, and -Fy3 and Murine Monoclonal Anti-Fy6

Enzyme	Tested phenotype	Human antisera*			Murine anti-Fy6
		Anti-Fy^a	Anti-Fy^b	Anti-Fy3	
None	Fy(a$^+$b$^+$)	16	16	512	1,024
Neuraminidase (10 U/ml)	Fy(a$^+$b$^+$)	16	16	512	2,048
Trypsin (1 mg/ml)	Fy(a$^+$b$^+$)‡	16	4	2,048	4,096
Chymotrypsin (1 mg/ml)	Fy(a$^+$b$^+$)§	0	0	2,048	0
Ficin (1 mg/ml)	Fy(a$^+$b$^+$)	0	0	2,048	0
Papain (1 mg/ml)	Fy(a$^+$b$^+$)	0	0	2,048	0

Cells at 1% concentration were incubated with each enzyme solution at 37°C, washed and tested as described in Table I.

* Human reagents were adsorbed with Fy(a$^-$b$^-$) neuraminidase treated red cells before testing to remove their content of anti-T agglutinins.

‡ Trypsin-treated red cells were incubated for 10 min at room temperature with 1 mg/ml soybean trypsin inhibitor.

§ Erythrocytes were incubated for 2 h at 37°C with chymotrypsin, washed and incubated for 10 min at room temperature in enzyme inhibitor (PMSF).

quantitative immunoferritin electron microscopy suggested that normal adult red cells of the Fy(a$^+$b$^-$) type have ~13,300 Fy^a sites per cell, compared to about 6,900 sites on Fy(a$^+$b$^+$) heterozygous cells. Using a directly iodinated preparation of anti-Fy6, the number of binding sites at equilibrium was estimated using different concentrations of antibody and a constant number of red cells. The antibody used in those assays was first shown to be highly active after iodination: 84–90% of the radioactivity was specifically removed by adsorption with Fy(a$^+$b$^-$), Fy(a$^-$b$^+$) or Fy(a$^+$b$^+$) cells, and 95% of this binding was inhibited by an excess of unlabeled anti-Fy6. Binding data at equilibrium was obtained for the different antibody concentrations and for cells of the four common Duffy types, and the number of sites per cell was then calculated using a Langmuir adsorption isotherm (21), as shown in Fig. 1. The mean number of sites on Fy6 cells was estimated at 12,200 ± 1,260 per cell, with a mean binding constant at equilibrium of 3.1 (±0.22) × 10^8/M, with no visible heterogeneity of binding. The differences in point estimates for these values, obtained with erythrocytes of the three positive types, Fy(a$^+$b$^-$) Fy(a$^-$b$^+$), and Fy(a$^+$b$^+$), were quite small. We must yet, however, define site density and K_o values with red cells known from genetic studies to be heterozygous for the Fy6 epitope.

FIGURE 1. Langmuir equilibrium adsorption studies of ^{125}I-labeled anti-Fy6 mAb. Human red cells of five different individuals were tested: Fy(a$^+$b$^+$),** and OO; Fy(a$^+$b$^-$), ++; Fy(a$^-$b$^+$), ××; and Fy(a$^-$b$^-$). The latter, not shown, adsorbed <3% of applied antibody. Duplicated aliquots of 5 × 10^8 red cells were incubated with 0.6, 1.2, 1.8, and 2.4 pM of labeled anti-Fy6. Free antibody is based on mean dpm adjusted for both fluid volume and mean corpuscular volume of the tested erythrocytes was subtracted from total antibody applied to obtain bound antibody. However, mean antibody bound by Fy(a$^-$b$^-$) cells was considered nonspecific and was subtracted from antibody bound by Fy6$^+$ red cells. For each of the four Fy6$^+$ cells, the reciprocal of bound antibody (minus the nonspecific) was plotted against the reciprocal of free antibody to obtain point estimates of intercept and slope. Assuming 150,000 M_r for antibody and a one-to-one correspondence between bound antibody and antigen, numbers of antigen sites per cell were calculated from the reciprocal of the intercept value. The average constant of binding at equilibrium (K_o) was obtained by dividing the intercept value by the slope value. Point estimates for each individual cell type are shown in inset. The line shown is based on intercept and slope values derived from the mean values for sites per cell, and K_o.

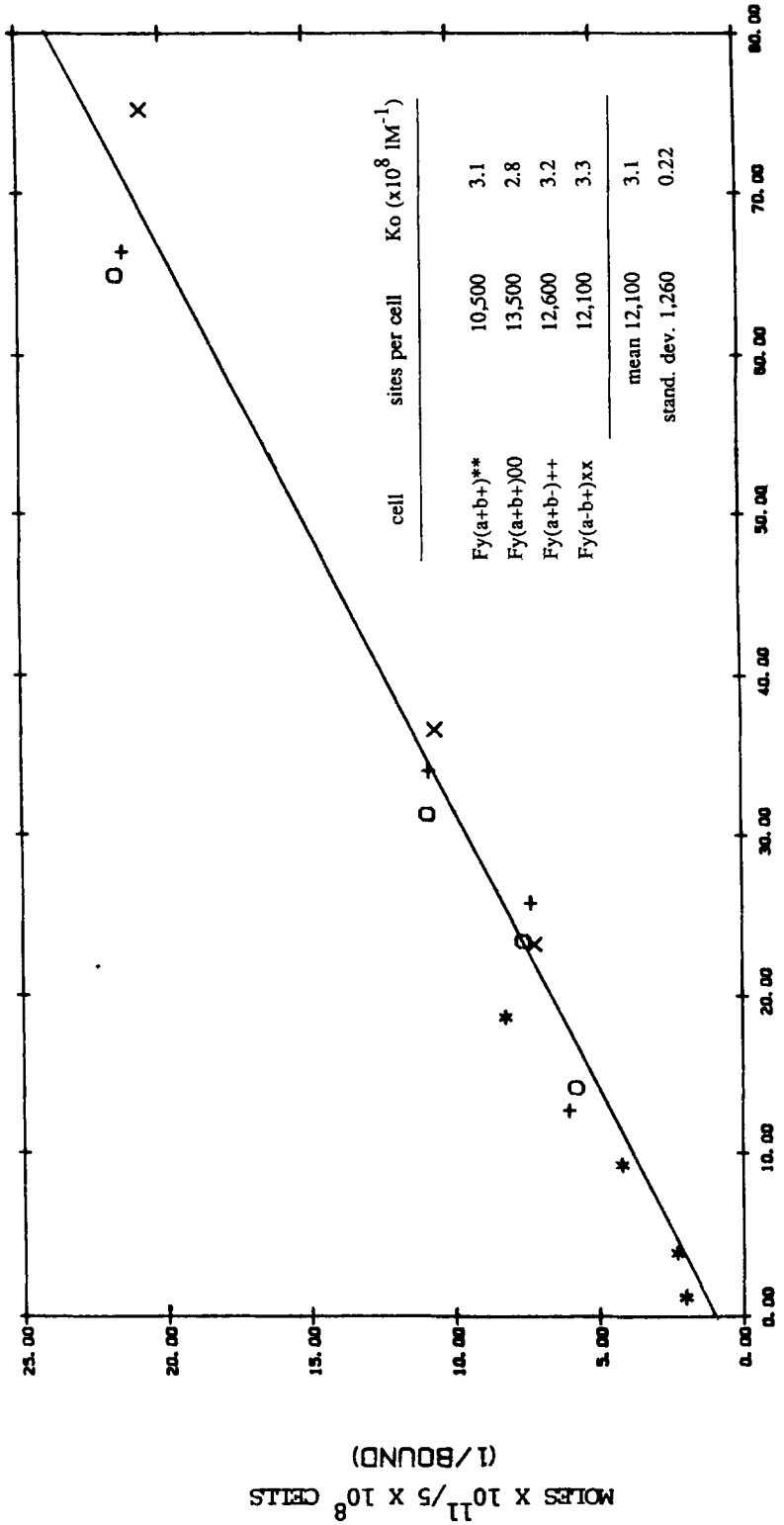

FIGURE 1.

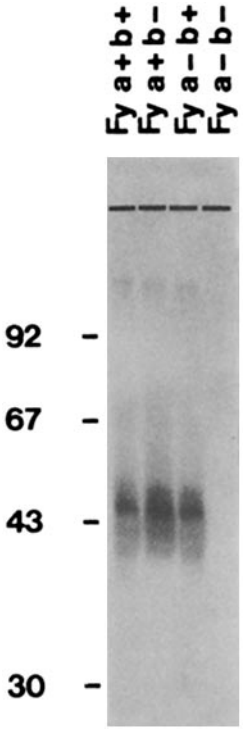

FIGURE 2. Western immunoblot analysis of anti-Fy6 mAb. Electrophoretically separated (SDS-PAGE) membrane proteins from red cells of the four major Duffy types were blotted onto nitrocellulose membranes and incubated overnight with anti-Fy6 mAb. A 1:1,000 dilution of HRP-conjugated rabbit anti-mouse IgG was used as second antibody. Colorimetric identification of the bound mAb was obtained by the addition of 4-chloro-1-napthol. Molecular mass markers are indicated at left (kD).

TABLE III

Summary of Duffy/Anti-Duffy Reactions with Primate Red Cells

Primate		Human antisera for:				Reference	Invasion by *P. knowlesi*	Reference	Invasion by *P. vivax*	Reference	Murine anti-Fy6‡
Common name	Species	Fy[a]	Fy[b]	Fy3	Fy5						
Man	*Homo sapiens*	+	0	+	+	5, 7‡	+	§	+	11‖	+
		0	+	+	+	5, 7‡	+	27	+	11‖	+
		+	+	+	+	5, 7‡	+	10	+	11, 13	+
		0	0	0	0	5, 7‡	0	§	0	11, 13	0
Great Apes											
Gorilla	*Gorilla gorilla beringei*	0	+*	+	+	25, 26	NT		NT		+*
Chimpanzee	*Pan troglodytes*	0	+	+	0	26§	+	§	+	28	+
Gibbon	*Hylobates agilis*	0	+	+	NT	26§	+	§	+	30	+*
Old World monkeys											
Rhesus	*Macaca mulatta*	0	+	+	0	26§	+	§	0	29	0
Cynomolgus	*Macaca fascicularis*	0	+*	+	0	26§	+	§	0	28	0
Baboon	*Papio sp.*	+*	+	+	NT	25‡	+	28	NT		0
New World monkeys											
Squirrel	*Saimiri sciureus*	0	0	+	NT	‡§	+	§	+	28‖	+
Capuchin	*Cebus apella*	0	0	+	NT	‡§	0	§	0	28‖	0
Dourocoli	*Aotus trivirgatus*	0	+	+	NT	26‡§	+	§	+	32	+

* Serologically weak positive.

‡ Data from current study.

§ Marsh, W. L. [2]

‖ Barnwell, J., M. E. Nichols, and P. Rubenstein, manuscript in preparation.

Apparent Molecular Mass of Fy6 Protein. After Western blotting of membrane proteins from Fy(a^+b^-) cells, anti-Fy6 binds to a molecule of apparent molecular mass (by SDS-PAGE) in the same range as the protein immunoprecipitated with a strong anti-Fy^a alloantibody from Fy^{a+} red cells (1). This glycoprotein separates as a rather broad band (Fig. 2) within which several sharper bands may be visualized, the strongest of which appears to be 46 kD. Identical results were obtained with Fy(a^+b^+) and Fy(a^-b^+) red cells, but no binding of the anti-Fy6 monoclonal reagent was detectable on blots from Fy(a^-b^-) cells.

Duffy Types and Malaria Susceptibility of Nonhuman Primates. Tests of nonhuman primates indicated not only that Fy6 differs from previously recognized Duffy specificities but that the presence of Fy6 coincides with susceptibility of red cells to invasion by *P. vivax*. This is summarized in Table III, which includes pertinent references. Anti-Fy6 was negative with red cells of two *Macaque* species, both of which were positive with anti-Fy^b and anti-Fy3, and of *Papio*, which was also positive, albeit weakly, with anti-Fy^a. The opposite pattern, positive reactions with anti-Fy6 but reportedly negative with both anti-Fy^a and anti-Fy^b, was observed with the New World monkey *Saimiri*. Fy6 and Fy^b, which are similar in serological behavior and sensitivity to enzymes, may be present independently of each other or together, but either or both are associated with the presence of Fy3 in each species thus far studied. From these available though limited observations, Fy3 might be thought of as, perhaps, the "older" specificity of the system. Be this as it may, the data are important in assessing the possible significance of the Fy6 epitope in the susceptibility of red cells to invasion by malarial parasites. As shown in Table III, the New World monkey species whose erythrocytes can be invaded by *P. knowlesi* are Fy(a^-b^-) but $Fy6^+$. On the other hand, Old World monkeys, whose red cells are Fy^{b+} and are susceptible to *P. knowlesi* lack the Fy6 specificity and are not invaded in vitro by *P. vivax*, suggesting that the precise Duffy epitope used by this parasite is distinct from that used by *P. knowlesi*. These relations become more obvious when contingency analysis is applied. If man is divided into two categories, Duffy positive and Duffy negative, the evaluated nonhuman primates provide seven additional categories, only three of which are positive both for Fy6 and susceptibility to invasion by *P. vivax* merozoites. Analyzed by Fisher's exact method, the probability of lack of association is $p < 0.008$ (126:1). In distinction, the presence or absence of Fy^a, Fy^b, and/or Fy3 cannot correspond to *P. vivax* susceptibility because rhesus, cynomolgous, squirrel, and capuchin are exceptional. Fy^a, Fy^b, and/or Fy3, however, correspond well with susceptibility to *P. knowlesi*, where of ten categories, only capuchin is exceptional.

Summary

A new Duffy specificity, Fy6, defined by a murine monoclonal antibody of the IgG1κ class, is related to susceptibility to malarial invasion. In humans, Fy6 is present on the red cells of all persons except those of the Fy(a^-b^-) type, a distribution resembling that of Fy3. However proteolytic enzyme treatment of red cells enhances the reactivity of Fy3, whereas Fy6, like Fy^a and Fy^b, is susceptible to degradation by this process. The number of Fy6 sites on human red cells was found to be 12,200 per cell, in close agreement with earlier estimates of the number of Fy^a sites. Anti-Fy6 reacted in western blots with a membrane

glycoprotein of ~46,000 M_r, not significantly different from that of a molecule known to bear the Fy^a determinant. The Fy6 epitope is shown to be present on the red cells of some but not all nonhuman primate species, where it has a distribution not only distinctly different from Fy^a, Fy^b, and Fy3, but in close accordance with susceptibility to penetration by *Plasmodium vivax*. Thus, the red cells of two species of macaques (*Macaca mulatta* and *M. fascicularis*), which are invaded by *Plasmodium knowlesi* but not by *P. vivax* are shown to have other Duffy antigens but to be devoid of Fy^6. It appears, therefore, that the red cell epitopes used by these closely related species are distinct, and that susceptibility to *P. vivax* merozoite penetration is dependent on the presence of Fy^6.

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