Single Locus (rol) Control of Extreme Resistance to Red Cell Osmotic Lysis: Intrinsic Mode of Gene Action

Catherine A. Schaefer and Michael J. Dewey

Department of Biology, University of South Carolina, Columbia, South Carolina 29208

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ABSTRACT

Previous work has indicated that inbred mouse strains C57BL/6 and DBA/2 produce red cells differing in their sensitivity to osmotic lysis and that the trait is under multigene control. A recombinant inbred strain (BXD-31), produced from C57BL/6 and DBA/2, has red cells manifesting resistance to osmotic lysis far greater than that of either progenitor. We demonstrate here that the fragility difference between BXD-31 and DBA/2 is the consequence of allelic variation at a single autosomal locus, termed rol. The resistance allele (rol') is almost completely recessive to the sensitive one (rol). Results of bone marrow chimera analyses indicate that (1) the mode of rol gene action is by a direct influence on the properties of the red cells rather than an indirect influence on their extracellular milieu, and (2) rol does not affect erythrocyte production and turnover. The fragility difference caused by rol variation is likely to involve the erythrocyte membrane or underlying cytoskeleton, since various red cell properties sensitive to ion metabolism differences are unaffected by the gene.

Genetic variation among animals often provides tools to study a variety of functions. For example, molecular geneticists are currently exploiting genetic variation among mouse inbred strains at the biochemical level (Paigen et al. 1975) to learn more about gene regulation. Similarly, the study of mouse mutants influencing blood formation has contributed much to our understanding of hematopoiesis (Russell 1979; Van Zant et al. 1983). Yet this genetic approach is relatively unused to study the factors that maintain the structural integrity of cells and their membranes.

It is toward this goal that we have been studying the genetic factors influencing red cell osmotic fragility in mice (Dewey, Brown and Nallaseeth 1982; Norman and Dewey 1985). Although many factors such as diet (Ehrstrom, Harms-Ringdahl and Alling 1981) and liver function (Cooper and Jandl 1968) are known to extrinsically influence osmotic fragility. It is also reasonable to assume that intrinsic factors directly controlling membrane structure and integrity will also affect osmotic fragility.

In 1982 we initially described a strain difference for osmotic fragility (Dewey, Brown and Nallaseeth 1982). Red cells from strain C57BL/6 were considerably more sensitive to lysis than those from DBA/2. To determine the number and possible identity of loci responsible for this difference we undertook an analysis of recombinant inbred mouse strains (Swank and Bailey 1973; Taylor 1976) derived from C57BL/6 and DBA/2 (Norman and Dewey 1985). The results indicated that differences at two to four distinct loci account for the fragility differences between C57BL/6 and DBA/2 mice. Thus, the phenotypes of almost all 24 recombinant inbred lines corresponded to either that of one of the progenitors or fell in between the two. One line, BXD-31, was exceptional, however. BXD-31 displayed a resistance to lysis that was much in excess of that of the resistant progenitor line (DBA/2). This observation suggested the possibility that strain C57BL/6, although exhibiting a susceptible phenotype, contains a gene (or genes) conferring some resistance. The converse would be true for DBA/2. BXD-31 would then be a strain bearing all the resistance alleles of strain DBA/2 along with one or more from C57BL/6.

In the present report evidence is presented which indicates that the fragility difference between BXD-31 and DBA/2 is indeed the consequence of allelic variation at a single locus. For its effect on the resistance to osmotic lysis of erythrocytes we term this locus rol. Furthermore we demonstrate that the mode of rol gene action is intrinsic to the erythrocytes themselves rather than an indirect influence on their extracellular milieu.

MATERIALS AND METHODS

Mice: Mice of strains C57BL/6 and DBA/2 were obtained from The Jackson Laboratory (Bar Harbor, Maine). The recombinant inbred line BXD-31/Ty, and H-2 congenic B6.C-H-24/a/By were obtained from the colonies of Benjamin Taylor and Donald Bailey, respectively, both of The Jackson Laboratory. The congenic (n = 9) line DBA/2. Gpi-1" was produced at the University of South Carolina by repeated backcrosses to DBA/2 from the initial (C57BL/6 × DBA/2)F1 hybrid.

During the course of these studies it was discovered that
BXD-31 was genetically contaminated about January 1984 (B. Taylor, personal communication). This was probably by an accidental outcross to another BXD recombinant inbred line. Our original characterization of BXD-31 (Norman and Dewey 1985) was with mice obtained prior to January 1984, whereas the results reported here are from BXD-31 mice purchased soon after that date. Those animals have been maintained at this Institution by successive brother/sister matings and the rol congenic lines are on that background. The possible contamination of BXD-31 does not affect any of the conclusions in this report.

Erythrocyte fragility: Retro-orbital sinus blood was taken in heparinized hematocrit tubes and washed in 0.85% phosphate-buffered saline (pH 7.4) and adjusted to a final volume eight times the initial one with the same buffer. Fifty microliters of the cell suspension were added to 5 ml of each of graded salt solutions. Preparation of the stock salt solution was as previously described (Dewey, Brown and Nallathamby 1982). As soon as all additions were complete, the tubes were centrifuged to pellet unlysed cells and membranes. The relative amount of hemoglobin released into the supernatant was determined spectrophotometrically at 420 nm.

Irradiation chimeras: (DBA/2 × B6.C-H-2"/a)F1 mice were used as host mice for the bone marrow chimera experiments. Mice of that genotype were chosen for (1) being of the identical genotype at H-2 as both of the donor animals and (2) being hybrid at the Gpi-1 locus so as to produce a heterodimeric glucose phosphate isomerase (GPI) band not produced by either of the donor genotypes. The host mice received a lethal dose (950 R) and were immediately injected with 1 × 106 bone marrow cells composed equally of bone marrow cells from mice of strains DBA/2.Gpi-11 and BXD-31.

Mosaic analyses: Donor takeover and strain proportions were monitored by analyses for strain-specific electrophoretic variants of GPI (Eppig et al. 1977). The actual composition was determined by comparison with standard gels derived from artificial mixtures of known proportions from pure strain animals (Behringer, Eldridge and Dewey 1984). Blind control experiments indicated that the mosaic composition estimated by this means was usually within five percentage points of the true value. Platelets for analysis were harvested from the supernatant of blood diluted with phosphate buffered saline and centrifuged for 10 min at 750 × g to pellet the red and white cells. The platelet rich supernatant was then centrifuged for 10 min at 4500 × g to pellet the platelets (Van Zant et al. 1983). Lymphocytes were isolated from blood samples fractionated on step gradients of iso-osmotic Percoll at the phosphate buffered saline-1.09 g/ml Percoll interface.

Hematological studies: Standard procedures were used to determine erythrocyte counts and hemoglobin content in a Coulter particle counter and hemoglobinometer; to determine the hematocrit by centrifugation of blood in microhematocrit tubes; and to calculate the mean cell volume. Reticulocytes were estimated from the percentage of erythrocytes staining with brilliant cresyl blue. Cell water content was based on weight differences of blood samples before and after overnight exposure to 100%.

RESULTS

BXD-31 is a recombinant inbred strain derived from the progenitor strains C57BL/6 and DBA/2. Osmotic fragility profiles of BXD-31 red cells exhibit a striking resistance to osmotic lysis in comparison with DBA/2 red cells (Figure 1). Fragility curves from the two strains reveal that their 50% lysis points differ by 0.15 percentage points of NaCl concentration. This difference between DBA/2 and BXD-31 is threefold greater than the original difference measured between DBA/2 and C57BL/6 red cells (see Figure 1 in Norman and Dewey 1985). Furthermore, red cells from BXD-31 are markedly more resistant to osmotic lysis than red cells from any of the other inbred strains tested including BALB/c, CBA, C3H, RF, ICR, A, SJL, and AKR (not shown). Thus, the BXD-31 fragility phenotype is an extreme one and uncommon among mouse strains.

Genetic analyses: Based on considerations outlined in the introduction, we thought it possible that allelic variation at a single locus could be responsible for fragility differences between BXD-31 and DBA/2. The results of the segregation analysis between these two strains are presented in Figure 2. BXD-31 mice crossed to DBA/2 mice produced progeny whose red cells were all phenotypically comparable to DBA/2 red cells. Thus, susceptibility is the dominant phenotype. The hybrids were subsequently backcrossed to BXD-31 and produced two phenotypically distinct classes of offspring, one of which corresponded to the susceptibility phenotype of the (BXD-31 × DBA/2)F1 hybrid and the other to the resistance phenotype of BXD-31. Susceptible phenotype animals from the initial backcross were then serially backcrossed to BXD-31 and the results were identical to those of the first backcross (for example, see backcross 5, Figure 2). From the composite results approximately equal numbers of animals of each phenotype (195 resistant and 222 susceptible) were produced from 9 successive backcross generations.
influenced by factors external to the erythrocytes and which are possibilities we turned to bone marrow chimeras (Figures 3) containing red cells of the two genotypes. Such mosaic animals produce a mixture of red blood cells respectively, these fragility differences could be the result of erythrocyte osmotic fragility could be the direct consequence and DEWEY 1985).

Such segregation results clearly indicate single locus control for the fragility trait. Furthermore, during the serial backcrossing some of the resistant phenotype mice were crossed to each other and produced offspring of only the resistant phenotype. Animals with the hybrid phenotype crossed with each other produced both hybrid and resistant offspring (not shown). This single locus is henceforth designated rol.

In Figure 1 are presented fragility profiles for resistant and susceptible phenotype segregants from the fifth backcross. It can be seen that the phenotypes of the backcross animals differ little from the phenotypes of the progenitor strains. Therefore, virtually all the fragility difference between BXD-31 and DBA/2 can be accounted for by rol variation.

The rol locus is not likely to reside on the non-pairing region of the X chromosome since in the sequential backcrossing susceptible phenotype males crossed to BXD-31 segregated both resistant and susceptible phenotypes of both sexes. BXD-31 and DBA/2 do not differ at the locus for the hemoglobin beta chain (BEN TAYLOR, personal communication). Furthermore, congenic strains of mice with either the diffuse or the single alleles of Hbb produce erythrocytes with comparable fragility characteristics (NORMAN and DEWEY 1985).

Mode of rol gene action: The difference in erythrocyte osmotic fragility could be the direct consequence of rol gene action in erythrocytes. Alternatively, these fragility differences could be the result of factors external to the erythrocytes and which are influenced by rol. To distinguish between these two possibilities we turned to bone marrow chimeras (Figure 3) containing red cells of the two genotypes. Such mosaic animals produce a mixture of red blood cells from genotypically distinct populations of precursor cells, but would be expected to exhibit a homogeneous environment bathing the erythroid progenitor cells as well as mature erythrocytes. If the mode of rol gene action is indeed intrinsic to the red cells then the rol" and rol" red cells in chimeras would still retain their difference in osmotic fragility. On the other hand, if genetically controlled environmental differences are responsible, there would be no difference in fragility between red cells of the two genotypes that mature and circulate in the same animal.

As outlined in Figure 3 and MATERIALS AND METHODS host mice were irradiated and reconstituted with bone marrow cells containing equal contributions of DBA/2 and BXD-31 cells. Care was taken to ensure that both donor strains and the host animals were of the same genotype at the H-2 locus so as to avoid possible difficulties arising from hybrid resistance (CUDKOWICZ 1961). Strain-specific electrophoretic variants of GPI provided a marker system with which to monitor and quantify red cell mosaicism within the bone marrow chimeras. The same marker was used to establish the completeness of reconstitution since the host mice were Gpi-1 hybrid and therefore expressed the electrophoretically distinct heterodimer. Figure 4, lanes 1-4, illustrates the utilization of the GPI marker system to monitor the donor takeover of the host hematopoietic system. By 5-6 weeks after reconstitution host cells were still detectable, but by 8 weeks only donor cells of the two genotypes were detectable. All of the bone marrow chimeras exhibited a preponderance of susceptible strain (DBA/2) erythrocytes.

Relative fragilities of rol" and rol" erythrocytes were determined on blood samples taken from a number of bone marrow chimeras four months after reconstitution. Mosaic composition determinations were made of either the total red cells or ones surviving
isozymes in blood cells from bone marrow chimeras. The donor strain are DBA/2 (Gpi-1<sup>+</sup>), the sensitive strain, and BXD-31 (Gpi-1<sup>/-</sup>), the resistant one. The host genotype is Gpi-1<sup>+</sup>. Lanes 1-4 show the utilization of this GPI marker system to monitor donor strain takeover of the hybrid host strain as well as the shift over time in the mosaic composition in favor of DBA/2. Shown are results from red cells taken from a bone marrow chimera at 5 weeks (lane 1), 6 weeks (lane 2), 9 weeks (lane 3), and 40 weeks (lane 4) after reconstitution. In lanes 5-8 are shown red cell determinations from another chimera. Blood from this animal was analyzed without prior treatment (lane 5) or as the cell fraction surviving prelysis in 0.45% (lane 6), 0.40% (lane 7), and 0.35% (lane 8) NaCl. In lanes 9-11 are shown GPI determinations from erythrocytes (lane 9), platelets (lane 10), and lymphocytes (lane 11) from a chimera taken 4 months after reconstitution.

lysis with graded concentrations of saline. Results of these experiments are shown in Figure 4, lanes 5-8, and in Figure 5. Clearly, as the osmolality of the lysing solution decreases to 0.40-0.35% NaCl, the surviving cells are increasingly enriched in the proportion of resistant strain (BXD-31) cells. If the experiment is carried out to 0.30 or 0.25% NaCl the trend reverses itself, however. This is presumably due to contaminating white cells, whose GPI composition is at least 10 fold that of red cells (VAN ZANT et al. 1983) and which would be expected to contribute to the GPI readings when the bulk of the red cells have been lysed. Furthermore, evidence for two populations of red cells that differ in susceptibility to osmotic lysis could be observed in the osmotic fragility curves derived from the bone marrow chimeras (not shown). Together these results are a clear indication that the rol mediated erythrocyte fragility difference is an intrinsic red cell property.

**Comparative hematopoietic kinetics of BXD-31 and DBA/2:** Radiation chimeras are also potentially useful for detecting lineage-specific genetic differences in hematopoietic kinetics that might otherwise go unnoticed in studies of pure strain animals (BARKER, MCFARLAND and BERNSTEIN 1982; VAN ZANT et al. 1983). To test the possibility that rol may preferentially influence red cell production the mosaic composition of erythrocytes, lymphocytes, and platelets from several chimeras were compared. In no case was there more difference than 5 percentage points in the composition of cells from the three lineages (for example, see Figure 4, lanes 9–11). Therefore, it does not appear that rol or any other gene difference between BXD-31 and DBA/2 exerts a lineage specific effect on erythrocyte maturation. However, all of the bone marrow chimeras in this study did show a preponderance of DBA/2 blood cells from all three lineages and in some animals a gradual shift over time favoring the DBA/2 genotype even more. This could indicate that DBA/2 stem cells manifest some competitive advantage over stem cells from BXD-31.

Red cell turnover in BXD-31 and DBA/2 was compared to determine whether red cell age differences between the two strains could account for the fragility results. Erythrocyte lifespan, assessed by the rate of disappearance from the circulation of <sup>51</sup>Cr-labeled erythrocytes (NORMAN and DEWEY 1985), was the same for the two strains (not shown). Both had released 50% of the label between 18 and 20 days. This value is consistent with the published data on red cell life span evaluations for DBA/2 (19.7 days; GOODMAN and SMITH 1960).

**Other red cell properties:** Blood smears stained with Wright solution revealed no apparent shape abnormalities of BXD-31 erythrocytes or differences from DBA/2 ones. Red cells from both strains of the semicongenic pair were indistinguishable and appeared in the scanning electron microscope to exist predominantly in the characteristic biconcave disc form (not shown). Similarly between the semicongenic strains no major differences were evident in reticulocyte counts, hematocrits, hemoglobin content, mean cell volumes, and cell water content (Table 1).

**DISCUSSION**

Mouse genetic analyses have benefited greatly with the development and use of recombinant inbred
strains. Questions regarding the number and linkage of loci governing particular traits can be answered with considerably less time and effort compared to traditional segregation analyses. This report underscores a novel and potent application of recombinant inbred strains. This relates specifically to phenotypes under multigene control where, as we demonstrate here, it is possible to genetically isolate and study single genes responsible for modulating a particular characteristic. The characteristic under study is erythrocyte osmotic fragility, one we previously demonstrated to be controlled by several loci differing between C57BL/6 and DBA/2 (Norman and Dewey 1985). From those several it was relatively simple to isolate a single locus (rol) exerting a major effect on fragility. With the same strategy it should be possible to use the BXD recombinant inbred strains to dissect other single loci from the complex mix and study their effects.

We designate this gene rol signifying resistance to osmotic lysis. The dominant allele (rol^{d}) is present in DBA/2 and imparts osmotic susceptibility. The resistance allele (rol^{r}) is present in BXD-31 and imparts resistance to osmotic lysis. A consequence of the serial backcrossing described in this report will be the eventual production of a congenic pair of BXD-31 strains differing only for alleles at rol. This pair of strains will be essential for subsequent studies of rol gene action.

The rationale for studying genes controlling erythrocyte osmotic fragility phenotypes is that some such genes may likely provide tools to supplement ongoing biochemical studies of cellular architecture. Once a gene is defined that influences osmotic fragility, however, it is of vital importance to determine whether the red cell is its target of gene action before any biochemical studies are initiated. For rol that question was posed here, and results from bone marrow chimeras indicate that the rol-directed influence on osmotic fragility is indeed an intrinsic property of the red cell. It is not known whether rol influences characteristics of cells other than mature erythrocytes. The bone marrow chimera results suggest that rol does not specifically affect erythrocyte maturation or turnover.

Other genes have been described in the mouse that influence red cell osmotic fragility and these include ones associated with thalassemia (Anderson et al. 1982) and other genes that cause hemolytic anemia (Bernstein 1980). The rol phenotype differs from these other genes both in its lack of effect on red cell production and turnover as well as the abnormal resistance (rather than susceptibility) produced by the variant.

Since the rol phenotype is an intrinsic erythrocyte property the way is now clear for a concerted biochemical study to determine its mechanism of action. Rol may exert its influence in at least one of two general ways. One would involve effects on ion metabolism, while the other would be direct alteration of the structural integrity of the membrane itself. Perturbations of ion metabolism should be manifest by alterations in cell volume, cell water and cell shape. We have observed effects on none of these properties. On the other hand, preliminary lipid analyses indicate likely differences in fatty acid composition between the congenic strains (R. Keith, C. Shafer and M. Dewey, unpublished data). Thus, rol is likely to directly influence the structure of the erythrocyte membrane.

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LITERATURE CITED


that have completed the first meiotic division. Nature 269: 517–518.


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