NATURAL IMMUNIZATION IN PREGNANT GOATS AGAINST RED BLOOD CELLS OF THEIR SHEEP × GOAT HYBRID FOETUSES

By G. Alexander,* D. Williams,* and L. Bailey†

Summary

Female goats readily conceived when mated with male sheep but the foetuses usually died within 2 months. Examination of living hybrid foetuses about 2 months old suggested that death was due to haemolytic disease. Haemolytic antibodies against red blood cells of the sire usually appeared in the maternal goat sera shortly after the time of cotyledonary attachment at about 30 days gestation.

These findings lead to the hypothesis that the foetuses die from haemolytic disease when antibodies against their own red blood cells reach them from the maternal circulation. Features of the hybrid placenta suggest that it may more readily allow the passage of foetal red cells into the maternal circulation and the return passage of maternal antibodies into the foetal circulation than the normal ruminant placenta.

I. Introduction

Attempts to hybridize sheep (*Ovis aries*) with goats (*Capra hircus*) have been mostly unsuccessful (Warwick, Berry, and Horlacher 1934; Quinlan *et al.* 1941; Hancock 1964). Fertilization usually occurred when female goats were inseminated with sheep semen but the foetuses rarely survived for more than 2 months. On the other hand, Bratanov and Dikov (1961) reported birth of hybrids after parents had been subjected to a course of intramuscular injections of blood of the other species. The cause of death and the mechanism whereby the injections of blood prevented death were not explained satisfactorily.

In this paper we present evidence that the hybrid foetuses die as a result of haemolytic disease when antibodies to their own red blood cells cross the placenta from the maternal circulation.

Examination of maternal sera for haemolytic antibodies to hybrid or sheep red cells was prompted by the finding of jaundiced hybrid foetuses reminiscent of human offspring affected by haemolytic disease due to maternal Rh isoimmunization.

II. Materials and Methods

(a) Production of Hybrids

Attempts to produce hybrids were made in 1965 and 1966 using polled or horned female Saanen goats and a Merino or Suffolk ram. A few individuals of other types were also included in 1966 (see Fig. 1). The goats were purchased from a variety of sources and their ages varied widely.

*Division of Animal Physiology, CSIRO, Ian Clunies Ross Animal Research Laboratory, Prospect, N.S.W.

†Department of Genetics, University of Adelaide.

Does were exposed to a vasectomized male goat twice daily during most of the breeding season (February to August) in 1965 and once daily in 1966. Does that permitted mounting were immediately mated with a male sheep; it was usually
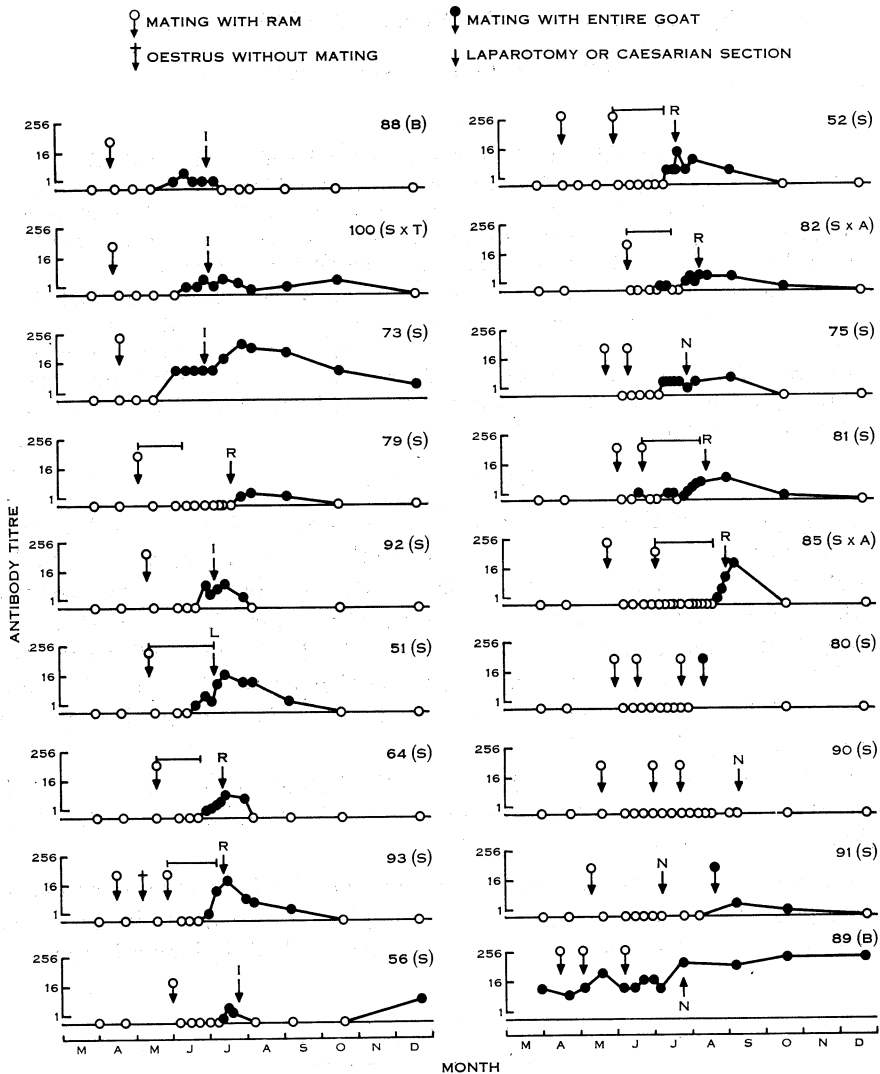

Fig. 1.—Antibody titres of maternal sera with time. Titres are plotted on a logarithmic scale. Absence of detectable antibody is indicated by an open circle, presence of a titre by a closed circle. Animals are arranged in chronological order of date of conception, and the breed is indicated thus: S, Saanen; B, British Alpine; T, Toggenburg; A, Anglo-Nubian. Findings at laporotomy or Caesarian section are indicated by L, live foetus; R, recently dead foetus; I, uterus involuting (foetus dead for some time); N, uterus completely involuted. Horizontal bars indicate minimum period of foetal survival.

necessary to hold the doe by the head and sometimes to lift the tail to permit intromission. The Merino ram used in 1965 and the Suffolk used in 1966 were selected for their indiscriminate mating habits. Mated does were returned to the flock and daily testing for oestrus was continued. Does coming into oestrus again were usually not remated with the ram until the second oestrus after the first mating.

Between 7 and 12 weeks (usually 50–60 days) after mating, does that had not exhibited oestrus again were either killed and the conceptus collected (1965) or subjected to laparotomy or Caesarian section (1965 and 1966).

(b) Detection of Antibodies and Determination of Blood Groups

Haemolytic antibodies against red blood cells of the rams and of the hybrids were detected in maternal goat sera by a simplification of the haemolytic test described by Stormont and Cumley (1943) and Rendel (1957).

Maternal sera were prepared from samples of jugular blood collected at widely differing intervals, but usually weekly during pregnancy (Fig. 1). Ram and foetal red cell suspensions were prepared from heparinized blood obtained by jugular puncture from the rams and through a hypodermic needle inserted into the umbilical vessels in the foetuses. Guinea-pig complement was prepared by diluting pooled sera of four or five male guinea-pigs with saline in the ratio 1 to 15.

Tests were carried out in the wells of a Perspex tile, and haemolysin titres in maternal sera were determined by testing serial dilutions of serum against a standard 3% (v/v) suspension of washed red cells. One drop of red cell suspension was added to two drops of maternal serum undiluted or diluted 1 in 2, 1 in 4, 1 in 8, etc.; one drop of fresh guinea-pig serum (complement) was then added and the mixture was shaken. Haemolysis was recorded after incubation at 37°C for 2 hr. The titre of antibody was taken as the reciprocal of the highest dilution at which haemolysis was readily detected. Agglutination of red cells which sometimes occurred in the absence of haemolysis was not used in scoring antibody titre.

The specificity of haemolysins in maternal sera was examined by the standard haemolytic test using R and O sheep red cells separately. When different titres were observed, the presence of specific anti-O or anti-R haemolysins was confirmed by repeating the test after removal of non-specific haemolysins by absorption with sheep R or O red cells. Absorption was done by first inactivating goat complement by heating serum to 56°C for 20 min and then incubating with an equal volume of R or O packed sheep red cells for 30 min at 37°C, followed by centrifugation to remove the red cells.

The R and O blood groups of rams were determined using the inhibition test described by Rendel (1957) for the detection of R and O substances in serum.

(c) Foetal Haematology

Haematocrits were determined by the conventional method, and haemoglobin content of blood by estimation of oxyhaemoglobin in a dilute ammonia solution (see Wintrobe 1961).

TABLE 1

DESCRIPTION OF FOETAL HYBRIDS ALIVE AT COLLECTION

Year	Doe No.	Foetal Body Weight (g)	Age Estimated from Weight* (days)	Actual Age (days)	Liver Weight (g)	Liver Weight (as % of body wt.)	Haematocrit (%)	Haemoglobin in Blood (%)	Degree of Jaundice	Remarks
1965	12	80	63	63	14	18			Marked	Abdomen distended; contained several millilitres of blood-stained fluid
	5	68	61	63	11	16	17·0	7·9	Not noted	
	28	45 }†	57	59	6	13			Marked	
		49 }	57	59	5	10			Marked	
	32	32 †}	53	57	5	16			Marked	Pale
		— }	—	57	—	—			—	Red, autolysing
	49	55 }†	59	61	7	13			Slight	
		55 }	59	61	9	16			Slight	
1966	51	24	50	55	6	17	18·0	3·8	Slight	Pale; allantoic fluid yellow
Controls (1966):										
6 normal goat foetuses		37–59	—	59–65	3–5	8–11	25–27‡	6·0–13·3§	Nil	Allantoic fluid almost colourless
18 normal sheep foetuses		44–70	—	59–61	3–6	7–10	22–33	7·6–11·5	Nil	Allantoic fluid almost colourless

*See Cloete (1939).　†Twin foetuses (second foetus of doe No. 32 not weighed).　‡Two values only.　§Five values only.

III. RESULTS

(a) Conception Rate

Conception rate was judged by the failure of mated does to return to oestrus, normal intervals in the goat ranging from 12 to 24 days (Asdell 1964).

Eleven does were mated with the ram in 1965 and all apparently conceived (9 at the first mating, and 1 each at the second and third matings). In 1966, 18 does were mated, but only 14 appeared to conceive (10 at the first mating and 4 at the second). The remaining four came into oestrus regularly throughout the breeding season. Thus conception most frequently occurred at the first mating.

There was no significant evidence of mortality of very young hybrid embryos because the incidence of longer than normal oestrous cycles that followed mating with the ram was the same as in unmated does. In both cases, five cycles out of 16 were longer than 25 days.

(b) Survival of Foetuses

When examined 7–12 weeks after mating, two does (one from each season) that had not come into oestrus again showed no evidence of a conceptus; resorption was either complete or the does had not become pregnant. In the other 23 does the uterine contents were extremely variable, ranging from apparently healthy twin foetuses through foetuses in various stages of debilitation and degeneration to advanced resorption, as set out in the following tabulation:

	1965 Season	1966 Season
No. of does with:		
Twins, both alive	2	0
Twins, one recently dead	1	1
Single alive foetus	2	0
All foetuses recently dead	1	2
All foetuses dead for some time	3	9
Conceptus almost completely resorbed	1	1

Thus only six does contained living foetuses. There were traces of membranes of a second foetus present in both does bearing single alive foetuses in the 1965 season.

(c) Description of Living Foetuses

Morphologically, the living foetuses were indistinguishable from sheep or goat foetuses of the same age except that the tail was of an intermediate length (see also illustrations of Warwick and Berry 1949). There was no evidence of malformation. However, all living hybrid foetuses inspected showed some degree of jaundice (Table 1) and in most the liver was disproportionately large, representing about 15% of body weight as compared with less than 10% in normal sheep and goat foetuses (Table 1). The abdomen of one foetus was distended and contained several millilitres of blood-stained fluid. When gestational ages of the foetuses (Table 1) were estimated from the chart of Cloete (1939) for ovine foetuses, estimated age was equal to actual age in one foetus and was 2 days less than actual in five. In two others that appeared pale and anaemic, the estimated ages were 4 and 5 days less than actual.

(d) Description of Dead Foetuses

Foetuses classified in the above tabulation as having died recently were purplish red in colour and the outer layers were gelatinous. Foetuses classed as having been dead for some time were brown and tended to be friable; their lengths corresponded with gestational ages of 34 days or more (Table 2).

TABLE 2

APPARENT AGE AT DEATH OF HYBRID FOETUSES IN RELATION TO APPEARANCE OF ANTIBODIES IN MATERNAL SERA

Not all uteri were opened

Doe No.	Time Antibodies First Detected* (days after mating)	Apparent Foetal Age at Death† (days after mating)	Time of Laparotomy or Caesarian Section (days after mating)	Condition of Foetus
51	32–39	50	55	Alive at collection
52	38–41	40	53	Dead for some time
64	36–44	34	55	Dead for some time
79	74–85	34	77	Dead for some time
81	c.35	45	53	Recently dead
82	38–43	36	59	Dead for some time
85	47–51	43	59	Dead for some time
93	28–36	41	47	Dead for few days

*The first figure indicates the time of the previous test.
†Calculated from length according to the chart of Cloete (1939) for sheep.

(e) Placentae of the Living Hybrid Conceptuses

The cotyledons were similar in size, shape, and arrangement to those in a normal goat. However, the foetal surface, instead of being smooth, was markedly irregular with small elevations and depressions several millimetres across; blood clots, some several centimetres in diameter, occurred on the foetal surface of most cotyledons.

In at least three does unusual patches of small outgrowths of the chorion, 2–3 mm in diameter, appeared to be invading the intercotyledonary endometrium and there was some blood between the chorion and endometrium.

The amniotic and allantoic membranes were markedly oedematous and the volume of allantoic fluid associated with each foetus was abnormally large, usually exceeding 1 litre, in contrast with a volume of less than 200 ml in normal sheep and goats [Alexander and Williams (1966) and unpublished data]. Amniotic volumes appeared normal (approx. 200 ml).

The colour of allantoic fluids was usually not noted but in the last foetus collected it was distinctly yellow.

(f) Foetal Haematology

Haematocrit and haemoglobin levels were determined in two hybrid foetuses. Both haematocrit values (Table 1) were below the range found in normal 60-day sheep and goat foetuses from allied studies. One haemoglobin level was just above the lower end of the normal range but the other was exceedingly low.

(g) Haemolysins in Maternal and Foetal Sera

Only one sample of the maternal serum (goat 51) was examined in 1965 but during 1966 haemolysins against red cells of the ovine sire (R-O-i type R) were detected in the sera of all 13 goats in which traces of a conceptus were found (Fig. 1). These antibodies were detected for the first time in samples collected between the 35th and 51st day after mating except in doe 79 which is discussed below. Following the appearance of haemolysin, titres usually increased gradually until about 60 days after mating, irrespective of the month of mating (Fig. 1); titres then declined slowly until antibodies were no longer detectable. The constancy of these changes in relation to the time of mating suggests that the pattern is not simply a reflection of any normal seasonal fluctuations in serum antibody. Maximum titres ranged from 2 to 128, and titres persisted for intervals ranging from less than 4 weeks to more than 7 months.

In 11 of the 13 does sera collected when titres were high contained one or more antibodies that lysed sheep red cells both of type R and type O, i.e. contained heterohaemolysin. O antibodies as well were also detected in one of these does (doe 100). In the remaining two does (79 and 56) titres were low and satisfactory tests of antibody type were not made. High titres of R antibodies were displayed throughout the study by one goat that did not conceive (doe 89, Fig. 1).

The foetal age at death, estimated from length, is related in Table 2 to the time of appearance of haemolysins. Where the foetus appeared to have been dead for only a few days (does 81 and 93), haemolysins were clearly detected before foetal death. In three other does (52, 64, and 82), the apparent development of titres after foetal death can be explained by post-mortem shrinkage of the foetus and a reduced rate of growth prior to death, as suggested in Table 1; alternatively, the resorption of a dead conceptus might have stimulated antibody production.

The time relationships are more equivocal in the doe (85) in which haemolysin was first detected 51 days after mating and in which a foetus, dead for some days, was collected 59 days after mating. In this doe foetal death presumably followed closely on the development of detectable antibody. This is in contrast with doe 51 in which the foetus was still alive 16 days after haemolysin was first detected.

The findings in doe 79 are anomalous (Fig. 1; Table 2). At a laparotomy 60 days after the mating shown, a healthy pregnant uterus was found but of a size appropriate to between 40 and 50 days of gestation. Either the growth of the hybrid conceptus and development of antibodies was considerably retarded, or a mating 2–3 weeks after that shown (Fig. 1) was not recorded as a result of human error. If the latter were correct the development of a titre after Caesarian section remains anomalous, although the significance of so low a titre is doubtful.

In two does (50 and 90) of the remaining five that did not appear to conceive, no haemolysin was detected throughout the sampling period from March to December. In another (91) low titres of 1–2 were recorded in September and October, and in a fourth doe (89) high titres (anti-R) were recorded throughout sampling. These results confirm that the rise and fall in haemolysin titre following conception (Fig. 1) were not normal seasonal changes in blood group antibodies such as discussed by Rendel (1957).

Red cells of the hybrid twin foetuses from doe 49 (1965) and of the single foetus from doe 51 (1966) were also lysed by the respective maternal sera collected coincidentally. Titres were 2 against the twins' cells and 4 against the single's cells. The corresponding titre against the cells of the sire of the single was 8.

IV. Discussion

On the basis of the circumstantial association between death and appearance of the foetuses and the development of haemolysins in maternal blood, we advance the hypothesis that the hybrid foetus dies from haemolytic disease when antibodies against its own red blood cells reach it from the maternal circulation. A similar suggestion was made by Eyquem, Millot, and Robin (1952) but without experimental evidence. We suggest that the mechanisms involved are analogous to those in Rh isoimmunization in man (see Roberts 1959). However, the term "isoimmunization", as defined by Mollison, Mourant, and Race (1948), may not be appropriate to the present situation since two different genera are involved.

Detailed pathological examinations of the hybrid conceptuses were not carried out in 1965, but were planned for 1966. However, only one foetus was obtained in that year. Nevertheless, during the two years, a number of similarities with human and other species of newborn affected by haemolytic disease (see Potter 1964) was noted. Thus, jaundice was present and allantoic fluid was stained yellow in the one case noted. Foetal growth was retarded and the liver, which is the main haemopoietic organ in early foetal life (Wintrobe 1961), was apparently enlarged. Blood-stained fluid was found in the peritoneal cavity of one foetus. This was a regular finding in piglets suffering from haemolytic disease (Buxton and Brooksbank 1953). Haematocrits and haemoglobins tended to be low in the two foetuses in which they were determined. Finally, the placenta was grossly oedematous.

Although anti-globulin tests (Coombs and Roberts 1959) were not applied to foetal blood for detection of maternal anti-red-cell antibodies, the appearance of haemolysins in maternal sera shortly before foetal death, coupled with the apparent nature of foetal damage, is strongly suggestive that the damage resulted from haemolysins of maternal origin.

In the sheep, and presumably in the goat, the trophoblastic invasion of the cotyledons commences about the 30th day of gestation (Cloete 1939). Hence, the antibody titres in maternal sera developed soon after the maternal and foetal circulations came into intimate contact, when it would appear that the opportunity for leakage of foetal blood into the maternal circulation is presented for the first time. This close time relationship suggests that leakage does occur. In man leakage of foetal red cells into the maternal circulation has been amply demonstrated by Zipursky et al. (1959), but whether it occurs normally in other species is not known. Nor are there reports of foetal damage due to isoimmunization in species other than man, except after experimental production of antibodies in the maternal circulation (Stone and Irwin 1963). The hybrid placenta with its haemorrhagic appearance and abnormal cotyledonary surface may be more permeable to foetal red cells than the normal ruminant placenta.

Similarly, the passage of haemolysin from mother to hybrid foetus may result from placental defects since there is considerable evidence that maternal antibodies do not normally reach the ungulate foetus (Brambell, Hemmings, and Henderson 1951). However, evidence is equivocal that haemolytic antibodies produced by artificial immunization of pregnant sheep, cattle, and horses do reach the foetus (Laing and Blakemore 1951; Eyquem, Millot, and Robin 1952; Girard et al. 1956; Rendel 1957; Kiddy et al. 1958).

Other possible explanations of death of the hybrid foetuses have been advanced. Hancock (1964) suggested that progesterone deficiency could play a role, and indeed very large volumes of allantoic fluid, such as recorded above, are associated with progesterone deficiency in the sheep (Alexander and Williams 1966). Hancock also suggested that some intrinsic foetal factor could be involved but there were no obvious developmental abnormalities and no evidence of chromosomal non-disjunction that cannot be ascribed to inadequate technique. Chromosomes of the hybrids have been studied by Berry (1938), Buttle and Hancock (1966), Hancock and Jacobs (1966), and Ilbery, Alexander, and Williams (1967). Moreover, these explanations could not account for the failure of sheep and goat foetuses to develop in intergeneric ova transfer experiments (Warwick and Berry 1949; Hancock 1964; Loginova 1964). Much of this failure could be accounted for by the present hypothesis although in some cases death may have occurred before the 30th day of gestation (Warwick and Berry 1949).

Our findings suggest that the success of Bratanov and Dikov (1961) in producing living hybrid offspring should be explained by immunological phenomena. The frequent injection of sheep red cells into the goat and *vice versa* may have resulted in elimination of incompatible animals, immunologic paralysis, absorption of haemolysin by injected red cells, or in production of an antibody that does not cross the placenta and that destroys foetal cells leaking into the maternal circulation. Our hypothesis thus provides a rational basis for the investigation of possible immunologic avenues of producing living hybrids. Success of these approaches would provide the actual proof of the validity of the hypothesis.

V. ACKNOWLEDGMENTS

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