Immunology, 1974, **26**, 613.

Tetraparental Sheep Chimaeras Induced by Blastomere Transplantation

CHANGES IN BLOOD TYPE WITH AGE

ELIZABETH M. TUCKER*, R. M. MOOR† and L. E. A. ROWSON†

*Agricultural Research Council, *Institute of Animal Physiology, Babraham, Cambridge, and †Unit of Reproductive Physiology and Biochemistry, 307 Huntingdon Road, Cambridge*

(*Received* 16*th July* 1973; *accepted for publication* 13*th August* 1973)

Summary. The blood groups are described of three sheep chimaeras produced by injection of blastomere cells into fertilized eggs of different parental origin. Evidence is presented that these chimaeras had a blood type which could only have been derived from both sets of parental genes. Two antigenically distinct red cell populations were identified in all three sheep and chimaerism of serum transferrin and albumin types was also present in two of them. One sheep had a mixture of red cell potassium types and another of haemoglobin types. Soluble R and O blood group substances were present together in the saliva of all three sheep and on the red cells of two of them. The relative proportions of the two red cell populations changed with age of the sheep, the blastomere-derived population becoming less. In one sheep one transferrin type also declined. A fourth sheep studied was not a blood chimaera but was apparently composed of blood derived entirely from that of the transplanted blastomere cells. All four sheep were phenotypically male but studies of cultured leucocytes showed that female cells were present in two of the chimaeras (34 per cent and 89 per cent respectively).

INTRODUCTION

It is now well established that tetraparental (allophenic) chimaeras can be produced experimentally in mice by egg fusion (Tarkowski, 1961; Mintz, 1965) or by the injection of embryonic cells into host blastocysts (Gardner, 1970). There have, however, been no confirmed reports of the successful production of chimaeras by egg manipulation in any other species. In this paper we present data on the blood groups found in three sheep chimaeras produced experimentally by blastomere transplantation and describe age-related changes in the blood of these animals. In addition, a fourth sheep is described whose blood group was apparently derived entirely from that of the injected blastomeres.

MATERIALS AND METHODS

Sheep

Finnish Landrace, Suffolk and Welsh Mountain breeds of sheep were used. All parent sheep were fully blood-typed and where possible, a Finnish Landrace ram, homozygous for the rare albumin allele *W*, was used as the sire for the blastomere donors.

Blastomere transplantation

Fertilized eggs were flushed from the reproductive tracts of sheep, hereafter referred to as the blastomere donors, 3–7 days after the onset of oestrus by the method of Hunter, Adams and Rowson (1955). Individual blastomeres were obtained by removing the zona pellucida mechanically from each egg using a micromanipulator (de Fonbrune-Masson et Cie, Paris) and then disaggregating the blastomere mass using a 0·025 per cent solution of EDTA in calcium- and magnesium-free tyrode solution. Fertilized eggs from a second group of sheep, the egg donors, were obtained 72 hours after oestrus and one to four blastomeres were injected into each of these eggs using the microsurgical procedure of Lin (1969). The injected eggs were transferred to non-pregnant recipient sheep and allowed to develop to term.

Serology

Red cell antigens were detected using different sheep blood grouping reagents in a haemolytic test employing rabbit complement (Tucker, 1965). Twelve of these reagents were isoimmune antisera prepared by suitable absorptions and standardized in an International Sheep Blood-Grouping Comparison Test. The reagents were used at a dilution at which they always gave complete haemolysis with red cells from normal sheep possessing the corresponding antigen. The latter were always included in a test as a positive control. When only partial haemolysis occurred, the presence of two antigenically distinct red cell populations was suspected and the red cells were then tested with the reagent at strengths ranging from undiluted to 1 in 512. If only partial haemolysis was still obtained then chimaerism was confirmed. R-positive sheep cells were identified using cattle anti-J and O-positive cells by cattle anti-O antisera. Soluble R and O substances in serum and saliva were detected by inhibition tests (Tucker, 1965). Separation of the two red cell populations in a chimaera was carried out by differential haemolysis (Dain and Tucker, 1970).

Electrophoresis

Separation of the blood proteins was carried out by horizontal starch gel electrophoresis using a Tris–EDTA–borate continuous buffer system at pH 8·9 (Gahne, Rendel and Venge, 1960) for haemoglobins, and a Tris–citric acid discontinuous buffer system (Kristjansson, 1963) at pH 7·6 for the separation of serum transferrins and at pH 6·2 for the separation of serum albumins (Tucker, 1968).

Potassium types

These were determined using a flame photometer (Drury and Tucker, 1963).

Karyotyping

A chromosomal analysis of the peripheral leucocytes of the chimaeric lambs was carried out using the *in vitro* technique of McFee, Banner and Murphree (1965). A total of fifty suitable metaphase figures were photographed and analysed for each lamb.

RESULTS

BLASTOMERE TRANSPLANTATION

Blastomeres were injected into ninety-two eggs and these were subsequently transferred to fifty-seven recipient sheep. Lambs were carried to term by twenty-eight of the recipients;

a total of forty-seven eggs had been transferred to these twenty-eight recipient sheep, and thirty-three lambs were subsequently born. Blood samples were taken from each lamb 60 days after birth.

BLOOD TYPING

Of the thirty-three lambs born, twenty-nine had blood groups which could be attributed only to those of the egg donors. Presumably the injected blastomeres had not survived or had not become involved in the development of the blood system in these lambs. However, four single-born lambs arising from one injection series (i.e. using eggs from the same two sets of parents) had a blood phenotype which could not have been derived from the egg donor alone. Table 1 shows the blood groups of these four lambs together with those of the four possible parents).

In the serum of lamb number 8 (albumin type SW), the W albumin band was present (Fig. 1) and this could only have been derived from the sire of the blastomere donor

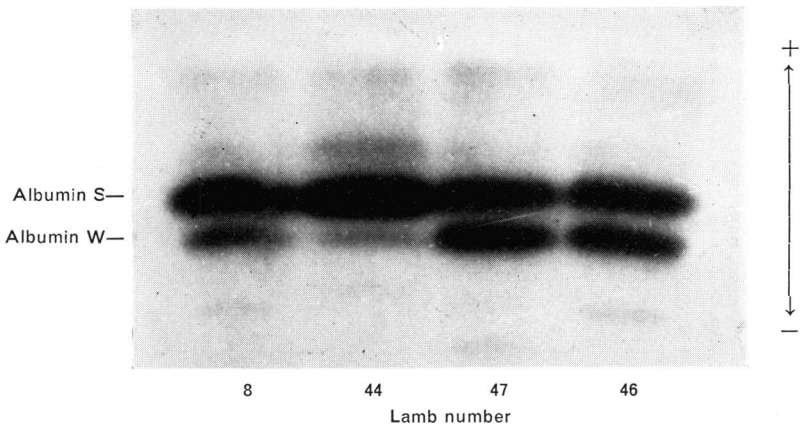

FIG. 1. Serum albumin phenotypes in the four lambs. Note the faint W band in lamb numbers 8 and 44. Starch gel electrophoresis in Tris–citric acid buffer, pH 6·2.

(ram E34). Moreover, unlike the normal phenotypic expression of an SW heterozygote, the S band in this case was much stronger in staining intensity than the W band. This would suggest a superimposition of an SW type (blastomere donor) on an SS type (egg donor). Also in this lamb, the transferrin C band was stronger than the A band (Fig. 2), suggesting admixture of transferrin type CC (blastomere donor) with AC (presumably egg donor). There were two antigenically distinct populations of red cells present. In population number 1, blood factors A, B′ and R could only have come from the parents of the egg donor and in population number 2, factors K and N′ only from those of the blastomere donor. The red cell potassium concentration in this lamb was not the usual distinct high potassium (HK) or low potassium (LK) type (Evans, 1954), but was of an intermediate value (67 mmoles/litre packed cells). When the two red cell populations were separated by differential haemolysis using selected blood-grouping reagents, it was found that the N′ negative population was LK type and the A-negative population HK type. The haemoglobin type was of the normal heterozygous AB pattern and could have been derived from either or both sets of parents.

TABLE 1

THE BLOOD TYPES OF FOUR LAMBS AND THEIR POTENTIAL PARENTS

	Sheep	Albumin	Transferrin	Red cell antigens	Potassium	Haemoglobin	Conclusion
Blastomere donor	W952 dam Welsh Mountain	SS	AC	K U N′ O′x Cx M M′ L O	Low	AB	
	E34 sire Finnish Landrace	WW	CD	Cx M M′ P O	High	AA	
Egg donor	W827 dam Welsh Mountain	SS	AB	U Cx M M′ P R	High	AA	
	517 sire Suffolk	SS	BC	A B′ O′x Cx M M′ L R	Low	BB	
Lamb	Number 8	SW	AC	* (1) A B′ Cx M M′ L O R	Low	AB	Chimaera for serum and red cells
				† (2) K U N′ O′x Cx M M′ P O R	High	AB	Blastomere and egg donor implicated
Lamb	Number 44	SW	ABC	(1) Cx M M′ O	High	AB	Chimaera for serum and red cells
				(2) U O′x Cx M M′ O	High	AB	Blastomere and egg donor implicated
Lamb	Number 47	SW	CC	(1) B′ Cx M M′ L O R	Low	AB	Chimaera for red cells
				(2) U N′ O′x Cx M M′ L P O R	Low	AA	Blastomere and egg donor implicated
Lamb	Number 46	SW	CD	K U N′ O′x Cx M M′ P O	High	AB	Not a chimaera Blastomere donor implicated.

* Population number 1.
† Population number 2.

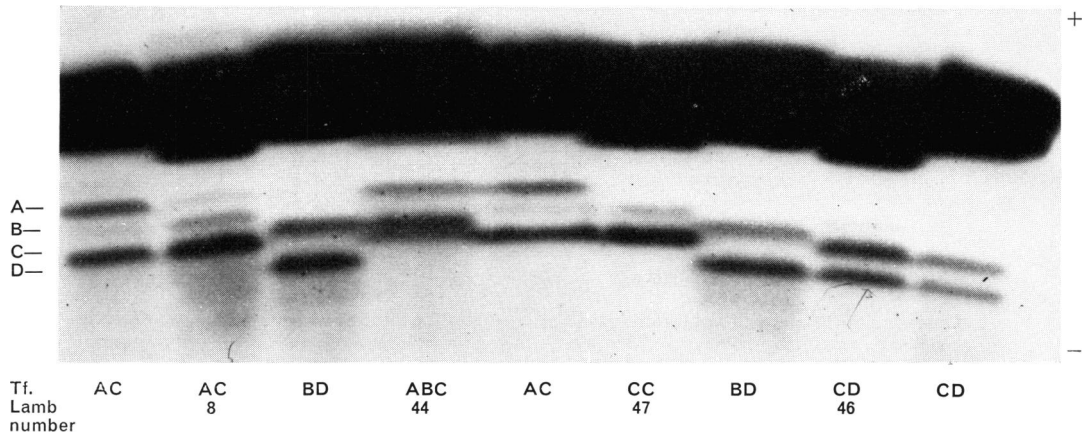

FIG. 2. Serum transferrin phenotypes in the four lambs. Each test sample is placed alongside an appropriate control sample. Starch gel electrophoresis in Tris–citric acid buffer, pH 7·6.

The second lamb, number 44, had a similar albumin phenotype to that of lamb number 8 (Fig. 1) and the same arguments attributing this type to the blastomere donor as well as the egg donor, could be applied. The transferrin phenotype was unusual, in that instead of the two main bands found in normal heterozygotes, three distinct bands (A, B, C) were present (Fig. 2). This could be a mixture of an AC (blastomere donor) with a BB, BC or AB (egg donor) or more likely from the intensity of the stained bands a CC (blastomere donor) with an AB (egg donor). Two distinct red cell populations were identifiable in this lamb by antigen differences but, since all the factors in both populations could have come from either set of parents, it was not possible to say from the red cell antigens whether either or both blastomere donor and egg donor were involved. Similarly, the potassium and haemoglobin types were normal, and could have been derived from either set of parents.

The third lamb, number 47, had a normal SW albumin phenotype (Fig. 1) but the presence of the W band indicated that parental blastomere donor genes must be involved. The transferrin type was a normal homozygous CC type (Fig. 2) which could only have been derived from the blastomere donor parents. Two distinct populations of red cells were present. Factor B′ (population number 1) could only have come from the egg donor, and N′ (population number 2) from the blastomere donor parents. The red cells of this lamb also gave a very weak R-positive reaction. The potassium type was a normal LK, which could be attributed to either set of parents. The haemoglobin pattern was abnormal (Fig. 3). in that instead of the usual heterozygous AB pattern, the A band was stronger than the B. Differential haemolysis followed by starch gel electrophoresis showed that the B′ negative population of cells was Hb type AA (blastomere donor) and the other population was type AB (Fig. 3).

Although there was no evidence that the fourth lamb (number 46) was a chimaera, the blood group data indicated that it was composed only of blastomere donor genetic material. The albumin and transferrin types, and the red cell factors N′ and K could only have been derived from the blastomere donor parents, and there was no need to implicate the egg donor parents in any of the other blood groups possessed by this lamb.

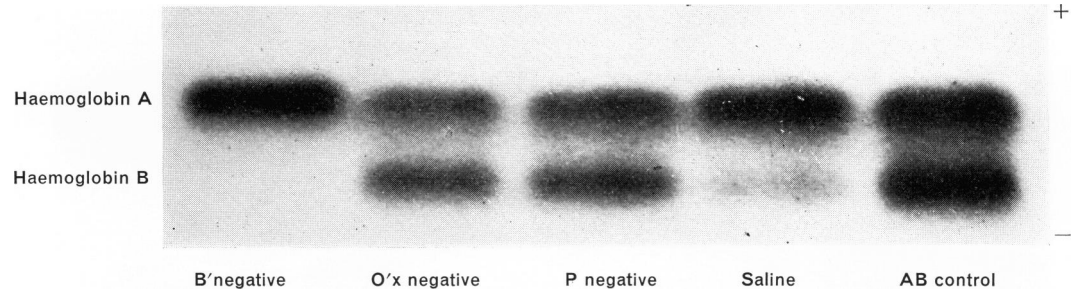

FIG. 3. Haemoglobin pattern of lamb number 47 after separation of the two red cell populations by differential haemolysis using reagencts B′, O′x and P. The saline sample shows the haemoglobin pattern of the red cells before separation; note the faint Hb B band. Starch gel electrophoresis in Tris–EDTA–borate buffer, pH 8·9.

CHANGES IN BLOOD TYPE WITH AGE

The four lambs were tested at the ages of 2, 13, 17, 20 and 25 months and the relative proportions of the two populations of red cells were determined by differential haemolysis (Table 2). In lamb numbers 8 and 47 the blastomere donor population decreased, and this was especially marked in lamb number 8. In this lamb, the potassium values also confirmed this decrease for they fell from 67 mmoles at 2 months to 35 mmoles/litre red cells at 17 months and thereafter maintained this level. The serum transferrin pattern did not change. In lamb number 44, since it was not possible to distinguish blastomere from egg donor-derived red cells, it is not known if the slight shift found was in the direction of egg or blastomere donor type majority. However, in this lamb the transferrin C band became weak, indicating that the blastomere-derived transferrin was, as had been suspected previously, type CC, and that this was disappearing relative to the egg donor

TABLE 2

CHANGE IN RED CELL POPULATIONS WITH AGE OF LAMBS

Age (months)	Lamb number 8		Lamb number 44		Lamb number 47		Lamb number 46
	Blastomere donor per cent (A negative)	Egg donor per cent (N′ negative)	Percentage (O′x negative)	Percentage (O′x positive)*	Blastomere donor per cent (B′ negative)	Egg donor per cent (P negative)	Blastomere donor per cent
2	39	53	62	38	71	46	100
13	14	75	36	64	55	43	100
17	7	94	25	75	58	55	100
20	8	87	23	77	51	64	100
25	7	93	21	79	45	58	100

* Second population percentage inferred; it could not be directly measured because no antibody was available which would lyse the O′x negative population.

Because of the unavoidable variability of the method of differential haemolysis, the above percentages are only approximate. A, N′ negative, etc. refers to the percentage of cells which were negative for that particular reagent.

transferrin type AB. There was no evidence that the albumin pattern changed in any of the lambs.

INHIBITION TESTS FOR BLOOD GROUP SUBSTANCES IN SERUM AND SALIVA

O substance was present in the serum and saliva of all four lambs. R substance was detectable in the saliva of lambs numbers 8, 47 and 44, but not in that of number 46, It was also found in the serum of lamb number 8 and there was a slight inhibition of anti-R by the serum of lamb number 44.

KARYOTYPES

All four lambs were phenotypically male, but cytogenetic studies, carried out when they were 6 months of age, showed female cells to be present as well as male cells in lamb number 47 (34 per cent female cells) and in lamb number 8 (89 per cent female cells).

DISCUSSION

The blood group results clearly established that three of the four lambs were composed of genetic material which could only have been derived from two sets of parents, while the blood of the fourth lamb was apparently composed only of blastomere donor material.

In naturally-occurring sheep twin chimaeras which result from placental vascular anastomosis *in utero*, the admixture of blood groups only extends to the red cells, and chimaerism of serum proteins is not found (Dain and Tucker, 1970). In two of the lambs in the present study, a mixture of transferrins or albumins was apparent and this indicates that, as would be expected, the chimaerism involved tissues other than blood. The red cell factors R and O in sheep are primarily soluble substances which only secondarily become attached to the red cells from the plasma (Rendel, 1957). All normal group R sheep have R substance present on their red cells and in their serum and saliva, whereas in group O sheep, O substance is similarly distributed. In naturally occurring chimaeras the 'foreign' red cells acquire the R and O specificity from their host's secretions and both populations in secretor chimaeras are therefore always R- or O-positive but never both. In the allophenic lamb numbers 8 and 47 both populations of red cells were both R and O positive, indicating that both the R and O substances were being secreted by these lambs. This was confirmed when R as well as O substance was found in their saliva. Although the red cells of the third lamb (number 44) were classified as R-negative, soluble R substance was detected in its saliva and there were probably also trace amounts in its serum. No R substance wa sfound in the saliva of lamb number 46 whose blood was composed only of blastomere donor material, and this lamb was the only one to have the naturally-occurring antibody, anti-R in its serum. It could be concluded therefore that the three allophenic lambs were secreting R substance as well as O, once more indicating that the chimaerism involved tissues other than blood.

In contrast to the situation in human (Race and Sanger, 1968) and cattle (Stone, Friedman and Fregin, 1964) 'natural' chimaeras, the relative proportions of the two red cell populations in sheep chimaeras have not been found to change with age (Dain and

Tucker, 1970). However in the three allophenic sheep in the present study one population is declining and in at least two of them it is the blastomere donor population which is decreasing. One hesitates to suggest that this change has an immunological basis, although there is evidence from studies with mice that certain chimaeras undergo a graft-versus-host reaction (Barnes, Tuffrey, Kingman, Thornton and Turner, (1972) and that the lymphocytes from tetraparental chimaeras react *in vitro* against parental cell lines (Wegmann, Hellström and Hellström, 1971). The change could be due to competition and selection, but if so, it is not apparently simply the result of a majority population crowding out a minority, because in the case of lambs numbers 44 and 47 it is the population which started out as a majority which has declined. Mintz and Palm (1969) suggested a possible selective advantage of C56Bl/6 over C3H mouse erythropoietic tissue, and it is likewise possible that the egg donor sheep phenotype has a marginal superiority over that of the blastomere donor.

In tetraparental mice a preponderance of males over females has been noticed (Mystkowska and Tarkowski, 1968), and it may be significant that the three allophenic sheep were all phenotypically male, although two of them were sex chromosome mosaics. By analogy with the mouse results one might expect to find that the ram which is not a sex-chromosome mosaic would breed according to either set of parental genes, whereas those with sex chromosome admixture would breed according to the genetic type of the male cells only. Breeding experiments are in progress to test this.

ACKNOWLEDGMENT

We wish to thank Miss Y. Pearce for capable technical assistance.

REFERENCES

Barnes, R. D., Tuffrey M., Kingman, J., Thornton, C. and Turner, M. W. (1972). 'The disease of the NZB mouse. I. Examination of ovum fusion-derived tetraparental NZB : CFW chimaeras.' *Clin. exp. Immunol.*, **11**, 605.

Dain, A. R. and Tucker, E. M. (1970). 'Cytogenetic, anatomical and blood group studies of sheep twin chimeras.' *Proc. roy. Soc. B*, **175**, 183.

Drury, A. N. and Tucker, E. M. (1963). 'Red cell volume, potassium and haemoglobin changes in lambs.' *Res. vet. Sci.*, **4**, 568.

Evans, J. V. (1954). 'Electrolyte concentrations in red blood cells of British breeds of sheep.' *Nature (Lond.)*, **174**, 931.

Gahne, B., Rendel, J. and Venge, O. (1960). 'Inheritance of β-globulins in serum and milk from cattle.' *Nature (Lond.)*, **186**, 907.

Gardner, R. L. (1970) 'Manipulations on the blastocyst.' *Advances in the Biosciences*. (Ed. by G. Raspe), volume 6, p. 279. Pergamon Press, Oxford.

Hunter, G.L., Adams, C. E. and Rowson, L. E. A. (1955). 'Interbreed ovum transfer in sheep.' *J. agric. Sci. (Camb.)*, **46**, 143.

Kristjansson, F. K. (1963). 'Genetic control of two pre-albumins in pigs.' *Genetics*, **48**, 1059.

Lin, T. P. (1969). 'Microsurgery of inner cell mass of mouse blastocysts.' *Nature (Lond.)*, **222**, 480.

McFee, A. F., Banner, M. W. and Murphree, R. L. (1965). 'Chromosome analysis of peripheral leucocytes of the sheep.' *J. Anim. Sci.*, **24**, 551.

Mintz, B. (1965). Genetic mosaicism in adult mice of quadriparental lineage. *Science*, **148**, 1232.

Mintz, B. and Palm, J. (1969). 'Gene control of haematopoiesis. I. Erythrocyte mosaicism and permanent immunological tolerance in allophenic mice.' *J. exp. Med.*, **129**, 1013.

Mystkowska, E. T. and Tarkowski, A. K. (1968). 'Observations on CBA-p/CBA-T6T6 mouse chimaeras.' *J. Embryol. exp. Morph.*, **20**, 33.

Race, R. R. and Sanger, R. (1968). *Blood Groups in Man*. Fifth Edn, p. 475. Blackwell Scientific Publications, Oxford.

Rendel, J. (1957). 'Further studies on some antigenic characters of sheep blood determined by epistatic action of genes.' *Acta agric. scand.*, **7**, 224.

Stone, W. H., Friedman, J. and Fregin, A. (1964). Possible somatic cell mating in twin cattle with erythrocyte mosaicism. *Proc. nat. Acad. Sci. (Wash.)*, **51**, 1036.

Tarkowski, A. K. (1961). 'Mouse chimaeras devel-

oped from fused eggs.' *Nature* (*Lond.*), **190**, 857.
TUCKER, E. M. (1965). Further observations on the i blood group in sheep. *Vox Sang.* (*Basel*), **10**, 195.
TUCKER, E. M. (1968). Serum albumin polymorphism in sheep. *Vox Sang.* (*Basel*), **15**, 306.
WEGMANN, T. G., HELLSTRÖM, I. and HELLSTRÖM, K. E. (1971). Immunological tolerance. Forbidden clones allowed in tetraparental mice (serum blocking factor). *Proc. nat. Acad. Sci.* (*Wash.*), **68**, 1644.