SERUM ANTIGENS OF CATTLE. I. IMMUNOGENETICS OF A MACROGLOBULIN ALLOTYPE¹

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THERE has been considerable work on allotypes in several species, but relatively little in cattle. ASHTON (1958) reported the rare absence of a slow α_2 -globulin in cattle serum detected by differences in electrophoretic patterns on starch gels. GAHNE (1964) produced isoprecipitins by immunizing "negative" cattle with whole serum from cattle containing the α_2 -globulin. This protein is a normal constituent of most cattle sera; its absence is apparently very rare. More recently, MILLOT (1966) presented preliminary results describing an allotypic system in cattle. He found that 2 to 3% of cow sera contained naturally occurring antiglobulins which agglutinated cattle erythrocytes previously sensitized with isoimmune sera. Using an inhibition test similar to that used to detect the Gm and Inv types in humans, he showed that some normal cattle serum could inhibit this agglutination reaction. He concluded that the normal serum contained an allotypic factor called A. In the second phase of this work, he described another factor, called B. It was detected using an antiglobulin serum prepared in goats against precipitates of ovalbumin with cattle anti-ovalbumin serum. Normal cattle serum capable of inhibiting the agglutination of sensitized cells coated with this heteroimmune antiglobulin serum contained the B factor. No data on the physical and chemical nature of the two factors were reported, but by analogy with the human allotypes, MILLOT suggested that they were γ -globulins.

Our attempts to detect allotypes in cattle began in 1961. In our earlier studies (IANNELLI, HIRSCHFELD and STONE 1966), we detected what appeared to be an α -globulin specificity using a heteroimmune antiserum produced in rabbits against pooled cattle serum. After suitable absorption with individual cattle sera, the antiserum gave precipitation in gel with some cattle sera. This allotypic specificity was not constant *in vivo*. Some serum samples obtained at different times had lost or gained the specificity, so we could not study the inheritance of this factor. The explanation of this variation is still unknown.

The present paper is the first in a series in which we will describe several allotypic systems in cattle. The first system, call Mc (Macroglobulin cattle) is a

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FIGURE 1.—Starch gel electrophoretic patterns of ammonium sulphate fraction of serum (top) used for isoimmunization and of normal cattle serum (bottom). Direction of migration from left (cathode) to right (anode).

macroglobulin detected by isoimmune antibodies produced against an ammonium sulphate fraction of serum.

MATERIALS AND METHODS

Preparation of Antigen: An ammonium sulphate fraction of serum was used rather than whole serum to reduce the total volume as well as the amount of albumin used in the immunizations and to provide dry, weighed samples. To fresh adult cattle serum an equal volume of saturated ammonium sulphate was added. The resulting precipitate was dissolved in water, dialyzed and re-precipitated three times. The final product was lyophilized and stored under vacuum in the cold until used as antigen. Nitrogen determinations of the antigen preparation using the Folin-Ciocalteu Method (KABAT and MAYER 1961) showed that it contained an average of 70% protein by dry weight. Starch gel (Figure 1) and immunoelectrophoretic analyses (Figure 2) showed that the preparation contained high concentrations of gamma and beta globulins, lesser concentrations of alpha globulins and lipoproteins and traces of albumin.

Immunizations: Each of six adult Holstein-Friesian cows was immunized with an antigen preparation obtained from different donors of either the Brown Swiss or Ayrshire breeds. In general, each recipient received intramuscular injections at three-week to six-week intervals. Each injection consisted of an equal mixture of adjuvant (Difco) and antigen preparation con-

FIGURE 2.—Upper—The electrophoretic pattern of the ammonium sulphate fraction of serum used for isoimmunization compared to the pattern obtained with normal cattle serum. Lower—The immunoelectrophoretic patterns obtained with these same preparations tested against the isoimmune serum detecting the Mc1 specificity. Direction of migration from left (cathode) to right (anode).

taining approximately one gram of antigen. Two of the six recipients were immunized with complete adjuvant and four with incomplete adjuvant. Later, these four received complete adjuvant with the antigen and one began to produce precipitins. Unfortunately, it was killed prior to bleeding. Only the recipients originally immunized with complete adjuvant produced useful antisera, but only after seven injections during a period of six months. Generally, antibodies did not appear until after the fourth or fifth injection (16–20 weeks). The titers of the antibodies drawn at different times after each injection were very erratic. Therefore, it was necessary to obtain serum samples every other day after the last injection to determine the time of the peak titer. Usually, the best antisera were obtained between seven and twelve days after the last injection. Blood was drawn from the recipient, the serum harvested, and stored frozen until used.

Gel Precipitation Tests: The antisera were tested against individual cattle serum by a double diffusion precipitation test on microscope slides using a modification of the technic described by HIRSCHFELD (1963). Because the reliability of the test required exact conditions, we will describe the procedure in detail.

The agar gel was prepared by dissolving 0.86 g of Hemagglutination Buffer (Bacto, #0512, Difco Laboratories, Detroit, Michigan) in 100 ml of deionized water. One gram of special agar (Noble, #0142-01, Difco Laboratories) was added to the buffer solution and the mixture placed in a boiling water bath for 35 min. Immediately after heating, merthiolate was added to a concentration of 0.01%. While the mixture was still hot, 2 ml was pipetted onto the surface of a microscope slide (1 × 3 in.). The slides were then placed in a moist chamber at 4°C overnight. The next day, wells were cut into the gel using a plastic template and a sawn-off hypodermic needle as a punch. A variety of templates with different well patterns was tried, but for routine typing the pattern containing a center well with six evenly-spaced peripheral wells was superior. In this regular hexagonal pattern, each well was 2.5 mm in diameter, and each peripheral well was equidistant (4.0 mm) from the edges of its adjacent wells and from the edges of the center well (Figure 3).

FIGURE 3.—Isoimmune antiserum (106S) detecting the Mc1 specificity tested by gel precipitation with cattle normal sera. Upper—(a) Actual test using unabsorbed antiserum. (b) Diagram of results in (a). Lower—(c) Actual test using absorbed antiserum. (d) Diagram of results in (c). Note that the unabsorbed antiserum detects at least three different specificities.

Using finely drawn out Pasteur pipettes, the peripheral wells were filled just over the top with antigen (serum). Next, the center well was filled just to the top with undiluted antiserum (some antisera could be used diluted as high as $\frac{1}{4}$, but undiluted antisera gave unequivocal results and were generally used). Antisera were frequently used in an absorption procedure as follows: the center well was filled to the top with the absorbing serum. This serum was allowed to diffuse out completely (30–60 min.). The antigen was then added to the peripheral wells as already described. Finally, the antiserum was added to the center well.

The tests were incubated in a moist chamber at room temperature ($24^{\circ} \pm 3^{\circ}\text{C}$) and read after 4–6 hrs. Additional readings were made at about 18, 45 and 72 hrs. The precipitin bands were weakly developed at 4–6 hrs., but usually fully developed at 18 hours. For permanent records, some slides were blotted with filter paper, washed with distilled water and saline to remove excess protein and then stained with Amido Black (Naphthol Blue Black, E. Merck, Darmstadt, Germany). All readings were made using a magnifying lens ($1.5\times$) and blue-filtered light from a microscope lamp with a small aperture.

Immuno-electrophoresis: A modification of the immuno-electrophoretic procedure described by HIRSCHFELD (1960) was used. One gram of Special Noble agar and 33 ml of distilled water were heated in a boiling water bath for 35 min. Immediately after removing the agar mixture from the water bath, 66 ml of 0.064 M Veronal lactate buffer (pH 8.68 previously heated to 56°C), and 1 ml of 0.1% merthiolate were added and thoroughly mixed. Approximately 2.2 ml of the solution was pipetted onto level glass slides. The final pH of the cooled agar was 8.49. The slides were stored at 4°C overnight, then the pattern was cut for immuno-electrophoresis (Figure 2) using a plastic template. Four pieces of #50 filter paper 2.5 cm wide were used at each side of the row of slides. The voltage used was actually 5–6 v/cm with a running time of 2 to 3 hours. However, the applied voltage was reduced when necessary to keep the current below 50 ma, to avoid artifacts and overheating. When an absorption was performed, after electrophoresis, the trough was first filled level with the absorbing serum, followed an hour later by the antiserum.

RESULTS

Serologic studies: One isoimmune serum (166, produced against the fraction of serum from 106S) was used throughout these studies. As shown in Figure 3(a,b) it contained more than one factor (see DISCUSSION) and therefore had to be absorbed for routine testing. The same absorbing serum (708) was used throughout. After absorption, only a strongly reactive factor was detected as shown in Figure 3(c,d). Clearly, this factor was present in some, but not all, cattle normal sera. It was possible to establish that this absorbed antiserum was detecting a single antigenic factor, since every reactive serum was capable of absorbing out the reaction for every other reactive serum. In other words, the absorbed antiserum (reagent) could not be further fractionated by absorption, thus satisfying the serologic criterion of a unit antigenic factor.

Genetic studies: Data from 560 matings established that the serum factor behaved as a unit in inheritance. As shown in Table 1, the data are consistent with the hypothesis that the Mc^1 factor is controlled by a dominant gene, called Mc^1 . As expected of dominant inheritance, matings of positive parents gave at least 75 percent positive offspring and matings between positive and negative parents gave at least 50 percent positive offspring.

A study of specific matings in which one parent was heterozygous (Mc^1/Mc^0) (ascertained by pedigree analysis; Mc^0 is used to designate the absence of a detectable allelic product) and the other homozygous negative (Mc^0/Mc^0) provided

TABLE 1

Inheritance of allotype Mc1 in cattle

Type of mating	Reactions with anti-Mc1		
	No.	+	—
+ × +	47	37	10
+ × —	223	126	97
— × —	290	0	290
Totals	560	163	397

evidence of dominant inheritance. The segregation ratios from 109 of these matings gave 57 positive and 52 negative offspring which is very close to the expected 1:1 ratio ($P > 0.6$). These data represent the progeny of nine different sires and were corrected for ascertainment by excluding from the progeny ratios the one negative progeny that established heterozygosity of each sire. These matings gave positive and negative offspring of both sexes ruling out sex-linkage. Taken together, these data are consistent with the hypothesis that the Mc1 factor is controlled by a dominant allele at an autosomal locus.

Breed distribution of Mc1 factor: The frequency distribution of the Mc1 factor among 2,548 cattle of seven different breeds is depicted in Figure 4. It is clear that there are wide breed differences ranging from the most frequent among the Charolais (75.9%) and Brown Swiss (73.2%) to the least frequent among the Holstein-Friesian (10.4%) and Guernsey (2.2%). Thus, the autosomal dominant gene Mc^1 shows a considerable range in frequency from about 0.5 to about 0.01.

Identification of Mc1 antigen: Since the ammonium sulphate fraction of serum used for immunization was not very different from normal serum (Figures 1, 2), it was impossible to predict the serum components against which the antibodies would be directed. The Mc1 antigen gave consistent results when the same serum sample was tested at different times even after prolonged storage in the freezer and after repeated freezing and thawing. Also, serum samples taken at different times (days or months) from the same animal gave consistent results, so the antigenic factor was constant *in vivo* and *in vitro* (frozen). On the other hand, the Mc1 reaction was markedly weakened after heating the serum at 56°C for one hour and completely lost after two hours. Prolonged storage at room temperature also markedly weakened the reaction.

The results of immunoelectrophoresis suggested that the Mc1 antigen was either a macroglobulin or a lipoprotein. As shown in Figure 2, the precipitin bands of the untreated normal serum and the ammonium sulphate fraction occurred on the anode side close to the antigen well in the region containing the macroglobulins and lipoproteins.

To test for the possibility that Mc1 was a lipoprotein, the serum was fractionated by ultracentrifugation. The lipoproteins were isolated by flotation in a preparative ultracentrifuge using KBr as a solvent. The solvent density was adjusted to 1.169 by diluting 2.5 ml of serum with an equal volume of KBr of density

FIGURE 4.—Frequency distribution of the Mc1 factor in various cattle breeds. Fractions represent number positive over total tested. CH—Charolais, BS—Brown Swiss, AY—Ayrshire, JY—Jersey, HE—Hereford, HF—Holstein-Friesian, GY—Guernsey.

1.334. The sample was centrifuged for 24 hours, using a Spinco 40.3 rotor (39,000 rpm at 8°C). The top yellow layer, containing most of the lipoproteins in the serum, was carefully removed and tested in the double diffusion test with the anti-Mc1 serum. No precipitation bands were visible indicating that the Mc1 antigen was not lipoprotein. In contrast, the serum fractions recovered from the bottom of the tube, following ultracentrifugation, reacted strongly with the anti-serum.

The serum containing the Mc1 antigen was treated with 2-mercaptoethanol (2-ME) (0.1 M). After reduction, the serum was alkylated with iodoacetamide (0.1 M) in 0.25 M Tris buffer (pH 8.0) when dialyzed. This treatment destroyed the capacity of the serum to form a precipitin band when tested with antiserum. This lability to 2-ME is typical of macroglobulins.

Additional evidence that the Mc1 antigen was a macroglobulin was obtained from tests on fractions of normal serum separated on G200 Sephadex. Normal

FIGURE 5.—Chromatography of normal cattle serum containing the Mc1 antigen on a G200 Sephadex column. The exclusion volume for blue dextran was at fraction number 4. Antigen was found only in fractions 5 through 13 representing the first peak. See Figure 6 and text for details.

serum containing the Mc1 antigen was run through a G200 Sephadex column 26×1 inch, at room temperature. The flow rate was 26–30 ml per hour. Approximately 1.8 ml of normal serum was added to the column and 60 fractions were collected at nine minute intervals. The eluant was 0.15 M KCl. UV absorption measurements were done on the individual fractions with a Beckman spectrophotometer (Figure 5). Following the run, pooled fractions were electrophoresed and also tested with the anti-Mc1 serum in double diffusion tests. As shown in Figure 6, only the pooled fractions one through three showed reactivity. These pooled fractions consisted of individual fractions obtained within the first peak, representing fractions numbered 5 through 13 in Figure 5. These results strongly indicate that the Mc1 antigen is a macroglobulin with a molecular weight over 200,000.

To further determine the fraction of the serum proteins in which the Mc1 antigen resided, the serum was run on starch gel electrophoresis (Figure 1). Sections of the gel were cut, extracted and tested for activity. The sera were vertically electrophoresed (SMITHIES 1959) for 18 hrs. at 5 v/cm using Borate buffer (pH 8.9). No difference in band pattern was evident between sera containing the Mc1 antigen and those lacking it. Consequently, either the antigen did not migrate into the gel or it migrated into the gel to a position corresponding to another protein band or was too faint to be detected. Three identical starch gels were run simultaneously. After electrophoresis, the bottom third of each gel was sliced and stained. The remaining part was sectioned into 21 equal strips about 0.7 mm wide (perpendicular to the direction of migration), 16 on the side of the

FIGURE 6.—Electrophoresis (left) and diagram of double diffusion test results (right) of cattle normal serum (106S) and serum fractions obtained with G200 Sephadex. Left, top to bottom,—Normal serum, Fractions 1, 2, and 3. Each fraction represents a pool of three individual fractions obtained in the first peak (fractions numbered 5 through 13) as shown in Figure 5.

gel toward the anode and five toward the cathode. The corresponding strips from each of the three gels were frozen and thawed and the protein eluted by squeezing them in disposable syringes. The resulting samples were filtered through a millipore prefilter to remove excess gel, and then dialyzed. Since the eluted serum protein was extremely dilute, each sample was concentrated in Aquacide (Calbiochem, Los Angeles, California). These concentrated eluates were tested by double diffusion with the Mc1 antiserum. The antigen samples were added twice and the antiserum once to the appropriate wells. Only the eluate obtained from the first strip of gel on the anode side gave a precipitin band.

From these results it was not clear whether the reactive fraction had, in fact, migrated into the gel (albeit, an exceedingly short distance) or whether it was present on the edge of the slot (origin) having been unable to migrate into the gel. To answer this question, we combined the use of starch gel and agar gel double diffusion. Untreated serum was electrophoresed on starch gel. Then, a square of gel was cut from the starch, such that one side of the square included the anode edge of the gel slot, and the other side of the square extended about 1 cm into the gel toward the anode. A similar square was taken from the cathode side of the slot. Each of the squares was placed on a 3 × 1 inch microscope slide. Two ml of agar was poured around the piece of starch and allowed to cool. Wells were cut on three sides of the square piece of starch gel (in front of the slot slide and the two adjacent sides) and filled with antibody. A strong precipitin band appeared only between the anode side of the slot and the well in front of it. From this it was concluded that the antigen did not migrate into the starch gel but collected on the anode edge of the slot. These data provided convincing evidence that the Mc1 antigen was a macroglobulin since the size and asymmetry of macroglobulins typically exclude their migration into starch gels, so that they collect on the edge of the slot nearest the anode.

The Mc1 antigen in twins: Tests of the Mc1 antigen in twins of various breeds

indicated that it was a useful genetic marker for diagnosing zygosity. Thus, the serum of each of 25 pairs of twins judged monozygotic by blood type, hemoglobin and transferrin type, had identical reactions with anti-Mc1. In other words, there was 100 percent concordance of twin pairs with respect to the presence or absence of Mc1. In contrast, ten of 38 pairs of dizygotic twins gave discordant reactions with anti-Mc1. Significantly, 37 of these 38 pairs of twins were chimeras, yet seven pairs of them were discordant for the Mc1 antigen. These data argue strongly against chimerism of the Mc1 factor. In this connection, there is yet no convincing evidence for chimerism of other serum proteins such as transferrins in cattle chimeric twins (DATTA and STONE 1963).

Development of the Mc1 antigen: The offspring of 62 matings in which at least one parent was positive for the Mc1 antigen were studied to determine the development of the antigen in the newborn. Most of the calves were first bled within 24 hrs. after birth and periodically thereafter until they were about eight weeks old. Of the 62 calves, 26 ultimately became positive and remained so indefinitely. At birth, calves were either negative or weakly positive. Generally the antigen was first detectable within the first three days of life and within about seven days the normal adult reaction was developed. The Mc1 antigen was not found in milk, therefore, as expected, there were no calves who showed the antigen transiently.

DISCUSSION

The results reported here demonstrate an allotypic polymorphism in cattle. This is not surprising, because such intraspecies variation is one of the levels of heterogeneity typical of serum proteins in every species studied (COHN and PORTER, 1964). However, it is surprising that this allotypic system in cattle should turn out to be a macroglobulin. No allotypic determinants have been found unique to the macroglobulin (IgM) molecules of mice and humans. The human Inv determinants and the major rabbit allotypic determinants are present on all three classes (IgA, IgG and IgM) of immunoglobulins (see COHEN and PORTER 1964 for references). The allotypes in cattle reported by MILLOT (1966) are presumably IgG but no chemical evidence has been presented to support this.

Allotypic determinants present on IgM and not on the other immunoglobulin classes have been described only in rabbits (KELUS and GELL 1965; SELL 1966). These polymorphisms, called Ms1 and Ms2, were detected using antisera produced by isoimmunization with antibody-coated bacteria. The evidence that both Ms1 and Ms2 are carried by IgM molecules is convincing because unlike cattle, the immunoglobulins of rabbits have been separated and characterized extensively. The evidence we have presented here establishes that Mc1 is a macroglobulin, but whether or not it is an IgM remains to be determined. Our preliminary attempts to demonstrate the Mc1 antigen on 19S antibodies have only begun but seem promising. We are fully aware that more data must be obtained to establish whether or not this macroglobulin is an immunoglobulin. For example, we will explore whether or not the Mc1 specificity is associated with the μ -chain. However, our evidence that Mc1 is a macroglobulin seems unequivocal and will

be summarized briefly. The preparation used for isoimmunization contained a high concentration of macroglobulins (Figure 2); therefore it might be expected that some of the antibodies produced would be against IgM. On immunoelectrophoresis the precipitation reaction occurred on the anode side very close to the antigen well (Figure 2). This is the characteristic position for immunoelectrophoretic reactions of human, rabbit and mouse IgM (FAHEY 1962). In addition, the Mc1 antigen failed to migrate into starch gel, but collected on the edge of the slot nearest the anode as expected of large asymmetric macroglobulin molecules (FAHEY 1962).

More direct evidence that the Mc1 specificity was carried by macroglobulin molecules was obtained from ultracentrifugation fractionation of serum. Only the fraction recovered from the bottom of the tube contained antigen. Further, the Mc1 reaction was destroyed after treating normal serum with 2-mercaptoethanol. This lability is also characteristic of macroglobulins (COHEN and PORTER 1964). Finally only the high molecular weight fraction ($> 200,000$) recovered from chromatography on Sephadex G200 (Figures 5 and 6) contained the antigen. Taken together these results leave little doubt that the Mc1 determinant is carried on macroglobulin molecules.

Our serologic studies strongly suggest that the Mc1 reagent is detecting a single antigenic specificity because it has not been possible to fractionate the reagent by absorption. However, as studies proceed it is entirely possible, indeed expected, that additional specificities will be found belonging to phenogroups as with the IgG allotypes of humans, rabbits and mice (OUDIN 1966). Our genetic studies indicate that the Mc1 allotype is controlled by an autosomal locus, the *Mc1* allele being transmitted as a dominant. All of the allotypes so far described in all species have been similarly controlled (DRAY 1964; OUDIN 1966), although MILLOT (1966) did not present any genetic evidence for the allotypes he described in cattle. The preliminary results of the genetic studies on the IgM allotypes of rabbits suggest that Ms1 and Ms2 are controlled by co-dominant allelic genes (KELUS and GELL 1965; SELL 1966). As already noted (see Figure 3), the original anti-serum containing the anti-*Mc1* antibody also contained at least two other specificities (showing medium and weak activity). Preliminary studies suggest that one of these is also detecting determinants on macroglobulin molecules and may represent an allele of the *Mc1* locus.

The wide range of frequency distributions of the Mc1 factor in various breeds (Figure 4) is a feature of this allotype which makes it very useful for gene frequency analyses of cattle breeds. In addition, our data indicated that the gene frequencies among different unrelated herds of the same breed were remarkably similar. It is interesting to note that the strength of reactions varied markedly among the different cattle breeds.

The development of the Mc1 factor in the newborn is as expected of IgM molecules (GOOD and PAPERMASTER 1964). They are either absent or weakly present at birth and do not ordinarily pass through the placenta. Our data show that the Mc1 factor is not fully developed until after birth. Since the Mc1 factor was not present in colostrum or milk, it cannot be passively acquired by the

suckling newborn. Furthermore, dizygotic twins exhibiting chimerism of the red cell antigens were not chimeric for the Mc1 factor. This characteristic adds to the usefulness of the Mc1 factor as a parameter for distinguishing the zygosity of twins.

It remains to be determined if the determinants described by MILLOT (1966) are related at all to the Mc system described in this paper.

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SUMMARY

An allotypic system, called Mc1, has been described in cattle. The antigenic determinant is carried by macroglobulin molecules. This allotype is controlled by an autosomal dominant gene and occurs with variable frequencies in different cattle breeds.

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