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Studies on Metatherian Sex Chromosomes VIII.* Evidence for an Absence of Dosage Compensation at the Glucose-6-phosphate Dehydrogenase Locus in Cultured Cells of *Macropus rufogriseus*

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Abstract

The level of the sex-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) has been measured in cultured fibroblasts of *M. rufogriseus* and several other kangaroo species. The data obtained support the conclusion obtained from other evidence that there is a failure of dosage compensation at this locus in fibroblasts of females.

Introduction

In several species of kangaroos the enzyme glucose-6-phosphate dehydrogenase (EC 1.1.1.49; G6PD, locus symbol *Gpd*) exhibits intraspecific variation in electrophoretic mobility which is controlled by an X-linked locus (Richardson *et al.* 1971; Johnston *et al.* 1975; Johnston and Sharman 1975). In these species the *Gpd* locus is subject to dosage compensation by paternal X inactivation. Most of this work has been carried out on erythrocytes. Where other tissues of *Gpd* heterozygotes have been examined no evidence for any activity of the paternally derived allele has been found (Johnston *et al.* 1978). In blood and other tissues males and females have either F or S allozymes, never both. Thus dosage compensation appears to be complete in the living animal. However, when fibroblast cells from heterozygotes are cultured *in vitro* and examined electrophoretically for their G6PD type a third pattern, designated I, is obtained (Johnston *et al.* 1978). This pattern indicates that both alleles are active within the one cell and so raises the question whether there is any dosage compensation for G6PD in cultured fibroblasts. In this paper data are reported which show that in fibroblasts of *Macropus rufogriseus* it is probably absent.

Materials and Methods

Animals

The principal species used was *Macropus rufogriseus* (Desmarest) which has two races, *M. r. rufogriseus* (Bennett's wallaby) and *M. r. banksianus* (the red-necked wallaby). Bennett's wallaby is found in Tasmania and on King Island, Bass Strait. It is uniformly G6PD-F on electrophoresis. The red-necked wallaby is found in New South Wales and Queensland and is polymorphic for G6PD-F and G6PD-S (Johnston *et al.* 1975). The *M. rufogriseus* animals used in this investigation are from two populations. One group consists of the two subspecies and hybrids between them. These animals have been kept in captivity in Canberra for several generations, and there is some degree of common ancestry amongst them. The other group is a sample of animals from a red-necked population in Queensland. The remaining macropodid species and subspecies were *Macropus*

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giganteus Shaw (the eastern grey kangaroo), *Macropus parma* Waterhouse (the parma wallaby), *Megaleia rufa* (Desmarest) (the red kangaroo), and hybrids of *Macropus robustus erubescens* ♀ × *Macropus robustus robustus* ♂. Details of origins of individual animals are given in the tables which contain the enzyme level data.

Blood

Blood was collected from a lateral tail vein of animals kept in captivity and put into citrated saline (5 g NaCl, 17·4 g trisodium citrate $2H_2O$ made up to 1 litre). Blood from the Queensland population of red-necked wallabies was collected from the heart or throat of animals shot in the field. The red cells were washed once in physiological saline and stored in an equal volume of freezing solution [40% (v/v) ethylene glycol, 6% (w/v) sodium citrate in distilled water] to packed red blood cells at −30°C. Enzyme assay was performed within 1 week of collection if the blood was kept in citrated saline or else the samples stored in freezing solution were used. To prepare a haemolysate for enzyme assay a blood sample was centrifuged at 3000 rev/min and the packed red blood cells were lysed in distilled water at a dilution of 1:30.

Table 1. Comparison by *t*-tests of the activity of G6PD in erythrocytes of two populations of red-neck wallabies classified according to various criteria

Values in parentheses are the numbers of animals. Values in italics are 1·96 × s.e.

Population	Mean activity ±s.e. (units/g haemoglobin)		t or t^1	P
	(*a*) Allozymic type			
	F	S		
Warwick, Qld	6·3±0·6 (12)	12·1±1·2 (9)	$t^1 = 4{\cdot}34$*	<0·01
	1·18	*2·35*		
Canberra	7·7±0·6 (12)	4·0±0·9 (3)	$t = 2{\cdot}72$*	0·01–0·02
	1·18	*1·76*		
	(*b*) Sex			
	♀	♂		
Warwick, Qld	6·7±0·7 (9)	10·3±1·3 (12)	$t^1 = 2{\cdot}52$*	0·01–0·05
	1·37	*2·55*		
Canberra	6·7±1·0 (8)	7·3±0·8 (7)	$t = 0{\cdot}43$	0·6–0·7
	1·96	*1·57*		
	(*c*) Sex within the F allozymic type			
	♀	♂		
Warwick, Qld	6·2±0·8 (7)	6·4±0·9 (5)	$t = 0{\cdot}15$	0·8–0·9
	1·57	*1·76*		

* Means are significantly different.

Cell Culture

Cells were cultured as described in Cooper *et al.* (1977). The cells were removed from the culture vessel with 0·05% (w/v) pronase. The total number of cells present in the culture was determined by counting the number of cells in a haemocytometer. After centrifuging at 1000 rev/min for 10 min the supernatant medium was removed with a Pasteur pipette. A known volume of distilled water (20 or 40 µl per 10^5 cells) was then added to suspend the cells. The cells were lysed by freezing and thawing twice in a deep-freeze unit.

Enzyme Assay

The G6PD assay procedure was modified from the World Health Organization (1967). The content of the reaction mixture was as follows (volumes expressed as millilitres per millilitre of reaction mixture): 0·75 ml buffer [0·05 M tris–maleate, pH 8·4, 0·1 M $MgCl_2$ (Unilab)], 0·1 ml 2·0 mM NADP (Calbiochem), 0·50 ml lysate, 0·1 ml 6·0 mM glucose-6-phosphate (Calbiochem). 3-ml cuvettes with a 1-cm light path were used in assaying G6PD in haemolysates. Microcuvettes holding

0·6 ml were used for cultured fibroblasts. The increase in optical density at 340 nm was measured with a Shimadsu MPS-50L or Varian Model 635 recording spectrophotometer at 22°C. For the pH optimum curve 0·05 M tris–maleate was used for pH 5·8–8·7 and 0·05 M glycine–NaOH for pH 8·6–10·2. All buffers were made 0·1 M $MgCl_2$. In fibroblasts enzyme activity was expressed as units per 10^5 cells and in blood as units per gram of haemoglobin. The amount of haemoglobin in the lysate was measured by the cyanomethaemoglobin method (Faulkner and King 1970).

Table 2. G6PD activity of cultured fibroblasts and erythrocytes from individual animals of *Macropus rufogriseus*

Animal (passage No. of cultured cell)	Age	G6PD allozymes[A]	Origin (and pedigree)	G6PD activity Fibroblast (units × 10^4/ 10^5 cells)		Erythrocyte (units/g haemoglobin)
			(*a*) Females			
n79 (1)	Adult	F	Tasmania (n20 × n30)	174		7·4
n87 (1)	Adult	F	Tasmania (n58 × n62)	58		11·9
n99 (1)	Adult	I	Warwick (n85 × n62)	116		5·2
n2 (0)	Adult	I	Warwick, Qld	121		n.t.[B]
n5 (1)	Adult	F	Warwick, Qld	97		4·3
n6 (0)	Adult	I	Warwick, Qld	108		4·2
n7 (1)	Adult	F	Warwick, Qld	133		11·6
n14 (0)	Adult	F	Warwick, Qld	60		3·6
n19 (0)	Adult	I	Warwick, Qld	77		6·1
n20 (0)	Adult	I	Warwick, Qld	173		n.t.
n13 (0)	*c.* 10 months	I	Warwick, Qld	99		n.t.
			(*b*) Males			
n1 (5)	Adult	F	Flinders Island	58		n.t.
n2 (0)	Adult	F	Flinders Island	34		n.t.
n95 (0) (1)	Adult	F	Captains Flat, N.S.W.	52, 62	57	8·1
n100 (0) (1)	Adult	F	Tasmania (n79 × 62)	49, 35	40	9·5
n109 (0)	Adult	F	N.S.W. and Qld (n86 × n95)	52		12·9
n10 (0) (1)	*c.* 10 months	F	Warwick, Qld	59, 129	94	10·7
n27 (0)	*c.* 4 months	S	Warwick, Qld	40		28·6

[A] The I pattern is found only in females of the heterozygous genotype Gpd^S/Gpd^F. See Johnston *et al.* (1978) for further explanation.
[B] Not tested.

Results

Initial experiments were carried out with *M. giganteus* erythrocytes because they were readily available. Dilution of the haemolysate below 150 µl/ml resulted in a decrease in enzyme activity per gram of haemoglobin. All assays were accordingly carried out with concentrations of haemolysate at or greater than this value. There was a broad pH optimum between 7 and 9. In subsequent experiments it was assumed that these results also held for the other species in the study.

A series of *t*-tests was performed to reveal the presence of any significant differences in erythrocytic G6PD activity between males and females and between the G6PD-F and G6PD-S allozymes for the two *M. rufogriseus* samples. These *t*-tests are presented in Table 1. Where there was a significant difference between the variances of the two

characteristics a modified *t*-test, giving t^1, was done as described by Snedecor and Cochran (1967). A significant difference was found between the G6PD-F and G6PD-S allozymes in the Queensland population, G6PD-S having greater activity than G6PD-F. Males also had significantly higher activity than females in the Queensland population. This is probably because of the greater number of G6PD-S males than G6PD-S females, i.e. in this population the more active G6PD-S enzyme was unequally distributed between the two sexes. In order to obtain an unbiased estimate of the sex differences a *t*-test was done for males and females within the G6PD-F group and no significant difference in activity was found. In the Canberra population the G6PD-F and G6PD-S activities were also significantly different, but here G6PD-F had greater activity than G6PD-S. However, there were only three G6PD-S animals in this group so this may be an unreliable estimate of the difference between G6PD-F and G6PD-S activities. No significant difference was found between male and female enzyme activities in the Canberra population. Thus there is no evidence against the hypothesis of dosage compensation for G6PD activity being present in erythrocytes of *M. rufogriseus*.

Table 3. Comparison by *t*-tests of the activity of G6PD in cultured fibroblasts and erythrocytes of red-neck wallabies (data in Table 2) classified according to various criteria

Values in parentheses are the numbers of animals. Values in italics are 1·96 × s.e.

Cell type	Mean activity ± s.e.		t or t^1	P
	(*a*) Sex for fibroblasts			
	♀	♂		
Fibroblast[A]	111 ± 11·81 (11)	54 ± 7·60 (7)	$t = 3·39$*	0·01–0·001
	23·15	*14·90*		
	(*b*) Sex for erythrocytes			
	♀	♂		
Erythrocytes[B]	6·8 ± 1·16 (8)	14·0 ± 3·74 (5)	$t^1 = 1·84$	>0·05
	2·27	*7·33*		
	(*c*) Allozymic types (F or I) for fibroblasts within females			
	G6PD-F	G6PD-I		
Fibroblast[A]	104 ± 22·18 (5)	116 ± 13·10 (6)	$t = 0·43$	0·6–0·7
	43·47	*25·68*		
	(*d*) Race for fibroblasts			
	Tasmania (*M. r. rufogriseus*)	Mainland (*M. r. banksianus*)		
Fibroblast[A]	70 ± 21·19 (6)	98 ± 10·73 (12)	$t = 0·81$	0·4–0·5
	41·53	*21·03*		

[A] Units × 10^4 per 10^5 cells. [B] Units per gram haemoglobin.
* Means are significantly different.

The data for activity in cultured fibroblasts of *M. rufogriseus* are shown in Table 2, together with data on activity in erythrocytes of some of the same animals. There is large variation between animals for both measures. For the fibroblasts there is a significant departure from the expectation of equality of means in the two sexes; the ratio of values between the two sexes is very nearly 2:1 for female:male (Table 3*a*). For the same animals in erythrocytes the ratio is reversed, but the difference in this

case is not significant (Table 3*b*). The high activities in the males are in the two young animals, which is to be expected since erythrocytic G6PD activity is elevated in very young animals (Kronfield *et al.* 1967). For fibroblasts there does not seem to be any association between allozyme pattern and G6PD activity; in females the F animals had a mean of 104 units and the heterozygous I animals 116 units (Table 3*c*). The means for the fibroblasts of the two races do not differ significantly (Table 3*d*). The fibroblast cultures were all primary cultures or the first passage (except n1 (5), Table 2). There is no significant difference between G6PD activity in the primary cultures and the first and subsequent passages (for primary cultures the mean G6PD activity $\pm$s.e. is 77 ± 12 units $\times 10^4$ per 10^5 cells; for first and subsequent passages it is 96 ± 15 units $\times 10^4$ per 10^5 cells; $t_{19}= 0{\cdot}95$, $0{\cdot}2 < P < 0{\cdot}4$).

Table 4. G6PD activity in fibroblast cultures from males and females of four kangaroo species

For females the mean G6PD activity $\pm$s.e. is 92 ± 20 units $\times 10^4$ per 10^5 cells; for males it is 60 ± 11 units $\times 10^4$ per 10^5 cells. On the hypothesis of 1:1, $t_{10}= 1{\cdot}28$, $0{\cdot}2 < P < 0{\cdot}4$. On the hypothesis of 2:1, $t_{10}= 0{\cdot}27$, $P\simeq 0{\cdot}8$

Species	Animal (passage No. of cultured cell)	Origin[A]	Sex	Allozymes	G6PD activity[B] (units $\times 10^4/10^5$ cells) (1)	(2)
(*a*) *Macropus giganteus*	SG60 (1)	M.U.	♀	S		42
	SG14 (1)	M.U.	♀	S		34
	SG62 (1)	M.U.	♂	S		24
	SG56 (2)	M.U.	♂	S		39
(*b*) *Macropus parma*	P6 (0)	M.U.	♀	S	58	
	(1)				110	92
	(2)				116	
	(4)				67	
	P178 (1)	M.U.	♂	S	64	
	(2)				125	90
	(4)				81	
(*c*) *Macropus robustus erubescens* (♀) × *M. r. robustus* (♂)	ew1 (3)	M.U.	♀	I		157
	ez22 (6)	M.U.	♀	I		93
	ew82 (0)	CSIRO	♂	n.t.[C]	61	69
	(2)				77	
	ew73 (0)	CSIRO	♂	n.t.	107	90
	(3)				74	
(*d*) *Megaleia rufa* (≡ *Macropus rufus*)	K2 (2)	M.U.	♀	S	61	
	(3)				204	135
	(4)				174	
	K4 (0)	M.U.	♂	S		50

[A] M.U.= Macquarie University Fauna Park; CSIRO = Division of Wildlife Research, CSIRO, Canberra.

[B] (1) Indicates values for individual passages of the cultures, (2) indicates average values for one or more passages.

[C] Not tested.

Further data for four other species of kangaroos are shown in Table 4. Although there is considerable variation between species, a comparison by a *t*-test between sexes across species is valid because there are equal numbers of the two sexes within each species. The mean G6PD activities for females and males are respectively 92 and 60 units $\times 10^4$ per 10^5 cells. The *t*-tests given in Table 4 show that these data

are compatible with both the hypothesis of equality and the hypothesis that females have double the activity of males. The cultures from these four species range from the primary culture to the sixth passage. There is large variation between different passage numbers of the same culture, which could be related to the length of time the cells have been in culture. The two *Macropus parma* cultures (Table 4*b*) both show a peak in G6PD activity after two passages, with a subsequent decline. More data are required to investigate this problem. The data in Table 4 are presented as a preliminary comparison of G6PD activity in males and females of these species.

Discussion

The data reported here for activity of G6PD in blood support the conclusion from earlier work summarized above, namely that there is dosage compensation for G6PD level. While some association between sex and activity in blood is evident in our data, there is no suggestion that this can be explained in terms of a failure of dosage compensation. Where there is a departure from equality, males have the greater activity.

For fibroblasts, *M. rufogriseus* females have twice the activity of males. One possible explanation is that in fibroblasts there is an association between allozymic type and activity. Such an association is evident in erythrocytes (Table 1). But since there is no significant difference between F and I animals in female *M. rufogriseus*, and all but one of the males was F, this explanation is not supported by the data (Tables 2 and 3).

It is worth noting that variation in G6PD level in erythrocytes but not in other tissues has been previously described. This arises because the erythrocyte is a long lived cell which does not synthesize protein, and there may be differences in long-term stability of different forms of the enzyme. In some Africans there are three forms of G6PD: A, B and A^-. In erythrocytes the unstable A^- has 8–20% of the activity of A or B but in white cells, in some other tissues, and for the purified enzyme, A^- has the same activity as A or B (World Health Organization 1967; Kirkman 1971). The erythrocyte population of young animals would contain a greater proportion of young cells than the erythrocyte population of adult animals. As the activity of G6PD is decreased in older erythrocytes (Fornaini *et al.* 1969) young animals have a greater amount of active enzyme in their blood than adults. The effect of cell age and long-term enzyme stability is probably not a factor which affects G6PD level in fibroblasts, because these cells are rapidly dividing and manufacture G6PD at one or more stages of the cell cycle (reviewed in Mitchison 1971).

An explanation which should still be entertained is that the difference is racial, i.e. between *M. r. rufogriseus* and *M. r. banksianus*, since our females were mainly *rufogriseus* and our males mainly *banksianus*. The difference between the two races, however, was not significant (Table 3*d*).

In view of the allozyme evidence that there is expression of both alleles in cultured fibroblasts (Johnston *et al.* 1978) we conclude that at present for our data the most acceptable explanation is that there is an absence of dosage compensation at the *Gpd* locus in kangaroos. More data from less genetically heterogeneous populations of macropodids are needed to test the validity of this conclusion, in particular from *M. robustus* where the allozyme data also suggest a breakdown of dosage compensation.

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