WARFARIN RESISTANCE AND DT DIAPHORASE ACTIVITY IN THE RAT

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Received 14 September 1973

1. Introduction

The indirect anticoagulant warfarin is believed to prevent the synthesis of the vitamin K-dependent blood clotting factors by inhibiting the activity of vitamin K oxide reductase. This inhibition leads to an accumulation of vitamin K oxide which has, in turn, been proposed to be the true competitive inhibitor of vitamin K in warfarin-treated animals [1]. Rats showing monogenic resistance to warfarin and other 4-hydroxycoumarins such as dicoumarol have been shown to have a distinctively increased requirement for the vitamin [2, 3] and, in one study, an equally distinctive decrease in the activity of the dicoumarol-sensitive enzyme DT diaphorase has been observed [4]. It was suggested from these findings that DT diaphorase might be implicated in the biochemical mechanism of warfarin resistance by way of its possible involvement in the reduction of vitamin K oxide [4]. In the DT diaphorase study [4], however, the warfarin-resistant rats (Tolworth HW strain [3]) were more than 98% derived from Wistar stock, whereas the non-resistant rats with which they were compared were of the Sprague–Dawley strain [4]. There was thus a possibility that the observed difference in DT diaphorase activity was due to a difference in genotypic background between the two strains and not to the resistance gene per se.

The present report shows that in animals with a balanced genotypic background, different levels of liver DT diaphorase activity were not significantly associated with particular resistance genotypes, and hence that the enzyme does not appear to be involved in the biochemical mechanism of monogenic resistance to warfarin.

2. Methods

Animals which differed in resistance and hence, putatively, in DT diaphorase activity, but which were otherwise balanced genotypically, were obtained by breeding an F2 generation between the resistant HW strain and the non-resistant Wistar strain as follows. First a male rat homozygous for the resistance gene (Rw^2) [5] and heterozygous for albinism (c) was crossed with Wistar females. The resultant F1 animals were all heterozygous for resistance (i.e. Rw^1Rw^2); about half were also heterozygous for albinism (i.e. Cc) while the remainder were homozygous for albinism. These F1 rats were inter-bred to produce 173 F2 animals, some of the matings being intercrosses ($Rw^1Rw^2Cc \times Rw^1Rw^2Cc$) and others mixed crosses ($Rw^1Rw^2cc \times Rw^1Rw^2cc$).

The resistance genotypes of the F2 rats at six weeks of age were determined by measuring the activity of the vitamin K-dependent blood clotting factors under conditions of controlled vitamin K administration. This technique, which is based upon observations of the vitamin K requirement of different types of rat [3], was adopted in preference to a direct test of the response to warfarin in order to differentiate between

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Table 1
Segregation of resistance and coat colour in 173 F2 rats.

Mating type	Phenotypic class					
	Rw^2Rw^2		Rw^2Rw^1		Rw^1Rw^1	
	C	*c*	*C*	*c*	*C*	*c*
Inter-cross	11	2	17	1	9	8
Mixed cross (*Rw* inter-crossed)	21	10	26	38	2	28

resistant homozygotes and heterozygotes, as well as to avoid the possibility that treatment with warfarin might influence DT diaphorase activity subsequently. Details of the experimental procedure have been given elsewhere [3]. Briefly, each rat was maintained on a vitamin K-deficient diet for four days during which it received 4.0 μg/100 g of vitamin K_1 subcutaneously each day; rats whose clotting factor activity was reduced to less than 15% of normal during this period were classified as resistand homozygotes (Rw^2Rw^2) as were three rats that died of vitamin K deficiency as a result of this experiment. The remaining rats, after a recovery period of three days were returned to the vitamin K-deficient diet for a further four days and this time they received no supplementary vitamin K; rats whose clotting activity fell to less than 15% of normal in this second experiment were classified as heterozygous (Rw^1Rw^2) while the remainder were taken to be non-resistant homozygotes (Rw^1Rw^1).

The dicoumarol-sensitive DT diaphorase activity of liver postmicrosomal supernatants was assayed for 48 out of the 173 F2 rats, using NADH and 2,6-dichlorophenolindophenol as electron donor and acceptor respectively, as previously described [4].

3. Results and discussion

The data given in table 1 indicate that resistance segregated in the expected 1:2:1 ratio ($\chi^2 = 0.572$; $p = 0.7–0.8$). Recombination between the resistance (*Rw*) and albinism (*c*) loci was 0.234 ± S.E. 0.051, a value consistent with that of 0.216 ± S.E. 0.023 previously obtained from backcross data, where survival of a normally lethal dose of warfarin was the criterion of resistance [5]. The dispersion of the data on clotting factor activity (fig. 1) suggests that some animals may have been wrongly classified though such errors, if they occurred, were evidently too few to disturb the

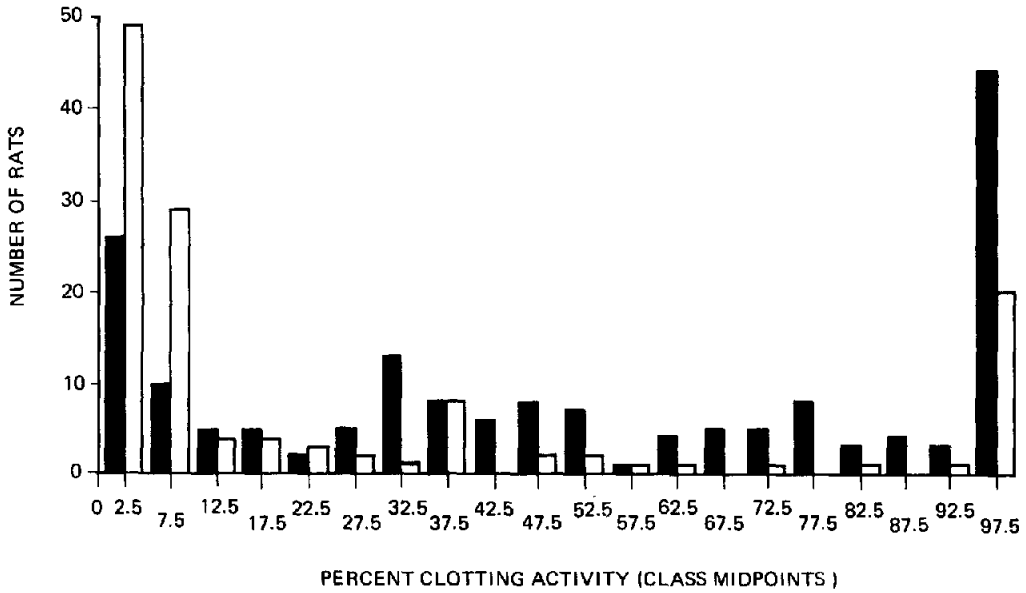

Fig. 1. Percent activity of vitamin K-dependent clotting factors in F2 rats after four days on the vitamin K-deficient diet. Solid bars: Rats received 4.0 μg/100 g of vitamin K_1 daily. Open bars: Rats received no supplementary vitamin K.

Table 2
Liver DT diaphorase activity* in 48 offspring of an inter-cross (F2) between rats heterozygous for warfarin resistance.

DT diaphorase activity	Type of rat		
	Non-resistant (Rw^1Rw^1)	Resistant	
		Heterozygous (Rw^1Rw^2)	Homozygous (Rw^2Rw^2)
Low			
Males	5.6, 3.8, 6.8, 4.7, 4.2, 4.5,	6.5, 4.7, 5.7, 5.5, 5.6, 5.5, 5.3, 6.5	5.8, 5.6, 4.5, 4.0, 4.2
Females	18.2, 17.5	16.9	17.4
Intermediate			
Males	13.7, 13.8, 15.4, 8.4	15.3, 9.4, 12.5, 11.6, 9.0, 10.3	10.5, 15.0, 9.6, 8.1, 11.3
Females	20.0	23.3, 21.4	19.5
High			
Males	21.2	29.9, 33.6	–
Females	26.4, 26.2	25.9	–

* μ moles of dichlorophenolindophenol reduced per minute per gram of liver.

segregations significantly.

DT diaphorase activity (table 2) was consistently lower than had previously been reported for non-resistant Sprague–Dawley rats [4]. Nevertheless in the F2 males the activity of the enzyme was more variable than had been found in the grandparental resistant strain [4] ($F = 22.6; p<0.001$) while in females it was significantly more active than in the grandparental strain [4] ($t = 3.110; p<0.01$). The enzyme activity did not appear, however, to segregate in a readily recognisable manner. The results for the 48 animals were therefore divided into the three categories shown in table 2, one showing low DT diaphorase activity, within the limits previously found for homozygous resistant rats [4] and two arbitrary categories showing intermediate and higher levels of activity. A chi squared analysis of this classification did not indicate that the different levels of enzyme activity were significantly associated with particular resistance genotypes ($\chi^2 = 2.734; p = 0.5–0.7$).

These observations appear to show conclusively that DT diaphorase is not involved in the biochemical mechanism of monogenic resistance to warfarin. Further research will be required to elucidate the mode of inheritance of DT diaphorase activity and to determine whether the large inter-strain differences previously reported [4] are connected with the subsidiary variations in warfarin susceptibility [6] and vitamin K requirement [7] that occur in rats.

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