BLOOD GROUPS IN SHEEP. I. THE X-Z SYSTEM¹

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PREVIOUS studies of blood groups in sheep, including those of ANDERSEN (1938), RENDEL (1957) and SPRAGUE (1957), have been primarily concerned with the serology and genetics of the R-O system, and with its relationship to the A-B-O system of man, the J system of cattle, and the A-O system of swine. Several investigators (ANDERSEN 1938; YCAS 1949; and DUJARRIC DE LA RIVIÈRE, MILLOT and EYQUEM (1952) have reported that immunizations with sheep erythrocytes may give rise to hemolysins which distinguish differences in sheep bloods other than those seen in R-O typing, but there have been no reports of definitive systems other than R-O. It is the purpose of this report to present evidence for a second system of ovine blood groups, the X-Z system.

MATERIALS AND METHODS

The reagents used in typing for X and Z were developed from heteroimmune antisera which were prepared by immunization of rabbits with erythrocytes of sheep as well as other species in the families Bovidae and Antilocapridae. Certain of the rabbit antisera used in the present study were the same as those prepared and used by STORMONT and SUZUKI (1958). When more than one blood was used to immunize any one rabbit, equal amounts of blood from each donor were pooled. During a period of three weeks, nine intravenous injections were made (on alternate days) of 0.5 ml whole blood or the equivalent in erythrocytes suspended in isotonic saline (0.91 percent sodium chloride solution). The antisera were collected four to seven days after the last injection, and were kept at -20° C when not in use.

The antisera were absorbed with erythrocytes of individual sheep to determine whether they contained specific fractions of antibodies defining individual differences in sheep. Blood for the absorptions was obtained from an experimental flock of 40 sheep. The sheep were kept at the Armstrong Tract of the University of California at Davis, for use in studies of blood groups. They were bled by puncture of the jugular vein, and the blood was collected in 250 ml evacuated bottles containing 100 ml Alsever's solution (2.05 percent dextrose, 0.8 percent sodium citrate and 0.42 percent sodium chloride adjusted to a pH of 6.2 with ten percent citric acid). Samples of the antisera were diluted $\frac{1}{8}$ with isotonic saline

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and heated at 56° C for 30 minutes before absorption. Ordinarily, three successive absorptions (10 min, 30 min, and 30 min) were required for complete removal of antibodies for the absorbing cells when a ratio per absorption of 0.75:1.0 of washed, packed erythrocytes to diluted serum was used. The decanted serum from the third absorption was kept at -20° C if it was not used immediately, and was heated for 15 minutes at 56° C, just prior to testing.

In addition to the blood from the experimental flock, citrated blood samples were obtained for genetic studies from the flock of purebred Shropshires of Mrs. Ida M. Groverman and Son at Petaluma, California, from the flocks at the University of California at Davis which included Suffolks, Hampshires, Rambouillets, Corriedales and Targhees, and from Targhees and Rambouillets at the U. S. Sheep Experiment Station at Dubois, Idaho. The samples were collected in 15 ml tubes containing 3 ml isotonic citrate solution (2.0 gm sodium citrate plus 0.5 gm sodium chloride per 100 ml). The blood samples were refrigerated until used.

The bloods were tested in a hemolytic test. The reaction of antigen (red blood cells) and antibody (antiserum) in the presence of complement was observed. The complement used was either fresh guinea pig serum diluted 1/15 to 1/20 or undiluted absorbed fresh rabbit serum. Often duplicate tests were set up, one with guinea pig and one with rabbit complement. The rabbit complement was absorbed with washed packed erythrocytes from several sheep to remove natural heterohemolysins for sheep red cells. Two absorptions of ten minutes each at refrigerator temperatures, and centrifugation in a refrigerated centrifuge were adequate to remove normal antibodies, using a ratio of cells to serum of 1:4 per absorption.

The blood typing tests were set up as follows: to a 10 × 75 mm tube containing 0.10 ml of the diluted antiserum, 0.05 ml of a two to three percent suspension of washed erythrocytes was added. The tubes containing antiserum and red cells were shaken and allowed to stand a few minutes before the addition of 0.05 ml of complement. The test was read at one half hour, when slight (tr), incomplete ($\pm$) and complete (+) hemolysis was recorded. At 2½ to 3 hours and 4½ to 5 hours, degrees of hemolysis from 0 (no hemolysis), 0+, tr, 1, 2, 3 and 4 (complete hemolysis) were recorded. All tests were performed at room temperature (24° ± 3° C) and the tubes were shaken after the first two readings.

EXPERIMENTAL RESULTS

Preparation of anti-X

Tests of absorptions of an antiserum produced in a rabbit (X3) against the erythrocytes of eight Karakul sheep showed that absorption with the red cells of any one of nine out of ten sheep exhausted all of the hemolysins (Table 1).

Absorption with red cells of No. 1457 failed to remove hemolysins for the red cells of seven of the nine sheep whose red cells seemed to exhaust all the antibodies. Since the erythrocytes of sheep No. 572 and No. 668 absorbed all the antibodies but were nevertheless not lysed by antibodies remaining in the serum following absorption with No. 1457, it seemed probable that dosage reactions

TABLE 1
Tests of absorptions of Karakul (X3) antiserum

Test cells	Absorbing cells										Rabbit complement control
	559	572	595	630	668	999	1457	2055	2083	2355	
559	0	0	0	0	0	0	3	0	0	0	0
572	0	0	0	0	0	0	0	0	0	0	0
595	0	0	0	0	0	0	4	0	0	0	0
630	0	0	0	0	0	0	4	0	0	0	0
668	0	0	0	0	0	0	0	0	0	0	0
999	0	0	0	0	0	0	4	0	0	0	0
1457	0	0	0	0	0	0	0	0	0	0	0
2055	0	0	0	0	0	0	4	0	0	0	0
2083	0	0	0	0	0	0	1	0	0	0	0
2355	0	0	0	0	0	0	3	0	0	0	0

were involved. The antibodies remaining in the serum following absorption with the red cells of No. 1457 might be of proper quantity and quality to lyse erythrocytes from individuals homozygous for a blood factor X, but not those heterozygous for this factor. This anti-X serum stimulated a search for a complementary blood factor, and the discovery of such a blood factor, designated Z, made it easier to study systematically the possible dosage reactions.

Preparation of anti-Z

Anti-Z was discovered in several antisera prepared in rabbits against erythrocytes of individual species, including springbok, blesbok, eland, pronghorn antelope and bison. These antisera were individually absorbed with erythrocytes of two type-X sheep. Selected antisera produced in rabbits against red cells of these species showed rather marked hemolytic activity for erythrocytes of sheep No. 1457, as well as some hemolytic activity for erythrocytes of sheep No. 572 and No. 673 (Table 2).

An antispringbok serum seemed most promising as a source of antibodies against Z, and by individual absorptions of this serum, an appropriate absorbing cell of type X was selected for preparation of a Z reagent.

Effects of dosage and complement

Reactions of the original X and Z reagents were compared in parallel tests on the bloods of the 40 sheep in the experimental flock, and three blood types were defined, as follows: 29 were type X, ten were type XZ, and one was type Z. Twenty rabbits were immunized with erythrocytes of sheep No. 1457, the only type-Z sheep in the flock, and usable Z reagents were prepared from 14 of these antisera. Of 14 rabbits immunized with erythrocytes of type-X sheep, two produced antisera from which usable X reagents were prepared. Over 1000 sheep were tested with X and Z reagents and the existence of three types, X, XZ and Z was confirmed. Not one of the bloods was negative for both X and Z.

TABLE 2

Summary of tests of absorptions of antisera containing anti-Z

Absorbing cells: Test cells	Antisera produced in rabbits against red cells of										Guinea pig complement control
	Bovidae								Antelocapridae		
	Eland (10P)		Blesbok (W)		Springbok (B)		Bison (X32)		Pronghorn (X4)		
	595	630	595	630	595	630	595	630	595	630	
595	0	0	0	0	0	0	0	0	0	0	0
630	0	0	0	0	0	0	0	0	0	0	0
511	0	0	0	0	0	0	0	0	0	0	0
559	0	0	0	0	0	0	0	0	0	0	0
572	2	tr	1	1	3	4	tr	tr	tr	tr	0
634	0	0	0	0	0	0	0	0	0	0	0
673	1	tr	1	1	4	4	tr	tr	tr	tr	0
999	0	0	0	0	0	0	0	0	0	0	0
1457	2	2	4*	4*	4*	4*	2	2	4*	4*	0
2355	0	0	0	0	0	0	0	0	0	0	0

* Complete hemolysis in $\frac{1}{2}$ hour.

The Z reagents consistently lysed type-Z erythrocytes much more rapidly than those of type XZ. The type of complement used in the test was important in contrasting erythrocytes of type X and type XZ. When guinea pig complement was used, type-Z cells were rapidly and completely lysed by the Z reagents, whereas type-XZ cells were lysed more slowly, and the hemolysis was rarely complete at the end of five hours. When rabbit complement was used, type-Z cells were usually lysed as rapidly as if guinea pig complement was used, but the type-XZ cells were often not lysed at all; if there was any hemolysis, it was slight and took place slowly. Z reagents from more than 20 different antisera were studied, and although this difference between rabbit complement and guinea pig complement was more marked with some of them than with others, it was always apparent. In order to clearly differentiate cells of type XZ and Z by the use of Z reagents, each potential reagent was titrated and a dilution of the antiserum was chosen which most clearly differentiated between cells of the two types. The antiserum was then used, at the dilution chosen, as a dosage reagent.

Similarly, the dilution at which the X reagents most clearly differentiated cells of type X and XZ were determined, for the preparation of dosage reagents. As a general rule, the X reagents lysed type-X and XZ cells with equal rapidity when guinea pig complement was used in the test. When rabbit complement was used, almost no lysis was observed in tests with one of the X reagents, but two others prepared from two different antisera lysed most type-X erythrocytes more rapidly than most cells of type XZ. However, the degree of hemolysis varied considerably in different families of sheep, and although these X reagents made it possible in most cases to distinguish between X and XZ erythrocytes from sheep of the same family, it was not dependable for this purpose when erythrocytes of more than one family were tested in the same test. Thus if cells of type X from one family were lysed as slowly as the cells of type XZ from another family, these cells of

type X and type XZ could not be differentiated, even though cells of type X and type XZ within one family were lysed at different rates.

One striking exception to the dosage effects was seen in the tests of the erythrocytes from one of the sheep in the experimental flock. The cells of sheep No. 577 were negative when tested with the X reagent when rabbit complement was used, but were type X rather than type XZ.

Genetic analysis

The serological evidence strongly suggested that X and Z were acting as a pair of complementary blood factors, and that two alleles, X and X^z , controlled the expression of the three phenotypes X, XZ and Z. The distribution of these phenotypes among 40 sheep in our experimental flock checked exactly with genetic expectations based on the Hardy-Weinberg Law: 29 were type X, ten were type XZ and one was type Z. These 40 sheep were of a variety of breeds, pure and mixed, and might be considered a representative sample of California sheep, although certainly not a random sample.

Additional data are given in Table 3. The "white-faced" breeds included Corriedales, Targhees and Rambouillets, as well as some Rambouillet-Merino cross-breeds. It was only in the white-faced breeds that both alleles appeared to be segregating and the distribution of the three phenotypes in this population was in agreement with expectation. The allele X was completely fixed in the "black-faced" breeds studied (primarily Shropshires and Suffolks).

TABLE 3

Frequency of R, O, X, XZ and Z in sheep of white-faced breeds

Blood type	X	XZ	Z	Totals
R	112	79	12	203
O	102	51	6	159
Totals	214	130	18	362
Chi-square of test for independence of X-Z and R-O = 3.23				
d. f. = 2 p > 0.05				

Most of the blood samples which were typed for X and Z were also typed with R and O reagents in stock in this laboratory, and no association of blood factor X or Z with R or O was noted, as is shown in Table 3, which summarizes tests for R, O, X and Z in white-faced breeds, where all of the expected types RX, RXZ, RZ, OX, OXZ and OZ were found.

The gene responsible for production of the R phenotype is dominant to that for O (STORMONT 1951; RENDEL, NEIMANN-SØRENSEN and IRWIN 1954). The symbol R is used for the gene responsible for the blood type R, and r^o for the gene which when homozygous ($r^o r^o$) results in blood type O. The offspring of a ram of genotype $Rr^o XX^z$ and ewes of genotype $r^o r^o XX$ were of all four types expected in progeny from a backcross, and the numbers of each type observed were: one

$Rr^o XX$, two $Rr^o XX^z$, two $r^o r^o XX$ and two $r^o r^o XX^z$. These data exclude the possibility that the genes responsible for the six phenotypes **RX**, **RXZ**, **RZ**, **OX**, **OXZ** and **OZ** are members of the same allelic series.

Because of the relative rarity of type-Z sheep in the families studied, most of the data involved matings of type-X and XZ sheep. Results of tests on the bloods of 259 lambs, their dams and sires are shown in Table 4. These data are in accord with the hypothesis of a two-allele system.

TABLE 4
Inheritance of X-Z blood groups in 259 lambs

Kind of mating	X(genotype XX)	Number of offspring of type	
		XZ(genotype XX^z)	Z(genotype $X^z X^z$)
$XX \times XX$	172	0	0
$XX \times XX^z$	24	34	0
$XX \times X^z X^z$	0	4	0
$XX^z \times XX^z$	4	12	7
$XX^z \times X^z X^z$	0	1	1

DISCUSSION

The X-Z system of sheep resembles the F-V system of cattle described by STORMONT (1952). In both of these systems, only a single pair of alleles appears to be involved at present, and in both systems dosage reactions were observed and were of initial importance in the elucidation of each system. In neither were they necessary for complete genotypic and phenotypic classification if reagents for both F and V or X and Z were available. In the F-V system, dosage reactions were observed with most F reagents produced from rabbit antisera, but not with V reagents. In the X-Z system, dosage reactions were observed with both anti-X and anti-Z.

In discussing the F-V system, STORMONT (1952) stated that the usual explanation for these differences in sensitivity of the blood cells was that two doses of the gene produced more of the same antigen than did one dose. He also cited OWEN's (1948) work as evidence for demonstrating the principle of competition of alleles or their products for the same substrates. The differences in reactivity of cells from homozygotes and heterozygotes for the alleles X and X^z may be explained on the basis of either of the above hypotheses, when a reagent for X or Z is tested with a single type of complement and dosage reactions are observed. However, the two X reagents described in this report showed dosage effects with rabbit complement, whereas cells of type X and type XZ were indistinguishable when guinea pig complement was used. If a difference in the amount of antigen produced in heterozygotes and homozygotes were the sole basis for the dosage effect, it might be expected that it would be just as apparent with guinea pig complement.

The most pronounced differences in rates and degrees of hemolysis were usually observed when the reagents were quite dilute. When less dilute, the reagents

lysed erythrocytes from both heterozygotes and homozygotes more rapidly, and differences were obscured. However, the differences between the tests with X reagents when different sources of complement were used were maintained regardless of how greatly the reagent was diluted. Similarly, the dosage reactions with the Z reagents were more marked when rabbit complement was used, regardless of the dilution of the antiserum.

Although at present only two alleles are known to be involved in the X-Z system, occasional cells of type X (such as those of sheep No. 577 described above) failed to react with the dosage reagent when used with rabbit complement and were more slowly lysed by the X reagents used in this study than were other type-X cells when used with guinea pig complement. Such reactions suggest that subtypes of X may exist, or that other reagents might be developed which could detect new phenotypes in the X-Z system.

As already pointed out, no XZ or Z phenotypes were observed in sheep of the black-faced breeds. More than 200 sheep, primarily Shropshires and Suffolks, were tested. In data for 362 sheep of white-faced breeds (Table 3, which includes Corriedales, Rambouillets and Rambouillet-Merino crossbreeds) the frequency of the allele X^z was 0.23. The greatest deviation in frequency of this allele from the average frequency was observed in small lines of Rambouillets, where it varied from approximately 0.5 to 0.0. The frequency of the allele X^z was close to 0 in three small families, and close to 0.5 in two. No typing for X and Z had been done previously, but these data suggested that as expected, allele X^z may be lost or may be increased in frequency in small families, and repeated testing for X and Z in flocks in which the alleles X and X^z are segregating would help, in conjunction with other markers, to measure progress in inbreeding.

SUMMARY

1. Evidence is presented for a second system of ovine blood groups, the X-Z system, which identifies a genetic locus different from the locus for the R-O system.

2. The X-Z system comprises three phenotypes, X, XZ and Z, which appear to be controlled by a pair of alleles, X and X^z . These phenotypes are detected by means of reagents prepared from antisera produced in rabbits against erythrocytes of sheep as well as certain related species in the families Bovidae and Antilocapridae.

3. The Z reagents differentiate bloods of type XZ from those of type Z by means of differences in rates and degrees of hemolysis. In the hemolytic test erythrocytes of homozygotes (type Z) are rapidly and completely lysed whereas those of heterozygotes (type XZ) are more slowly and less completely lysed by Z reagents. These differences are more pronounced if rabbit complement rather than guinea pig complement is used in the test.

4. The X reagents may be used to differentiate bloods of type X from those of type XZ, but the dosage effect is much less pronounced than that obtained with Z reagents, and is apparent only when rabbit complement is used.

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