Altered Dihydrofolate Reductase Associated with Drug-Resistance Transfer between Rodent Plasmodia

Abstract. *Resistance to pyrimethamine in strains of* Plasmodium vinckei *and of* Plasmodium berghei *is attributed to changes in amounts and properties of the dihydrofolate reductases. The resistant strain of* Plasmodium berghei *was isolated from an experimentally induced mixed infection of drug-resistant* Plasmodium vinckei *and drug-sensitive* Plasmodium berghei, *through biological filtration in hamsters. The drug resistance in* Plasmodium berghei *appears to have been acquired through transfer of part of the gene or genes coding for dihydrofolate reductase from the resistant* Plasmodium vinckei *to the sensitive* Plasmodium berghei.

Although the transference between cells of inheritable biological traits, including resistance to drugs, has been well established in bacteria, few examples can be found among the protozoa (*1*). Yoeli *et al.* (*2*) reported the transfer of resistance to the antimalarial pyrimethamine between two species of rodent malaria in vivo. A strain of *Plasmodium vinckei* resistant to pyrimethamine was inoculated into mice concurrently with a strain of *P. berghei* sensitive to pyrimethamine, and the mixed infection was allowed to develop. Because *P. vinckei* was incapable of growth in hamsters and because *P. berghei* was sensitive to the drug, it was possible to select for a strain of *P. berghei* resistant to pyrimethamine by passing the mixed infection from the mice to a hamster and then by treating with high doses of drug. Thus, through this method of biological filtration, a strain of plasmodia was obtained, identified as *P. berghei* by its morphological characteristics and biological behavior, which had attained resistance to pyrimethamine. The properties of the genetic marker transferred, that is, pyrimethamine resistance, can now be investigated biochemically. This is possible because of studies demonstrating (i) that pyrimethamine in *P. berghei* inhibits the enzyme dihydrofolate reductase (*3*), and (ii) that changes in the amounts and properties of the dihydrofolate reductase of a pyrimethamine-resistant strain of *P. berghei* were found (*4, 5*).

The details of the history and of the development of the strains used are included elsewhere (*2*). Materials and methods used in the preparation of extracts and assay of dihydrofolate reductase have been described (*3*). Briefly, extracts were prepared by passing washed parasites, freed from infected erythrocytes by saponin treatment, through a French pressure cell. Dihydrofolate reductase was assayed by following the decrease in optical density at 340 nm from the reaction of dihydrofolate + reduced nicotinamide-adenine dinucleotide phosphate, $\rightleftharpoons$ tetrahydrofolate + nicotinamide adenine dinucleotide phosphate (*6*).

The parameters investigated with the enzymes from all four strains were (i) the specific activity (V_{max}); (ii) the Michaelis constant K_m) for dihydrofolate; (iii) the 50 percent inhibitory concentration of pyrimethamine; (iv) the effect of prior incubation of dihydrofolate with enzyme on inhibition by pyrimethamine; and (v) the effect of KCl on enzyme activity. The V_{max} and K_m values for dihydrofolate for each enzyme were obtained by plotting the reciprocals of dihydrofolate concentration against velocity (*7*). The effect of prior incubation of dihydrofolate on pyrimethamine inhibition was studied because it had been observed that under this condition inhibition by pyrimethamine was reversed with the enzyme from strains of *P. berghei* sensitive to pyrimethamine, but not from a resistant strain (*5*). Similarly, it had been found that KCl markedly activated the enzyme of sensitive strains but had only a slight stimulatory effect on the dihydrofolate reductase of a *P. berghei* resistant to pyrimethamine (*4, 5*).

The enzyme from the sensitive *P. vinckei* differed from that of the sensitive *P. berghei* in all tests (Tables 1 and 2). Because this enzyme from the sensitive *P. berghei* was almost identical to the dihydrofolate reductases of two other pyrimethamine-sensitive *P. berghei* strains investigated (*3, 5*), the characteristics of the *P. vinckei* enzyme apparently reflect true species differences.

The development of resistance to pyrimethamine in *P. vinckei* was accompanied by changes in the dihydrofolate reductase similar to those reported for the enzyme from a pyrimethamine-resistant *P. berghei* (*4, 5*). The differences noted in the resistant strain were: (i) increases in the V_{max}, in the K_m value for dihydrofolate, and in the 50 percent inhibitory concentration of pyrimethamine; (ii) loss of activation by 0.15*M* KCl; and (iii) lack of effect of prior incubation of dihydrofolate on pyrimethamine inhibition (Tables 1 and 2). The same mechanism of resistance seems to be operative in *P. vinckei* (*5*); the resistant strain possesses increased concentrations of a dihydrofolate reductase which has altered properties, including decreased affinity for the drug.

Thus, the dihydrofolate reductase of the "donor" strain (resistant *P. vinckei*) was markedly different from that of the "recipient" strain (sensitive *P. berghei*). If we assume that the transfer of resistance involved transference of the genetic material responsible for the coding of dihydrofolate reductase of the resistant *P. vinckei* to the sensitive *P. berghei*, the characteristics of the en-

Table 1. Comparison of properties of the dihydrofolate reductases of strains of *P. vinckei* and *P. berghei* resistant to and sensitive to pyrimethamine. The standard enzyme assays were performed as described (*3*). The 50 percent inhibitory concentrations of pyrimethamine (I_{50}) were derived from plotting the log of the concentration of the drug against the activity of the enzyme. To determine the effect of prior incubation of substrate on inhibition by the drug, dihydrofolate was first incubated with the mixture of enzyme and pyrimethamine (50 percent inhibitory concentration). A minus (−) indicates that percent inhibition was essentially unchanged; a plus (+) indicates that the inhibition was decreased to 20 percent or less.

Strain	V_{max} (nmole min^{-1} mg^{-1} protein)	Dihydrofolate K_m (μM)	Pyrimethamine I_{50} (nM)	I_{50} reversal
		P. vinckei		
Sensitive	4.8	7.9	2.0	±*
Resistant	17.4	68	450	−
		P. berghei		
Sensitive	0.8	4.0	0.5	+
Resistant	7.2	39	10	−

* Complete reversal was observed during the first 2 minutes after the reaction was initiated; the rate then changed to that of the 50 percent inhibited reaction.

Table 2. Effect of KCl on the activities of dihydrofolate reductases from strains of *P. vinckei* and *P. berghei* resistant to and sensitive to pyrimethamine. The assays were performed as described (*3*) in the presence of buffer [0.015*M* tris(hydroxymethyl)aminomethane-hydrochloride (tris-HCl) (*p*H 7.0; 37°C)] and the indicated concentrations of KCl, which were added to the buffer. In each case the activity in 0.015*M* tris-HCl was set equal to 100 for ease of comparison.

KCl (*M*)	*P. vinckei*		*P. berghei*	
	Sensitive	Resistant	Sensitive	Resistant
None	100	100	100	100
0.15	156	96	300	133
0.21	116	92	280	119
0.51	114	74	214	75

zyme from the strain selected as a product of the mixed infection (the resistant *P. berghei*) should be readily discernable. The data support this assumption; major differences were found between the dihydrofolate reductases of the sensitive and resistant *P. berghei* strains (Tables 1 and 2). The enzyme from the resistant strain had a ninefold higher V_{max}, a tenfold higher K_m value for dihydrofolate, and a 20-fold increase in the 50 percent inhibitory concentration for pyrimethamine, as compared to the enzyme from the sensitive *P. berghei* (Table 1). Also, the resistant *P. berghei* enzyme was stimulated less effectively by KCl than the enzyme from the resistant strain (Table 2), and the inhibition of the enzyme by pyrimethamine was unaffected by prior incubation with dihydrofolate (Table 1).

The possibility that extracts of the resistant *P. berghei* contained a mixture of separate molecules of the enzyme from the resistant *P. vinckei* and the sensitive *P. berghei* was investigated. Inspection of curves of percentage inhibition plotted against the logarithm of pyrimethamine concentrations for the enzymes from the latter two strains revealed that there is a range of pyrimethamine concentrations (0.8×10^{-8} to $4.0 \times 10^{-8}M$) in which the sensitive *P. berghei* enzyme is completely inhibited and the resistant *P. vinckei* is unaffected. The 50 percent inhibitory concentration of pyrimethamine found for the enzyme from the resistant *P. berghei* ($1 \times 10^{-8}M$) fell within this range of concentrations; thus, if it were a mixture, it would have to contain equal amounts of the two enzymes, and a graph of the percentage inhibition versus the logarithm of pyrimethamine concentration would be expected to show a broad "plateau" at this range of concentrations. Such a plateau was in fact observed when a prepared mixture (1 : 1) of the enzyme from the resistant *P. vinckei* and from the sensitive *P. berghei* was tested for inhibition by pyrimethamine. However, the curve for the resistant *P. berghei* did not show this plateau. A straight line was obtained with the same slope as in the case of the other three enzymes; thus it was concluded that the dihydrofolate reductase was not a mixture of the enzyme from the "donor" and "recipient" strains.

The properties of the dihydrofolate reductase from the resistant *P. berghei* were intermediate in character between those of the resistant *P. vinckei* and the sensitive *P. berghei*. The marked differences among the enzymes from the various strains suggest that the protein structures of these enzymes are not identical. In the resistant *P. vinckei* this is presumably due to a mutant gene or genes coding for an altered dihydrofolate reductase, selected during the development of resistance to pyrimethamine. It seems likely that only part of the gene or genes responsible for coding of the enzyme of the donor strain would be transferred to the recipient. Hotchkiss and Evans (*8*) and Sirotnak *et al.* (*9*) have reported on multiple gene sites involved in drug resistance in bacteria, in which different degrees of resistance could be transferred in transformation experiments. Also, altered dihydrofolate reductases are present in mutant strains of antifolate-resistant bacteria (*8, 10*). The experiments reported here, and previously (*2*), indicate that these phenomena can occur in plasmodia. It may be difficult to determine the mechanism by which the transfer occurred in view of the absence of techniques for genetic analysis in plasmodia. However, by arranging conditions to allow for biological filtration, and by detailed analysis of the genetic marker chosen, our studies support the contention that transfer of genetic material among plasmodia is possible.

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References and Notes

1. J. Greenberg and H. L. Trembley, *J. Parasitol.* **40**, 667 (1954); B. M. Honigberg, *J. Protozool.* **15**, 223 (1968); P. J. Walker, in *Infectious Blood Diseases of Man and Animals*, D. Weinman and M. Ristic, Eds. (Academic Press, New York, 1968), vol. 1, p. 367.
2. M. Yoeli, R. S. Upmanis, H. Most, *Parasitology* **59**, 429 (1969).
3. R. Ferone, J. J. Burchall, G. H. Hitchings, *Mol. Pharmacol.* **5**, 49 (1969).
4. R. Ferone, *Fed. Proc.* **28**, 847 (1969).
5. ———, *J. Biol. Chem.*, in press.
6. F. M. Huennekens, *Biochemistry* **2**, 151 (1963).
7. H. Lineweaver and D. Burke, *J. Amer. Chem. Soc.* **56**, 658 (1934).
8. R. Hotchkiss and A. H. Evans, *Fed. Proc.* **19**, 912 (1960).
9. F. M. Sirotnak, G. J. Donati, D. J. Hutchison, *J. Biol. Chem.* **239**, 4298 (1964).
10. R. Berberich and M. Levinthal, *Bacteriol. Proc.* **1969**, 54 (1969).
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Mixed Lymphocyte Reactions and Tissue Transplantation Tolerance

Abstract. *The induction of tolerance of Lewis histocompatibility antigens in BN rats inoculated at birth with BN/Lewis F_1 hybrid bone marrow cells, as revealed by the prolonged survival of Lewis skin grafts, is accompanied by markedly decreased reactivity in the mixed lymphocyte interaction. Blood lymphocytes from animals inoculated with lymph node cell suspensions also display diminished proliferative reactivity to hybrid BN/Lewis cells in the interaction. However, these recipients are not tolerant of Lewis skin grafts. Blood lymphocytes from BN rats inoculated neonatally with Lewis thymocytes fail to display any level of unresponsiveness in vitro, and such animals are not tolerant of Lewis skin grafts. The results suggest that in rats skin and marrow cells have histocompatibility antigens that are absent or poorly expressed on lymph node cells and thymocytes.*

In rats, bone marrow cells constitute the only effective means by which neonatal recipients can be rendered tolerant of Ag-B incompatible skin grafts (*1*), that is, skin grafts that differ from their host at the major histocompatibility locus of the species (*2*). By comparison, cells derived from the spleen and lymph nodes are poor in inducing tolerance of these grafts and thymocytes are ineffective (*3*). Nonetheless, there is evidence that rats inoculated at birth with Ag-B incompatible lymph node or splenic cells are persistent cellular chimeras. Blood leukocytes from these animals will immunize isologous recipients against skin homografts from the donor strain (*4*); and a high pro-