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A New Immunoglobulin Sub-Class in the Sheep

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Summary. A new immunoglobulin sub-class, $IgG1_A$, has been defined in the sheep with the aid of an antibody prepared by immunizing goats with sheep IgG. The sub-class antigen is located on the Fc fragment and is carried by approximately 20 per cent of the IgG1 molecules. $IgG1_A$ is lacking in the serum of some sheep and this appears to be under X-linked genetic control. No marked breed distribution was found for $IgG1_A$ negativity.

INTRODUCTION

The immunoglobulins of all the mammalian species so far studied have been divided into several classes on the bases of their molecular weights, and the chemical and immunological properties of their heavy chains. In the sheep, four immunoglobulin classes have been defined: two analogues to human IgM, and IgA and two antigenically related 7S immunoglobulins, IgG1 and IgG2 (Silverstein, Thorbecke, Kraner and Lukes, 1963; Aalund, Osebold and Murphy, 1965).

In the course of an investigation into the phylogenic relationships between the immunoglobulins of the domesticated ruminants, injection of goats with ovine IgG1 produced a precipitating antibody which reacted with approximately 20 per cent of the molecules of the IgG1, thus defining a new ovine immunoglobulin sub-class $IgG1_A$.

This paper describes some of the properties of this new immunoglobulin sub-class and its defining antibody.

MATERIALS AND METHODS

Animals

The sheep used as a source of immune globulins and lymph nodes were adult (4–6 years), cross-bred Merino wethers maintained in pens at the C.S.I.R.O. Animal Health Research Laboratory, Parkville, Victoria, Australia. Serum samples were also obtained from sheep of a number of breeds kept on various properties in south-eastern Australia. The goats used for the production of antisera were of the Saanen strain, purchased from various commercial breeders and maintained in pens at the Parkville laboratory.

Immunoglobulin preparation

A mixture of IgG1 and IgG2 was separated from the serum of individual sheep by chromatography on DEAE-cellulose (Whatman DE11) by the method of Levy and Sober (1960). IgG1 and IgG2 were separated from the mixture by gradient-elution chromatography on DEAE-cellulose. The gradient used was from 0·01 M Na_2HPO_4 to 0·3 M NaH_2PO_4 and the column was loaded with 10 mg protein/1 ml of wet DEAE-cellulose. The two fractions were homogeneous as judged by immunoelectrophoresis against an antiserum prepared in rabbits to the whole mixture.

^{125}I precipitin assay

One-millilitre volumes of a 10 per cent (w/v) solution of IgG1 labelled with ^{125}I by the method of Hunter and Greenwood (1962) to a level of 1·4 atom of iodine per molecule of protein were incubated for 10 hours under sterile conditions at 37° with 0·5-, 1·0-, 1·5 and 2·0 ml of antiserum. The precipitate was collected by centrifugation at 30,000 ***g*** for 20 minutes at 4° and washed four times at 4° by suspending in 3 ml of 0·14 M NaCl and centrifuging for 20 minutes at 30,000 ***g***. The radioactivity in the precipitate and the combined supernatant and wash fluids was determined in a Packard Autogamma Scintillation Spectrometer.

Papain digestion

Papain digestion of immunoglobulin was carried out by the method of Edelman, Heremans, Heremans and Kunkel (1960).

Immunoelectrophoresis

This was carried out in agar using the method of Scheidegger (1955).

Ouchterlony immunodiffusion

Ouchterlony immunodiffusion was carried out using Hyland 'Immuno-plates', pattern D.

Fluorescent antibody technique

The various antibodies were purified on DEAE-cellulose, conjugated with either fluorescein isothiocyanate or tetraethylrhodamine isothiocyanate and purified by chromatography on DEAE-acrylate gel as described previously (Curtain and Baumgarten, 1965). Five-micron frozen sections were cut from fresh lymph nodes, fixed and treated with the fluorescent antibodies, as described by Curtain and Baumgarten (1966).

In vitro *culture of peripheral blood lymphocytes*

Sheep peripheral blood lymphocytes were cultured in the presence of phytohaemagglutinin (Difco PHA-P) as described previously (Lobb, Curtain and Kidson, 1967). The lymphocytes were also cultured in the presence of the various anti-sheep immunoglobulin antisera.

Injection of animals

The purified immunoglobulins were either adsorbed on to aluminium hydroxide at pH 6·8 (Boyd, 1956) or emulsified with complete Freund's adjuvant (Difco) and injected intramuscularly into the gluteal muscles. In some cases, the primary injection was of aluminium hydroxide-adsorbed antigen intravenously into the jugular vein. Although sheep were found to withstand subsequent intravenous injections, goats were prone to generalized anaphylaxis. From 10 to 100 mg of antigen were injected weekly for up to 10 weeks.

RESULTS

INJECTION OF GOATS WITH SHEEP IMMUNOGLOBULIN

Three goats were injected with a mixture of IgG1 and IgG2. All were given initially 50 mg of aluminium hydroxide-adsorbed antigen intravenously and then 20 mg of the adsorbed antigen in Freund's adjuvant weekly. After 3 weeks, an antibody appeared in the serum of the goats which gave a sharp precipitin line with the sera of four out of five sheep tested from the experimental flock (Fig. 1). On testing isolated IgG1 and IgG2

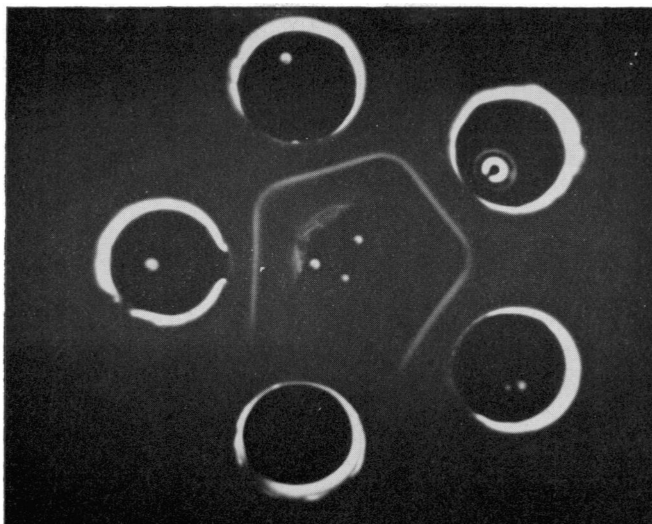

FIG. 1. Reaction of four positive and one negative sheep sera with goat anti-sheep immunoglobulin.

from the positive sera on the Ouchterlony plate against the goat antisera, and by immunoelectrophoresis of whole serum, it was found that the antigen was carried by the IgG1 fraction only (Fig. 2). In the immunoelectrophoretic pattern a reaction of partial identity occurred between the line produced by the goat antiserum and the line produced by the rabbit antisera with the IgG1. The three goat antisera had identical properties. Two more goats were immunized, one with IgG1 and the other with IgG2. Only the goats immunized with IgG1 gave an antibody response, and this was identical with that given by the goats immunized with the whole IgG fraction.

The reaction of partial identity between the IgG1 molecules precipitated by the goat antisera and the whole IgG1 precipitated by the rabbit antisera suggests that the former is a sub-class of the IgG1. In the rest of the paper, this sub-class will be called $IgG1_A$ and the defining antibody anti-$IgG1_A$.

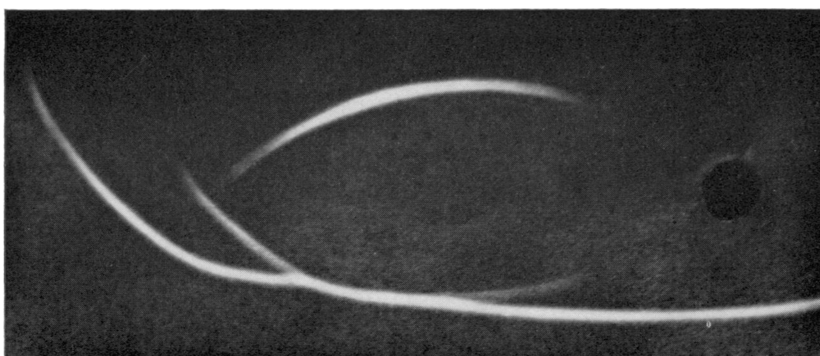

FIG. 2. Immunoelectrophoresis of sheep serum (Ig region). Upper well, Goat anti-$IgG1_A$; lower well, rabbit anti-sheep-IgG.

PROPORTION OF $IgG1_A$ MOLECULES IN SHEEP IgG1

This was determined by measuring the amount of ^{125}I-labelled sheep IgG1 precipitated by varying amounts of anti-$IgG1_A$, as described above. It was found that the 1·0-, 1·5- and 2·0-ml volumes of goat antiserum precipitated 20 per cent of the labelled IgG1. All of the radioactivity in the original IgG1 could be precipitated by the addition of 4·0 ml of rabbit anti-sheep IgG.

IMMUNIZATION OF SHEEP NEGATIVE FOR $IgG1_A$

Pooled aluminium hydroxide-adsorbed sheep IgG1, containing $IgG1_A$, was injected with Freund's adjuvant into an $IgG1_A$-negative sheep. After four weekly injections a precipitating antibody appeared in the serum of the sheep. This antibody gave a reaction of identity with the goat anti-$IgG1_A$ against the IgG1 on the Ouchterlony plate.

LOCALIZATION OF ANTI-$IgG1_A$ IN SHEEP AND GOAT ANTISERA

IgG1 and IgG2 were separated from the antiserum raised in the $IgG1_A$-negative sheep and from pooled goat anti-IgG_A. In both antisera, the antibody activity appeared to be equally distributed in the two fractions.

STABILITY OF ANTI-$IgG1_A$

The activity of the antibody was unaffected by heating at 56° for 50 minutes. It was stable for 9 months on storage at −70°.

ABSENCE OF $IgG1_A$ FROM THE SERA OF OTHER BOVIDS

Sera from twenty-four goats, twenty-six shorthorn and thirty Hereford steers and six Benteng cattle gave no precipitin reaction with either goat or sheep anti-$IgG1_A$.

ABSENCE OF $IgG1_A$ FROM COLOSTRUM

Colostrum was collected from ewes with $IgG1_A$ positive sera. The casein and fat were removed by acidification to pH 3·8 and the supernatant concentrated fourfold by pressure dialysis against pH 7·0, $I = 0{\cdot}1$ sodium phosphate buffer. The concentrate was tested by immunodiffusion and $IgG1_A$ was absent in all cases.

DEVELOPMENT OF $IgG1_A$

$IgG1_A$ was absent from foetal and newborn lamb sera and could be first detected at about 16 weeks of age, after weekly testing.

IMMUNOCYTOCHEMICAL LOCALIZATION OF $IgG1_A$

Using rhodamine-conjugated goat anti-$IgG1_A$ and fluorescein-conjugated rabbit antibody to IgG obtained from $IgG1_A$-negative sheep, it was possible to show that $IgG1_A$ occurred in approximately 10 per cent of the immunoglobulin-containing cells in frozen sections of sheep lymph nodes. The lymph nodes of sheep negative for $IgG1_A$ did not contain cells reacting with the labelled goat antiserum.

PERIPHERAL BLOOD LYMPHOCYTE CULTURE

Incubation of sheep blood lymphocytes with rabbit anti-sheep IgG resulted in blast transformation of approximately 80 per cent of the cells. Approximately 15 per cent of the

cells from $IgG1_A$-positive sheep were transformed by culture in the presence of sheep or goat anti-$IgG1_A$. Serum from non-immunized goats did not cause transformation. On culture in the presence of PHA and subsequent staining with fluorescent antibodies, it was found that approximately 20 per cent of the cells from $IgG1_A$-positive sheep contained $IgG1_A$. None of the PHA-stimulated cells from $IgG1_A$-negative sheep reacted with fluorescent anti-$IgG1_A$.

DISTRIBUTION OF $IgG1_A$ IN DIFFERENT FLOCKS

A total of 411 sera from sheep of different breeds was tested (Table 1). Overall, 90 per cent of the sheep were $IgG1_A$-positive, although wide fluctuations occurred from flock to flock. No marked variation with breed was noticed when the figures for all the flocks were taken together. More rams than ewes were negative for $IgG1_A$.

TABLE 1

DISTRIBUTION OF $IgG1_A$ IN SHEEP OF DIFFERENT BREEDS

Breed	Rams		Ewes	
	+	−	+	−
Dorset Horn				
Flock 1	5	1	9	0
Flock 2	12	2	11	0
Corriedale				
Flock 1	28	5	10	2
Southdown				
Flock 1	16	0	4	0
Flock 2	9	4	6	1
Flock 3	8	1	7	0
Romney Marsh				
Flock 1	15	2	19	1
Flock 2	12	4	15	0
Merino				
Flock 1	45	5	37	1
Flock 2	18	3	39	0
Flock 3	33	4	16	1

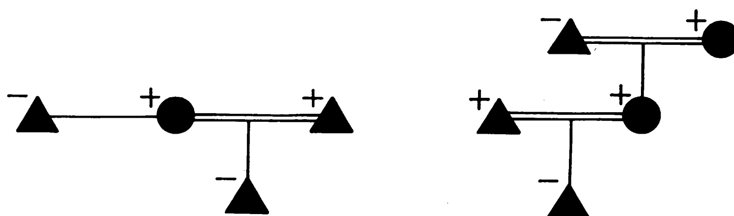

FIG. 3. Pattern of X-linked inheritance of absence of $IgG1_A$ in two families of Romney Marsh sheep. ▲, Ram; ●, ewe.

GENETIC CONTROL OF $IgG1_A$

From an examination of the records of a stud flock (Romney Marsh) in which 10 per cent of the sheep were negative for $IgG1_A$, it seemed that the absence of $IgG1_A$ from the serum was controlled by an X-linked gene (Fig. 3).

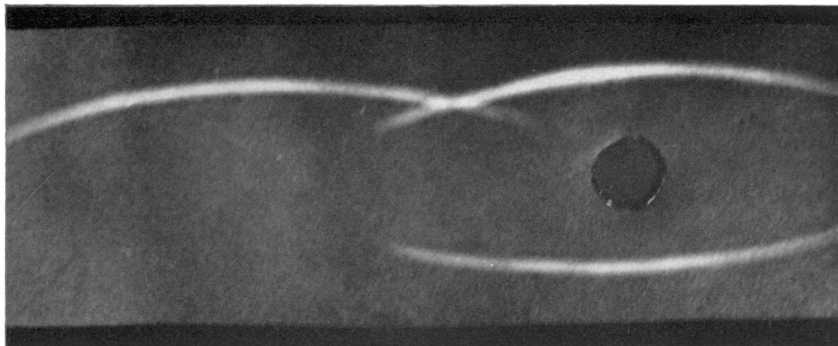

FIG. 4. Immunoelectrophoresis of papain-digested sheep IgG1. Lower well, Goat anti-$IgG1_A$; upper well, rabbit anti-sheep-IgG.

MOLECULAR LOCALIZATION OF THE $IgG1_A$ ANTIGEN

Immunoelectrophoresis of papain-digested sheep IgG1 was carried out against goat anti-$IgG1_A$ and rabbit anti-IgG1. Only the fast fragment of the papain digest reacted with the anti-$IgG1_A$ (Fig. 4). Since it has been observed (Curtain, unpublished) that the fast and slow fragments of ovine immunoglobulins are analogous to the Fc and Fab pieces observed in other species it appears that the sub-class-specific antigen is localized on the Fc fragment.

DISCUSSION

In the mouse, a close similarity is found between the antigenic determinants and the peptide maps of $IgG2_A$ and $IgG2_B$, and a lack of similarity between these and the IgG1 and IgA classes of that species (Potter, Apella and Geisser, 1965). The close relationship in the mouse between $IgG2_A$ and $IgG2_B$ has led to their being defined as sub-classes of IgG2. Similarly, it appears from the reaction of partial identity shown in the immunoelectrophoresis pattern that sheep $IgG1_A$ shares many antigenic determinants with the other molecules in the IgG1 region of the sheep serum protein pattern. That two populations of molecules are involved is shown by the fact that increasing concentrations of anti-$IgG1_A$ would not precipitate more than 20 per cent of ^{125}I-labelled sheep IgG1 although all the radioactivity could be precipitated by rabbit anti-IgG1. We are, therefore, justified in regarding $IgG1_A$ as a sub-class of IgG1.

Although breeding experiments over several generations will be necessary to confirm the observation, the apparent X-linked inheritance governing the absence of $IgG1_A$ from the serum of sheep is of interest in view of the evidence that the regulatory genes controlling human immunoglobulin synthesis are possibly also X-linked, as suggested by the overwhelming proportion of males amongst human agammaglobulinaemic patients (Lyon, 1962). Selective agammaglobulinaemia, where only one immunoglobulin class is missing, is also known in man (see reviews by Stiem and Fudenberg, 1966; Hobbs, 1968) although the mode of inheritance of these selective agammaglobulinaemias is not clear cut.

The finding that PHA did not stimulate the synthesis of $IgG1_A$ in cultured peripheral lymphocytes from $IgG1_A$-negative sheep is in accord with the finding of Fudenberg and Hirschhorn (1964) that culture in the presence of PHA did not lead to immunoglobulin synthesis although it still stimulated blast-transformation in peripheral blood lymphocytes from agammaglobulinaemic human subjects.

The improbable alternative to the regulatory gene hypothesis is the location of the structural gene for the IgG1$_A$ H chain in the X-chromosome. The available evidence for mouse, rabbit and man suggests that the autosomal gene loci determining the H chains are clustered in a chromosome region in close linkage (Mårtensson, 1966). In view of the conservatism in the major features of antibody synthesis of the range of species so far studied, it is hardly likely that the sheep has acquired a locus for an immunoglobulin sub-class on the X-chromosome.

Finally, the results throw an interesting light on the relationship between the sheep and the goat. It is significant that all five goats injected with sheep IgG readily produced anti-IgG1$_A$ antibodies, all of which gave reactions of identity with each other. No other antibody was formed. Sheep, goats and cattle are members of the family *Bovidae* of the genus *Bovoidea*. The development of the bovids took place at a relatively recent date. They were probably derived from the tragulids of the Oligocene but the first fossils, *Eotragus* and other rare forms, appear only towards the end of the Miocene (Romer, 1945). Eurasia appears to have been the main centre of dispersal and a large number of forms have been described from the Old World Pliocene where the two main lines of bovid evolution into sheep and goats on the one hand, and cattle and bison on the other, are discernible. Pointing the way to the sheep and goats are the so-called goat antelopes, such as the chamois. It would be of considerable interest to test for IgG1$_A$ in the sera of the surviving wild sheep, the moufflon of Europe and Asia Minor, the urial of Afghanistan and western Asia, the argali of central Asia and the bighorn of northern Asia and North America. Without this information, it is difficult to say whether the gene for IgG1$_A$ arose early in the evolution of the sheep, or as a result of selective processes in the course of domestication.

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