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21. If the following criteria were not satisfied, tests of Purkinje cells were discarded: (i) regular, spontaneous neuronal activity at rates less than 70 per second; (ii) continuous neutralization of iontophoretic currents within 0 to 15 na; (iii) constant spike amplitude without evidence of injury. Since the interpretation of negative results with iontophoresis is difficult, a classification of "no effect" was given a cell only when the drug barrel had been "warmed up" by several pulses of at least 100 na applied over a 2- to 5-minute period (*11, 12*), and when the neuron then did not respond by a change in rate of a least 10 percent to at least two consecutive pulses of 200 na each applied for at least 1 minute. Had large ejection currents and long durations of application not been used, many responses to cyclic AMP would have been overlooked. These considerations could in part account for the low frequency of cyclic AMP-induced depressions of Purkinje cells reported by Lake and Jordan (*10*).
22. Several unidentified cells were tested which did not have the characteristics of Purkinje cells, such as climbing fiber bursts and regular firing patterns of simple spikes at rates greater than 10 to 15 per second. Of 33 complete tests with several of the more potent derivatives, only 6 percent of these neurons displayed depressions of rate, 39 percent were speeded, and 55 percent were uneffected. Here again the effects of the derivatives parallel the results seen previously with cyclic AMP (*23*), and emphasize the necessity of identification of cell types for meaningful interpretation of iontophoresis studies.
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Erythrocyte Receptors for (*Plasmodium knowlesi*) Malaria: Duffy Blood Group Determinants

Abstract. *Duffy blood group negative human erythrocytes (*FyFy*) are resistant to infection by* Plasmodium knowlesi, *a simian malaria that infects Duffy positive human erythrocytes. The* P. knowlesi *resistance factor, Duffy negative erythrocytes, occurs in high frequency in West Africa, where the people are resistant to vivax malaria. This suggests that Duffy blood group determinants (*Fy^a *or* Fy^b*) may be erythrocyte receptors for* P. vivax.

West Africans (*1*) and approximately 70 percent of American blacks (*2*) are resistant to infection by *Plasmodium vivax*, although they are susceptible to the other three species of human malaria. The vivax resistance factor completely blocks infection; sickle cell trait which decreases mortality from *P. falciparum* does not block infection (*3*). The resistance to *P. vivax* is evident after sporozoite induced infection (*1*) or after inoculation of infected erythrocytes (*2*). Since parasitized erythrocytes initiate infection in the recipient without an exoerythrocytic cycle, the resistance factor must interfere with the merozoite's ability to invade erythrocytes or to develop once within them.

In a search for erythrocyte receptors for malaria parasites (merozoites) (*4*), we tested erythrocytes that lacked various antigenic determinants for susceptibility to invasion by a simian malaria (*P. knowlesi*). This species invades human erythrocytes in culture (*5*) and infects man (*6*). Of the erythrocytes tested, one type which lacked Duffy a and b antigens, Duffy negative (*FyFy*) (*7*), was resistant to invasion. This genotype is present in approximately 90 percent of West Africans (*8*); it is extremely rare in other racial groups (*9*) who are susceptible to *P. vivax*. This striking association suggested that the Duffy negative genotype (*FyFy*) might be the factor involved in resistance to *P. vivax* in West Africans and that invasion by *P. vivax* could require a Duffy positive erythrocyte (Fy^a or Fy^b).

To explore these questions, we studied the relation between Duffy blood group determinants and invasion by *P. knowlesi*. Human erythrocytes were obtained from five whites and five blacks, positive for either or both of the Duffy antigens (a and b) and from 11 blacks negative for these antigens. The erythrocytes were washed three times with culture medium (*10*), and mixed with *P. knowlesi* (*11*) infected rhesus erythrocytes that contained primarily schizonts. This suspension (5000 human erythrocytes and 500 parasitized erythrocytes per cubic millimeter) was added to 16-mm flat-bottom Linbro tissue culture wells (0.5 ml per well) and incubated for 3 hours at 35°C in an atmosphere containing 2 percent CO_2. The merozoite invasion frequency was determined from Giemsa-stained smears prepared from erythrocytes in the wells and counted (under code) (*5*). Duffy positive and negative erythrocytes were studied at the same time under identical conditions.

The average invasion frequency for Duffy positive erythrocytes was 80.3 parasitized erythrocytes per 1000 erythrocytes

Table 1. The effect of various proteolytic enzymes on Duffy blood group determinants and malaria invasion of Duffy positive human erythrocytes. Removal of Duffy blood group determinants is accompanied by reduced invasion by *Plasmodium knowlesi*. Abbreviation: RBC, erythrocytes.

RBC phenotype	Enzyme*	Duffy† typing	Titer after‡ adsorption	Infected§ RBC per 10,000 RBC
Fy(a+ b–)	None	+++	–	610
	Trypsin (1 mg/ml)	+++	–	870
	Chymotrypsin (0.01 mg/ml)	+++	1 : 1	380
	Chymotrypsin (0.1 mg/ml)	–	1 : 8	46
	Chymotrypsin (1 mg/ml)	–	1 : 8	17
Fy(a– b+)	None	++++	–	850
	Trypsin (1 mg/ml)	++++	–	1110
	Chymotrypsin (0.01 mg/ml)	++++	–	840
	Chymotrypsin (0.1 mg/ml)	++++	1 : 1	78
	Chymotrypsin (1 mg/ml)	–	1 : 8	25

*See (*5*) for details of enzymatic treatment. †Scoring: ++++, macroscopic clumps, clear supernatant; +++, macroscopic clumps, slightly reddish supernatant; –, no microscopic clumps. ‡ Antiserum to Duffy antigen was absorbed with RBC as follows: three drops of packed erythrocytes which had been washed with 0.85 percent NaCl were mixed with four drops of antiserum to Duffy antigen (titer = 1 : 16), and the suspension was incubated for 40 minutes at 37°C. The supernatant was used for Duffy typing with the appropriate Duffy positive erythrocytes. A titer of 1 : 8 indicates no adsorption of antibody, since mixture with cells caused a 1 : 2 dilution of the original antiserum. §The counts were the sums of duplicate chambers. At high rates of invasion (> 100 infected RBC per 10,000 RBC), less than 10,000 RBC were counted, and the numbers in the table were estimated.

Table 2. The effect of antiserums to Duffy antigen and to Rh_0 (D) antigen on invasion of Duffy positive, Rh_0 (D) erythrocytes by *Plasmodium knowlesi*. Erythrocytes (100,000/mm³ in culture medium) were incubated with an equal volume of antiserums to Duffy antigens (anti-Fy^a, 1 : 128, Spectra Biologics; anti-Fy^b, titer 1 : 16, American Hospital Supply; anti-Rh_0 (D), Ortho Diagnostic) for 30 minutes at 37°C. After the cells were washed five times with culture medium, they were incubated with parasitized erythrocytes. The invasion ratio was the invasion frequency of antibody treated erythrocytes divided by the invasion frequency of untreated erythrocytes. Abbreviation: RBC, erythrocytes.

Exp. No.	RBC phenotype	Invasion* frequency in untreated RBC	Invasion ratio			
			Anti-Fy^a	Anti-Fy^b	Anti-Rh_0	Anti-Fy^a + Anti-Fy^b
1	Fy(a+ b–)	960	0.28		0.91	
2	Fy(a+ b–)	40	0.15	0.88	0.80	
	Fy(a– b+)	70	1.22	0.82	0.80	
	Fy(a+ b+)†	70	0.92	1.08		1.00
3	Fy(a+ b–)	630	0.21	1.10	0.98	
	Fy(a– b+)	730	1.46	1.04	1.18	
	Fy(a+ b+)†	570	0.54	1.22		0.48

* The counts were the sums of duplicate chambers. At high rates of invasion (> 100 infected RBC per 10,000 RBC), less than 10,000 RBC were counted and the numbers in the table were estimated. The variation in invasion frequency between experiments reflects the differences in the quality of the parasites. Invasion frequencies can only be compared in the same experiment. † Duffy typing with anti-Fy^b: Fy(a+ b+) in experiment 2, macroscopic agglutination; Fy(a+ b+) in experiment 3, microscopic agglutination.

(range, 53 to 99). The invasion rates for the three Duffy positive phenotypes, Fy(a+ b–, Fy(a– b+), and Fy(a+ b+), were similar. Only 2.2 parasitized erythrocytes per 1000 erythrocytes (range, 0 to 5) was observed in Duffy negative erythrocytes. No consistent differences ascribable to other blood groups were observed between the Duffy positive and negative erythrocytes (*12*). One of the 11 Duffy negative donors had hemoglobin S.

The susceptibility of other negative and null erythrocytes from the collection of frozen cells at the Blood Bank, National Institutes of Health Clinical Center, was evaluated. Rh null, Lewis (Le a– b–), and Lutheran (Lu a– b–) erythrocytes were invaded normally. Bombay (ABO null), Kell null (Ko), and Kidd (Jk a– b–) erythrocytes could not be evaluated because the freezing process severely damaged the erythrocytes (many crenated spheres and lysed erythrocytes), and no fresh cells were available for testing.

We have shown (*5*) that chymotrypsin (≥ 0.1 mg/ml) and Pronase treated erythrocytes were resistant to invasion by *P. knowlesi* merozoites; trypsin had no effect on invasion. The loss of susceptibility to invasion in these studies was directly correlated with enzymatic removal of the Duffy blood group determinant from the erythrocyte surface (Table 1).

We measured the parasite invasion frequency of Duffy positive erythrocytes coated with antiserum to Duffy antigens (abbreviated anti-Fy^a and anti-Fy^b) (Table 2). Anti-Fy^a caused a marked reduction in invasion of Fy(a+ b–) erythrocytes while it had no effect on Fy(a– b+) erythrocytes. Anti-Fy^b had no effect on Fy(a– b+). The lower titer of anti-Fy^b (1 : 16 as compared to anti-Fy^a (1 : 128) may have accounted for the failure of anti-Fy^b to block invasion of Fy(a– b+) erythrocytes. Anti-Fy^a reduced invasion of Fy(a+ b+) erythrocytes in experiment 3 but not in experiment 2 (Table 2).

The interaction of merozoites with erythrocytes was recorded on video tape with the use of a low light intensity video microscope system (*13*). This permitted a direct comparison between the interaction of merozoites with Duffy positive and negative erythrocytes. A significant difference was apparent. In both cases, following contact by a merozoite, the erythrocyte was markedly deformed. This was followed, in the case of Duffy positive erythrocytes, by a localized invagination of the erythrocyte around the merozoite and the subsequent interiorization of the parasite (*13*). However, with Duffy negative erythrocytes, the critical second stage in the invasion process, that is, localized invagination of the erythrocyte, did not occur; the merozoite eventually detached and was capable of interacting with other erythrocytes. Thus, although *P. knowlesi* merozoites can attach to and interact with both Duffy positive and negative erythrocytes, the complete invasion process is only possible with Duffy positive erythrocytes. One explanation for these observations is that two receptors, one for attachment and one for interiorization, may be required for the complete invasion process and the Duffy blood group determinants are receptors for the interiorization phase of the interaction.

These in vitro observations probably account for the failure of some blacks to develop a patent infection after intravenous inoculation of *P. knowlesi* infected blood for the then accepted treatment of neurosyphilis with malaria (*6*). The resistance does not appear to be caused by other membrane defects in Duffy negative erythrocytes, since destruction of Duffy blood group determinants by proteolytic enzymes and blocking of the antigen with antiserum greatly reduces invasion. Presumably each of these affects only the surface structures, although they also affect surface structures other than Duffy blood group antigen.

The resistance of West Africans and some American blacks to *P. vivax* corresponds to the unique distribution of Duffy negative erythrocytes (*FyFy*) in the world. Other African populations with a higher incidence of Duffy blood group positive erythrocytes (*14*) could account for the presence of *P. vivax* in East Africa and Madagascar. If the Duffy blood group determinants are the receptors for *P. vivax*, Africans infected with *P. vivax* should all have Duffy positive erythrocytes while Duffy negative individuals would be resistant to challenge by *P. vivax*.

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Neuronal Locus Specificity: Trans-Repolarization of *Xenopus* Embryonic Retina After the Time of Axial Specification

Abstract. *Signaling within an embryonic* Xenopus *eye comprised of two fused eye fragments can reprogram, in turn, the anteroposterior and dorsoventral axes of one of the fragments. The responding fragment, subsequently isolated and allowed to round up and innervate the brain, shows corresponding inversions in its retinotectal map. This is the first evidence for trans-repolarization of presumptive retina and provides an assay system for analysis of positional signaling within the retinal field.*

Differentiating retinal ganglion cells undergo position-dependent diversification, acquiring properties (*locus specificities*) that enable each cell's axon to reach its appropriate locus in the retinotectal map (*1, 2*). Such differentiations presumably continue to occur throughout tadpole life in *Xenopus*, as new ganglion cells are added to the ciliary margin of the growing retina (*3*). Yet, even before the first optic axons appear, a developmental program is finalized in the stage 28 to 31 optic cup which *specifies* the permanent anteroposterior (AP) and dorsoventral (DV) reference axes for positional information (*4*) in the retinal field, and establishes the spatial blueprint for patterning of locus specificities across the entire future ganglion cell population (*5*). Axial specification occurs in two steps (AP first) over 5 hours, and is triggered under retinal control (*6*). Before specification, a rotated eye can interact with the axial cues of the embryo, rapidly replace its labile retinal axes with a new pair of (properly aligned) axes (*7*), and assemble a normally oriented retinotectal map from the rotated position (*7, 8*). After specification, the stage 31 eye is unaffected by the embryo's axial cues and, even when grafted in rotated orientation into a pre–stage 28 host, retains its specified axes and assembles a correspondingly rotated retinotectal map (*5, 8*).

This developmental program is exceedingly stable and was expressed with fidelity when stage 31/32 eyes were submitted to a variety of serial transplantation procedures, prolonged tissue culture, chronic deprivation of tectal connections, or temporary suppression of retinal growth (*5, 9*). Likewise, individual stage 31/32 nasal, temporal, or ventral eye fragments, which round up and form morphologically "whole" eyes, are insensitive to the extraocular microenvironment, and retain (or, occasionally, reduplicate) their specified axes (*10*). The first hint of modifiability came from surgically constructed eyes, formed by fusing specific stage 31/32 eye fragments together (*11, 12*); but without knowing which regions of the adult retina arose from which fragments of the recombinant eye, inferences about frank modification of retinal axes remained speculative (*2*).

Here we show that when allowed to fuse and interact with a right-nasal fragment, a left-temporal fragment (subsequently isolated, allowed to round up, and assayed after it has mapped into the tectum alone) undergoes a stepwise reprogramming of first its AP and then its DV axis. This is the first clear evidence for trans-repolarization of retinal tissue and for axis reversal after the time of specification, and provides an assay system for analysis of positional signaling within the retinal field. Published accounts exist for all methods used, including those for staging, surgical management, and rearing of *X. laevis* clawed frog embryos (*5*); preparation of eye fragments and recombinant eyes (*10, 11*); testing of visually guided strike responses of the frogs during metamorphosis [to confirm the existence of functional synapses between the experimental eye and the brain (*2, 5*)]; and electrophysiologic analysis of the visual field projection from the experimental right eye to the left optic tectum, 5 to 18 weeks after eye surgery, in the juvenile frog (*2*).

Four control series were prepared, concurrent with the experimental series and using siblings of the experimental embryos. Normally oriented retinotectal maps (Fig. 1a) developed in all 11 frogs whose right eye was removed and replaced intact in normal orientation at stage 27 ± 1, 31/32, 38/39, or 43/44. Thus, simple surgical intervention at these stages did not produce map inversions. Normally oriented maps developed in seven frogs after grafting a stage 27 ± 1 right eye, in 180°-rotated orientation, into the completely vacated right orbit of a stage 27 ± 1 host; but the map was inverted in both axes in all eight frogs, after grafting a stage 31/32 right eye in 180°-rotated orientation into the vacated right orbit of a stage 27 ± 1 host. These controls confirm that the stage 31/32 eyes used in our experiments (since their axes were not modified by interaction with host embryos of proven competence) had in fact undergone axial specification prior to stage 31/32. Finally, the retinotectal map was normally oriented in the AP axis but inverted in the DV axis in all but 11 frogs after grafting a stage 31/32 left eye (in AP-normal, DV-inverted orientation) into the vacated right orbit of a stage 31/32 host, with no further surgery or with subsequent (after 15 to 30 minutes or after 13 to 14 hours) extirpation of its nasal region (see Fig. 1b). Thus, the retinal axes of a specified left eye are stable in left-temporal fragments, isolated in the right orbit, and allowed to map into the left tectum. Reversal of these axes in the experimental series (in which the left-temporal fragments were similarly isolated after contact with a right-nasal fragment) must have resulted from interaction with the right-nasal fragment.

In the four experimental series, a (donor) stage 31/32 left-temporal eye fragment was apposed to a (host) stage 31/32 right-nasal fragment, by grafting the donor fragment in place of the extirpated temporal region to the host right eyes. The right-nasal (host) fragment was either (i) completely removed after 15 to 30 minutes (sham fusion); (ii) left undisturbed as part of a permanent recombinant eye; (iii) completely removed after 13 to 16 hours (host stage 39 ± 1; 22°C) when the two fragments were composite halves of a "dumbbell-shaped" eye; or (iv) completely removed after 30 to 32 hours (host stage 43/44), when the two fragments were no longer visibly discrete but remained easily separable by cutting along the fusion scar. Nasal extirpations were confirmed histologically (*10*).

The first two experimental series, which define the boundary conditions for the time of fragment interaction (sham fusion and indefinite fusion), gave consistent results: all seven frogs in the sham-fusion series developed AP-normal, DV-inverted maps (Fig. 1b), identical to those seen in the fourth control group; with only minor vari-