BLOOD GROUPS IN SHEEP. III. THE A, C, D AND M SYSTEMS¹

BENJAMIN A. RASMUSEN,² CLYDE STORMONT AND YOSHIKO SUZUKI

The School of Veterinary Medicine, University of California, Davis, California

Received May 4, 1960

IN the course of studying the inheritance of phenogroups in the X-Z and B systems of blood groups in sheep (RASMUSEN 1958, 1960) it became apparent that some of the reagents used for blood typing of sheep were reacting in neither of these two systems nor in the R-O system. This report presents evidence for four additional systems of ovine blood groups detected by these reagents.

MATERIALS AND METHODS

The blood samples and the techniques used in performing the blood-typing tests and absorptions in this study were the same as those used for the studies of the X-Z and B systems (RASMUSEN 1958, 1960). Both isoimmune and hetero-immune antisera were used as sources of reagents.

Preparation of reagents

The A reagent: The A reagent (Table 1) was an unabsorbed, isoimmune anti-serum. It was produced in a sheep named A, which received five intrajugular injections, at five-day intervals, of 5 ml of citrated blood from a sheep named B. The antiserum was collected five days after the last injection. Sheep A was of blood-group R in the R-O system, and a preimmunization sample of her serum contained neither natural hemolysins nor agglutinins for the red cells of other sheep. Immune hemolysins were first demonstrated in the sample of serum collected just prior to the fifth or last injection. The antiserum, coded S3, was collected five days later.

This antiserum had a uniform titer of 1/128 in tests with the reactive bloods of different sheep, suggesting that it might contain a single, nonfractionable population of antibodies in tests with the red cells of sheep. Numerous absorptions were performed in which bloods from individual sheep were utilized, and in each instance the reactive bloods exhausted all of the hemolysins. The S3 antiserum was used routinely at a dilution of $\frac{1}{4}$ as the A reagent of this study. At this dilution regular and complete hemolysis was usually effected within one and one half hours after the tests were set up. A rare exception to the observation of complete hemolysis with this reagent occurred in tests of the red cells of a pair of twins, and in that instance it was possible to show that the incomplete hemo-

¹ Prepared in part from a dissertation submitted by the senior author in partial satisfaction of the requirements for the degree of Doctor of Philosophy, University of California. This study was aided in part by support of the Sheep, Goat and Fur Animal Section, Agricultural Research Service, United States Department of Agriculture.

² Present address: Department of Animal Science, University of Illinois, Urbana.

TABLE 1

Reactions of the erythrocytes of 15 rams with reagents for the A, C, D, M, R-O and X-Z systems

		Reagents and antiserum numbers*											
		A S 3	C S 66	C C 58	C _x S 33	D S 21	M S 67	M _x S 57	M _x RTG 18	R C 86	O C 45	X RS 9	Z RS 19
Red cells of rams													
Shropshire	114	+	+	+	+	0	+	+	+	+	0	+	0
Shropshire	30	+	+	+	+	0	+	+	+	+	0	+	0
Suffolk	669-0	0	0	0	+	0	+	+	+	+	0	+	0
Suffolk	76650	0	0	0	+	0	+	+	+	0	+	+	0
Suffolk	105335	0	0	0	+	0	±	+	+	+	0	+	0
Grade Suffolk	21	+	+	+	+	0	0	0	0	+	0	+	0
Corriedale	412	0	0	0	±	0	0	0	0	+	0	+	0
Targhee	5020T	+	+	+	+	+	0	0	0	+	0	+	+
Targhee	53035T	0	0	0	+	0	0	0	0	0	+	+	0
Targhee	9501T	+	0	0	+	+	0	0	0	+	0	+	+
Rambouillet	4458RW	0	0	0	+	0	0	0	0	0	+	+	0
Rambouillet	2516XW	+	0	0	+	0	0	0	0	0	+	+	+
Rambouillet	4375RW	+	0	0	+	+	0	0	0	+	0	+	0
Rambouillet	2435XW	+	0	0	+	0	0	+	+	+	0	+	+
Rambouillet	396XW	+	+	+	+	+	+	+	+	0	+	+	0

* Reagents C (S66), C_x (S33), M (S67), and M_x (S57), prepared from ovine isoimmune antisera and reagent C (C58) prepared from a bovine isoimmune antiserum, were tested using rabbit complement (absorbed at 4°C with sheep red cells). Reagent A (S3), prepared from a sheep isoimmune antiserum and reagent M_x (RTG18) prepared from a rabbit anti-Tahr-goat antiserum, were tested using guinea pig complement. The D reagent was used in agglutination tests. For tests involving the X and Z reagents see RASMUSEN (1958).

lysis was a result of erythrocyte mosaicism (STORMONT, WEIR and LANE 1953).

Sheep A was given a booster of 25 ml of the blood of sheep B approximately three months after antiserum S3 was collected, and eight days later a second antiserum was collected. This antiserum, coded S9, paralleled the S3 antiserum in titer and degree of reaction and reacted with the same erythrocytes as S3. All immunizations of A-negative sheep with the blood of A-positive sheep (seven trials) resulted in the production of A antibodies. Consequently, in subsequent series the immunization of A-negative sheep with A-positive blood was avoided.

The C and C_x reagents: The C reagent (S66, Table 1) was obtained from an antiserum prepared by injecting 10 ml of washed citrated blood from sheep 2083 into the jugular vein of sheep 575 (group O in the R-O system) twice a week for two weeks. This antiserum was collected four days after the last injection. Following absorption of S66 with human erythrocytes of group A to remove natural anti-sheep R, it behaved as if it contained a single, residual population of antibodies. The reagent was used at a dilution of 1/4 in the present studies. Antisera S20, S24, S27, S37 and S68 provided other sources of C reagents. Another C reagent (C58, Table 1), was prepared from a bovine isoimmune antiserum. An additional C reagent was obtained from an antiserum produced by two injections, one week apart, of 10 ml of pooled washed erythrocytes from black-tailed deer (*Odocoileus hemionus columbianus*) into the jugular vein of sheep 658. This

antiserum, SBT1, was collected seven days after the last injection. It was not yet available when the bloods of the rams listed in Table 1 were tested.

The reagent named C_x was an antiserum, S33, produced in sheep 469604. This sheep received two 10 ml injections, eight days apart, of erythrocytes of sheep 311, followed by a 10 ml booster of the pooled bloods of sheep 999, 1000 and 1318 approximately one and one half years later. The antiserum was collected one week after the booster injection. Trial absorptions yielded no evidence for more than a single population of antibodies. A dilution of $\frac{1}{4}$ was chosen as optimal or near-optimal for C_x in the tests.

The D reagent: The D reagent (Table 1) was prepared by immunization of sheep 3539 (group O in the R-O system) with washed erythrocytes of sheep N772, N773, O831 and O832. Six injections, five days apart, of 10 ml of pooled erythrocytes were made, and the antiserum, S21, was collected one week after the last injection. It contained three distinct populations of antibodies against red cells of sheep: (1) natural anti-R; (2) a population called D which acted as agglutinating antibodies and which also caused some hemolysis in conjunction with rabbit complement but not with guinea pig complement; and (3) a population called E' which reacted with certain phenogroups in the B system (RASMUSEN 1960) and produced hemolysis in conjunction with either rabbit or guinea pig complement.

This antiserum, after absorption with human red cells of group A to remove the natural anti-R, was used only with guinea pig complement and served as a D plus E' reagent. Agglutination appeared much more rapidly after the tests were set up than did hemolysis, so that the reactions due to the D population of antibodies (observed as agglutination) were clearly distinguishable from those due to the E' population (observed as hemolysis), inasmuch as guinea pig complement did not participate in the D antigen-antibody system. Erythrocytes reactive with both populations of antibodies (D and E') were first agglutinated and then lysed. This report is concerned only with the reactions of the D population. These D antibodies were unique among the battery of reagents used for blood typing of sheep in these studies inasmuch as they were the only sheep blood-typing antibodies which reacted regularly as agglutinins. Reagents for all the other blood-group systems reacted as hemolysins (see footnote to Table 1 and also RASMUSEN 1958, 1960).

The M and M_z reagents: Three additional antisera were produced from reimmunizations of sheep 3539, and from each of these at least two reagents could be prepared. Fourteen months after the S21 antiserum was collected, sheep 3539 was given a single injection of 10 ml of pooled erythrocytes from sheep 999, 1000 and 2960. A second booster injection was made with cells of sheep 2355, and finally, a third booster injection, again with cells of sheep 2355, resulted in an antiserum, S57, which contained a population of antibodies not previously encountered. This new population was named M, and by appropriate absorption the M reagent was isolated. Another reagent, similar to the M reagent prepared from S57, was obtained from antiserum S67. This antiserum was produced by injection of 10 ml of washed erythrocytes of sheep 2083 into the jugular vein of sheep 652 twice a

week for three weeks and was collected four days after the last injection. S67 did not react with all of the bloods lysed by the original M reagent (S57), but the bloods lysed by S67 were always lysed by S57. After tests of several hundred blood samples were examined for comparison of the reactions of these two M reagents, the original M (S57) was designated M_x and that from S67 was designated M (Table 1).

A second source of M_x reagent was a rabbit antiserum, coded RTG18, produced by immunization with the red cells of Tahr goats (see STORMONT and SUZUKI 1958). The reagent was prepared by absorption of antiserum RTG18 with red cells from M-negative sheep.

EXPERIMENTAL RESULTS

Table 1 summarizes the reactions of the red cells of 15 rams with reagents for the A, C, D, M, R-O and X-Z systems. The rams headed the sire families which were used in the studies of the genetics of the blood-group systems. Their B phenogroups are given in Table 3 of the report on the B system (RASMUSEN 1960).

Inheritance studies

A study of the inheritance of the phenogroups reactive with the reagents A, C, C_x , D, M and M_x was made using the same bloods as those used in the studies of the X-Z and B systems (RASMUSEN 1958, 1960).

To determine whether any of these six reagents contained antibodies reactive with any of the numerous phenogroups in the B system, with phenogroups R and O of the R-O system, or with phenogroups X and Z of the X-Z system, their reactions with the red cells of lambs sired by rams known to be heterozygous for alleles at the B, R-O, and X-Z loci were examined. There was no evidence that any of the six reagents contained antibodies reactive with phenogroups in these three systems. On the contrary, the reactions of these six reagents with the erythrocytes of the lambs appeared to be completely independent of the reactions due to the B, R-O and X-Z phenogroups inherited from the rams. Consequently, the reactions of the reagents A, C, C_x , D, M and M_x must be ascribed to a system or systems other than B, R-O and X-Z.

It was thus necessary to determine the number of new systems that must be postulated to account for the reactions of the reagents A, C, C_x , D, M and M_x . If, for example, any two of these reagents, as A and C, are reacting in the same system, the phenogroup formulas with respect to those reactions must be either A/C or AC/— (— = no-A and no-C) in animals known to be heterozygous, i.e., animals with some offspring whose erythrocytes were not lysed by the reagent or reagents in question. For example, if a ram were of formula A/C, he would be expected to transmit A and C as alternatives. If he were of formula AC/—, he would be expected to transmit AC as an alternative to no-AC (—). If, however, the A and C reactions are due to phenogroups in different systems, then the appearance among the progeny of any three of the four possible types: (1) A and C; (2) A and no-C; (3) no-A and C; or (4) no-A and no-C; or all four of

these types, as transmitted by the ram alone, would establish that separate systems are involved in the reactions of A and C.

Data in Table 2 for 13 offspring of a Targhee ram, 5020T, show that the phenogroups A as contrasted with no-A (—), C as contrasted with no-C (—) and D as contrasted with no-D (—) are in three separate systems and are not in the B, R-O or X-Z systems. Data in Table 3 for six selected offspring of Shropshire ram 114 show that phenogroup M contrasts with no-M (—) in a system separate from the B, R-O, A and C systems. In Table 4, results are presented which show that the M_x reagent is not reacting with phenogroups in the X-Z system. While the data in Table 5 show that the M system is separate from the D system, they are

TABLE 2

Inheritance of B, R-O, X-Z, A, C and D phenogroups of ram 5020T (Targhee) by 13 of his offspring

Offspring number	Phenogroups from sire in blood-group system					
	B	R-O	X-Z	A	C	D
6	Q1†	*	X	*	—	*
11	Q1	*	X	—	—	D
14	Q1	O	Z	*	C	—
15	Q1	R	Z	*	—	D
19	Q1	R	Z	—	C	—
27	Q1	R	X	A	C	D
38	Q1	O	Z	*	C	—
39	Q1	R	X	*	—	*
49	Q1	O	X	*	C	*
67	Q1	O	*	—	—	D
33	I5‡	O	X	—	*	—
50	I5	O	*	*	C	*
66	I5	R	*	*	*	D

* Contribution of sire not determined.

† Q1 = $BB'ENO'_xQ$.

‡ I5 = $BB'E_xI_1Y$.

TABLE 3

Inheritance of B, R-O, A, C and M phenogroups of ram 114 (Shropshire) by six of his offspring

Offspring number	Phenogroups from sire in blood-group system				
	B	R-O	A	C	M
423	Q9†	R	A	—	M
428	*	*	A	—	—
431	*	O	*	C	—
464	Q9	O	—	—	—
475	Q'2‡	O	—	*	—
476	O'2	O	—	C	M

* Contribution of sire not determined.

† Q9 = O'_xQ .

‡ O'2 = $BB'EO'_1T_3U_1$.

TABLE 4

Inheritance of M and X-Z phenogroups of ram 2435XW (Rambouillet) by four of his offspring

Offspring number	Phenogroups from sire in blood-group system	
	M	X-Z
3917XW	M _x	Z
3918XW	M _x	X
3722XW	M _x	X
2048US	—	Z

TABLE 5

Inheritance of D and M phenogroups of ram 396XW (Rambouillet) by three of his offspring

Offspring number	Phenogroups from sire in blood-group system	
	D	M
3535XW	—	—
3724XW	—	M
3986XW	—	—

TABLE 6

Frequency of D and M in 98 white-faced ewes

	M	—	Total
D	5	56	61
..	4	33	37
Total	9	89	98
Chi-square = 0.18 d.f. = 1 P > 0.05			

TABLE 7

Frequency of D and M in 75 black-faced ewes

	M	—	Total
D	5	4	9
..	39	27	66
Total	44	31	75
Chi-square = 0.04 d.f. = 1 P > 0.05			

perhaps somewhat inconclusive due to lack of D-positive offspring of ram 396XW in matings to D-negative ewes. The two-by-two distributions shown in Tables 6 and 7 indicate that the D and M reactions must involve separate systems.

In Table 6 no attempt was made to distinguish between the subtypes M and M_x in the M system. In the C system, only phenogroups C and — (no-C) were distinguished in Tables 2 and 3. Actually, phenogroup C_x was not included in the analysis of sire families because of its high frequency and the consequent difficulty in finding matings of heterozygous rams with C_x-negative ewes for family

studies. It was placed in the C system primarily as a result of the following criteria: (1) Bloods that were lysed by the C reagent were invariably lysed by C_x , thereby indicating a relationship between C and C_x . (2) The cross-reactions of C_x with bovine erythrocytes were entirely limited to phenogroups in the C system of cattle just as were the cross-reactions of the C reagent C58. (S66 did not react with cattle blood). (3) The limited genetic data available were also in accord with the hypothesis that C_x was reacting in the C system.

Table 8 summarizes the blood groups of the ewes from the flocks studied, including 75 Shropshires, 23 Suffolks, 8 Corriedales, 29 Targhees and 71 Rambouillets (including some Rambouillet-Merino crossbreds).

The two phenotypes, A-positive and A-negative, in the A system were encountered in all breeds studied, and in no breed was the A-negative phenotype observed in more than half of the blood samples for that breed (Table 8).

The frequency of phenotypes C and C_x differed considerably between the Shropshires, which were largely of phenotype C, and the other four breeds, which were largely C_x . The frequency of C was the most striking difference between the two black-faced breeds (Shropshires and Suffolks) observed in these studies (Table 8).

In the D system, D-positive animals were rather infrequent in the black-faced breeds but were more frequent in white-faced breeds (Corriedales, Targhees and Rambouillets). Conversely, in the M system, the M-positive phenotypes were more frequent in the black-faced breeds (Table 8).

The data of Table 8 may not reflect accurately the frequencies of blood groups

TABLE 8

Phenotypes of ewes in six blood-group systems

System	Phenotype	Black-faced breeds		White-faced breeds		
		Shropshire	Suffolk	Corriedale	Targhee	Rambouillet
R-O	R	62	5	4	14	39
	O	13	17	4	12	39
	i*	0	1	0	3	0
A	A	49	15	4	21	58
	—	26	8	4	8	13
C	C	67	0	0	0	8
	C_x	2	22	8	29	61
	—	6	1	0	0	2
D	D	7	2	3	24	32
	—	68	21	5	5	39
M	M	43	18	0	4	5
	M_x	0	0	0	0	3
	—	32	5	8	25	63
X-Z	X	75	23	5	19	39
	XZ	0	0	2	10	29
	Z	0	0	1	0	3

* i=no-R, no-O.

for the entire breed for any of these blood-group systems, since only a few flocks were studied.

As is shown in Table 8, two phenotypes may be distinguished in the A system, three in the C system, two in the D system and three in the M system. If a small letter is used for the allele which when homozygous results in a negative blood type, the symbols for the alleles at each of the four loci may be written as follows (in descending order of dominance): (1.) *A, a*. (2.) *C, C^x, c*. (3.) *D, d*. (4.) *M, M^x, m*.

DISCUSSION

The C system of sheep appears to be homologous to the complex C system of cattle, on the basis of studies of cross-reactions of bovine and ovine isoimmune sera (STORMONT, SUZUKI and RASMUSEN 1957). In sheep our present knowledge of the C system is limited to the observation, by the use of two reagents, of only three phenotypes (*C, C_x* and no-*C*). Further studies with new reagents may reveal that this system in sheep is as complex as the C system of cattle (see STORMONT, OWEN and IRWIN 1951).

Of the other three systems (*A, D* and *M*) described in this paper, the *M* system appears to be the most complex. The reactions of the *M*-system reagents available clearly differentiated three phenotypes (*M, M_x* and no-*M*), and there was evidence which suggested that several more phenotypes exist, which for the present have been considered together simply as *M*. For example, one of the hetero-immune antisera produced in sheep by immunization with cattle blood contained antibodies which reacted almost exclusively with homozygotes for an unclassified subtype of *M* found in Shropshires.

Preliminary studies of cross-reactions of the *M*-system reagents indicate that the *M* system of sheep may be homologous to the S-U system of cattle, but the data are as yet inconclusive.

SUMMARY

Evidence is presented for four systems of ovine blood groups: *A, C, D* and *M*. These, in conjunction with *B, R-O* and *X-Z*, bring to seven the number of blood-group systems currently recognized in sheep.

Presently, there are two phenogroups in the *A* system, *A* as contrasted with no-*A*. Antibodies against phenogroup *A* are regularly engendered in *A*-negative sheep immunized with the blood of *A*-positive sheep. Isoimmune antisera have provided the sole source of *A* reagents.

Similarly, there are at present two phenogroups in the *D* system, *D* as contrasted with no-*D*. A single ovine isoimmune antiserum provided the source of *D* antibodies which acted typically as agglutinins in contrast with the reagents utilized in the studies of the other systems, which reacted typically as hemolysins.

Multiple alleles appear to be involved in the control of phenogroups in the *C* and *M* systems. Both ovine and bovine isoimmune antisera have provided sources of reagents reactive with phenogroups in the *C* system. Reagents reactive with phenogroups in the *M* system have been obtained primarily from ovine iso-

immune antisera, but certain M reagents were also prepared from heteroimmune antisera.

The C system of sheep appears to be homologous to the C system of cattle.

LITERATURE CITED

- RASMUSEN, B. A., 1958 Blood groups in sheep. I. The X-Z system. *Genetics* **43**: 814-821.
1960 Blood groups in sheep. II. The B system. *Genetics* **45**: 1405-1417.
- STORMONT, CLYDE, R. D. OWEN, and M. R. IRWIN, 1951 The B and C systems of bovine blood groups. *Genetics* **36**: 134-161.
- STORMONT, CLYDE, and YOSHIKO SUZUKI, 1958 The distribution of Forssman blood factors in individuals of various Artiodactyl species. *J. Immunol.* **81**: 276-284.
- STORMONT, CLYDE, YOSHIKO SUZUKI, and B. A. RASMUSEN, 1957 Cross-reactions of ovine and bovine isoimmune antibodies in cattle and sheep blood-typing studies. (Abstr.) *J. Animal Sci.* **16**: 1102.
- STORMONT, CLYDE, W. C. WEIR, and L. L. LANE, 1953 Erythrocyte mosaicism in a pair of sheep twins. *Science* **118**: 695-696.