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Glutathione reductase activity deficiency in homozygous *Gr1*^{a1Neu} mice does not cause haemolytic anaemia†

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Summary

A glutathione reductase (GR) mutant with approximately 50% residual enzyme activity in blood compared with wild-type was detected amongst offspring of isopropyl methanesulphonate-treated male mice. Homozygous mutants with only 2% residual enzyme activity were recovered in progeny of *inter se* matings of heterozygotes. Results of linkage studies indicate a mutation at the *Gr1* structural locus on chromosome 8. The loss of GR activity was evident both in blood and in other tissue extracts. Erythrocyte and organo-somatic indices did not show differences between wild-types and homozygous mutants, indicating no association between the GR deficiency and haemolytic anaemia in this potential animal model.

1. Introduction

The enzyme glutathione reductase (GR) (EC 1.6.4.2) catalyses the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as a cofactor. This reaction is particularly important in red blood cells, where, in the absence of nuclei or mitochondria, it is one of the few NADP-generating systems. Equally important is the role of GR in maintaining the level of thiol groups in the cell, the absence of which provokes haemolysis (Jacob & Jandl, 1966). The glutathione produced by GR combines with hydrogen peroxide in the presence of glutathione peroxidases to regenerate GSSG, thereby preventing the harmful build-up of hydrogen peroxide. Erythrocyte GR deficiency in man has been reported in association with clinical syndromes of haemolysis and neurological disease.

Using an electrophoretic variant for GR, Nichols & Ruddle (1975) were able initially to map the gene for this enzyme to chromosome 8 in the mouse; the estimated map position is 18·0 cM (Ceci & Mills, 1997). The homologous human gene GSR is located at 8p21·1 (Jensen *et al.*, 1984). The present paper describes the genetical characteristics as well as some biochemical and physiological properties of the mutant GR 180 (allele designation *Gr1*^{a1Neu}) with decreased GR enzyme activity.

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† Dedicated to Jack Favor on the occasion of his fiftieth birthday in appreciation of his many contributions to mammalian genetics.

2. Material and methods

(i) *Mutation induction*

A detailed description of the mutation induction by isopropyl methanesulphonate (iPMS) is given by Ehling & Neuhäuser-Klaus (1995). F1 offspring of this experiment were screened for activity variants of 10 different enzymes (Pretsch *et al.*, 1994). All mice used were obtained from colonies maintained in Neuherberg.

(ii) *Biochemical and physiological characterization of the GR mutation*

Ten-week-old animals of both sexes were used. Heterozygous mutant offspring were selected and backcrossed at least 15 generations to the inbred C3H/El wild-type strain in order to transfer the mutant gene to a defined inbred genetic background. Heterozygotes originating from such backcrosses were mated *inter se* to recover homozygous mutants.

Genetic and physiological characterization of the mutants followed essentially the procedures previously described in detail (Merkle & Pretsch, 1989), including sampling and preparation of blood and tissues, as well as examination of haematological and other physiological parameters.

Enzymatic activity was determined using the automatic analysers ACP 5040 and EPOS 5060 (Eppendorf, Hamburg, Germany). The standard reaction mixture for measuring GR activity contained TRA buffer (0·1 M triethanoleamine/1 mM EDTA buffer, pH 7·5), 5 μM flavin-adenine dinucleotide (Na_2 salt), 0·23 mM NADPH and 2·4 mM GSSG. The GR activity was assayed at 25 °C and monitored by changes in absorbance at 334 nm, which reflects loss of NADPH.

(iii) *Mapping studies*

Heterozygous *Gr1*^{a}/*Gr1*^{a1Neu} mice on the C3H/El background were outcrossed to C57BL/6 partners. One male and one female *Gr1*^{a}/*Gr1*^{a1Neu} animals were subsequently backcrossed to C57BL/6 mice. The backcross progeny were examined for GR activity and recombination fractions were determined between the *Gr1* locus and the markers *D8Mit335*, *D8Mit190* and *D8Mit305*. The map on chromosome 8 flanking the *Gr1* locus was constructed with RI Manager, a program designed by Manly & Elliot (1991). The best gene order was determined by minimizing the total number of crossovers.

(iv) *Statistics*

For statistical comparisons of means between the different genotypes Student's *t*-test was used.

3. Results

(i) *Original mutant*

Altogether 1707 offspring derived from iPMS-treated post-spermatogonial cell stages have been screened for enzyme activity mutations. Two mutants with altered erythrocyte enzyme activity were detected: (1) mutant GR 180 with approximately 50% GR activity (described in this study) and (2) mutant G6PD 199 with about 150% glucose-6-phosphate dehydrogenase as well as GR activity compared with the wild-type.

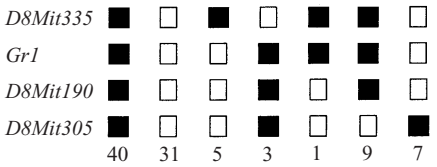

Fig. 1. Haplotype data for the intraspecific backcross showing the markers flanking the *Gr1* locus segregating on chromosome 8. The numbers of backcross progeny for each haplotype are identified by the numbers beneath the columns. C57BL and C3H alleles are designated by the black and white squares, respectively.

(ii) *Genetic characterization and mapping of* Gr1

The results of the matings are summarized in Table 1. In backcrosses of heterozygous mice having roughly 50% GR residual activity in blood with wild-type C3H/El animals, a ratio of approximately 1:1 was seen between wild-type and heterozygous offspring. In matings between two heterozygotes, homozygous mutants with approximately 2% of wild-type GR activity were obtained. There was no significant deviation from the 1:2:1 ratio for wild-type, heterozygous and homozygous mutants. Mean litter sizes of wild-type *inter se* crosses, backcrosses, heterozygous and homozygous mutant *inter se* crosses did not differ significantly.

It is known that GR is coded for by a single locus on chromosome 8 of the mouse (Nichols & Ruddle, 1975). *Gr1*^{a1Neu} was localized by haplotype analysis of 96 progeny from an intraspecific backcross employing three chromosome 8 microsatellite markers (Fig. 1). The deduced map position is: Centromere – *D8Mit335* (8·3 ± 2·8) – *Gr1* – (1·0 ± 1·0) – *D8Mit190* – (16·7 ± 3·8) – *D8Mit305* (distances in cM). The genetic distances are in agreement with published data (Ceci & Mills, 1997).

Table 1. *Genetic characterization of the GR-deficient mouse mutant* Gr1^{a1Neu}

Cross *Gr1* genotype	Mean litter size ± SD (no. of litters)	No. of animals		
		a/a	a/a1Neu	a1Neu/a1Neu
a/a × a/a	6·5 ± 1·9 (20)	130	—	—
a/a × a/a1Neu	6·5 ± 2·1 (66)	210	208	—
a/a1Neu × a/a1Neu	6·5 ± 2·0 (33)	50	112	45
a1Neu/a1Neu × a1Neu/a1Neu	5·0 ± 1·7 (15)	0	0	75

a/a, wild-type; a/a1Neu, heterozygous mutant; a1Neu/a1Neu, homozygous mutant.

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Table 2. *Physiological and biochemical characterization of the GR-deficient mouse mutant* Gr1^{m1Neu}

	a/a	a/a1Neu	a1Neu/a1Neu
Haematocrit (%)	46·8 ± 1·7 (9)	46·8 ± 2·2 (21)	46·1 ± 2·2 (17)
Haemoglobin (g/100 ml)	15·60 ± 1·49 (10)	15·39 ± 1·75 (21)	14·75 ± 0·55 (17)
RBC ($10^4/\mu$l)	827 ± 17 (4)	810 ± 41 (7)	907 ± 143 (13)
MCH (pg)	18·58 ± 1·15 (4)	19·24 ± 1·59 (7)	16·19 ± 2·95 (15)
MCV (fl)	54·72 ± 0·25 (4)	57·82 ± 2·32 (7)	52·25 ± 8·54 (14)
MCHC (g Hb/100 ml)	32·61 ± 4·92 (9)	32·87 ± 2·93 (21)	32·03 ± 2·24 (17)
Body weight (g)	21·7 ± 2·2 (10)	22·0 ± 2·0 (21)	22·7 ± 1·9 (17)
Liver SI	5·31 ± 0·33 (10)	5·62 ± 0·26 (21)	5·44 ± 0·28 (17)
Lung SI	0·59 ± 0·06 (10)	0·61 ± 0·06 (21)	0·56 ± 0·06 (17)
Kidney SI	1·26 ± 0·11 (10)	1·34 ± 0·12 (21)	1·42 ± 0·11 (17)
Spleen SI	0·41 ± 0·06 (10)	0·38 ± 0·08 (21)	0·38 ± 0·17 (17)
Heart SI	0·41 ± 0·03 (10)	0·41 ± 0·03 (21)	0·39 ± 0·03 (17)
GR activity (% of wild-type) in			
Blood		48 ± 10* (21)	2·3 ± 1·5* (17)
Liver		49 ± 7* (18)	8 ± 1* (16)
Lung		52 ± 6* (21)	9 ± 3* (16)
Kidney		51 ± 6* (17)	3 ± 1* (17)
Spleen		49 ± 5* (18)	8 ± 3* (17)
Heart		62 ± 4* (19)	21 ± 10* (14)
Brain		59 ± 5* (19)	14 ± 5* (16)

For the genotypes, the same symbols are used as in Table 1. Values are mean ± SD, with the number of animals tested in parentheses. SI, somatic index (organ weight × 100/body weight); MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; Hb, haemoglobin.
* Significant difference ($P \leqslant 0{\cdot}1$) between wild-types and mutants.

(iii) *Physiological characterization*

Routine haematological tests were performed to determine the possible effect of the GR deficiency on erythrocyte metabolism and to exclude the possibility that the reduced GR activity in blood results indirectly from altered erythrocyte dynamics. In heterozygous as well as in homozygous mutants no deviations from the wild-type values were observed for the haematocrit, haemoglobin, number of red blood cells, mean corpuscular haemoglobin, mean corpuscular volume and mean corpuscular haemoglobin concentration. Other physiological traits, such as body weight and organo-somatic indices (organ weight × 100/body weight) of liver, lung, kidney, spleen and heart also indicated no differences between wild-types and mutants (Table 2). These findings demonstrate that the mutation in either the heterozygous or homozygous state does not affect physiological functions. Moreover, the results obtained in the haematological tests indicate that the reduction in GR activity is not a result of an alteration in haematopoiesis.

(iv) *Blood enzyme activities and GR activity in several tissues*

The level of GR activity in blood and tissues of wild-type, heterozygous and homozygous mice are shown in Fig. 2. GR levels in liver, lung, kidney and spleen of heterozygous and homozygous animals was reduced to levels comparable to those observed in blood. In contrast, brain and heart exhibited smaller reductions in GR activity.

The activities of the erythrocyte enzymes glucose-6-phosphate dehydrogenase, glucose phosphate isomerase, triose phosphate isomerase, gluceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, phosphoglyceromutase, pyruvate kinase and lactate dehydrogenase were determined in blood lysate. There were no significant alterations in any of these parameters for the three genotypes (data not given).

4. Discussion

In mice, typical indications of haemolytic anaemia are a marked reduction in the number of red blood cells, reduced haematocrit, and a lowered concentration of haemoglobin. In several series of mutagenicity experiments we have detected enzyme activity mutations causing haemolytic anaemia. A mutation of lactate dehydrogenase (LDH) (Pretsch & Charles, 1980) resulted in an extreme erythrocyte LDH deficiency as well as in a severe haemolytic syndrome in homozygous individuals indicated by an enormously enlarged spleen (Kremer *et al*., 1986, 1987). Merkle & Pretsch (1993) also described the first two mutations causing hereditary glucose-6-phosphate isomerase (GPI) deficiency associated with chronic non-spherocytic haemolytic anaemia. As in humans, the hae-

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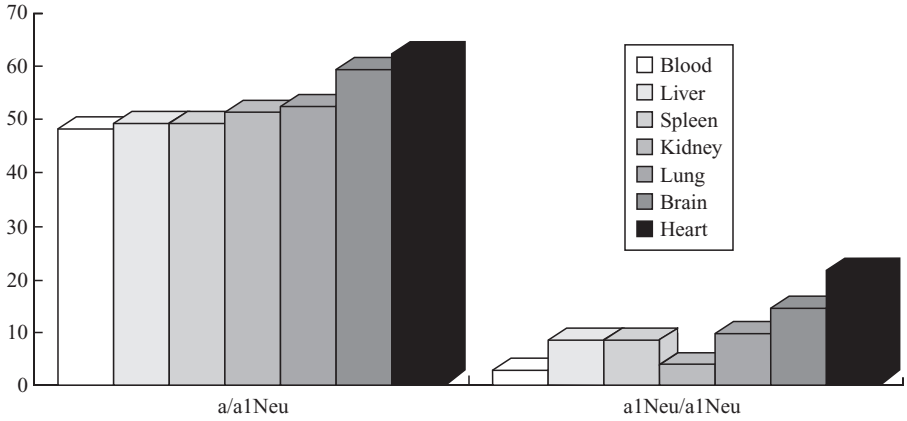

Fig. 2. Levels of GR activity (in % of wild-type) in blood and several tissues of heterozygotes (a/a1Neu) and homozygotes (a1Neu/a1Neu) of the GR-deficient mouse mutant *Gr1*.

molytic syndrome was exclusively manifest in homozygous mutants. Anaemic mice showed hepatosplenomegaly and typical adaptations to hypoxia, such as elevated heart-somatic and lung-somatic indices. Further, these animals were characterized by a marked reduction in body weight and an increase in lethality. By way of contrast, Padua *et al.* (1978) reported a mutant *Gpi1* allele that caused only mild haematological symptoms (increased autohaemolysis) in homozygotes. The enzyme activity level of these mice is comparable with that of human variants who show signs of haemolytic anaemia.

An X-linked glucose-6-phosphate dehydrogenase (G6PD) deficiency in the mouse reduced G6PD activity levels to about 15% in the blood of hemi- and homozygotes as compared with the wild-type (Pretsch *et al.*, 1988). Haematological properties were investigated and the erythrocyte indices exhibited no significant differences between the different genotypes. This is in accordance with human G6PD variants. In most subjects the haematological phenotype is practically normal, and acute haemolysis, which can be severe and even life-threatening, is seen only in response to an exogenous trigger.

In man, deficiency of erythrocyte GR has been reported in association with diverse clinical syndromes, including haemolysis and neurological disease. Löhr & Waller (1962) reported a 'novel' form of enzyme-deficiency haemolytic anaemia, where decreased GR activity was postulated to result in a strongly diminished content of erythrocyte glutathione. A syndrome including deficiency of GR activity in blood cells and other haematological disorders as well as neurological disturbances was studied by Waller (1968) in 47 patients. Kurz & Hohenwallner (1970) described a 12-year-old boy who was mildly anaemic and had a palpable spleen. Erythrocyte GR activity of about half the normal level was detected in both the patient and his mother. In other reports GR deficiency has been accompanied by thrombocytopenia or pancytopenia without evidence of haemolytic anaemia, and in one family by mental deficiency (Waller *et al.*, 1965); while Pohl and co-workers (1976) described the occurrence of haemolytic anaemia in five members of a family in three generations where the GR activity was about half the normal and where there appeared to be abnormal membrane phospholipid metabolism, including markedly decreased lysolecithin-acyl-transferase activity.

It is unclear whether these syndromes represent specific genetic entities or, as Beutler (1969*a*, *b*) has shown, GR deficiency results indirectly from a deficiency of the cofactor flavine adenine dinucleotide, probably as a consequence of dietary deficiency of riboflavin. The difficulty in establishing that GR deficiency could have been responsible for the haemolytic anaemia or cytopenia in the cases cited above stems from two considerations (Dacie, 1985): first, it has been shown that GR activity is dependent upon the state of riboflavin nutrition and, secondly, a family has been described by Loos *et al.* (1976) where a virtually complete absence of GR activity was found in the erythrocytes of all three children (one male, two females) from a consanguineous marriage. Clinically, this deficiency was manifested by a haemolytic crisis precipitated by eating fava beans in the eldest daughter.

$Gr1^{a1Neu}/Gr1^{a1Neu}$ mutants exhibit approximately 2% wild-type enzyme activity without evidence of haemolytic anaemia. Mutant animals had normal haematological parameters and no evidence of the characteristic enlargement of the spleen.

G6PD and GR are metabolically linked: the pentose phosphate pathway is the only source of NADPH in red blood cells because they lack mitochondria, and so the production of NADPH is markedly diminished by G6PD deficiency. The major role of NADPH in red blood cells is to reduce the disulphide form of GSSG to the sulphhydryl form catalysed by GR. The reduced form of glutathione, GSH, a tripeptide with a free

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sulphhydryl group, serves as a sulphhydryl buffer that maintains the cysteine residues of haemoglobin and other red-cell proteins in the reduced state. It also plays a role in detoxification by reacting with hydrogen peroxide and organic peroxides and is essential for maintaining the normal structure of red blood cells and for maintaining haemoglobin in the ferrous state. GR deficiency *per se* does not produce haemolytic anaemia, as demonstrated by Loos *et al*. (1976) and in the GR-deficient mice described here; but it seems that red blood cells with a lowered level of GSH may be more predisposed to haemolysis. Investigations are considering whether haemolytic anaemia could be induced by a dietary deficiency of riboflavin in GR-deficient mice, as has been postulated for man.

In summary, it can be concluded that GR deficiency in man does not necessarily cause haemolytic anaemia and that many cases of haemolytic anaemia attributed to a genetic deficiency of the enzyme will be of other origin.

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