

## Immunogenetics of a sheep (*Ovis aries*) serum antigen

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### SUMMARY

An allotypic specificity (A1) of sheep serum is described. The antigenic determinant is located on a low molecular weight glycoprotein which has its isoelectric point at pH 5 and is capable of interacting with the lectin concanavalin A.

Family studies showed that the allotype is inherited in a simple Mendelian manner.

### 1. INTRODUCTION

At the present moment considerable attention is being paid to the phenomenon of allotypy since allotypes have proved extremely useful in a number of immunological and genetic studies such as the molecular structure of plasma proteins (Porter, 1963; Kabat, 1966) and the cellular and molecular aspects of antibody formation (Pernis *et al.* 1965; Kabat, 1966). By now numerous authors have contributed towards the identification and description of allotypes in a number of species including those in sheep (Rapacz, Hasler & Pope, 1970; Curtain, 1971; Bash & Milgrom, 1972).

This publication describes a sheep allotype, its mechanism of inheritance and the physico-chemical properties of the component carrying the allotypic determinant. The investigation forms part of a broader project directed toward the identification of allotypes in ruminants (in particular cattle, water buffaloes, sheep and goats) by means of isoimmune sera, with the objective of using allotypes as markers to monitor the phylogenetic relationships of serum proteins within this cluster of closely related species.

### 2. MATERIAL AND METHODS

(a) *Double diffusion* (D.D.). Five ml of 1% agarose (Behringwerke Ag, Marburg, Germany) in glycine buffer pH 7.8 were poured into Petri dishes of diameter 7 cm to form a layer 2 mm thick: the distance between the central well and the four peripheral ones was 6 mm. The central well (8 mm in diameter) was filled with 60  $\mu$ l of antiserum and each of the peripheral ones (6 mm in diameter) with 40  $\mu$ l of individual serum samples.

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(b) *Immuno-electrophoresis*. The procedure described by Hirshfeld (1960) was followed using the 2117 Multiphor apparatus (LKB, Bromma, Sweden). Electrophoresis was carried out by applying 7 V/cm for 1 h. Precipitation lines developed fully after a 2-day incubation period at room temperature.

(c) *Immuno-electrofocusing*. Isoelectric focusing of the antigen was performed in the 2117 Multiphor apparatus (LKB, Bromma, Sweden) following the manufacturer's instructions and using carrier ampholytes pH range 3.5–9.5. The resulting pH gradient was measured with a surface pH electrode (Ingold, Zurich, Switzerland). To ensure a medium of known pH value where antigen-antibody reaction could occur, before adding the antiserum to the troughs, the gel was dipped for 30 min in glycine buffer pH 7.8. Precipitation arcs were clearly visible after a 2- to 3-day incubation period at room temperature.

(d) *Sephadex G-200 gel filtration*, *Ultragel AcA22 gel filtration* and *ConA-Sepharose affinity chromatography* were performed as described by Iannelli *et al.* (1977); *DEAE-A50 ion exchange chromatography* was as described by Fahey & Terry (1967).

(e) *Immunization*. Sheep used as donors or as recipients were obtained by cross-breeding (Gentile di Pugliax Ile de France × Württemberg). Equal amounts of serum from two donors were pooled and emulsified with an equal volume of complete Freund's adjuvant. Each sheep received 5 ml of emulsion intramuscularly at 1-month intervals. Serum samples were collected from the immunized animals 7 days after each injection and tested against the individual donor sera by D.D.

### 3. RESULTS

#### (i) *Identification and characterization of the antigen*

Alloimmunizations of sheep with whole serum caused the production of antibodies which reacted with some but not all tested sheep sera. The antiserum used throughout the experiments reported here was produced in one of the immunized animals 7 days after the fifth injection. This antiserum was termed anti-A1; the antigenic marker detected with it A1; the gene controlling the synthesis of A1 was *A1* and the corresponding locus *A*. Classification of the animals into A1(+) (those having A1 specificity) and A1(−) (without A1 specificity) was performed by D.D. (Plate I, Fig. 1). The precipitation bands developed after 24 h and further incubation did not reveal additional ones. These were uniform for all reactive sera and coalescence was often observed between A1(+) sera when they happened to be in adjacent wells.

Absorption of anti-A1 antibodies with 30 individual positive sera (500  $\mu$ l of antiserum plus 100  $\mu$ l of A1(+) serum) were kept for 30 min at 37 °C and overnight at 5 °C; the mixture was then centrifuged for 15 min at approximately 2000 g exhausted the antiserum completely, thus suggesting that it could be monospecific, at least under the testing conditions used throughout this particular study.

In order to estimate the molecular weight of the molecule carrying the A1

determinant, one of the A1(+) sera was subjected to gel filtration through Sephadex G-200 and AcA22. The material with A1 antigenic activity eluted with albumin on both resins. The behaviour of A1 antigen was also studied by means of ion exchange chromatography on DEAE-A50 and was shown to adsorb onto the resin at pH 8.0 in 0.1 M Tris-HCl and to elute on addition of 1.0 M-NaCl.

Since concanavalin A (ConA) reacts with various serum components (Harris & Robson, 1963), presumably because of its specificity for their non-reducing mannosyl and *N*-acetyl-D-glucosaminyl end groups (Goldstein & Iyer, 1966), it seemed worth investigating the interaction of the A1 antigen with ConA in a further attempt to outline some of the characteristics of the former. When the A1(+) serum was chromatographed on ConA Sepharose in 0.1 M-Na acetate buffer at pH 7.8 containing 1 M-NaCl and  $10^{-3}$  M-Mn<sup>2+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup>, A1 bound to the resin. It was eluted on addition of 1.0 M  $\alpha$ -methyl-D-glycoside, thus suggesting that the molecule on which the antigen resides contains the sugar residues necessary for interaction with ConA.

To determine the electrophoretic mobility of the antigen, the same serum as used in the previous experiments was submitted to immunoelectrophoresis and the resulting pattern is shown in Plate I, Fig. 2.

Finally, the pI of the component carrying A1 was also determined by immunoelectrofocusing and shown to be approximately at pH 5.0 (Plate I, Fig. 3).

#### (ii) Genetic studies

Family data (Table 1) indicate the dominant inheritance of A1, as is the case for the great majority of antigenic markers: negative offspring are observed from +  $\times$  + matings, suggesting that some of the parents are heterozygous for this marker; no positive offspring are observed from -  $\times$  - matings, as must be expected when the absence of the factor is recessive. Segregation ratios from 21 matings between homozygous negative ewes and heterozygous rams (the heterozygosity of the three rams involved in the matings was ascertained by pedigree analysis) gave 10 positive and 11 negative offsprings, which is very close to the expected 1:1 ratio ( $\chi^2 = 0.05$  for 1 D.F.), thus confirming the dominant inheritance of the antigen. These matings produced both positive and negative offspring of both sexes, indicating that the A locus is not sex linked.

#### 4. DISCUSSION

Alloimmunization of sheep with whole serum resulted in the production of an antiserum (anti-A1) specific for an allotype marker (A1) carried by a serum component of low molecular weight.

In an attempt to delineate some of the physico-chemical characteristics of the protein carrying the determinant, A1(+) serum was submitted to gel filtration (Sephadex G-200 and AcA22), ion exchange chromatography (DEAE-A50), affinity chromatography (ConA-Sepharose), immunoelectrofocusing and immunoelectrophoresis. As a result of these studies it has been established that A1 antigen

Table 1. *Inheritance of A1 antigen*

Mating	Number of offspring			$\chi^2$	D.F.
	Total	A1(+)	A1(-)		
+ × +	8	6 (6.47)*	2 (1.53)*	0.068	1
+ × -	39	21 (21.92)*	18 (17.08)*	0.077	1
- × -	31	0	31		

\* Expected number of progeny in each class (figures in parentheses) was calculated on the basis of a gene frequency of  $A1 = 0.22$ .

upon gel filtration elutes along with albumin, possesses on its surface the sugar residues (mannosyl and/or *N*-acetyl-D-glucosaminy) required for interaction with ConA, and has its isoelectric point at pH 5.

More data on the chemical features of this protein (its carbohydrate and amino acid composition) are being collected and will be reported on at a later date.

Segregation data (Table 1) leave little doubt that A1 antigen is inherited as though controlled by a single dominant gene (*A1*), whilst its absence is controlled by a recessive allele (*a1*). The A1(+) animals included many 1- to 4-week-old lambs which would appear to suggest that the antigen is fully expressed either at birth or very soon after. It was excluded that maternal transfer of the antigen might have taken place since the phenotype of 4- to 5-month-old lambs was the same as that established at 1-4 weeks.

Group specific component (Gc) proteins in man are capable of binding vitamin D<sub>3</sub> under physiologically normal conditions (Daiger, Schanfield & Cavalli-Sforza, 1975). Their molecular weight is around  $5 \times 10^4$  (Giblett, 1969), i.e. close to that found for A1. In addition, proteins immunologically similar to the human Gc are known to occur in several other mammals (Reinskou, 1968). However, autoradiography of A1(+) serum, radiolabelled with [<sup>14</sup>C]vitamin D<sub>3</sub> as described by Daiger *et al.* (1975), proved that the molecule carrying the determinant A1 is not a Gc-like protein and its function is still entirely unknown.

At present we are directing our research toward the identification of the product of a hypothetical gene allelic to *A1* so as to ascertain whether in the heterozygous animal the products of the two allelic genes are both present on the same molecule. These data and what is even more significant their comparison with those obtained for immunoglobulin, macroglobulin and lipoprotein allotypes in other species, will be helpful in elucidating the biosynthesis and structure of serum proteins.

Another question which also remains to be clarified is whether the antigenic specificity is located on the protein portion of the molecule - as, for example, in the case of light and heavy chain allotypes of rabbit immunoglobulins (Reisfield, Dray & Nisonoff, 1965) - or on the carbohydrate portion - as recently found by the present author (unpublished results) for McA1, initially named Ci(a) (Iannelli, Masina & Zacchi, 1968), and McA2, two cattle macroglobulin markers. This latter possibility (i.e. that A1 might reside on the carbohydrate portion of the molecule) should also in fact be considered, in view of evidence that the protein carrying

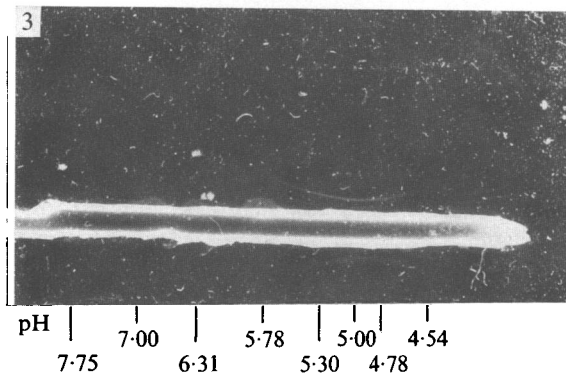
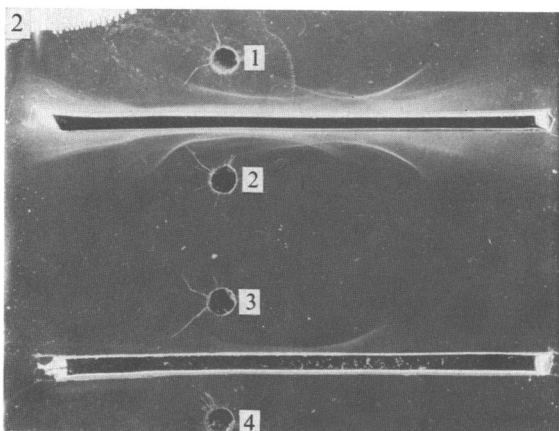
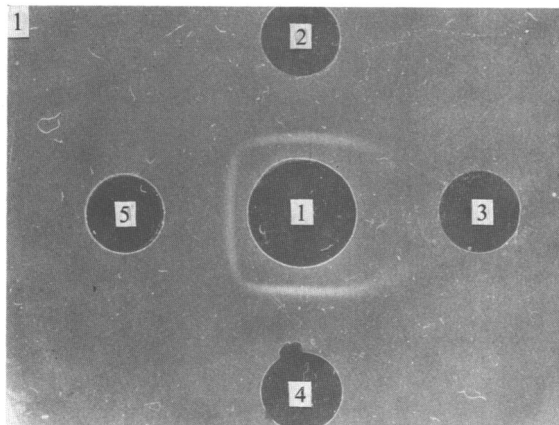


Fig. 1. Double diffusion pattern of A1 specificity. Well 1 was filled with 60  $\mu$ l of anti-A1 antiserum; wells 2, 4, 5 with 40  $\mu$ l of three different A1(+) sera and well 3 with 40  $\mu$ l of A1(-).

Fig. 2. Immunoelectrophoretic pattern of A1 antigen. Electrophoresis was conducted at 7 V/cm for 60 min. The anode was on the right. Upper trough contained rabbit antiserum to sheep whole serum; lower trough, anti A1 antiserum. Wells 1 and 4 contained an A1(-) serum and wells 2 and 3 an A1(+) serum.

Fig. 3. Analytical immunoelectrofocusing of A1 antigen. Peak III from Sephadex G-200 chromatography was concentrated by ultrafiltration up to 5 ml, then 15  $\mu$ l were adsorbed onto Whatman 3MM filter paper (size: 5  $\times$  10 mm) and focused for 3 h at 1000 V using carrier ampholytes pH 3.5-9.5. After electrofocusing and before application of antiserum the gel was dipped in glycine buffer pH 7.8 for 30 min. The precipitin arc formed approximately at pH 5.

A1 has been shown to interact with concanavalin A and, consequently, to contain carbohydrates which are strongly antigenic (Giblett, 1969).

Finally since the determinant A1 is located on a low molecular weight glycoprotein, it must be different from the  $\alpha_2$ -macroglobulin, the  $\beta$ -lipoprotein and the immunoglobulin sheep allotypes already identified by Curtain (1971) and by Rapacz (1970) as well as from the C, E and H markers described by Bash & Milgrom (1972) which are also carried by  $\alpha_2$ -macroglobulin molecules.

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