Ontogeny, expression and molecular characterization of the A blood group system of the chicken

Janet Elizabeth Fulton

Iowa State University

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Ontogeny, expression and molecular characterization of the A blood group system of the chicken

Fulton, Janet Elizabeth, Ph.D.
Iowa State University, 1989
Ontogeny, expression and molecular characterization of the A blood group system of the chicken

by

Janet Elizabeth Fulton

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Interdepartment Program: Immunobiology
Major: Immunobiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Professor-in-Charge
Program of Immunobiology

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa
1989
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ABBREVIATIONS

CAA  chicken adult antigen
CFA  chicken fetal antigen
ELISA enzyme-linked immunosorbent assay
HA   direct hemagglutination assay
IF   immunofluorescence assay
IgG  immunoglobulin, isotype G
IgM  immunoglobulin, isotype M
IHA  indirect hemagglutination assay
ISU-cA Iowa State University monoclonal antibody specific for chicken blood group A antigens
ISU-JI one particular monoclonal antibody
Kd   kilodaltons
MHC  major histocompatibility complex
PBL  peripheral blood lymphocytes
PBS  phosphate buffered saline
pI   iso-electric point
RBC  red blood cells (erythrocytes)
INTRODUCTION

Blood group antigens are molecules on the surface of cells capable of eliciting an immune response upon injection into an individual with a different allelic form (gene) of that antigen. Although originally described on erythrocytes, blood group antigens may also be found as soluble substances in serum or on lymphocytes or various tissues of the body. The latter are usually referred to as 'tissue antigens'. Blood groups are divided into independent genetically controlled systems, most of which are highly polymorphic. Blood group systems have been identified in many animal species, including humans.

Antigenic differences between chicken erythrocytes were first detected by Landsteiner and Miller (1924). However, it was not until intensive studies by Briles and others, beginning in the late 1940s, that the chicken blood groups, as recognized today, were formally identified. Within a 14 year span (1948-1962), a total of 10 blood group loci were identified in the chicken (Briles et al. 1948; Briles et al. 1950b; Gilmour 1954; Briles 1958; Briles 1962; Briles et al. 1963). In 1961, Schierman and Nordskog determined that the B blood group identified the chicken major histocompatibility complex (MHC). Chicken blood group research then concentrated on the MHC and its role in disease resistance, effects on production traits and its genetic composition.
With few exceptions, study of the other chicken blood groups was discontinued.

The literature review includes a brief discussion of production and limitations of conventional antisera and monoclonal antibodies. Chicken blood group alloantigen systems are reviewed, with particular emphasis on the A blood group system. Detailed review is also included on the developmental antigens CFA (chicken fetal antigen) and CAA (chicken adult antigen), and on molecular characterization of those chicken MHC molecules expressed exclusively on erythrocytes (B-G).

Explanation of Thesis Format

This thesis has been prepared in the 'alternate format' in which separate manuscripts are prepared for submission to research journals. There are five separate manuscripts. The first and second manuscripts entitled 'Monoclonal antibody differentiates chicken A alloantigens' and 'Ontogeny and expression of chicken A blood group antigens' are included in their entirety as originally submitted to 'Animal Genetics'. Both of these manuscripts have been accepted for publication by 'Animal Genetics', the first one with considerable editorial condensation and the second one virtually unchanged. The information in these two manuscripts was also published as part of the proceedings of the Avian Immunology Research Group Meeting held at Lelystad, the Netherlands in
1988 (Fulton and Lamont 1989). The third and fourth manuscripts entitled 'Cellular location of chicken A blood group antigens' and 'Molecular characterization of A blood group antigens' will be combined for submission for publication under the authorship of J. E. Fulton, W. E. Briles and S. J. Lamont. The fifth manuscript entitled 'Production and characterization of ISU-J1 monoclonal antibody' was included in the thesis to provide complete documentation of the initial development of the ISU-1 monoclonal antibody. These five manuscripts are then followed by an overall discussion in which the various research aspects are synthesized.

The appendix at the end of the thesis is intended as a technical guide. It was prepared in considerable detail so that the techniques described in the thesis could be repeated by an individual with limited experience with these methods.
LITERATURE REVIEW

Conventional and Monoclonal Antibodies

Antisera are produced by injecting individuals with an immunizing agent (antigen). If this antigen is foreign to the individual, specific cells (B cells) are stimulated to proliferate and secrete antibody into the blood. These antibodies are found in the plasma or serum portion of the blood and are collectively referred to as antiserum. Each stimulated B cell secretes an antibody specific for one region or epitope of the antigen. In the immunized animal, many B cells are stimulated, each secreting their own unique antibody, resulting in a heterogeneous population of antibodies in the antiserum. Immunizations of antigens (e.g., RBC) between individuals of the same species result in production of alloantisera. These alloantisera frequently detect blood group differences between individuals, and are the reagents used for blood typing for virtually all species. These conventional antisera have severe limitations on their usefulness. They are difficult to reproduce, not only between immunized animals but also within one animal between subsequent immunizations, as different populations of B cells may be stimulated. Their heterogeneous nature results in high levels of cross-reactivity between many different antigens. Absorptions to remove cross-reacting antibodies
may be done, but frequently result in a very low residual antiserum.

The development of monoclonal antibodies (Kohler and Milstein 1975) eliminated many of the problems encountered with conventional antiserum. Monoclonal antibody production involves fusion of an antigen-stimulated B cell with a neoplastic myeloma cell. The resulting hybrid cell secretes one specific antibody and can be perpetuated in tissue culture, thus resulting in an unlimited supply. These antibody sources are highly homogeneous, recognizing only one epitope of an antigen, but they may still be cross-reactive with similar epitopes on other non-related antigens. Because of their high degree of specificity, monoclonal antibodies may not recognize antigens if they are altered in any way, such as by denaturation. Monoclonal antibodies are of only one isotype and are therefore limited in use to the restricted functions of that particular isotype, e.g., IgM works well for agglutination assays and IgG works well for immunoprecipitation assays. For discussion on production of monoclonal antibodies and conventional antisera, please refer to Goding (1986).

**Chicken Blood Groups**

The first report of chicken blood group antigens was by Briles et al. (1948) in which they briefly described the identification of two independent, autosomal and multiple
allelic series of antigenic differences of red blood cells. These two loci were initially designated as 'B' and 'D' loci. In a subsequent more lengthy description of this work, these loci were renamed as A and B, respectively (Briles et al. 1950b; Briles 1984). By using alloantisera produced by erythrocyte immunizations between closely related individuals, 9 alleles of the A system and 5 alleles of the B system were identified (Briles et al. 1950b). Two more chicken blood group systems, C and D, were soon identified (Briles et al. 1950a; Briles et al. 1963). A fifth multiple allelic blood group system, E, was detected and subsequently found to be very closely linked (.49 ± .09 m.u.) to the A locus (Briles 1958; Briles 1968; Reevey et al. 1969).

Gilmour, studying British poultry flocks, independently identified the A, B, and C loci, and two new loci designated L and N (Gilmour 1954, 1959; Briles 1984). Six additional blood group systems have since been identified in chickens, including H, I, J, K, P, (Briles 1962) and R (Crittenden et al. 1970; Crittenden and Briles 1971).

Early studies of blood group loci in various poultry populations detected high levels of polymorphism which suggested some selective advantage associated with heterogeneity of the various blood group alleles (Briles and McGibbon 1948). Gilmour (1954, 1958) studied blood group allelic frequencies within inbred lines. After 14
generations of brother x sister matings they found continued segregation of six blood group loci. There was evidence of heterozygote advantage with respect to various reproductive traits, including fertility, egg production and viability.

Function of Chicken Blood Groups

The association of the B blood group with the MHC (Schierman and Nordskog 1961) clearly established the importance of this particular blood group locus. Subsequent work has uncovered the complexity of the chicken MHC, and subdivided the complex into three subregions, defined as B-G, B-F and B-L. Each of these subregions is responsible for antigenic expression on various sub-populations of cells. There has been much research done on the B blood group because of its association with the MHC (see review, Crone and Simonsen 1987). There has been limited study of other chicken blood groups. The Ç blood group antigens are expressed on both erythrocytes and lymphocytes (as are B blood group antigens) (Schierman and Nordskog 1962). Evidence suggests that the Ç locus may be a minor histocompatibility locus (Schierman and Nordskog 1965). The R blood group was originally identified in association with resistance to avian leukosis sarcoma virus infection (Crittenden et al. 1970). The antigen appears to provide a site for viral binding. The K blood group is involved with
hemagglutination induced by vaccinia virus (Brown et al. 1973).

Molecular Characterization of Chicken Blood Group Antigens

The most studied chicken blood group antigens are those of the B blood group. Molecular characterization of only those B antigens expressed exclusively on RBC (i.e., B-G) will be discussed. Miller et al. (1984) used monoclonal antibodies and alloantisera to immunoprecipitate B-G antigens. The various B antigens were extremely heterogeneous in both the degree of complexity and in molecular structure. Early studies of B-G antigens identified several chains of varied mass. Size estimates varied considerably between research labs. Pink et al. (1977) reported reduced chains of approximately 42 Kd and 31 Kd. Wolf et al. (1984) isolated bands of 47 Kd and 42 Kd under reducing conditions and a molecule of 104-109 Kd under non-reducing conditions. Salomonsen et al. (1987) detected B-G antigens of 46-48 Kd when reduced and either dimers of 85 Kd or trimers of 130 Kd under non-reducing conditions. This considerable inconsistency between laboratories has been explained by a high susceptibility of the B-G antigens to proteolytic degradation during isolation (Salomonsen et al. 1987). Early reports suggested that B-G antigens were glycosylated (Nielsen et al. 1982, cited in Salomonsen et al. 1987). Subsequent study has revealed that they are not
(Salomonsen et al. 1987). A recent molecular structure proposed for B-G consists of a homodimer consisting of 48 Kd subunits and a heterodimer composed of 40 Kd and 44 Kd subunits (Kline et al. 1988a) with expression of each of these molecules dependent on the differentiation stage of the erythrocytes.

In summary, the structure of the B-G antigen is still not fully understood, but it appears to be composed of non-glycosylated molecules, that are monomers of 40 Kd to 48 Kd, linked by disulfide bonds into either dimers or trimers. There is considerable variation in molecular size between different alleles.

Limited molecular characterization of the chicken R blood group antigens has also been reported. They appear to have sialic acid as part of their immunodominant structure and are cross reactive with human blood group MN reagents (Springer et al. 1974).

**Developmental Antigens of Chickens**

Two chicken developmental antigens, chicken fetal antigen (CFA) and chicken adult antigen (CAA) have been well studied. They are considered as developmental antigens because they are detectable in various forms on differentiating erythrocytes and their precursors. They are useful as markers to identify and characterize subpopulations of normal erythrocytes as well as hematopoietic-lymphoid tumors.
(Sanders et al. 1982; Hartwell et al. 1985). CFA and CAA are different molecules that are reciprocally expressed on erythrocytes (Blanchet 1976a). Peptide mapping and co-cap analyses have indicated that CFA and CAA are distinct molecules (Kline et al. 1984).

**Chicken Fetal Antigen** Chicken fetal antigen (CFA) is a developmentally associated erythrocyte antigen first reported by Sanders (1968). It was initially found on erythrocytes from embryos of 12 days of incubation (Blanchet 1976a). Antigen expression gradually decreased as chicks aged and was undetectable by 17 weeks of age (Sanders 1968; Blanchet 1976a).

Although originally identified on erythrocytes, CFA has subsequently been found on subpopulations of B and T lymphocytes (Dietert et al. 1982) and peritoneal macrophages (Dietert et al. 1983). Lymphocytes and macrophages each have unique subsets of the total antigenic determinants found on erythrocytes. CFA has also been found on hematopoietic tumors induced by avian erythroblastosis virus (AEV) (Krsmanovic et al. 1983; Nelson et al. 1982), Marek's disease virus (MD) (Murthy et al. 1979) and avian leukemia virus (ALV) (Murthy et al. 1979).

Initial studies of CFA were done using rabbit anti-chicken erythrocyte polyclonal sera. These sera required exhaustive absorptions to attain the monospecificity needed
for detailed characterizations and developmental studies of CFA. Mouse monoclonal antibodies specific for different CFA determinants have since been developed (Sanders et al. 1982, Miller et al. 1982; Hartwell et al. 1985; Trembicki and Dietert 1985), enabling more detailed study of subsets of cells expressing specific CFA determinants.

The CFA is antigenically complex. Thirteen antigenic determinants have been recognized on chicken embryonic erythrocytes (Dietert and Sanders 1978). These individual CFA determinants are sequentially lost from erythrocyte membranes as birds age (Dietert and Sanders 1979). Various subsets of these determinants are found on erythrocytes from both embryos and adults of 14 different avian species including turkey, Japanese quail, ringneck pheasant and pigeon (Dietert and Sanders 1978). Single gene control for the acquisition of one antigenic determinant (CFA10) was found for pigeon erythrocytes (Dietert and Sanders 1979). Evidence from enzymatic digestion (Dietert et al. 1981), immunoprecipitation with monoclonal antibodies (Kline et al. 1984) and hapten inhibition of microcytotoxicity and agglutination (Lewin and Dietert 1982, 1983), suggested that CFA may be associated with carbohydrate moieties. Subsequent studies confirmed this carbohydrate association. The CFA10 has been shown to have a D-galactose component (Dietert et al. 1981; Lewin and Dietert 1983) and CFA5 and CFA11 have D-
mannose-like and D-glucose-like molecules respectively, as immunodominant structures (Lewin and Dietert 1983). Genetic influence on the incidence of another CFA determinant (CFA8) has also been found for the chicken (Qureshi et al. 1986). Immunoprecipitation experiments using CFA-specific monoclonal antibodies and rabbit antisera have detected CFA molecules with relative molecular weights of 48-50 Kd (Krsmanovic et al. 1979; Kline et al. 1982; Dietert et al. 1984; Kline et al. 1984).

The function of CFA is not known. Sindbis virus binds to and agglutinates erythrocytes carrying CFA determinant 9 (Sanders et al. 1980). The reappearance of CFA on neoplastic cells induced by a variety of viruses defines CFA as being an onco-developmental antigen (Dietert and Sanders 1978).

Chicken Adult Antigen Chicken adult antigen (CAA) is a developmentally associated antigen restricted to erythrocytes from post-hatch chicks. It is first detectable on erythrocytes from chicks 3 days after hatching. Antigen levels rapidly increase as birds age, and by 28 days of age, adult levels are reached (Blanchet 1976a, b). The CAA has since also been found on chemically induced erythroleukemia cells (Nelson et al. 1982) and on lymphocytes (Kline et al. 1984).

The CAA has been found to be antigenically complex. Eight antigenic determinants have been recognized. Various
subsets of these determinants are unique to erythrocytes from various avian species (Kline et al. 1982).

Molecular characterization of CAA has been done by Kline et al. (1982, 1984) and Krsmanovic et al. (1979). The CAA was found associated with several molecules ranging in size from 43 to 210 Kd with different subsets of these molecules being isolated by different sera. Some of these proteins appeared to be glycoproteins. Early studies by Miller et al. (1982) suggested that CAA may be identical with B-G antigens of the chicken MHC. More recent comparisons of molecules immunoprecipitated by B-F specific alloantisera and CAA specific rabbit anti-chicken antisera have shown that CAA molecules are the same as B-F antigens (Kline et al. 1988b).

Chicken A Blood Group Antigen

The chicken A blood group locus was identified concurrently with the B locus by both Briles (Briles et al. 1948) and Gilmour (1954). Although it is the second most studied chicken blood group locus, very little is known about it. In this section of the literature review, the A blood group system will be discussed in more detail.

Location of A Antigenic Products

Schierman and Nordskog (1962) first attempted to detect the presence of the A blood group antigens on the surface of lymphocytes. Using agglutination assays with lymphocytes and alloantisera, they found agglutination of lymphocytes with respect to the B and
Ç blood group loci, but not with the A, D or L blood group loci, suggesting that the presence of the latter blood group antigens was restricted to erythrocytes. The A blood group antigens were also not detectable on lymphocytes by immunofluorescence (Davidenas 1970).

The first evidence of the presence of A blood group antigens on lymphocytes was given by Wong et al. (1972) using an allofixation technique. In the presence of specific alloantisera, lymphocytes would adhere to glass or plastic. Two alleles were detected ($A^2$, $A^6$) and heterozygotes were distinguishable from homozygotes due to an additive gene dosage effect. This observation of A antigens on lymphocytes suggested involvement with transplantation and immunity (Wong et al. 1972).

**Time of Embryonic Appearance** The time of appearance of the A blood group antigens in the developing embryo was first studied by Briles et al. (1948). The A antigens were weakly detectable by the third day of incubation and resulted in strong agglutination by five days incubation. Early detection of A antigens was also reported by Poschl and Hala (1974) and Schjeide et al. (1978). Johnson (1956) (cited in Briles 1964) suggested that the A antigens reacted at levels equivalent to that of adults by three days of incubation. Briles (1964) suggested that the weak agglutination of very
young embryonic blood (Briles et al. 1948) was due to low cell numbers in cell suspensions.

**Agglutination by Normal Bovine Serum** Early studies of hemagglutination of chicken red blood cells showed that agglutination could be caused by normal bovine serum (Landsteiner and Levine 1932). This characteristic of chicken red blood cells varied between individuals and was found to be due to a single, partially dominant gene (Olson 1943). Briles and co-workers (1952) investigated this phenomenon in relation to known blood group antigens. They found that this hemagglutination of chicken red blood cells by normal bovine serum was due to alleles of the A blood group locus, with some allelic products reacting very strongly and others reacting weakly or not at all.

**Allelic Frequencies** Early studies of blood group allelic frequencies in various poultry populations indicated high levels of polymorphism for the A blood group locus (Briles and McGibbon 1948; Gilmour 1958). Four research institutions have inbred lines which have been recently characterized for the various blood group loci: Reaseheath (England), Regional Poultry Research Laboratory, East Lansing (Michigan), University of California, Davis (California) and Beckenham (England) (Hala, 1987). Continual inbreeding has resulted in most of these lines now being homozygous at the A locus, of the 26 lines described, 21 (80%) are fixed for the
$\text{A}^4$ allele. The closely linked $E$ locus, although also homozygous, varies considerably between the lines, suggesting that the commonality of $\text{A}^4$ allele is not due to a common genetic base, but rather to a selective advantage for the $\text{A}^4$ allele during the initial establishment of the lines.

**Association with Immune Response** Johnson and Edgar (1984) found differences in $A$ allelic frequencies between two lines that had been selected for resistance and susceptibility to coccidiosis. Dunnington and co-workers (1984) determined gene frequencies for $A$ alleles in lines that had been divergently selected for antibody response to sheep red blood cells. The $\text{A}4$ allele had a frequency of 96% in the low antibody response line, compared to 58% in the high antibody response line. These two studies suggest an association of the $A$ blood group locus with immune response. Cock and Clough (1956) and Crittenden et al. (1964) found that the $A$ locus did not appear to influence graft rejection. Thus there are conflicting reports as to whether the $A$ blood group locus influences immune responses. Perhaps it influences other systems, or is closely linked to genes controlling other systems involved in immune response such as macrophage function or lymphokine production.

**Conclusion**

There were many early investigations of the $A$ blood group locus and its biological significance. However, very little
research has been done within the last 20 years and many questions such as its function and location remain unanswered. This thesis represents one of the first applications of various recently developed biochemical techniques for the isolation, identification and characterization of the chicken A blood group antigens.
PAPER I.
MONOClonAL ANTIBODY DIFFERENTiATES
CHICKEN A SYSTEM ALLOANTIGENS
MONOCLONAL ANTIBODY DIFFERENTIATES
CHICKEN A SYSTEM ALLOANTIGENS

J.E. FULTON
DEPARTMENT OF ANIMAL SCIENCE AND IMMUNOBIOLOGY PROGRAM
IOWA STATE UNIVERSITY, AMES, IOWA, U.S.A.

R.W. BRILES
DEPARTMENT OF BIOLOGY
NORTHERN ILLINOIS UNIVERSITY, DEKALB, ILLINOIS, U.S.A.

S.J. LAMONT
DEPARTMENT OF ANIMAL SCIENCE AND IMMUNOBIOLOGY PROGRAM
IOWA STATE UNIVERSITY, AMES, IOWA, U.S.A.

Running Title: Monoclonal antibody differentiates A alloantigens

Key Words: monoclonal antibody, chicken, blood group A, erythrocyte antigen

1 Journal Paper no. J-13348 of the Iowa Agriculture and Home Economics Experiment Station, Ames; Project no. 2237.

2 To whom correspondence should be addressed.
ABSTRACT

A monoclonal antibody (ISU-cA) was produced that recognized certain alloantigens of the chicken A blood group locus. Antigens produced by alleles $A^3$, $A^4$ and $A^8$ were positive, and those produced by $A^2$ and $A^5$ were negative, by hemagglutination. The specificity of ISU-cA for chicken A blood group antigens was demonstrated by three different procedures: serologic analyses, genetic crosses and competitive inhibition of binding by anti-A polyclonal sera. To our knowledge, this is the first reported monoclonal antibody against a chicken alloantigen system other than the B complex.
INTRODUCTION

The development of monoclonal antibodies has enabled scientists to conduct detailed study of the development of the immune system and to recognize subsets of cells involved in various immune responses. Monoclonal antibodies have been produced against various chicken cell-surface markers and have enabled identification of cell subsets and studies of the ontogeny and function of the various hematopoietic cell types in the chicken. Monoclonal antibodies have been produced that are reactive with chicken T cells (Houssaint et al. 1985; Peault et al. 1982; Pink and Rijnbeek 1983; Chen et al. 1984; Kornfeld et al. 1983), B cells (Chen et al. 1984; Spencer and Benedict 1986; Kornfeld et al. 1983; Wolf et al. 1984; Pink and Rijnbeek 1983), leukocyte common antigens (Houssaint et al. 1987), erythrocytes (Sanders et al. 1982; Trembicki and Dietert 1985) and macrophages (Dietert et al. 1987). Particular emphasis has been placed on development of monoclonal antibodies specific for the B blood group antigens because the B system of haplotypes identifies the chicken major histocompatibility complex (MHC) (Schierman and Nordskog 1961). Monoclonal antibodies have been produced that are specific for antigens of each of the three classes of the MHC: B-G (Longenecker et al. 1979; Miller et al. 1982; Salomonsen et al. 1987), B-F (Pink et al. 1985; Crone
et al. 1985) and B-L (Crone et al. 1985; Guillemot et al. 1984; Ewert et al. 1984).

We report here a monoclonal antibody specific for certain alloantigens of the chicken A blood group system. This is the first report of a monoclonal antibody against a chicken blood group antigen other than those of the B complex.
MATERIALS AND METHODS

Monoclonal Antibody Production

Balb/c mice were immunized with 1 x 10^8 peripheral blood lymphocytes (PBL) (separated by Lympho-paque from whole blood) from a G-B1 (B^13/E^13) strain chicken and then boosted 3 weeks later with G-B1 bursal cells. Three days later, spleen cells from the immunized mice were fused with SP_2/0 myeloma cells following a modified procedure of Van Deusen and Whetstone (1981). Hybrid cells were selected by using HAT medium. Screening for reactivity to RBC and PBL was done by using hemagglutination (HA) and enzyme-linked immunosorbent assay (ELISA) assays, respectively. Positive hybridomas were then cloned by using limiting dilution. The isotype of the monoclonal antibody produced in this study was IgM (Hy-Clone Mouse Monoclonal Sub-Isotyping Kit, Logan, Utah). Ascites fluid was produced by injecting pristane-primed Balb/c mice with 5 x 10^5 actively growing hybridoma cells. The antibody was designated ISU-cA as an abbreviation for Iowa State University monoclonal antibody against chicken erythrocyte antigen A.

Chicken Stocks

The birds used in the study were from the partly inbred (F = 0.5) Iowa State University S1 White Leghorn chicken line (Nordskog and Cheng 1988). Birds of the S1 population were screened at 6 weeks of age for their indirect
hemagglutination (IHA) reactivity to the ISU-cA monoclonal antibody. Breeders used to initiate the homozygous lines were selected for subsequent matings on the basis of the agglutinability of their RBC and that of their full sibs. Any bird whose IHA score and that of their full-sibs was 4 was considered as a potential parent for establishing a positively reacting line. Negative birds were selected on the basis of their phenotype. The matings of individuals within phenotype were random except that full and half-sib matings were avoided. Mating was by artificial insemination, and all chicks were pedigreed.

Crosses were specifically made to test for linkage between the A blood group locus and the locus identified by ISU-cA. Blood samples of progeny from 12 backcross and 6 F₁ matings (with respect to their ISU-cA response) were tested at Iowa State University for their reactivity with ISU-cA and at Northern Illinois University for their A and B blood group genotypes.

Other Avian Species

Turkey blood was collected from mature birds and tested by the hemagglutination assays described previously. Eleven to 16 birds were sampled from each of 7 non-inbred, non-commercial, unrelated turkey strains. Cells from five commercial chicken hens of a DeKalb strain were tested concurrently with the turkey cells as a positive control for
antibody efficacy. This assay was conducted at the Department of Poultry Science, Oregon State University, Corvallis, Oregon. Blood samples from three ringneck pheasants (*Phasianus colchicus*) were tested at Northern Illinois University.

**Hemagglutination Testing with Monoclonal Antibody**

Blood was collected into phosphate-buffered saline (PBS) with 1% citrate by venipuncture from the jugular vein (hatch to 4 weeks of age) or the wing vein (5 weeks or older). Samples were stored at 4°C and tested within 48 hours of collection. Blood samples were washed once in PBS and spun at 130 x g for 10 min. Suspensions of 2% (v/v) packed red blood cells (RBC) in PBS were made for testing. Assays were carried out in 96-well round-bottom microtitre plates at room temperature. One drop (40 μl) of the cell suspension and one drop of ISU-cA antibody (1/200 dilution of ascites in PBS) were added to each well. The plates were briefly shaken on a microtitre plate shaker and incubated for 1h at room temperature. They were then reshaken, incubated for an additional hour, and observed for the direct hemagglutination (HA) score. The indirect hemagglutination (IHA) score was determined after addition of a second antibody (goat anti-mouse immunoglobulin [IgG, IgM, IgA, (H + L)] Cooper Biomedical, WestChester, Pennsylvania). The plates were
again shaken, incubated for 1h, shaken again and scored after an additional hour.

Scoring of the hemagglutination assays was conducted visually. Scores for the degree of agglutination were 0 for a button of non-agglutinated cells; 1 for a button of cells with slight agglutination; 2 for a small button of cells with agglutination; 3 for agglutination of most but not all cells; 4 for complete agglutination. All samples were scored by the same individual. A score of 0 was considered as negative, 4 was considered positive and all other values were classified as intermediate.

Blood samples for the antibody titration assay were obtained from 24 birds with negative cells, 20 birds with positive cells and from 38 birds from matings between negative x positive parents. All blood samples were obtained from mature birds (22 weeks of age). Both HA and IHA assays were carried out. The monoclonal antibody was titrated in doubling dilutions from 1/10 through 1/20,480. The last dilution in which agglutination occurred was considered the end point. For statistical analysis, the dilutions were transformed to numerical scores of n = 1 through 12, where n = \log_2[1/(10 \times \text{dilution})] + 1 (i.e., 1/10 dilution = 1; 1/20 = 2; ... 1/20,480 = 12).
Hemagglutination Testing with Alloantisera

All blood typing with alloantisera was carried out at Northern Illinois University (NIU), using reagents shown by immunogenetic analysis to be specific for antigens of the A, B, C, D, E, H, I, J, K, L or P alloantigen systems (Briles 1962; Briles et al. 1950; 1963). Each hemagglutination test was carried out using 2 drops (0.1ml) of typing reagent appropriately diluted according to titre and one drop (0.05ml) of 2% RBC suspension in 10mm X 75mm test tubes. Racks supporting the tubes were shaken, incubated 1.5h at room temperature, placed in refrigerator (3°C) overnight, shaken and incubated an additional hour at room temperature. Using a slit reading lamp, sedimentation buttons were visually scored 0 to 4; tubes were then individually shaken and suspended cells were scored for agglutination 0 to 4; suspensions exhibiting doubtful agglutination were transferred to a slide and observed microscopically for the presence of agglutination.

The monoclonal ISU-cA was similarly tested at a dilution of 1:600 on panels of cells from chickens of known genotype for each of the eleven alloantigen systems.

Competitive Binding Assay

A competitive binding assay was done between ISU-cA monoclonal antibody and two chicken anti-A4 alloantisera. Blood was collected in the same manner as for the
hemagglutination assay. The RBC were washed once, and 0.2% (v/v) suspensions were prepared. All samples were carried out in duplicate or triplicate by using 96-well round-bottom microtitre plates. Twenty μl of cell suspension was added per well, followed by 20 μl of either ISU-cA antibody (1/100 dilution in PBS) or of alloantisera (1/8 in PBS). The plates were then shaken and incubated on ice for 1h. Cells and antibody were resuspended by shaking twice during this incubation. The cells were then washed twice with PBS + 1% BSA + 0.1% NaN₃. Samples that had received ISU-cA were subsequently incubated with 20 μl of anti-A4 sera, and samples that had initially received anti-A4 sera were then incubated with ISU-cA monoclonal antibody. These samples were then incubated and washed as described. Twenty μl of the fluorescein isothiocyanate (FITC) conjugated second antibody (1/20 dilution in PBS) was added to each well [FITC goat anti-mouse IgA, IgG, IgM (H & L) Cooper Biomedical, WestChester, Pennsylvania]. The plates were covered with aluminum foil, shaken and incubated on ice for 1h. Plates were shaken twice during incubation to resuspend cells. The cells were washed twice with PBS + 1% BSA + 0.1% NaN₃ and once with PBS + 0.1% NaN₃ before analysis by flow cytometry. Total immunofluorescence was compared between these samples and controls that were incubated with ISU-cA monoclonal antibody only. The decrease in ISU-cA binding due to the
presence of anti-A4 antibodies was then determined. Red blood cells used were from an A^4/A^4 B^1/B^1 individual. Two noncompetitive alloantisera (anti-B1), previously produced and analyzed at ISU, were also tested to determine if steric hindrance was occurring. The binding of each chicken alloantisera to RBC was confirmed with FITC-labelled goat anti-chicken IgM (Bethyl Laboratories, Montgomery, Texas).

Flow Cytometric Analysis

Flow cytometric analysis was done by using an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, Florida) equipped with an argon ion laser (488 nm, 400 mW). Log fluorescence was collected for each RBC sample, and light scatter was used to gate out debris. Samples were counted until a total of 20,000 events was reached.
RESULTS

Breeding

The results of the breeding tests, conducted to determine the genetic basis for reactivity to the ISU-cA reagent, are shown in Table 1. The intermating of birds whose RBC showed strong indirect hemagglutination (positive) produced only positive progeny, and matings in which both parents were negative produced only negative progeny. The crosses of negative males x positive females and the reciprocal resulted in F₁ progeny with intermediate and somewhat varied hemagglutination scores. These birds varied considerably in the degree of direct agglutination. The more sensitive IHA assay resulted in higher agglutination scores which were used for classification.

The F₁ birds were intermated and produced progeny whose cells' response was in one of three different classes: negative, intermediate or positive for indirect hemagglutination. Segregation of progeny was analyzed by chi-square tests (Steel and Torrie, 1980). The ratio of progeny in each of these three classes did not differ significantly from the 1:2:1 ratio expected for a trait controlled by a single, autosomal, partially dominant gene (P > 0.05). The backcrosses of F₁ x negative and reciprocal resulted in two types of progeny, intermediate and negative in approximately equal numbers. The backcross mating of F₁ x
positive produced intermediate and positive progeny in a ratio of 52:53 but the reciprocal mating produced a ratio of 42 intermediate to 64 positive progeny which did differ significantly from expected ($X^2 = 4.57$, df = 1, $0.025 < P < 0.05$).

**Antibody Titration Test**

The results of the titration assay are summarized in Table 2. Differences between means were tested by using the t-test (Steel and Torrie 1980). None of the red blood cell samples from the homozygous negative birds was agglutinated by the ISU-cA monoclonal antibody, even at the highest antibody concentration used of 1/10. The mean end point titre at which the homozygous positive birds' cells were hemagglutinated significantly increased from $8.0 \pm 0.55$ for the HA assay to $11.1 \pm 0.89$ for the IHA assay ($p < 0.001$). End points were not identified for some IHA scores of homozygous positive samples. In those cases, a titre of 12 was assigned, thus underestimating the mean IHA titre for homozygous positive birds. The mean end point titre at which RBC of the heterozygous birds were agglutinated was $6.4 \pm 0.70$. This significantly increased to $7.7 \pm 0.62$ for the IHA score ($p < 0.001$). The homozygous positive birds showed a significantly greater ($p < 0.001$) mean end point titre for both the HA and IHA assays than the heterozygous birds.
Genetic Identity of ISU-cA Specificity

Initially, the ISU-cA monoclonal (1/600 dilution) was tested on cells from a panel of 46 birds selected primarily for analysis of antisera for B-system specificity, but considered to be representative, in general, for genetic types of the other alloantigen systems. In spite of genetic heterogeneity of the panel, the cells of all 46 birds were strongly agglutinated. Since some of the ISU S1 line were known to be negative in reactivity to ISU-cA, cell samples from negative and positive birds were then sent to the NIU laboratory. The first section of Table 3 shows the result of typing 4 ISU-cA negative and 4 ISU-cA positive birds with reagents for antigens of the A, B, C, D, E, H, I and L systems. Reactivity to ISU-cA could be explained by the antigen A4, or B21 or Ix, with A2, B19 and I2 resulting in no reactivity; however, among the NIU panel of cells already tested, birds with the genotype A4/A4 B19/B19 I2/I2-3 were positive to ISU-cA. Thus the A4 allele as expressed in the S1 line seemed to be the basis for the ISU-cA reactivity. In an effort to confirm this hypothesis, a special panel from the NIU colony, with a greater variety of A genotypes was tested with ISU-cA (last section of Table 3). Family B505, consisting of five individuals, was selected because all members were homozygous for B2 (also present in line S1) yet segregated for A2, A4 and A5. The reactivities shown by the
cells of the members of this family confirm that antigen A4 is positive and that A2 is negative; furthermore, since the cells of the two sibs possessing the genotype $A^2/A^5$ (B505-17602 and B505-17667) were non-reactive, the antigen A5 is also a nonreactor to ISU-cA. The inclusion of two other birds, C494-17340 ($A^3/A^3$) and A622-27304 ($A^2/A^8$), in the NIU panel demonstrated that antigens A3 and A8 are reactors to ISU-cA. No combination of alleles at any one of the other 10 loci appear to exclusively account for the reactivity of ISU-cA.

Final proof that the ISU-cA monoclonal antibody is specific for the $A$ system consists of simultaneous segregation from controlled matings showing that only those progeny inheriting $A^4$ reacted with the ISU-cA reagent. Table 4 shows the results of testing the progeny from backcrosses ($A^2/A^2 \times A^2/A^4$, $A^4/A^4 \times A^2/A^4$) and $F_1$ ($A^2/A^4 \times A^2/A^4$) matings for their reactivity to A2 and A4 alloantisera and to ISU-cA. Perfect agreement was found between negative ISU-cA reactivity and $A^2/A^2$ genotype and between positive ISU-cA reactivity and $A^4/A^4$ and $A^2/A^4$ genotype. This suggested that the ISU-cA monoclonal antibody was specific for alloantigens of the $A$ system.

Other Avian Species

None of the turkey blood samples was agglutinated by the ISU-cA monoclonal antibody by either the HA or more sensitive
IHA assay. The red blood cell samples from the five commercial chicken hens tested simultaneously with the turkeys were positive by both the HA and IHA assays. This shows that the antigenic determinant of interest was present on the RBC from the chickens and the antibody dilution used on the turkey cells possessed ample ISU-cA activity. The three pheasant blood samples included in the NIU panel of test cells were negative.

**Competitive Binding Assay**

The effect of incubation of RBC with alloantisera before or after ISU-cA incubation is shown in Table 5. Immunofluorescence of each sample due to the presence of the FITC-labelled goat anti-mouse immunoglobulin reflects the amount of ISU-cA bound to the RBC. Fluorescence of control samples that were incubated with ISU-cA and FITC-labelled anti-mouse antibody were used as a base line for comparisons with fluorescence of all other samples. Thus, fluorescence levels equivalent to controls would give values of 100%, whereas complete inhibition of ISU-cA binding would result in values of 0%.

Incubation of RBC with each of the two polyclonal chicken anti-A4 antisera (A4-138-967 and A4-260-747) resulted in decreases in subsequent ISU-cA binding to 67% and 81% of the control levels, demonstrating the occurrence of some competition. In the reciprocal samples in which ISU-cA was
allowed to bind to the cells before the putative competing polyclonal antibodies, smaller decreases in ISU-cA binding of 86% and 94%, respectively, of control levels was seen for these same two antisera. In contrast, incubation of irrelevant polyclonal chicken anti-sera, in this case two anti-B1 sera (B1-8700-85 and B1-8716-85), with RBC either before or after ISU-cA incubation caused no reduction in ISU-cA binding. The presence of chicken polyclonal antibodies on the surface of the RBC was confirmed by the binding of a FITC-labelled goat anti-chicken IgM second antibody.
DISCUSSION

The results of the breeding tests (Table 1) suggest that the expression of the erythrocyte antigen that is detected by the ISU-cA monoclonal antibody is under genetic control. Expression of the trait seemed to be determined by a single, autosomal gene that was partially dominant. The reactivity of cells from heterozygotes was intermediate in expression between that of the cells from homozygous positive and negative birds. The antibody titration test (Table 2) clearly distinguished heterozygous from homozygous positive individuals by the antibody dilution at which their RBC were agglutinated, thus confirming the phenomenon of dosage, a common characteristic of many cellular antigens.

The data strongly support the ISU-cA monoclonal antibody as recognizing certain A system alloantigens. Blood typing showed that all birds tested as ISU-ca positive were either $A^4/A^4$ or $A^2/A^4$ and the ISU-cA negative birds were $A^2/A^2$. The E blood group locus is known to be closely linked to the A blood group locus (Briles, 1968), however, the homozygosity for $E^2$ of the ISU birds and the B505 NIU family proves that the E locus cannot exclusively account for the reactivity of the ISU-cA monoclonal antibody.

The results from the competitive binding assay also support the hypothesis that the ISU-cA monoclonal antibody is recognizing the A4 blood group antigen. Prior incubation
with A4-specific alloantisera inhibited binding of the monoclonal antibody to its specific antigenic determinant. Inhibition of goat anti-mouse immunoglobulin binding was observed when RBC were incubated first with ISU-cA then with the polyclonal anti-A4 sera. This result may be explained by the nature of alloantisera versus that of monoclonal antibodies. Alloantisera are assumed to be polyclonal and contain antibodies of several different specificities. This array of antibodies would differentially bind to the various epitopes, most of which would likely not be recognized by the monospecific monoclonal antibody. The presence of these polyclonal antibodies on the A blood group antigenic molecule in close proximity to the ISU-cA bound monospecific site may physically interfere with the subsequent binding of the FITC-labelled anti-mouse second antibody to ISU-cA. Steric hindrance of binding of the second antibody to ISU-cA due to the presence of anti-chicken immunoglobulins on the RBC surface could explain the inhibition observed. However, no inhibition resulted from the presence of a putative non-competitive antibody (anti-B1) against the alloantigen B1 which would likely be separate from A4 antigens on the cell surface.

Three different approaches were used to examine the response of RBC to ISU-cA. Serological data showed an association between A blood group antigens and ISU-cA
reactivity. The genetic segregation tests demonstrated that the hemagglutination patterns seen due to ISU-cA and anti-A4 were due either to the same locus or to two tightly linked loci. This association and/or linkage was seen in birds from both the ISU and NIU flocks. The competitive binding assay showed that ISU-cA and polyclonal anti-A4 sera either bind to epitopes of the same molecule or to different molecules in extremely close proximity on the cell surface. Thus, the hypothesis that the ISU-cA monoclonal antibody recognizes chicken A blood group antigens has been supported by serologic, genetic and molecular evidence.

The seven turkey strains tested were unrelated and non-inbred and therefore would be expected to be reasonably representative of the genetic variability within the species. All turkeys tested being negative suggests that the specific epitope of interest is not present in the turkey. Though only three pheasants were tested, the negative scores also suggest that the antigenic determinant identified by the ISU-cA monoclonal antibody is not on pheasant erythrocytes.

The ISU-cA monoclonal antibody was demonstrated to identify the A\(^3\), A\(^4\) and A\(^8\) allelic products, but not those from A\(^2\) or A\(^5\) alleles. This reactivity pattern indicates that ISU-cA may recognize a public specificity shared by the A\(3\), A\(4\) and A\(8\) antigens or may simply be cross-reactive due to the presence of similar epitopes on the different A antigens.
One interesting anomaly regarding the ISU-cA monoclonal antibody is its specificity for erythrocyte antigens even though it was induced by lymphocyte immunization. There are conflicting reports in the literature regarding the presence of A antigens on lymphocytes. Blood group A antigens have not been detected on PBL by agglutination (Schierman and Nordskog 1962) or by immunofluorescence (Davidenas 1970). There is a report, however, of A allelic products being identified on lymphocytes by using an allofixation technique (Wong et al. 1972). We were unable to detect A antigens on PBL using ISU-cA and immunofluorescence with flow cytometric analysis. There are two possible explanations as to why ISU-cA is specific for antigens found on RBC yet was originally induced by PBL immunization. Either the immunizing PBL were contaminated with RBC, or there is a common epitope on both cell types that is at too low a level on PBL to be detected by our immunofluorescence assay. We are unable to distinguish between these two possibilities at this time, though the possible presence of some RBC in the immunizing suspension seems the most plausible. The ISU-cA production may have been stimulated by the A4 alloantigen on contaminating erythrocytes. The A4 alloantigen was found to be present in line G-B1 birds in a 1979 typing at the NIU laboratory.
The function of most blood group loci is not known. The R blood group is associated with susceptibility to avian leukosis-sarcoma virus (Crittenden et al. 1970), and the K blood group appears to determine agglutinability of chicken RBC by vaccinia virus (Brown et al. 1973).

The chicken A blood group was identified concurrently with the B blood group locus (Briles et al. 1948, 1950). Both these loci were independently identified by Gilmour (1954, 1958) (see review by Briles 1984). Extensive heterozygosity was observed for both of these loci within various inbred lines (Briles and McGibbon 1948; Gilmour 1954, 1958). This suggested that there was some selective advantage for heterozygosity of blood group loci and prompted further research into chicken blood groups. Subsequent work has shown that the B blood group identifies the major histocompatibility complex (MHC) of the chicken (Schierman and Nordskog 1961). The function of the A blood group locus is still not known.

Frequencies of A alleles in three inbred lines of chickens were reported by Briles (1972). Hala (1987) summarized the various blood group alleles present in inbred poultry lines from four different locations. Of the 26 lines described, 81% were fixed for the $A^4$ allele. Using non-inbred populations, Johnson and Edgar (1984) found differences in A allelic frequencies between two lines that
had been selected for resistance and susceptibility to coccidiosis. Dunnington et al. (1984) reported different A blood group allelic frequencies between high and low antibody response selected lines. These studies suggest some involvement of the A blood group locus in immunity and/or fitness. Recent studies of A-E congenic lines have indicated that A-E antigens may be involved in rejection of grafts and in response to RSV-induced tumors (H. Abplanalp, University of California, Davis, 1988, personal communication).

The availability of a monoclonal antibody specific for blood group A antigens should facilitate further investigations into the molecular nature and function of this polymorphic alloantigen system.
ACKNOWLEDGEMENTS

The authors acknowledge Lynne Wathen and Danielle LeBlanc for production of the ISU-ca hybridoma, Vickie Hall for assistance with flow cytometry, Dr. T.F. Savage, Dept. of Poultry Science, Oregon State University for allowing testing of the turkeys, and Dr. W.E. Briles for extensive editorial assistance with the manuscript.

This study represents a portion of a dissertation submitted by J.E. Fulton for partial fulfillment of the requirements for the degree of Ph.D. at Iowa State University.
REFERENCES


Table 1. Phenotypic response of parental erythrocytes to ISU-cA and two progeny generations

<table>
<thead>
<tr>
<th>Parental Phenotype</th>
<th>No. Sires</th>
<th>No. Dams</th>
<th>Progeny Phenotype</th>
<th>Expected Ratio</th>
<th>$\chi^2$</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neg.</td>
<td>Int.</td>
<td>Pos.</td>
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<tr>
<td>Pos. x Pos.</td>
<td>4</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>164</td>
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<tr>
<td>Neg. x Neg.</td>
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<td>101</td>
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<td>0</td>
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<td>97</td>
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<table>
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<th>2nd Generation</th>
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<th>Neg.</th>
<th>Int.</th>
<th>Pos.</th>
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<tr>
<td>Int. x Int.</td>
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<td>24</td>
<td>67</td>
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<td>1.19</td>
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<td>6</td>
<td>10</td>
<td>49</td>
<td>48</td>
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<td>Neg. x Int.</td>
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<td>43</td>
<td>0</td>
<td>1:1</td>
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<td>1</td>
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<td>Int. x Pos.</td>
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<td>0</td>
<td>52</td>
<td>53</td>
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<td>1</td>
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<tr>
<td>Pos. x Int.</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>42</td>
<td>64</td>
<td>1:1</td>
<td>4.57</td>
<td>1</td>
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</tbody>
</table>

$^a$Pos. = indirect hemagglutination (IHA) score of 4, Neg. = hemagglutination (HA) and IHA score of 0, Int. = HA and IHA scores between 0 and 4.

$^b$As is conventional for birds, sires are indicated first.
Table 2. Mean titration end points of response to ISU-cA for HA and IHA assay

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Tested</th>
<th>HA Endpoint</th>
<th>IHA Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous Positive</td>
<td>20</td>
<td>$8.0 \pm .55^a$</td>
<td>$11.1 \pm .89^b$</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>38</td>
<td>$6.4 \pm .70^*</td>
<td>$7.7 \pm .62^*$</td>
</tr>
<tr>
<td>Homozygous Negative</td>
<td>24</td>
<td>.0</td>
<td>0</td>
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$^a$ End-point dilutions were transformed; 1/10 dilution = 1; 1/20 = 2; ... 1/20480 = 12.

$^b$ Some samples did not reach an end point and were assigned a value of 12.

*Adjacent means are significantly different, ($P < 0.001$).
Table 3. Blood group genotypes of panel cells tested with ISU-cA monoclonal antibody

<table>
<thead>
<tr>
<th>Flock</th>
<th>Bird</th>
<th>Blood group system&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>ISU cA</th>
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<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
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<tr>
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<td>19/19</td>
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<td></td>
<td>C494-17340</td>
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<tr>
<td></td>
<td>A622-27304</td>
<td>2/8</td>
<td>23/23</td>
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</table>

<sup>a</sup>R<sup>2</sup> = Recombinant B-F<sup>2</sup> B-G<sup>23</sup>; R<sup>4</sup> = recombinant B-F<sup>21</sup> B-G<sup>23</sup>; R<sup>6</sup> = recombinant B-F<sup>21</sup> B-G<sup>23</sup>.

<sup>b</sup>Known to have one of these two alleles by pedigree analysis.

<sup>c</sup>Not tested.

<sup>d</sup>No response with reagent used.
Table 4. Segregation of reactivity to anti-A2 and anti-A4 alloantisera and ISU-cA

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>Progeny Genotype</th>
<th>Number</th>
<th>Antibody</th>
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<td></td>
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<td>anti-A2</td>
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<td>(A^2/A^4) x (A^2/A^4)</td>
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<td></td>
<td>(A^4/A^4)</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 5. Effect on ISU-cA binding to RBC of incubation of cells with alloantisera

<table>
<thead>
<tr>
<th>Alloantisera Specificity</th>
<th>Sequence of incubation</th>
<th>Alloantisera/ISU-cA</th>
<th>ISU-cA/Alloantisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>anti-A4 (A4-138-967)</td>
<td>67</td>
<td>86</td>
<td></td>
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<tr>
<td>anti-A4 (A4-260-747)</td>
<td>81</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>anti-B1 (B1-8700-85)</td>
<td>108</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>anti-B1 (B1-8716-85)</td>
<td>103</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Amount of fluorescence expressed as a percentage of fluorescence measured in the absence of alloantisera.
PAPER II.

ONTGENY AND EXPRESSION

OF CHICKEN A BLOOD GROUP ANTIGENS
ONTOGENY AND EXPRESSION
OF CHICKEN A BLOOD GROUP ANTIGENS

J.E. FULTON
DEPARTMENT OF ANIMAL SCIENCE AND IMMUNOBIOLOGY PROGRAM
IOWA STATE UNIVERSITY, Ames, IOWA, U.S.A.

V.J. HALL
DEPARTMENT OF BIOCHEMISTRY AND BIOPHYSICS
IOWA STATE UNIVERSITY, Ames, IOWA, U.S.A.

S.J. LAMONT
DEPARTMENT OF ANIMAL SCIENCE AND IMMUNOBIOLOGY PROGRAM
IOWA STATE UNIVERSITY, Ames, IOWA, U.S.A.

Running Title: Chicken A blood group antigen expression

Key Words: monoclonal antibody, chicken, A blood group, immunofluorescence, erythrocyte antigen

1Journal Paper no. J-13344 of the Iowa Agriculture and Home Economics Experiment Station, Ames; Project no. 2237.

2To whom correspondence should be addressed.
Expression of chicken red blood cell (RBC) surface antigens was studied by using a monoclonal antibody (ISU-cA) specific for chicken A blood group antigens. Erythrocytes were examined from embryos of 3 to 18 days of incubation and from chicks at hatch through 21 weeks of age. Specific antigens were detected on embryonic RBC surfaces by immunofluorescence as early as 3 days of incubation. Antigenic expression was examined by both hemagglutination and immunofluorescence and found to increase with age from embryos to mature birds. The antigen concentration on the cell surface was found to be affected by genotype; heterozygotes had an intermediate level of antigen between that of the two parental genotypes. These data confirm the co-dominance that is observed with most blood group antigens. Flow cytometric analysis allowed confirmation that the entire erythrocyte population gradually increased in antigenic expression over time, rather than having an antigen-negative subpopulation being replaced by a positive subpopulation.
INTRODUCTION

The chicken A blood group system was first described by Briles and co-workers (1948) using allogeneic polyclonal sera. It was identified concurrently with the chicken B blood group system, which was subsequently identified as the chicken major histocompatibility system (MHC) (Schierman and Nordskog 1961). Gilmour (1954, 1958) independently identified these same two blood group loci (Briles 1984).

The ontogeny of two chicken erythrocyte membrane antigens, chicken fetal antigen (CFA) and chicken adult antigen (CAA), has been studied extensively. The CFA is found on erythrocytes from embryos of 12 days of incubation. Antigen expression gradually decreases as chicks age and CFA is undetectable by 17 weeks of age (Sanders 1968; Blanchet 1976). Since its initial identification on erythrocytes, CFA has been found on lymphocytes (Dietert et al. 1982), peritoneal macrophages (Dietert et al. 1983) and on virus-induced hematopoietic tumours (Nelson et al. 1982; Krsmanovic et al. 1983; Murthy et al. 1979). These studies were done by using rabbit polyclonal antisera that had been appropriately absorbed. The presence of CFA was detected either by hemagglutination (erythrocytes), immunofluorescence (macrophages) or microcytotoxicity (macrophages and lymphocytes). Monoclonal antibodies specific for CFA have subsequently been developed (Miller et al. 1982; Sanders et
The CAA was originally found on erythrocytes (Blanchet 1976; Kline et al. 1982) by observing hemagglutination with absorbed rabbit polyclonal antisera. The CAA has subsequently been detected on splenic lymphocytes by immunofluorescence (Kline et al. 1984). It is first detectable by hemagglutination at low levels 4 days after hatching. Antigenic expression rapidly increases and reaches adult levels by 24 days of age (Blanchet 1976). There is evidence that suggests that CAA and antigens of the chicken major histocompatibility complex are the same molecule (Miller et al. 1982; Kline et al. 1984; Kline et al. 1988).

A monoclonal antibody (ISU-cA) has been characterized that is specific for chicken A blood group antigens (Fulton et al. 1989). The purpose of this study was to examine, by using the monoclonal antibody ISU-cA, the ontogeny and expression of the A blood group antigens on erythrocytes of chickens from embryonic stages through maturity.
MATERIALS AND METHODS

Blood Sample Collection

Blood samples were collected into phosphate-buffered saline (PBS) + 1% citrate either by venipuncture from the jugular vein (hatch to 4 weeks of age) or the wing vein (5 weeks and older). Embryonic red blood cells (RBC) were collected from major blood vessels of embryos by using heparin-coated capillary tubes. All samples were stored overnight in PBS at 4°C before testing.

Monoclonal Antibody Production

Production and characterization of the monoclonal antibody, ISU-cA, has been previously reported (Fulton et al. 1989). Briefly, Balb/c mice were immunized with peripheral blood lymphocytes (PBL) from a G-B1 (B\textsuperscript{13}/B\textsuperscript{13}) strain chicken and then boosted with G-B1 bursal cells. Spleen cells from the immunized mice were fused with SP\textsubscript{2}/O myeloma cells. The specificity of ISU-cA for several allelic forms of the chicken A blood group was confirmed by genetic, serological and molecular analysis. The RBC from birds with the A\textsuperscript{4} allele have been shown to bind ISU-cA, whereas RBC expressing A\textsuperscript{2}/A\textsuperscript{2} do not.

Birds

Birds used in this study were from the partially inbred (F = 0.5) Iowa State University S1 White Leghorn line
(Nordskog and Cheng 1988). All birds had either the \( A^2 \) and/or \( A^4 \) alleles of the A blood group locus.

**Hemagglutination Assays**

Blood samples were washed once in PBS and spun at 130 x g for 10 mins, and 2% solutions of packed RBC in PBS were made. Assays were carried out in 96-well round-bottom microtitre plates at room temperature. One drop (40 ul) of the cell suspension and one drop of ISU-cA antibody (1/200 dilution of ascites in PBS) were added to each well. The plates were briefly shaken on a microtitre plate shaker, then incubated for 1h. All incubations were done at room temperature. They were then reshaken and after an additional 1h incubation, the direct hemagglutination (HA) score was determined. The indirect hemagglutination (IHA) score was determined after addition of a second antibody [goat anti-mouse immunoglobulin (IgG, IgM, IgA, H + L chain specific, Cooper Biomedical, WestChester, Pennsylvania)]. The plates were again shaken, incubated for 1h, shaken again and scored after another 1h incubation.

Scoring of the hemagglutination assays was conducted visually. Scores for the degree of agglutination were 0 for a button of non-agglutinated cells; 1 for a button of cells with slight agglutination; 2 for a small button of cells with agglutination; 3 for agglutination of most but not all cells; 4 for complete agglutination. All samples were scored by the
same individual. A score of 0 was considered as negative, 4 was considered positive and all other values were classified as intermediate.

Blood samples were obtained from 58 heterozygous (A^2/A^4) chicks at 1 week of age. These same chicks were tested weekly through 21 weeks of age.

**Antibody Titration Test**

Blood samples were collected from 19 A^4/A^4 and 50 A^2/A^4 chicks at 2 weeks of age and from 20 A^4/A^4 and 38 A^2/A^4 birds at 22 weeks of age for the antibody titration test. Samples were prepared, and the HA and IHA assays were carried out as previously described. The monoclonal antibody was titrated in doubling dilutions from 1/10 to 1/20,480. The last dilution in which any agglutination was seen was considered the end point. For analysis, the dilutions were transformed to numerical scores of n = 1 through 12, where n = log_2(1/(10 x dilution)) + 1 (i.e., 1/10 dilution = 1; 1/20 = 2; ...1/20,480 = 12).

**Immunofluorescence Labelling**

Blood samples were washed once, and 0.2% packed RBC solutions were made. Assays were carried out in duplicate or triplicate using 96-well round-bottom microtitre plates. Twenty ul of cell suspension was added per well, 20 ul of ISU-cA antibody (1/100 dilution in PBS) was added to each well, and the plates were then briefly shaken then incubated
on ice for 1h. Cells and antibody were resuspended by shaking twice during this incubation. The cells were then washed twice with PBS + 1% BSA + 0.1% NaN₃. Twenty μl of the fluorescein isothiocyanate (FITC) conjugated second antibody (1/20 dilution in PBS) was added to each well [FITC-goat anti-mouse IgA, IgG, IgM (H & L), Cooper Biomedical, Westchester, Pennsylvania]. The plates were covered with aluminum foil, shaken and incubated on ice for 1h. Plates were shaken twice during incubation to resuspend cells. The cell solutions were washed twice with PBS + 1% BSA + 0.1% NaN₃ and once with PBS + 0.1% NaN₃ before analysis by flow cytometry.

Immunofluorescence was determined on samples taken from individuals at hatch and at regular intervals until they were 21 weeks of age. Mature individuals of each genotype were also assayed concurrently as controls.

Flow Cytometric Analysis

Flow cytometric analysis was done by using an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, Florida) equipped with an argon ion laser (488 nm, 400 mw). To each sample, 7.5 μm fluorescent beads (Flow Cytometry Standards Corp., Research Triangle Park, North Carolina) were added as an internal standard. Log fluorescence was collected for each RBC sample, and light scatter was used to gate out
debris. Samples were counted until a total of 20,000 events was reached.

Embryo Study

Embryonic blood was obtained from embryos of ages 3, 5, 7, 12 and 18 days of incubation. Blood was pooled from two or more genetically similar embryos within the 3-day and within the 5-day incubation samples to obtain sufficient sample volume. Five or six samples were tested at each age from each genotype. The immunofluorescence assay and flow cytometric analyses were done as previously described. All samples were analyzed on the same day to minimize data variation due to daily instrument fluctuations.
RESULTS

**Hemagglutination Assay**

The mean IHA score for heterozygous birds from 1 week to 21 weeks of age, in 4 week intervals, are shown graphically in Figure 1. An increase over time in mean IHA score was seen beginning at 1 week of age (0.9 ± 0.81) to 17 weeks of age (4.0 ± 0.16). At 21 weeks of age, all birds had the highest possible hemagglutination score of 4. The scores clearly increased with age; however, there was much variation within each week as shown by the large standard errors.

**Titration Assay**

The results of the titration assay are presented in Table 1. A significant increase (p < 0.001; t-test, Steel and Torrie 1980) from 9.4 ± 0.67 to 11.1 ± 0.89 was seen for \( A^4/A^4 \) birds tested at 2 weeks of age versus those tested at 22 weeks of age. End points were not identified for some IHA scores of 22-week-old birds, and in those cases, a titre of 12 was assigned, thus underestimating the mean titre of 22-week-old birds. A significant increase (p < 0.001) was also found for \( A^2/A^4 \) birds for this same time period (5.3 ± 0.50 and 7.7 ± 0.62 for 2 weeks and 22 weeks of age respectively). Comparisons between genotypes at each age also revealed significant differences. At 2 weeks of age, \( A^4/A^4 \) birds had significantly higher mean titration end points (p < 0.001) than did \( A^2/A^4 \) individuals. The mean titration end points
increased for both genotypes and were still significantly different at 22 weeks of age ($P < 0.001$). Blood from homozygous negative individuals ($A^2/A^2$) were also tested at 22 weeks of age (data not shown). No agglutination at any antibody titre was detected for all 24 samples.

**Immunofluorescence Assay**

The results of the fluorescence assay from mature birds of each of the three genotypes are depicted in Figure 2. The graphs show cell number versus log fluorescence of RBC. Figure 2a shows the results from an $A^2$ homozygous bird from which no fluorescence of RBC was detectable. An $A^2/A^4$ heterozygous sample shows more fluorescence (2b), and the greatest degree of fluorescence is detected from RBC of an $A^4/A^4$ bird (2c). The second peak detected in samples of RBC from homozygous $A^4$ birds is due to the greater fluorescence of occasional pairs of agglutinated cells. Mean log fluorescence values for these three genotypes are significantly different ($P < 0.001$; Duncan's multiple range test).

The effect of age on immunofluorescence of RBC is shown graphically in Figure 3. The first row of histograms shows the change in peak immunofluorescence of an $A^2/A^4$ individual. At hatch (3a) no fluorescence was detectable. Immunofluorescence had increased by 6 weeks of age (3b) and again by 22 weeks of age (3c).
Similarly, an increase of fluorescence with age, is seen for \( A^4 \) homozygous chicks (Figs. 3d-3f). The immunofluorescence of RBC from \( A^4/A^4 \) chicks at hatch is shown in Figure 3d. The amount of fluorescence for an \( A^4/A^4 \) chick is significantly greater than for the heterozygous hatch mate (3a). By 6 weeks of age (3e), a marked increase in fluorescence is noticeable, and by 22 weeks of age (3f), the amount of fluorescence detectable shows a further increase. Once again, a second peak due to pairs of agglutinated cells is observed.

The mean log fluorescence for each genotype at hatch, 6, and 22 weeks of age is depicted in Table 2. Because of the length of time over which the experiment was run, the mean fluorescence values can not be compared between weeks. Valid comparisons can, however, be made within each week and were analyzed by Duncan’s multiple range test.

All controls were from adult birds and were tested concurrently with the samples. At hatch, the fluorescence of \( A^2/A^4 \) birds was not significantly different from the homozygous \( A^2 \) (negative) control, and fluorescence of the homozygous \( A^4 \) sample was not significantly different from heterozygous \( (A^2/A^4) \) adult birds. By 6 weeks of age, there was an increase in the amount of fluorescence, particularly the homozygous \( A^4 \) samples. All samples and controls were significantly different from each other by 6 weeks of age,
with an increase in the amount of fluorescence for both A^2/A^4 and A^4/A^4 chicks. By 22 weeks of age, mean log fluorescence of homozygous A^4 and A^2/A^4 samples were no longer different from their respective controls.

**Embryo Study**

The fluorescence of embryonic RBC labelled with the monoclonal antibody is shown in Table 3. With the exception of the data from the 3-day-incubated embryos, which will be discussed later, the previously described effects of age and genotype on degree of fluorescence are seen here. The change in relative fluorescence ratio can be detected between 3 and 5 days of incubation for A^4/A^4 embryos, and this increase continues to 18 days incubation. These embryos also show greater fluorescence than their age-matched heterozygous counterparts.

The fluorescence detected on 3-day-incubated homozygous A^2 (negative) RBC was unexpected. Comparable levels of fluorescence were also detected on negative control samples of the same age which were treated with the FITC-labeled second antibody only (i.e., no ISU-cA antibody). To minimize the interference with data interpretation due to this non-specific fluorescence, the ratio of fluorescence detected on positive cells versus age-matched negatives were calculated (latter half of Table 3). These ratios were determined for both homozygous A^4 and heterozygous embryos. The ratios
increase with age, once again showing the effect of age on fluorescence. The ratios also indicate the greater fluorescence of \( A^4/A^4 \) cells versus age-matched \( A^2/A^4 \) cells.
DISCUSSION

The hemagglutination data (Figure 1) showed that antigen levels in blood samples from A^2/A^4 birds increased with age. Homozygous A^4/A^4 birds were not tested with this assay because the initial hemagglutination test used a single predetermined antibody dilution and did not indicate variation in their response. The titration assay used serial dilutions of antibody and confirmed the increase with age in antigen levels on erythrocytes from heterozygotes. It also showed that antigen levels increased in A^4/A^4 birds and that they maintained significantly higher levels than heterozygotes. The observation that A^2/A^2 individuals never showed hemagglutination even at the highest concentration of antibody used suggested that they do not have the epitope recognized by ISU-cA.

Comparison of the hemagglutination data and titration data emphasizes the limitations of the single-dilution hemagglutination assay. Once the maximum hemagglutination has been reached, the assay can no longer discriminate further increases in antigen level. A more dilute antibody concentration used during initial tests probably would have detected increasing antigen levels with age in A^4/A^4 birds. Thus, the original antibody dilution used delineates the sensitivity of a single-dilution hemagglutination assay.
The immunofluorescence assay clearly distinguished the heterozygous \( A^2/A^4 \) from homozygous positive \( A^4/A^4 \) genotypes by their level of fluorescence, which was indicative of their cell-surface antigen concentration. It also showed the increase in antigen level with age for both genotypes. The difference in antigenic expression between genotypes was evident even on early embryonic RBC (Table 3). The sample/negative ratio was much higher for homozygous positive samples than for samples from heterozygotes.

High levels of fluorescence were detected on erythrocytes from 3- and 5-day-incubated homozygous negative embryos. Similar levels of fluorescence were also detected on the early age-matched controls, which were incubated with FITC-labelled goat anti-mouse antibody only. Owing to the distinctive nature of early embryonic erythrocytes versus their more mature counterparts, the data given in Table 3 require interpretation. By 3 days of incubation, the chick erythrocyte population is composed of primary erythroblasts and primary polychromatic erythrocytes. These cells are considerably larger than subsequent erythrocyte populations (Lucas and Jamroz 1961). Flow cytometric analysis confirms that these cells are indeed larger (data not shown). Mature chicken erythrocytes autofluoresce after fixation with glutaraldehyde, and these fixed erythrocytes are routinely used as standards for flow cytometers (Herzenberg and
Herzenberg 1978). Thus the fluorescence observed is attributable to either autofluorescence or non-specific binding of the FITC-conjugated antibody. The greater size of these embryonic erythrocytes exaggerates any autofluorescence or non-specific binding.

Identification of A blood group antigens on erythrocytes from 3-day-incubated embryos confirms the early reports by Briles et al. (1948) in which anti-A reagents caused weak agglutination of embryonic RBC from 3-day-incubated embryos and strong agglutination of RBC from 5-day-incubated embryos. Johnson (1956) (cited by Briles 1964) suggested that the lower hemagglutination levels of erythrocytes from very young embryos may be due to low cell numbers in their cell suspensions. The immunofluorescence data presented here suggest that lower hemagglutination levels are due to lower antigen levels on early embryonic erythrocytes.

There are two possible explanations why the hemagglutination scores increase as birds age; either the cell population of younger birds that bear no or a low concentration of antigen is gradually replaced in older birds by a different cell population that bears a higher antigen concentration, or there is a gradual increase in age in the expression of the antigen on the cell surface of a single population of cells. Distinction between these two models is not possible by using hemagglutination data. However, the
histograms of the fluorescence of RBC (Figure 3) clearly show only one main peak, meaning that only one population of cells is present at any age. The fluorescence of these cells gradually and significantly increases, thus demonstrating that the concentration of A blood group antigens on the surface of all cells increases with age. Therefore, the model of a single type of erythrocyte with gradually increasing expression of A blood group antigens is most likely correct.

The titration and immunofluorescence data show that expression of the A blood group antigen in heterozygotes is intermediate in expression between that of homozygous individuals. This partial dominance in gene expression is common for blood group antigens.

All the studies reported here involved either $A^2$ (ISU-cA negative) or $A^4$ (ISU-cA positive) alleles. The effect of age and genotype on relative amounts of antigen therefore apply specifically to the $A^4$ allelic product, though similar effects with other A alleles are expected.

The data presented here suggest that the chicken A blood group antigens, although not differentiation antigens per se, are related to erythrocyte maturation. The ontogeny of A blood group antigens is different from either of the well-studied chicken differentiation antigens CFA or CAA, indicating that they are unrelated.
The function of the A blood group system is unknown. The influence of the A blood group antigens on graft rejection was studied by Cock and Clough (1956) and Crittenden et al. (1964). They found that the A locus did not seem to be involved in graft rejection. More recently, differences in A allelic frequencies have been reported in lines divergently selected for response to coccidial infection (Johnson and Edgar 1984) and to sheep red blood cell immunization (Dunnington et al. 1984). The E blood group locus is known to be closely linked to the A locus (Briles 1968). Current studies of A-E congenic lines have indicated that A-E antigens may be involved in rejection of grafts and in response to Rous sarcoma virus-induced tumors (H. Abplanalp, University of California, Davis, 1988; personal communication). Further study of A blood group antigens will define the role of the chicken A blood group antigens in immune response.
ACKNOWLEDGEMENTS

This study represents a portion of a dissertation submitted by J.E.F. for partial fulfillment of the requirements for the degree of Ph.D. at Iowa State University.
REFERENCES


Table 1. Mean titration end points\(^a\) for IHA assay for \(A^2/A^4\) and \(A^2/A^4\) chicks at 2 and 22 weeks of age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (weeks)</th>
<th>2</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A^2/A^4)</td>
<td></td>
<td>5.3 ± .50</td>
<td>* 7.7 ± .62</td>
</tr>
<tr>
<td>(A^4/A^4)</td>
<td></td>
<td>9.4 ± .67</td>
<td>* 11.1 ± .89(^b)</td>
</tr>
</tbody>
</table>

\(^a\)End point dilutions were transformed; 1/10 = 1; 1/20 = 2; ... 1/20,480 = 12.
\(^b\)Some samples did not reach an end point and were assigned a value of 12.

* Adjacent means are significantly different (\(P < 0.001\)).
Table 2. Mean peak log fluorescence (channel number) of ISU-cA labelled RBC from homozygous positive and heterozygous birds at hatch, 6 weeks and 22 weeks of age and from mature birds of each genotype assayed concurrently

<table>
<thead>
<tr>
<th>Sample Genotype</th>
<th>Age (wks)</th>
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<tr>
<td></td>
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<tr>
<td>A^4/A^4</td>
<td>52±5.2</td>
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<tr>
<td>A^2/A^4</td>
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</table>

<table>
<thead>
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<th>Control Genotype</th>
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</thead>
<tbody>
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<td>112±0.3</td>
<td>106±0.6</td>
<td>105±0.3</td>
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<tr>
<td>A^2/A^4</td>
<td>52±</td>
<td>57±1.3</td>
<td>38±6.7</td>
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<tr>
<td>A^2/A^2</td>
<td>11±</td>
<td>10±0.03</td>
<td>8±1.3</td>
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</tbody>
</table>

^a-eMeans within each week with the same superscript are not significantly different.
Table 3. Log fluorescence (channel number) of ISU-cA labelled RBC from embryos of various ages of each genotype. Relative levels of fluorescence of homozygous positive and heterozygous cells for the various ages.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Day of Incubation</th>
<th>Mean Fluorescence</th>
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<tbody>
<tr>
<td></td>
<td>3</td>
<td>5</td>
</tr>
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<td>A^4/A^4</td>
<td>30±3.5</td>
<td>24±3.0</td>
</tr>
<tr>
<td>A^2/A^4</td>
<td>ND^a</td>
<td>ND</td>
</tr>
<tr>
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<td>23±2.7</td>
<td>12±0.7</td>
</tr>
<tr>
<td>Controls^b</td>
<td>26</td>
<td>11</td>
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</table>

B. Ratio

<table>
<thead>
<tr>
<th></th>
<th>Pos./Neg.</th>
<th>Heterozygote/Neg.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1.28</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>3.41</td>
<td>4.72</td>
</tr>
<tr>
<td></td>
<td>4.72</td>
<td>10.52</td>
</tr>
</tbody>
</table>

^aND = not determined

^bControls were homozygous positive cells with FITC-labelled antibody only.
Figure 1  Mean indirect hemagglutination (IHA) scores of heterozygous birds from 1 to 21 weeks of age. Vertical lines indicate standard deviations.
Figure 2 Cytometric analysis of RBC labelled with ISU-cA monoclonal antibody. Log fluorescence (channel number) of cells from mature birds: a; homozygous negative. b; heterozygous. c; homozygous positive
Log Fluorescence (channel Number)

Relative Cell Number

a

b

c

Log Fluorescence (channel Number)
Figure 3  Effect of age on immunofluorescence of RBC labelled with ISU-cA monoclonal antibody from heterozygotes (a-c) and from homozygous positive individuals (d-f) at hatch (a,d), 6 weeks (b,e) and 22 weeks of age (c,f)
PAPER III.

CELLULAR LOCATION OF CHICKEN A BLOOD GROUP ANTIGENS
ABSTRACT

Attempts were made to identify chicken A blood group antigens on chicken lymphocytes using an A specific monoclonal antibody (ISU-cA) and alloantisera. Binding of A specific antibody was not detected with either immunofluorescence or enzyme-linked immunosorbent assay (ELISA). Immunoprecipitation of radiolabelled cell-surface molecules also did not detect A blood group antigens. Thus the data do not support the hypothesis that chicken A blood group antigens are on lymphocytes.
INTRODUCTION

The chicken A blood group antigenic system was first reported by Briles et al. (1950). Genetic and serologic tests identified 9 alleles of the A blood group whose products were detectable on erythrocytes.

Chicken A blood group antigens have not been detected by using alloantisera on peripheral blood lymphocytes (PBL) by agglutination (Schierman and Nordskog 1962), or by immunofluorescence (Davidenas 1970). Wong et al. (1972) reported that A specific alloantisera caused adherence of lymphocytes to glass or plastic surfaces. The results from this 'allofixation' technique suggested that chicken A blood group antigens were on lymphocytes, thus implying a role of A antigens in immunity (Wong et al. 1972).

Fulton et al. (1989a) characterized a monoclonal antibody (ISU-cA) specific for certain allelic products of the chicken A blood group. This paper describes attempts to identify A allelic products on chicken lymphocytes using the A specific monoclonal antibody ISU-cA and alloantisera.
MATERIALS AND METHODS

Chicken Stocks

All birds used in this study were from the partially inbred (F = 0.5) Iowa State University Sl White Leghorn chicken line (Nordskog and Cheng, 1988). Birds were of either A2/A2, A2/A4 or A4/A4 genotype as determined by alloantisera or ISU-ca hemagglutination response.

Antibodies

Production of the monoclonal antibody, ISU-ca, has been previously described (Fulton et al. 1989a). Briefly, Balb/c mice were immunized with PBL and boosted with bursal cells from a G-B1 line chicken. Spleen cells from the immunized mouse were fused with Sp2/0 myeloma cells. All assays described using ISU-ca were with ascites fluid diluted in phosphate buffered saline pH 7.2 (PBS). ISU-ca agglutinates cells carrying the A4 antigen. It does not bind A2/A2 cells. An irrelevant IgM monoclonal antibody, [(B78.18.2) against endotoxin from Treponema hyodysenteria, donated by M. Wannemuehler, Iowa State University] was used as an isotype control.

Chicken alloantisera were supplied by W.E. Briles (Northern Illinois University). Anti-4 alloantiserum (A4-84-296) was specific for A4 antigen-carrying erythrocytes (RBC), whereas anti-A2 alloantiserum (A2-165-321) was specific for all A allelic products except A4 (Briles,
personal communication). Alloantisera were used either unabsorbed, or were first absorbed three times with either A^2/A^2 or A^4/A^4 RBC to remove A specific antibodies.

**Blood Sample Collection**

Blood was collected either into PBS with 1% citrate or into heparin following venipuncture of the wing vein. Red blood cells were washed three times prior to use. The buffy coat was collected from centrifuged whole blood and layered over Ficoll-paque (Pharmacia, Piscataway, New Jersey) to separate the PBL. Cells were then washed three times before being used. All blood samples were obtained from birds over 20 weeks of age.

**Enzyme-linked Immunosorbent Assay (ELISA)**

ELISA assays were done using 96-well microtiter plates. Two birds of each genotype A^2/A^2, A^2/A^4, A^4/A^4 were tested and each sample was done in duplicate. PBL and RBC were obtained from the same birds. Assays were identical for PBL and RBC except that RBC were used fresh and PBL were fixed to the plate with 1% glutaraldehyde for 30 min and stored for 2 days at 4°C prior to use. Assays were done using 5 x 10^5 cells per well. Ascites fluid was diluted to 1/100, 1/1,000, 1/10,000 and 1/100,000 in PBS. A 50 ul volume of diluted antibody was used per well and incubated at RT for 2hr. Plates were washed three times with PBS-Tween (PBS + 0.05% Tween-20) then twice with PBS. Fifty ul of 1/20 dilution of
phosphatase-labelled goat anti-mouse (Cooper Biomedical, WestChester, Pennsylvania) was added and incubated for 1 h. Washes were repeated and 50 ul of substrate [5mg p-nitrophenyl phosphate disodium (Sigma, St. Louis, Missouri) in 6 ml 1M diethanolamine pH 9.3] was added. The color reaction was allowed to proceed for 30 min prior to stopping with 100 ul of 2N NaOH. ELISA scores were determined on an ELISA reader with 405 nm filter.

Negative controls, consisting of Sp2/0 ascites and B78.18.2 ascites (IgM isotype control), were used at the same dilution as ISU-cA monoclonal antibody. The ELISA reader was zeroed on the well with the lowest concentration of Sp2/0 ascites. Sample values were corrected for non-specific antibody binding by subtracting IgM isotype control for the appropriate dilution.

The assay to compare binding of ISU-cA to fresh versus fixed PBL as detected by ELISA was modified slightly from that previously described. PBL were obtained from either one or two birds of each genotype. Cells were washed in PBS and then each sample was divided into two volumes and used either fresh or fixed with 1% glutaraldehyde for 30 min. The fixed cells were washed three times remove excess glutaraldehyde. Assays were done in 12 x 75 mm glass tubes to allow for greater washing volume. ISU-cA was used at 1/100 dilution. To measure the ELISA reaction at the completion of the assay,
a 100 µl volume of supernatant was removed from each tube and transferred to wells of a 96-well plate for scoring. The ELISA reader was zeroed on the well with the lowest Sp2/0 ascites concentration. The isotype control antibody was not tested in this assay. ELISA values were mean scores for PBL from the same treatment.

The effects of pH on ELISA value was also tested. Wells containing PBL were washed with PBS of either pH 5.2, 6.2, 6.7, 7.2, 7.7, 8.2, 8.7, or 9.2. ISU-cA antibody was diluted in PBS of each pH. After incubation with the first antibody, wells were washed three times with PBS/Tween of the same pH in which their primary antibody was diluted. They were then washed with PBS of pH 7.2 and the assay was continued as previously described. The ELISA reader was zeroed on the Sp2/0 ascites well within each dilution and pH. All samples were tested in duplicate with cells from two birds of either A^2/A^2 or A^4/A^4 genotype.

Immunofluorescence

Immunofluorescence (IF) labelling was done as described by Fulton et al. (1989b). PBL from mature birds were incubated first with ISU-cA monoclonal antibody (1/100 dilution of ascites) and then with a fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG, IgM, IgA (H and L) second antibody (Cooper Biomedical, WestChester, Pennsylvania). Fluorescence was measured using
an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, Florida) equipped with an argon ion laser (488 nm, 400 mw).

**Radiolabelling and Cell Lysis**

Iodination was done following a modified procedure of Vitetta et al. 1971. Briefly, 4 x 10^8 PBL were enzymatically labelled with ^125^I (Amersham, Arlington Hts., Illinois) using lactoperoxidase. Radiolabelled PBL were lysed with 1 ml solubilizing buffer (0.5% Non-ident 40, 0.5% deoxycholate, 0.01% phenylmethylsulphonylfluoride in PBS) and incubated for 1 hr with gentle shaking. Nuclei were removed by centrifugation. Radioactivity levels obtained were 6-10 x 10^4 dpm/ul lysate.

**Immunoprecipitation**

A 500 ul volume of radiolabelled cell lysate was used per sample. One hundred ul of alloantisera or 10 ul of undiluted ascites was added and incubated for 1.5 hr with vigorous shaking. One hundred ul of either rabbit anti-mouse IgM (ICN Biomedicals Inc., Costa Mesa, California) or rabbit anti-chicken IgG (with cross reactivity to IgM) (Bethyl Laboratories Inc., Montgomery, Texas) was added, shaken vigorously for an additional 1.5 h then incubated overnight at 4°C. One hundred ul of a 10% solution of sepharose-protein A (Pharmacia, Piscataway, New Jersey) was then added and shaken for 2 h. Samples were washed five times, then dissociated with sodium dodecyl sulfate polyacrylamide gel
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electrophoresis (SDS-PAGE) sample buffer (0.125 M tris base, 20% glycerol, 4.6% SDS, 10% 2-mercaptoethanol, pH 6.8).

Polyacrylamide Gel Electrophoresis

SDS-PAGE separation of samples was done using discontinuous gels (Laemmli, 1970) with a stacking gel of pH 6.8 and 4.75% acrylamide and a resolving gel of pH 8.8 and 12% acrylamide. Molecular weight standards were obtained from BioRad (Richmond, California). Samples in volumes containing approximately $7 \times 10^3$ dpm were loaded per lane.

Autoradiography

Films (XAR; Kodak, Rochester, New York) were exposed at $-70^\circ C$ with intensifying screens for 3-8 days.
RESULTS

The effect of glutaraldehyde fixation of PBL on ISU-cA binding is shown in Figure 1. Peripheral blood lymphocytes from birds of each genotype ($A^2/A^2$, $A^2/A^4$, $A^4/A^4$) were tested. There was no difference within each treatment among the three genotypes ($F = 0.003$ and $0.010$ for fresh and fixed PBL respectively; df = 2,2). Also, there was no difference between the mean ELISA scores of fresh versus fixed PBL ($0.35$ and $0.33$ respectively; t-test = 1.714, df = 8).

The effect of pH on ISU-cA binding to PBL is shown in Figure 2. At each of the three antibody dilutions used (1/100, 1/1,000, 1/10,000) the amount of ISU-cA binding was not different between cells of each genotype. There was a decrease in ISU-cA binding with increasing pH for each antibody dilution. There was also a decrease in the level of ISU-cA binding with decreasing antibody concentration.

Figure 3 shows ELISA detection of ISU-cA binding to RBC (3a) and PBL (3b) at four different dilutions of ISU-cA. RBC from homozygous positive birds ($A^4/A^4$) have higher scores than those from homozygous negative ($A^2/A^2$) birds at antibody dilutions of 1/100 and 1/1000, with the heterozygotes ($A^2/A^4$) being intermediate. At 1/10,000 dilution of antibody, only RBC from homozygous positive birds ($A^4/A^4$) bind ISU-cA. No ISU-cA binding is detected at 1/100,000 dilution of ISU-cA for RBC of any genotype.
The slightly positive values seen for ISU-cA binding to PBL, were only at the greatest antibody concentration tested (1/100). These values were much lower than those seen with homozygous positive RBC (A^4/A^4) at the same antibody dilution. No differential response in ISU-CA binding was seen between the genotypes. At antibody dilutions greater than 1/100, ISU-cA binding to PBL was not detectable. ELISA values for PBL from birds of all genotypes were very similar to those for homozygous negative (A^2/A^2) RBC at each antibody dilution.

The results of the immunoprecipitations of radiolabelled PBL lysates with ISU-cA and alloantisera are shown in Figure 4. A^2/A^2 PBL immunoprecipitated by either ISU-cA (lane 1) or A2-specific alloantiserum (lane 3) are shown next to their respective controls (lanes 2 and 4) in which no immunoprecipitating antibody was used. Band differences are not detected, suggesting a lack of binding of ISU-cA and anti-A2 alloantiserum to molecules on the surface of PBL from A^2/A^2 birds. Lysates from A^4/A^4 PBL were immunoprecipitated by either ISU-cA (lane 5) or A4-specific alloantiserum (lane 7) and are shown adjacent to their respective negative controls (lanes 6 and 8) in which no immunoprecipitating antibody was used. Comparisons between each immunoprecipitated sample and its control reveal no differences in bands indicating a lack of binding of either ISU-cA or anti-A4
alloantiserum to cell-surface molecules on PBL from A\(^4\)/A\(^4\) birds. Lane 9 is a positive control consisting of A\(^4\)/A\(^4\) RBC lysate immunoprecipitated by A4-specific alloantiserum. The band indicated by the arrow (54.5 Kd) is the presumed A4 antigen immunoprecipitated from RBC.
DISCUSSION

Initial screening of ISU-cA was done using ascites dilutions of 1/100. All PBL tested were positive by ELISA, yet IF done at the same antibody dilution was negative. The series of ELISA tests examined the discrepancies between the ELISA and IF results. There are inherent differences between the two assays. Immunofluorescence is done with fresh cells, whereas ELISA is done with glutaraldehyde fixed cells. Comparison of ISU-cA binding to fresh and fixed PBL (Figure 1) clearly shows that fixation does not influence ISU-cA binding to PBL. The assay in which pH was varied was done to determine if ISU-cA binding was to a different molecule on \(A^2/A^2\) PBL than on \(A^4/A^4\) PBL. Incubations at different pH should have distinguished specific binding of ISU-cA to \(A^4/A^4\) PBL from non-specific binding to \(A^2/A^2\) cells. The lack of difference in response between cells of each genotype suggests that binding of ISU-cA is to the same molecule regardless of genotype. This implies that the genotypic differences detected by ISU-cA on RBC are not present on PBL.

ELISA is inherently more sensitive than IF since it is an enzymatic reaction and thus multiplicative. The ELISA with RBC demonstrated that genotypic differences in binding of ISU-cA are indeed detectable with the assay. At high ISU-cA concentrations (1/100) ELISA is too sensitive to detect
genotypic differences in expression between \( A^4/A^4 \) and \( A^4/A^2 \) individuals, but could distinguish these genotypes from negative individuals \( A^2/A^2 \). At lesser concentrations (1/1000) heterozygotes \( A^4/A^2 \) were easily distinguished from homozygous positives \( A^4/A^4 \), thus the multiplicative ELISA reaction can be used to detect the genotypic differences.

The ISU-cA is an IgM isotype antibody, and this large molecule could be binding non-specifically to PBL via Fc receptors. The irrelevant antibody was used as an isotype control to decrease false positive values due to non-specific binding of IgM. Values given for RBC and PBL in Figure 3 were corrected by subtracting the irrelevant antibody values. Positive values were obtained for RBC from homozygous positive and heterozygous birds confirming specific binding of ISU-cA to cells of these genotypes as earlier reported with immunofluorescence (Fulton et al. 1989b). The ELISA values of all PBL (for all genotypes) were similar to those obtained for homozygous negative RBC, even though both were corrected for non-specific binding of IgM. Quantification of antibody and/or protein levels in either the ISU-cA ascites or isotype control ascites were not done. It is possible that the low positive values detected reflect residual non-specific binding. The similarity in ELISA values between the PBL and homozygous negative RBC data suggests that low levels
of non-specific binding occurs for all cells, particularly at the high antibody concentration of 1/100.

The immunoprecipitation data show that the alloantisera do not bind to molecules from PBL. Both of these alloantisera can function in immunoprecipitation assays as they were successfully used in assays using RBC lysates (Figure 4, lane 9; Fulton, Department of Animal Science, Iowa State University, unpublished data). This lack of specificity for PBL is not surprising since these antisera were produced by RBC immunizations. The lack of detection by ISU-cA of molecules from A²/A² PBL is expected since ISU-cA is specific for A4 molecules. However, if A blood group antigens are on PBL, then the monoclonal antibody should have detected them from A⁴/A⁴ PBL lysates.

If ISU-cA was binding to a molecule common to all PBL and RBC, that molecule should have been detected by the immunoprecipitations. Perhaps the affinity of ISU-cA for this common molecule is too low to withstand the rigors of the immunoprecipitation technique. Or, as the ELISA results suggest, ISU-cA binding to PBL may not be specific.

There is considerable variation in the pattern of bands between the two lysates A2 and A4 (lanes 1-4 and 5-8 respectively). This pattern is consistent within each lysate and is presumably due to molecules precipitated by non-specific binding of the goat antimouse or goat antichicken
second antibodies used. The variation between birds is not surprising because they are from a strain of birds with an intermediate level of inbreeding and probably represent products of other polymorphic loci.

Both the ELISA and immunoprecipitation techniques are extremely sensitive assays for detection of molecules. Under conditions which clearly identified A antigens on RBC, neither of these assays detected chicken A blood group antigens on PBL. Thus these data do not support the hypothesis of the existence of chicken A blood group antigens on PBL.
REFERENCES


Figure 1  ELISA detection of glutaraldehyde fixation effects on binding of ISU-cA to PBL from birds of $A^2/A^2$, $A^2/A^x$ and $A^4/A^4$ genotypes

Figure 2  Effect of pH on binding of ISU-cA at three dilutions (1/100, 1/1000 and 1/10,000) to $A^4/A^4$ PBL as detected by ELISA
ELISA Value

PBL Treatment

ELISA Value

Ph
Figure 3  ELISA detection of ISU-cA binding at four dilutions (1/100, 1/1,000, 1/10,000 and 1/100,000) to RBC (a) and PBL (b) from birds of each genotype, $A^2/A^2$, $A^2/A^4$ and $A^4/A^4$.)
Figure 4  SDS-PAGE (reduced) of lysates from $A^2/A^2$ (lanes 1-4) and $A^4/A^4$ (lanes 5-8) PBL immunoprecipitated with the designated antibody, then with either rabbit antimouse antibody (lanes 1, 2, 5 and 6) or rabbit antichicken antibody (lanes 3, 4, 7 and 8). Lane 1, ISU-cA; lane 2, no first antibody (i.e. negative control for lane 1); lane 3, anti-A2 alloantiserum; lane 4, no first antibody (i.e. negative control for lane 3); lane 5, ISU-cA; lane 6, no first antibody (i.e. negative control for lane 5); lane 7, anti-A4 alloantiserum; lane 8, no first antibody (i.e. negative control for lane 7); lane 9, $A^4/A^4$ RBC lysate immunoprecipitated by anti-A4 alloantiserum (positive control for lanes 1-8)
PAPER IV.

MOLECULAR CHARACTERIZATION OF CHICKEN A BLOOD GROUP ANTIGENS
ABSTRACT

The molecular structure of two antigens of the chicken A blood group system (A2 and A4) was determined by using a monoclonal antibody (ISU-ca) that recognized A4 antigen and several alloantisera specific for chicken A blood group antigens. Molecules immunoprecipitated from erythrocytes were separated on SDS-PAGE under either reducing or non-reducing conditions. Molecules of relative molecular weights 53.0 and 54.5 Kd were identified under reducing conditions for A2 and A4 antigens, respectively. Non-reduced molecules migrated at a faster rate to give a relative molecular weight of 44.5 Kd for both antigens. Two-dimensional electrophoresis showed similar patterns for each antigen of a diffuse band near pH 6.5. The data are consistent with a glycosylated molecule with one or more intrachain disulfide bonds. Allelic differences between A2 and A4 antigens appear to be due to an additional section on A4 antigen with a net neutral charge. Molecules of identical size and two-dimensional pattern were immunoprecipitated by ISU-ca and anti-A4 alloantiserum, confirming earlier reports of ISU-ca specificity for chicken A blood group antigens (Fulton et al. 1989a).
INTRODUCTION

The chicken A blood group system was one of the first blood groups identified in poultry (Briles et al. 1950). It is highly polymorphic with 9 alleles being identified (Briles et al. 1950). There is disagreement in the literature as to whether A antigens are found exclusively on erythrocytes (Schierman and Nordskog 1962; Davidenas 1970) or are also present on lymphocytes (Wong et al. 1972). Early studies found no influence of A blood group locus on graft rejection (Cock and Clough 1956; Crittenden et al. 1964). Differences in A allelic frequencies have been found between lines divergently selected for response to coccidiosis infection (Johnson and Edgar 1984) and sheep red blood cell immunization (Dunnington et al. 1984), suggesting an association with immune response.

There has been limited molecular characterization of chicken blood cell antigens. Antigens of the B blood group locus have been well characterized due to their association with the major histocompatibility complex (MHC). The molecular structure has been described for antigens from each of the three regions of the MHC; B-G (Kline et al. 1988b), B-F (Kline et al. 1988a) and B-L (Guillemot et al. 1986). Two developmental antigens, chicken fetal antigen (CFA) and chicken adult antigen (CAA) have also been extensively characterized (Kline et al. 1984; Kline et al. 1982).
Determination of the structure of the chicken A blood group locus may provide some clues as to its function. The purpose of this paper is to describe molecular characteristics of two of the antigens of the A blood group system, A2 and A4.
MATERIALS AND METHODS

Chicken Stocks

All birds used in this study were from the partially inbred (F = 0.5) Iowa State University SI White Leghorn chicken line (Nordskog and Cheng 1988). Birds were of either $\text{A}^2/\text{A}^2$ or $\text{A}^4/\text{A}^4$ genotype as determined by alloantisera or ISU-cA hemagglutination response.

Antibodies

Production of the monoclonal antibody, ISU-cA, has been previously described (Fulton et al. 1989a). Briefly, Balb/c mice were immunized with PBL and boosted with bursal cells from a G-Bl line chicken. Spleen cells from the immunized mouse were fused with Sp2/0 myeloma cells. ISU-cA is an IgM isotype. All assays described using ISU-cA utilized ascites fluid. ISU-cA agglutinates cells carrying the A4 antigen. It does not bind A2-carrying cells.

Chicken alloantisera were supplied by W.E. Briles (Northern Illinois University). The anti-A4 alloantiserum (A4-84-296) was specific for erythrocytes carrying A4 antigen, whereas the anti-A2 alloantiserum (A2-165-321) was specific for all A allelic products except A4 (Briles, Department of Biological Sciences, Northern Illinois University, personal communication). Alloantisera were used either unabsorbed, or were first absorbed three times with either $\text{A}^2/\text{A}^2$ or $\text{A}^4/\text{A}^4$ RBC to remove A-specific antibodies.
Blood Samples

Blood from birds over 20 weeks of age was collected either into phosphate buffered saline pH 7.2 (PBS) with 1% citrate or into heparin following venipuncture of the wing vein. Whole blood was centrifuged, and the buffy coat (containing the lymphocytes) was removed. The remaining red blood cells (RBC) were washed three times prior to use. All blood samples were obtained from birds over 20 weeks of age.

Radiolabelling and Cell Lysis

Iodination was done following the procedure of Vitetta et al. (1971). Briefly, 2 x 10⁸ RBC were enzymatically labelled with ¹²⁵I (Amersham, Arlington Heights, Illinois) using lactoperoxidase. Following iodination, RBC membranes were ruptured with saponin (10% saponin + 0.01% phenylmethylsulphonylfluoride (PMSF) in PBS) and washed to remove hemoglobin. RBC membrane ghosts were lysed with 1 ml solubilizing buffer (0.5% deoxycholate and 0.5% NP-40 in PBS) and incubated for 1 h with gentle shaking. Nuclei were removed by centrifugation. Radioactivity levels obtained were 2.5 - 15 x 10⁴ dpm/µl.

Immunoprecipitation

To a 400 - 500 µl volume of radiolabelled cell lysate (approximately 2 x 10⁷ dpm), 100 µl of alloantisera or 10 µl of undiluted ascites was added and incubated for 1.5 h with vigorous shaking. One hundred µl of either rabbit anti-
mouse IgM (ICN Biomedical Inc., Costa Mesa, California) or rabbit anti-chicken IgG (with cross reactivity to IgM) (Bethyl Laboratories, Montgomery, Texas) was added, shaken vigorously for an additional 1.5 h and then left overnight at 4°C. One hundred ul of a 10% solution of sepharose-protein A (Pharmacia, Piscataway, New Jersey) was added and shaken for 2 h. Samples were washed five times, then dissociated with either O’Farrell’s lysis buffer (O’Farrell, 1974) for two-dimensional electrophoresis or SDS-PAGE sample buffer (either reducing or non-reducing) for SDS-PAGE.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done using discontinuous gels (Laemmli, 1970), with a stacking gel of pH 6.8 and 4.75% acrylamide, and a resolving gel of pH 8.8 and 12% acrylamide. Molecular weight standards were obtained from BioRad (Richmond, California). Samples containing approximately $1.4 \times 10^3$ dpm were loaded for each lane.

Two-dimensional Electrophoresis

Two-dimensional gel electrophoresis was done following O’Farrell (1974). Ampholytes (Pharmacia, Piscataway, New Jersey) were used at 1.6% pH 5-7 and 0.4% pH 3.5-10. Immunoprecipitated samples containing approximately $3 \times 10^4$ dpm were loaded for each tube. Separation on the second dimension was as described previously for SDS-PAGE. The pH
gradient established during the iso-electric focusing run was determined by slicing a tube gel (with no sample) into 5 mm sections, placing each section into 2 ml of distilled water, shaking for 4 h, then measuring the pH.

**Autoradiography**

Films (XAR5, Kodak, Rochester, New York) were exposed at -70°C with intensifying screens for 1-4 days for SDS-PAGE gels and 7-18 days for two-dimensional gels.

**Hapten Inhibition**

Hapten inhibition of hemagglutination assay was done using 96 well round-bottom microtitre plates. Blood was obtained from an A^4/A^4 bird and a 2% solution of packed RBC in PBS was prepared. Sugars tested were the monosaccharides D+galactose, D+mannose, L-mannose, L-rhamnose, D-arabinose, L-arabinose, D+glucose, D-fructose, L-fucose, D+fucose, D+xylose, D-ribose, the oligosaccharides lactose, trehalose, maltose, sucrose, palatinose, D+cellobiose, lactulose, D+raffinose and D+melezitose and the sugar derivatives n-acetyl-D galactosamine, n-acetyl-D glucosamine and n-acetyl-D mannosamine.

Sugars were diluted in PBS such that their final concentration during incubation with antibody was 100mM, 50mM, 25mM, 10mM, 5mM, 1mM, 0.5mM and 0.1mM. ISU-cA ascites was diluted in PBS to give antibody concentrations during
incubation with sugars of 1/500, 1/1000, 1/2500, 1/5000, 1/10000, and 1/20000.

Twenty ul each of antibody and sugar solution were mixed then incubated at 4°C for 1 h. Direct and indirect hemagglutination assay procedures and scoring were done as previously described (Fulton et al. 1989b).
RESULTS

Figure 1 shows the results of SDS-PAGE gel electrophoresis of molecules immunoprecipitated by ISU-cA and alloantisera from A\(^2/A^2\) and A\(^4/A^4\) RBC lysates. No band was detected that was unique to A\(^2/A^2\) RBC lysate immunoprecipitated by ISU-cA (lane 1), as compared with the negative control (i.e., no precipitating antibody, lane 2). In contrast, a unique molecule, with a relative molecular weight of 53 Kd, was immunoprecipitated from A\(^2/A^2\) RBC lysates by A\(^2\)-specific antiserum that was either unabsorbed (lane 3) or absorbed by A\(^4/A^4\) RBC (lane 4). This band was not immunoprecipitated by A\(^2\)-specific antiserum that had been absorbed with A\(^2/A^2\) RBC (lane 5) nor was it detected in the negative control (lane 6). Comparison between A\(^4/A^4\) RBC lysates immunoprecipitated by ISU-cA (lane 7) and the negative control (lane 8) showed a unique band of relative molecular weight 54.5 Kd (lane 7). The same relative weight molecule was immunoprecipitated by A\(^4\)-specific antiserum that was either unabsorbed (lane 9) or absorbed with A\(^2/A^2\) RBC (lane 11). This band was not detected in either the negative control (lane 12) or in the sample that was immunoprecipitated by A\(^4\)-specific antiserum that had been absorbed with A\(^4/A^4\) RBC (lane 10).

The separation of non-reduced samples by SDS-PAGE showed a prominent band of relative molecular weight 44.5 Kd for
A²/A² lysates immunoprecipitated by A2-specific antiserum and A⁴/A⁴ lysates immunoprecipitated by ISU-cA and A4-specific antiserum (Figure 2).

Figure 3 shows the two-dimensional gels of immunoprecipitated A2 and A4 antigens. Figure 3a is the presumed A2 antigen immunoprecipitated by A2-specific antiserum. The presumed A4 antigen immunoprecipitated by either A4-specific antiserum or ISU-cA is shown in Figures 3b and 3c respectively. The pH of this region was approximately 6.5. The three patterns are very similar between A genotype and between immunoprecipitating antibodies used.

The hapten inhibition assay did not show any inhibitory effects of sugar pre-incubation with antibody on the ability of ISU-cA to agglutinate RBC for any of the sugars or sugar derivatives tested (data not shown).
DISCUSSION

Molecules immunoprecipitated from erythrocyte membranes by chicken A blood group specific antibodies were detected by using SDS-PAGE (reducing conditions). These molecules were not precipitated by either goat antimouse or goat antichicken antibody only, or by alloantisera from which the specific A antibodies had been absorbed. Anti-A4 alloantisera and ISU-cA precipitated the same size molecule, confirming an earlier report that the ISU-cA monoclonal antibody is against A4 antigens (Fulton et al. 1989a).

The lower molecular weight of 44.5 Kd for the non-reduced A antigens versus 53.0 Kd and 54.5 Kd for reduced A2 and A4 antigens, respectively, suggests that both A antigens have at least one intra-chain disulfide bond. The occurrence of lower molecular weights of non-reduced proteins versus their reduced counterparts is an identifying feature of molecules with intra-chain disulfide bonds (Allore and Barber 1984).

The molecules resolved by two-dimensional electrophoresis are very similar between the two antigens. The diffuse band detected is suggestive of a glycosylated protein, as non-glycosylated proteins generally appear more tightly focused (Ledbetter 1979; Ledbetter et al. 1979). The two-dimensional electrophoresis patterns are also very similar between molecules immunoprecipitated by ISU-cA and by anti-A4.
alloantisera, confirming the specificity of ISU-cA for chicken A blood group antigens.

The two-dimensional electrophoresis technique is ideal for analyzing very complex antigens. Various cell surface molecules including the chicken major histocompatibility complex (MHC) (associated with the B blood group, Schierman and Nordskog 1961), have been studied with this technique and can be relatively complex and extremely polymorphic (Kline et al. 1988a, 1988b; Miller et al. 1984; Miller et al. 1988). In contrast, although only two allelic forms were analyzed, the chicken A blood group appears to be relatively simple.

Carbohydrate moieties are frequently involved in blood group differences, with the best example being the human A-B-O system in which the A and B blood types are distinguished by the substitution of galactosamine in B blood type by N-acetyl-galactosamine in A blood type (Watkins 1966). The hapten inhibition assay was used to determine if carbohydrate moieties may be playing a role in chicken A blood group antigenic differences. This type of assay has been particularly useful in studying differences in erythrocyte development antigens such as chicken fetal antigen (CFA) (Dietert et al. 1981; Lewin and Dietert 1982; Lewin and Dietert 1983). The lack of inhibition of hemagglutination by haptens for ISU-cA binding to A blood group antigens is not surprising. Perhaps the wide range of carbohydrates and
their derivatives tested did not include the specific carbohydrate recognized by the antibody. Or, as the antigenic binding site of antibodies is generally considered to be the size of 5-6 sugars (Kabat 1960), those mono- or disaccharides tested may not have been of sufficient size to block the binding site. Also, the antigenic differences may have been due to protein rather than carbohydrate differences. Thus although this assay did not confirm the involvement of carbohydrate moieties in the antigenic structure, neither did it exclude them.

The SDS-PAGE and two-dimensional electrophoresis results are consistent with a molecular structure of chicken A blood group antigens consisting of a single polypeptide with at least one intra-chain disulfide bond. The molecules are probably glycosylated. The A2 and A4 antigenic forms are distinguished by the presence of an additional section on A4 antigens. This additional section may be due to additional amino acids, additional carbohydrate moieties, or both. In any case, this addition to the A antigen does not appear to affect the net charge of the molecule and must be of net neutral charge.
REFERENCES


Figure 1 SDS-PAGE (reduced) of lysates from $A^2/A^2$ (lanes 1-6) or $A^4/A^4$ RBC (lanes 7-12) immunoprecipitated by ISU-ca or alloantisera. Lysates were immunoprecipitated with the designated antibody then with either rabbit anti-mouse antibody (lanes 1, 2, 7 or 8) or rabbit anti-chicken antibody (lanes 3-6 and 9-12). Lane 1, ISU-ca; lane 2, no first antibody (i.e., negative control for lane 1); lane 3, A2-specific antiserum; lane 4, A2-specific antiserum absorbed with $A^4/A^4$ RBC; lane 5, A2-specific antiserum absorbed with $A^2/A^2$ RBC; lane 6, no first antibody (i.e., negative control for lanes 3, 4, and 5); lane 7, ISU-ca; lane 8, no first antibody (i.e., negative control for lane 7); lane 9, A4-specific antiserum; lane 10, A4-specific antiserum absorbed with $A^4/A^4$ RBC; lane 11, A4-specific antiserum absorbed with $A^2/A^2$ RBC; lane 12, no first antibody (i.e., negative control for lanes 9, 10, and 11).

Figure 2 SDS-PAGE (non-reduced) of $A^2/A^2$ RBC lysates immunoprecipitated by A2-specific antiserum (lane 1), and $A^4/A^4$ RBC immunoprecipitated by ISU-ca (lane 2) or A4-specific antiserum (lane 3).
Figure 3  Two-dimensional electrophoresis gel of $A^2/A^2$ RBC lysates immunoprecipitated by A2-specific antiserum (a) and $A^4/A^4$ RBC immunoprecipitated by A4-specific antiserum (b) or ISU-cA (c)
PAPER V.
PRODUCTION AND CHARACTERIZATION OF ISU-J1 MONOCLONAL ANTIBODY
ABSTRACT

A monoclonal antibody (ISU-J1) was produced using chicken erythrocytes and peripheral blood lymphocytes as the immunizing antigens. This monoclonal antibody was IgM isotype. Direct and indirect hemagglutination assays and enzyme-linked immunosorbent assay (ELISA) failed to detect binding of ISU-J1 to erythrocytes. The ISU-J1 binding to peripheral blood lymphocytes (PBL) was not detectable by immunofluorescence, either microscopically or by flow cytometry. ELISA detected binding of ISU-J1 to PBL, but only at high antibody concentrations. Immunoprecipitation of \( ^{125}\text{I} \)-labelled PBL failed to identify molecules that were bound by ISU-J1. The results from these experiments suggest that the ISU-J1 monoclonal antibody does not bind to molecules either on erythrocytes or PBL. At high antibody concentrations, ISU-J1 appears to bind non-specifically to lymphocytes.
INTRODUCTION

The development of monoclonal antibodies has enabled scientists to conduct detailed study of the development of the immune system and to recognize subsets of cells involved in various immune responses. Monoclonal antibodies have been produced against various chicken cell-surface markers and have enabled identification of cell subsets and studies of the ontogeny and function of the various hematopoietic cell types in the chicken. Monoclonal antibodies have been produced that are reactive with chicken T cells (Houssaint et al. 1985; Peault et al. 1982; Pink and Rijnbeek 1983; Chen et al. 1984; Kornfeld et al. 1983), B cells (Chen et al. 1984; Spencer and Benedict 1986; Kornfeld et al. 1983; Wolf et al. 1984; Pink and Rijnbeek 1983), leukocyte common antigens (Houssaint et al. 1987), erythrocytes (Sanders et al. 1982; Trembicki and Dietert 1985) and macrophages (Dietert et al. 1987). Particular emphasis has been placed on development of monoclonal antibodies specific for the B blood group antigens (identified by Briles et al. 1948) because the B system of haplotypes identifies the chicken major histocompatibility complex (MHC) (Schierman and Nordskog 1961). Monoclonal antibodies have been produced that are specific for antigens of each of the three classes of the MHC: B-G (Longenecker et al. 1979; Miller et al. 1982; Salomonsen et al. 1987), B-F
(Pink et al. 1985; Crone et al. 1985) and B-L (Crone et al. 1985; Guillemot et al. 1984; Ewert et al. 1984).

Monoclonal antibody production was initiated for development of reagents useful for determination of B haplotype in chickens. One reactive monoclonal antibody was produced. It is called ISU-J1 and is IgM isotype. The purpose of this paper is to briefly describe the production and characterization of the ISU-J1 monoclonal antibody.
MATERIALS AND METHODS

Blood Samples

All blood samples were obtained from adult birds of the partially inbred \( F = 0.5 \) Iowa State University S1 White Leghorn chicken line (Nordskog and Cheng 1988). Blood was collected from the wing vein into either phosphate buffered saline, pH 7.2 (PBS) with 1% citrate or into heparin. Whole blood was centrifuged, and the buffy coat (containing the lymphocytes) was removed. Peripheral blood lymphocytes (PBL) were isolated from the buffy coat by Ficoll-paque (Pharmacia, Piscataway, New Jersey). All cells were washed three times in PBS prior to use.

Monoclonal Antibody Production

Balb/c mice were immunized with a cell suspension containing lymphocytes and erythrocytes (RBC) from an S1-B\(^{19}\) bird. Three weeks later they were boosted with a similar cell preparation prepared from another S1-B\(^{19}\) bird. Fusion of spleen cells and Sp2/0 myeloma cells was done following a modified procedure of van Deusen and Whetstone (1981). Isotype, as determined by Hyclone (Logan, Utah) monoclonal antibody isotyping kit, is IgM.

Hemagglutination Assay

One drop (40 \( \mu l \)) of a 2% (v/v) solution of packed RBC was mixed with an equal volume of ISU-J1 tissue culture supernatant in wells of a 96-well round bottom plate. After
RT incubation for 2 h, plates were examined and scored for direct hemagglutination. Cells were resuspended and one drop of goat antimouse IgG, IgM, IgA (H and L) (Cooper Biomedical, WestChester, Pennsylvania) was added and allowed to incubate for an additional 2 h at RT. Wells were scored again (indirect hemagglutination).

**Immunofluorescence Assay**

The immunofluorescence assay was done as earlier described (Fulton et al. 1989). Briefly, PBL from mature birds were incubated first with ISU-J1 antibody (1/100 ascites in PBS), then with fluorescein isothiocyanate (FITC) conjugated goat antimouse IgG, IgM, IgA (1/20 dilution) (H and L) (Cooper Biomedical, WestChester, Pennsylvania). Fluorescence labelling was examined either visually with a fluorescence microscope or using an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, Florida) equipped with an argon ion laser (488 nm, 400 mW).

**Enzyme-linked Immunosorbent Assay (ELISA)**

ELISA assays were done using PBL fixed by 1% glutaraldehyde in 96-well microtiter plates. ISU-J1 was tested in duplicate at dilutions of 1/100, 1/1000, 1/10,000, 1/100,000 (ascites in PBS) with PBL from 6 birds. Controls were ascites from Sp2/0 myeloma and B78.18.2 (an irrelevant IgM monoclonal antibody against endotoxin from Treponema hyodysenteriae, donated by M. Wannemuehler, Iowa State
University) used at the same dilutions as the ISU-J1 antibody. ELISA scores were determined on an ELISA reader with a 405 nm filter, zeroed on the well with the lowest concentration of Sp2/0 ascites. ELISA values were then corrected by subtracting the irrelevant IgM antibody scores for the appropriate dilution. Another ELISA was done under the identical conditions described above except that RBC were obtained from the same six birds.

To determine the effects of glutaraldehyde fixation on ISU-J1 binding, the assay was modified slightly. PBL were obtained from five birds, washed three times in PBS and then each sample was divided into two aliquots. One sample of each pair was fixed for 30 min with 1% glutaraldehyde, then washed three times prior to use. Assays were done in 12 x 75 mm glass tubes. To measure the ELISA reaction at the end of the assay, a 100 ul volume of supernatant from each tube was transferred to a well of a 96-well plate and then scored.

Radiolabelling and Immunoprecipitation

Whole PBL were radiolabelled with $^{125}$I using lactoperoxidase (Vitetta et al. 1971). Cells were lysed in solubilizing buffer (0.5% Nonidet P40, 0.5% deoxycholate, 0.01% phenylmethylsulphonylfluoride). Immunoprecipitations were done using 10 ul ISU-J1 ascites per 500 ul radiolabelled lysate. After incubation with a second antibody (rabbit antimouse IgM, ICN Biomedicals Inc., Costa Mesa, California)
the immune complex was precipitated by sepharose/protein A (Pharmacia, Piscataway, New Jersey). Negative control samples did not receive the ISU-J1 antibody. Separation of immunoprecipitated molecules was done by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (disc SDS-PAGE) (Laemmli, 1970). Gels were exposed to X-ray film at -70°C with intensifying screens for 3-8 days.
RESULTS

Hemagglutination and Immunofluorescence

No agglutination of RBC by ISU-J1 was observed by either direct or indirect agglutination (data not shown). The ISU-J1 binding to PBL was not detectable by immunofluorescence either microscopically or by flow cytometry (data not shown).

ELISA

Figure 1 shows ISU-J1 binding to PBL as detected by ELISA. The data have been corrected by subtracting IgM irrelevant antibody values. At high antibody levels (1/100, 1/1000) binding of ISU-J1 to PBL is detectable (ELISA = .65 and .28 respectively). Further antibody dilutions to 1/10,000 and 1/100,000 results in no detection of ISU-J1 binding. The data are very consistent between the six birds tested.

Figure 2 shows ISU-J1 binding to RBC as detected by ELISA. These data have also been corrected for non-specific IgM antibody binding. A slight amount of ISU-J1 binding is detected at 1/100 and 1/1000 dilutions, but there is considerable variation between birds. Mean ELISA values are .12 and .06 for antibody dilutions of 1/100 and 1/1000 respectively. These low values, combined with the variation between individuals are considered to be negative. At greater dilutions, no ISU-J1 binding is detectable.
Figure 3 shows the effect of glutaraldehyde fixation of PBL on binding of ISU-J1 monoclonal antibody. There is variation between birds within each treatment, however there is no difference between the fresh versus fixed cells.

**Immunoprecipitations**

The results of immunoprecipitation of PBL lysates with ISU-J1 antibody are shown in Figure 4. Lanes 2 and 4 were lysates from two different birds immunoprecipitated with ISU-J1 and lanes 1 and 3 are their respective negative controls in which no immunoprecipitating antibody was used. There are no differences in banding pattern between each sample and its negative control.
DISCUSSION

The direct and indirect hemagglutination assays did not detect binding of ISU-J1 to RBC. ELISA using RBC showed some variation between birds in detection of ISU-J1 binding. This variation may be a reflection of the technique in which cells were not fixed to a surface and may have been lost during the various washes. However, the mean ELISA values for these six birds were not high enough to be considered positive. Thus neither the direct and indirect hemagglutination assays, nor ELISA showed recognition of RBC surface molecules by ISU-J1.

ISU-J1 binding to PEL was not detected by immunofluorescence, yet positive values were obtained with ELISA. One major difference between these two assays is that immunofluorescence is done with fresh cells, whereas ELISA utilizes glutaraldehyde fixed cells. The ELISA comparing fresh and fixed cells did not indicate any change in ISU-J1 binding to PBL due to glutaraldehyde fixation of cells.

Binding of ISU-J1 to PBL was detectable only at high concentrations of antibody. This may be attributable to non-specific binding. ELISA values were corrected for non-specific binding by subtraction of the isotype control values. However, neither ISU-J1 nor the isotype control were assayed for protein and/or antibody concentration. It is possible that these low positive values may reflect residual non-specific binding to PBL. This has been seen with another
IgM isotype monoclonal antibody, ISU-ca (Fulton, Department of Animal Science, Iowa State University, unpublished data).

The immunoprecipitation technique is highly sensitive for detection of specific molecules. The lack of isolation of a specific molecule from PBL by ISU-J1 suggests lack of specificity of the monoclonal antibody.

The ISU-J1 monoclonal antibody does not appear to bind to cell surface molecules on either RBC or PBL. The ELISA assay appears to detect non-specific binding of ISU-J1 monoclonal to PBL at high antibody concentrations. This is probably due to the IgM isotype molecules of ISU-ca binding non-specifically to Fc receptors on PBL.
REFERENCES


Figure 1  ELISA detection of ISU-J1 binding (at dilutions of 1/100, 1/1,000, 1/10,000 and 1/100,000) to PBL from 6 birds

Figure 2  ELISA detection of ISU-J1 binding (at dilutions of 1/100, 1/1,000, 1/10,000 and 1/100,000) to RBC from 6 birds
Figure 3 Effect of glutaraldehyde fixation of PBL from 5 birds on binding of ISU-J1 as detected by ELISA
FRESH PBL Treatment

FIXED
Figure 4 SDS-PAGE (reduced) of lysates from PBL, from 2 birds immunoprecipitated with ISU-J1 (lanes 2 and 4) and their respective negative controls (no immunoprecipitating antibody) (lanes 1 and 3)
DISCUSSION

The monoclonal antibody ISU-cA was initially used as a positive control during testing of other monoclonal antibodies. However, although ISU-cA binding to PBL as determined by ELISA was always positive, RBC from all birds were not agglutinated by ISU-cA. Breeding studies to determine genetic control of RBC agglutination by ISU-cA showed that response to ISU-cA was due to a single autosomal gene, with partial dominance. The possible involvement of blood group locus was considered and subsequently tested by W.E. Briles at Northern Illinois University (NIU).

Comparisons of RBC from representative samples from NIU and ISU flocks agglutinated by ISU-cA and by alloantisera suggested an association with the chicken A blood group antigens. Genetic segregation tests failed to detect any recombination between loci controlling response to the two types of antibodies suggesting identity of the two loci. Competitive inhibition of binding between ISU-cA and alloantisera and the immunoprecipitation of identical molecules by both antibodies confirmed the identity of those molecules bound by ISU-cA and the A blood group antigens.

The ontogeny and expression study confirmed the presence of A blood group antigens on early erythrocytes as reported by other researchers (Briles et al. 1948; Poschl and Hala 1973; Schjeide et al. 1978). It also showed a previously
undetected increase in antigen expression on erythrocytes during post-hatching maturity. Flow cytometric analyses clearly showed that the increase in response was due to a gradual increase in antigen expression on the surface of individual erythrocytes. Hemagglutination by alloantisera, as used in the earlier ontogeny studies, would not have detected this. The co-dominance of A blood group antigens previously reported (Briles et al. 1950b) was confirmed, with heterozygotes identified as having antigen levels intermediate between that of the two homozygous parents.

There are conflicting reports as to whether A blood group antigens are restricted to erythrocytes (Schierman and Nordskog 1962; Davidenas 1970) or are also found on lymphocytes (Wong et al. 1972). All of these previous studies were done with alloantisera, produced in each laboratory. Alloantisera are notorious for their cross-reactivity and lack of reproducibility between laboratories. The availability of a monoclonal antibody specific for A blood group antigens greatly decreased the problems of cross-reactivity and allowed for detailed studies of antigen expression. Initial studies with ISU-cA and ELISA suggested that A antigen was on PBL, yet immunofluorescence did not confirm this. The increased sensitivity of ELISA versus immunofluorescence results in a greater chance of false positives. Further study suggested that initial ELISA
detection of ISU-cA binding to PBL was indeed a false positive, possibly due to non-specific binding of the ISU-cA IgM molecule to Fc receptors. Immunoprecipitation by ISU-cA of radiolabelled PBL surface molecules failed to detect specific binding to PBL. This observation combined with the lack of detection by immunofluorescence and probable non-specific binding detected by ELISA suggested that A antigens were not on PBL. It is very difficult to prove that something does not exist, as negative evidence may indicate technical limitations and/or difficulties or lack of appropriate assay. All of the assays used to attempt detection of A antigen on PBL, including the sensitive immunoprecipitation of radiolabelled cell-surface molecules, were used with success for detection of A antigens on RBC, thus eliminating technical problems as the likely explanation. Thus this research confirms the original reports of A antigens being exclusively on erythrocytes. The observations of Wong et al. (1972) in which they reported A antigen expression on lymphocytes are not explainable based on the data presented here. Further investigations of their 'allofixation' technique, perhaps with additional negative controls, may have implicated non-specific binding of ISU-cA to PBL as was detected by ELISA.

Molecules of 53.0 Kd and 54.5 Kd (reduced) were immunoprecipitated from A\(^2\) and A\(^4\) RBC lysates, respectively, by
ISU-cA and A specific alloantisera. Since ISU-cA recognizes the A4 antigen, it is hypothesized that the epitope recognized by ISU-cA is within the additional 1.5 Kd region found on A4 antigen. Is this 1.5 Kd additional region also found on the other ISU-cA positive antigens (A3 and A8) and not on other ISU-cA negative antigens (A5)? Can a subdivision of A antigens be made based on the presence of this region? Could this also represent a functional subdivision of A antigens? Molecular characterization of other A blood group antigens, including those whose agglutination response to ISU-cA was not tested, needs to be done to answer these questions. Peptide mapping by limited proteolysis and chemical cleavage will also yield more information of the structure of the A antigens. The diffuse two-dimensional gel electrophoresis pattern obtained suggested glycosylation of A blood group antigens. Enzymatic degradation using various saccharidases may confirm the presence of carbohydrates and indicate their carbohydrate linkage. Further hemagglutination studies using additional sugars and longer oligosaccharides may also determine whether ISU-cA binds to carbohydrates.

The serendipitous discovery of a monoclonal antibody specific for chicken A blood group antigens has resulted in broadening of knowledge of the chicken A blood antigens. It has allowed detailed study of expression, ontogeny and
molecular structure of the antigens, that could have been very difficult with alloantisera. Most chicken blood typing is done within the White Leghorn breed and the reagents are prepared with White Leghorns birds. Blood typing reagents show extensive cross-reactivity when used for blood typing outside of the breed in which they were prepared, and are thus virtually useless for blood type comparisons across breeds. Since monoclonal antibodies are monospecific, antigen recognition is far more definitive, making them extremely useful as blood-typing reagents.

The function of the chicken A blood group system is still unknown. Its discovery, by two independent researchers, during the early chicken blood group investigations, suggests that it is highly immunogeneic. Intriguing hints implying a role in immune function have been uncovered since its initial discovery. Further studies involving function assays may reveal the purpose of the chicken A blood group antigens.

Some of the pitfalls encountered with monoclonal antibody production and characterization were clearly shown in this study. Though both ISU-cA and ISU-J1 monoclonal antibodies initially showed similar reactivities, i.e., they were both IgM isotype and showed binding to PBL by ELISA, subsequent assays showed considerable differences between the two in their specificities. The ISU-cA was found to be specific for A blood group antigens on RBC, and ISU-J1 was found to bind
non-specifically to PBL. This demonstrated the importance of using various techniques for characterization of monoclonal antibodies. Both positive and negative detection of monoclonal antibody binding may be due to limitations of either the antibody or the assay. It is imperative that screening of hybridomas be done using the assay in which the antibody will be utilized. It is also imperative to use more than one assay to determine the specificity of monoclonal antibodies. As the initial screening of ISU-J1 clearly showed, positive values with one assay do not necessarily indicate detection of specific binding. Confirmation of antibody specificity by more than one assay is needed to ensure specific reactivity.
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Common Laboratory Solutions

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<td>200 U/ml</td>
</tr>
<tr>
<td>PBS</td>
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<td>Heparin (sodium salt) from porcine intestinal mucosa</td>
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<tr>
<td>(Sigma, cat. no. H-3125)</td>
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<td>Solution B: Na$_2$HPO$_4$.H$_2$O</td>
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<td>Add distilled H$_2$O to 1000 ml</td>
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<tr>
<td>Solution B</td>
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<td>NaCl</td>
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<tr>
<td>Add distilled H$_2$O to 4000 ml.</td>
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<td><strong>Phosphate Buffered Citrate (Citrate)</strong></td>
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<tr>
<td>Solution A</td>
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### PBS/\(\text{NaN}_3\)

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<td>(\text{NaN}_3)</td>
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Caution: \(\text{NaN}_3\) is extremely toxic and must be handled with care.

### PBS/Tween

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<td>Tween 20</td>
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Tween 20 = polyoxyethylene sorbitan monolaurate

### PBS/Tween/\(\text{NaN}_3\)

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<tr>
<td>Tween 20</td>
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<td>(\text{NaN}_3)</td>
<td>1 g 1%</td>
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### PBS/Tween/BSA

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</tr>
<tr>
<td>BSA</td>
<td>10 g 1%</td>
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</tbody>
</table>

BSA = bovine serum albumin

Filter solution through .2 um to remove debris.
Blood Sample Collection

1. For young birds (0-4 weeks), use syringe with 0.3 ml citrate. Collect blood from jugular vein.

2. For older birds (5 weeks - adult), use scalpel blade, nick wing vein, collect blood into tubes containing 2 ml citrate.

3. For embryos, blood can be collected at all ages using heparin coated capillary tubes. Nick a major blood vessel near the heart of young embryos or from the external membranes of older embryos and collect blood with capillary tubes. Each sample is then put into 0.5 ml PBS and centrifuged. Appropriate dilutions of packed RBC for HA and IF assays can then be made.

Solutions

Citrate

PBS
Isolation of Lymphocytes From Chicken Peripheral Blood

1. Collect 5 ml blood using 5 ml syringe with 0.5 ml heparin.

2. Put sample into 13 x 100 mm glass tubes. Spin (130 x g, 10 min), remove lymphocytes on top of packed cells (buffy coat) with pasteur pipette and resuspend buffy coat in 5 ml PBS.

3. Carefully layer buffy coat over 3.75 ml Ficoll-paque in a 15 ml conical tube. Centrifuge at 325 x g for 20 min. Remove lymphocytes from the interface layer.

4. Step 3 may be repeated if contaminating erythrocytes are still present.

5. Wash cells three times in PBS to remove Ficoll-paque.

Solutions

Ficoll-paque (Pharmacia, cat. no. 11-A-246-02)

Heparin

PBS
Erythrocyte Absorption of Alloantisera

1. Collect 15 mls blood per bird in 0.1 ml heparin.

2. Wash cells in PBS by spinning at 130 x g for 10 min. Remove supernatant and resuspend cells in PBS, then spin again. Repeat this wash step twice for a total of 3 washes. Do not remove the lymphocytes at the top of the packed cells (buffy coat). Add volume of PBS equal to volume of packed cells. Resuspend cells.

3. Put 1.5 ml cell suspension in 12 x 75 mm glass tube. Spin (130 x g, 10 min) and remove supernatant, leaving 0.75 ml of packed cells.

4. Add 0.75 ml serum to be absorbed to packed cells and resuspend cells. If agglutination occurs, spin samples immediately and remove serum for next absorption, otherwise leave for 30 min at RT.

5. Spin, then transfer serum to another tube of packed cells. Resuspend cells and incubate for 30 min at RT.

6. Repeat step 5 to give a total of 3 absorptions.

7. Spin, remove and freeze supernatant in small aliquots (.25 ml).

Solutions

Heparin

PBS
Direct and Indirect Hemagglutination Assays

1. Spin blood, 130 x g for 10 min. Remove buffy coat.
2. Make 2% solutions (v/v), (20 ul packed RBC in 1 ml PBS).
3. Put one drop of sample (40 ul) per well of a 96 well round-bottom microtiter plate. Then add one drop of appropriate dilution of monoclonal antibody per well.
4. Shake plate on microtiter plate shaker for 1 min or resuspend cells by pipetting.
5. Incubate at RT for 1 hr, score 1st Direct score
6. Shake, incubate another hour at RT, score 2nd Direct score
7. Resuspend cells again by shaking or pipetting, add one drop (40 ul) of goat antimouse antibody (1/200) to each well, and resuspend.
8. Leave at RT for 1 hr, score wells 1st Indirect score
9. Resuspend cells, leave at RT for 1 hr, score 2nd Indirect score

10. Scoring System

   0 = compact button of non-agglutinated cells in the center of the well (negative)
   1 = button of cells with an indistinct edge
   2 = small button of cells in center of well, with an indistinct mat of agglutinated cells surrounding the button
3 = large indistinct mat of cells, slightly denser center of mat
4 = large indistinct mat of cells, all cells agglutinated, no non-agglutinated cells in the center

Solutions

Citrate

Goat antimouse IgG, IgM, IgA (H and L) (Cooper Biomedical; cat. no. 0111-0231)

ISU-ca monoclonal antibody

PBS
Immunofluorescence Assay

1. Spin blood (130 x g, 10 min). Remove and discard buffy coat for immunofluorescence of RBC, or isolate PBL as previously described for immunofluorescence of PBL.

2. Make 0.2% solutions (v/v). Put 20 µl packed RBC into 1 ml PBS (2% solution), then put 100 µl of this 2% solution into 0.9 ml PBS. This gives a cell count of approximately 1.5 x 10^7 cells per ml.

3. Put 20 µl of cell suspension into each well of 96-well round-bottom microtiter plate. Put 20 µl of 1/100 dilution of ISU-ca in each well. Resuspend cells.

4. Incubate on ice for 1 hr. Resuspend cells twice during this incubation by shaking briefly.

5. Remove unbound antibody by adding 200 µl cold PBS/BSA/NaN_3 to each well. Spin at 130 g for 10 min. Remove and discard supernatant.

6. Repeat step 5.


8. Repeat steps 4-6.

9. Add 200 µl cold PBS/NaN_3, spin and remove supernatant. (Caution: NaN_3 is extremely toxic and should be handled with care.)

10. Resuspend cells in 50 µl cold PBS/NaN_3.
11. Examine under fluorescence scope or with flow cytometer.

Solutions

**Fitc-labelled goat antimouse IgA, IgG, IgM** (Cooper Biomedical, cat. no. 1211-0231)

**ISU-cA**

**PBS**

**PBS/BSA/NaN₃**

**PBS/NaN₃**
ELISA Plate Preparation

1. Dilute peripheral blood lymphocytes to $1 \times 10^7$ cells/ml in PBS.
2. Add 50 ul of cell suspension to each well of 96-well flat bottom plate.
3. Gently tap plate to ensure even distribution of cells. Leave at RT for 1 hr so that cells can settle.
4. Gently add 50 ul of a 1% solution of glutaraldehyde solution to each well without disturbing the cell layer. Incubate 30 min at RT to fix cells to plate.
5. Remove glutaraldehyde by inverting and shaking plate over sink. Wash three times with PBS or PBS/Tween.
6. Fill wells with PBS/Tween/NaN$_3$. Cover plate with parafilm and store at 4°C. May be stored up to 6 months prior to use.

<table>
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<tr>
<td>PBS</td>
<td>495 ml</td>
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</table>

Use within 2 weeks of preparation.

PBS

PBS/Tween

PBS/Tween/NaN$_3$
Enzyme-linked Immunosorbent Assay (ELISA)

1. Using plate prepared following Appendix 7, remove PBS/Tween/NaNO₃ from wells by flicking plate over sink and then washing plates three times with PBS/Tween, then twice with PBS.

2. Add 50 µl of antibody (either tissue culture supernatant or 1/100 dilution of ascites) to each well and allow to incubate at RT for 2 hr.

3. Remove antibody by inverting plate and flicking plate, wash wells three times with PBS/Tween, then twice with PBS. To ensure all unbound antibody is removed from upper margins of wells, make sure wells are completely filled at least once during the washing.

4. Add 50 µl of 1/20 dilution of phosphatase-labelled goat antimouse IgG, IgM, IgA. Incubate 1 hr at RT.

5. Repeat step 3.

6. Add 50 µl freshly made substrate to each well. Incubate at RT for 30 min.

7. Add 100 µl NaOH stop solution to stop reaction.

8. Read plates on ELISA reader immediately, using 405 nm filter.

9. Appropriate negative controls are particularly important for ELISA to decrease false positive readings due to non-specific binding. Negative controls consist of either tissue culture medium from non-antibody secreting-cells
(for monoclonal antibody testing) or normal serum from a non-immunized individual (for alloantisera testing). An additional isotype control consisting of an irrelevant monoclonal antibody of the same isotype should also be tested when available. The ELISA reader should be zeroed on a negative control well before scoring test samples.

### Solutions

#### NaOH Stop Solution

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<thead>
<tr>
<th>NaOH</th>
<th>Amount</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>40 g</td>
<td>2M</td>
</tr>
</tbody>
</table>

Add distilled H₂O to 500 ml.

#### PBS

#### PBS/Tween

#### Phosphatase labelled goat anti mouse IgG, IgM, IgA (H and L)

(Kirkegaard and Perry, cat. no 15-18-07)

#### Substrate

6 ml 1M diethanolamine pH 9.3

5 mg tablet p-nitrophenyl phosphate disodium (Sigma, cat. no. 104-105)
Iodination of Chicken Blood cells

1. Dilute fresh RBC or PBL to 4-5 x 10^8 cells/ml (keep cells at RT).

2. Put 25 μl of lactoperoxidase solution into 13 x 100 mm glass tubes. Then add 0.5 ml cell solution to each tube, mix by swirling.

3. Load 1 ml syringe with H_2O_2 solution. Put cells and syringe in hood.

4. Tap source vial with Na^125_I to dislodge liquid from top. Put iodine trap on vial. Using a Hamilton syringe, add 40 μl PBS to source vial. Mix, withdraw 50 μl volume.

5. Add 12.5 μl of this solution to each tube of cells, swirl cells.

6. Add 50 μl H_2O_2 solution to each tube and swirl cells. Repeat this every 2.5 min to give a total of 4 additions of H_2O_2 during 7.5 min. Periodically swirl cell solution during iodination. Allow 10 minutes for total reaction time.

7. Stop reaction by adding approximately 7 ml cold PBS. Plug tubes with cork stoppers and spin (130 x g, 10 min).

8. Remove and discard supernatant in radioactive wastes bottle.

9. Resuspend cells in 7 ml cold PBS, transfer to new tubes and use new corks. Spin (130 x g, 10 min).
10. Repeat steps 8-9 three times, to give a total of 4 washes. After the last wash resuspend cells in 1 ml in PBS/PMSF. Can store for up to one week at 4°C before lysing cells.

11. Count 1 ul of cell suspension in gamma counter. Expect approximately $5 \times 10^4$ dpm/ul for whole radiolabelled cells.

### Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Amount</th>
<th>Final Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_2\text{O}_2$ Solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{H}_2\text{O}_2$</td>
<td>20 ul</td>
<td>0.06%</td>
</tr>
<tr>
<td>PBS</td>
<td>10 ml</td>
<td></td>
</tr>
</tbody>
</table>

$\text{H}_2\text{O}_2$ comes as 30% solution. Dilute immediately prior to iodination.

### Lactoperoxidase

- lactoperoxidase 1 mg
- distilled $\text{H}_2\text{O}$ 110 ul

Lactoperoxidase is from Sigma, cat. no. L8257. Prepare immediately prior to iodination.

### $\text{Na}^{125}$I

5 mCi $^{125}$I, 350-600 mCi/ml (Amersham, cat. no. IMS-300)

### PBS
**PBS/PMSF**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>100 ml</td>
</tr>
<tr>
<td>PMSF Stock</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

**PMSF Stock**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>10 ml</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

PMSF = phenylmethylsulfonylfluoride

Since PMSF is not soluble in $H_2O$ it must first be dissolved in methanol before using.
Preparation of Erythrocyte and Lymphocyte Lysates

1. Transfer cells (2-2.5 x 10^8 in 1 ml volume) to 50 ml centrifuge tube.

2. For erythrocytes, add 1 ml cold saponin solution.
   Resuspend cells well, incubate at RT for 10 min with gentle shaking. Wash membrane ghosts three times with 25 ml cold PBS to remove hemoglobin.

3. For lymphocytes or erythrocyte membrane ghosts, pellet the cells then add 1 ml solubilizing buffer. Resuspend cells. Incubate at RT for 1 hr with gentle shaking.


5. For radiolabelled cells, expect 7 - 35 x 10^4 dpm/ul.

Solutions

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Amount</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PMSF Stock Solution

Saponin Solution

<table>
<thead>
<tr>
<th>saponin</th>
<th>10 g</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF (use PMSF stock sol.)</td>
<td>0.5 ml</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Add PBS to 100 ml. Filter through 0.2 um. Store sterile at 4°C.
## Solubilizing Buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonidet P40</td>
<td>0.5 g</td>
<td>0.5%</td>
</tr>
<tr>
<td>deoxycholate</td>
<td>0.5 g</td>
<td>0.5%</td>
</tr>
<tr>
<td>PMSF (use PMSF stock sol.)</td>
<td>0.5 ml</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Add PBS to 100 ml. Store at 4°C.
Immunoprecipitation of Cell Surface Proteins

1. Use aliquot of 200-500 μl of radiolabelled cell lysate in each micro-centrifuge tube. Want approximately $3 \times 10^5$ dpm/sample.

2. Add 10 μl of monoclonal antibody (undiluted ascites) or 100 μl of alloantisera to each sample. Shake vigorously for 1.5 hr.

3. Add 100 μl of rabbit antimouse IgM or IgG (depending on monoclonal antibody isotype) or 100 μl of rabbit antichicken IgG or IgM (depending on isotype of alloantisera) (The rabbit antichicken IgG obtained from Bethyl Laboratories showed cross reactivity with chicken IgM). Shake vigorously for 1.5 hr, leave overnight at 4°C.

4. Add 100 μl of 10% solution of sepharose/protein A to each sample. Shake vigorously for 2 hr.

5. Spin in micro-centrifuge 2 min. and keep precipitate.

6. Wash precipitate 5x in solubilizing buffer. Last wash transfer to new micro-centrifuge tube.

7. Spin and discard supernatant.

8. For samples to be reduced and run on SDS-PAGE gels, add 50 μl of 2x SDS sample buffer with 10% 2-mercaptoethanol and resuspend sample. Incubate at RT for 30 min, then resuspend sample and boil 10 min. For non-reduced samples, add 50 μl of 2x SDS sample buffer without 2-
mercaptoethanol. For samples for IEF, add 50 μl IEF sample buffer and resuspend. Incubate at RT for 2 hr resuspending periodically. Do not boil IEF samples. For all samples, resuspend, then spin 5 min in microcentrifuge and remove and keep supernatant as sample.

9. Check activity of sample. Expect .7 - 7 x 10^3 dpm/μl.

Solutions

<table>
<thead>
<tr>
<th>Alloantisera</th>
</tr>
</thead>
</table>

IEF Sample Buffer

ISU-cA monoclonal antibody

Rabbit antichicken IgG (Bethyl Labs, cat. no. A30-107)

Rabbit antimouse IgM (ICN, cat. no. 64-365)

SDS Sample Buffer (2x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>tris base</td>
<td>1.51 g</td>
<td>0.125 M</td>
</tr>
<tr>
<td>glycerol</td>
<td>20.0 g</td>
<td>20%</td>
</tr>
<tr>
<td>SDS (use 10% stock sol.)</td>
<td>46 ml</td>
<td>4.6%</td>
</tr>
</tbody>
</table>

Add distilled H_2O to 75 ml volume. Adjust pH to 6.8. Add distilled H_2O to 100 ml. Add 10% 2-mercaptoethanol to small volumes just prior to use.
Sepharose/Protein A

- protein A/sepharose Cl-4B: .2 g
- sterile PBS: 2 ml

Filter PBS through .2 um filter before adding to sepharose/protein A. Keep in sterile container.

Protein A/sepharose Cl-4B is from Pharmacia, cat. no. 17-1081-01.

Solubilizing Buffer

- Nonidet P40: 0.5 g, 0.5%
- deoxycholate: 0.5 g, 0.5%
- PMSF (use 2% stock sol.): 0.5 ml, 0.01%

Add PBS to 100 ml. Store at 4°C.
Discontinuous SDS-polyacrylamide Gel Electrophoresis (Disc SDS-PAGE)

1. Clamp together one notched and one un-notched plate with gel spacers in between. Seal with 1% agarose.
2. Prepare acrylamide for resolving gel. Pour gel to 12 cm below notch. Overlay with resolving gel overlay and allow to polymerize for 1 hr. Remove overlay and any unpolymerized acrylamide then overlay again with resolving gel overlay. Cover with plastic wrap and leave at RT overnight.
3. Remove resolving gel overlay and rinse with distilled H₂O. Prepare acrylamide for stacking gel. Pour gel up to top of notch. For SDS-page gels, insert Teflon comb for well formation. For IEF tube gels do not use comb, but overlay with stacking gel overlay. Allow to polymerize for 1.5 hr, then remove comb (if used) and rinse with distilled water to remove any unpolymerized acrylamide.
4. For standard SDS-PAGE gels, load gel onto electrophoresis tank. Then load samples into wells (10 - 30 µl volume) and overlay with SDS running buffer.
5. For samples in tube gels, first place a thin layer of overlay agarose on top of acrylamide gel to raise the level of the gel. Carefully place the tube gel on top, ensuring no air bubbles are trapped. Leave 5 mm space at
one end for molecular weight markers (see step 7). Load prepared molecular weight markers. Seal tube gel and molecular weight markers on with another layer of overlay agarose. Then load gels onto electrophoresis tank.

6. Fill upper and lower electrophoresis tanks with SDS running buffer. Add 100 µl bromophenol blue solution to upper tank. Run at constant current until dye front reaches bottom of gel.

7. Preparation of molecular weight markers. Add 4 µl of molecular weight marker and 5 µl bromophenol blue solution to 20 µl 2x SDS sample buffer (reducing). Place in boiling water for 5 min. Add 2 drops hot 1% agarose solution, mix and immediately pipet onto a piece of parafilm to form two droplets (ensure that no air bubbles are trapped). Each droplet can be cut in half and one piece can be loaded per gel.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Amount</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide Stock for SDS gels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acrylamide</td>
<td>150 g</td>
<td>29.2%</td>
</tr>
<tr>
<td>bis-acrylamide</td>
<td>4.1 g</td>
<td>0.8%</td>
</tr>
</tbody>
</table>

Add distilled H₂O to 500 ml. Filter and store in foil covered bottle at 4°C.
Agarose

agarose 1 g 1%

Add distilled $H_2O$ to 100 ml. Heat to boiling to melt agarose.

APS

ammonium persulfate 1 g 10%
distilled $H_2O$ 1 ml

Prepare fresh daily.

Bromophenol Blue

bromophenol blue 0.01 g 0.1%
distilled $H_2O$ 10 ml

Solution is red but turns blue after addition to running buffer.

Overlay Agarose

tris base 1.5 g 0.025 M
glycine 7.2 g 0.192 M
SDS (use 10% stock sol.) 5 ml 0.1%
agarose 2.5 g 0.5%

Add distilled $H_2O$ to 500 ml. Heat to melt agarose.

Store in 50 ml volumes at $-20^\circ C$. 
### Resolving Gel Acrylamide (12%)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>resolving gel buffer</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>acrylamide stock</td>
<td>12.0 ml</td>
</tr>
<tr>
<td>distilled H$_2$O</td>
<td>10.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 µl</td>
</tr>
<tr>
<td>APS</td>
<td>99 µl</td>
</tr>
</tbody>
</table>

Mix the first three ingredients by swirling gently. Add TEMED and APS immediately prior to use. This volume is for one gel. TEMED is N,N,N',N'-tetramethylethylenediamine.

### Resolving Gel Buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tris base</td>
<td>181.7 g</td>
<td>1.5 M</td>
</tr>
<tr>
<td>SDS (use 10% stock sol.)</td>
<td>40 ml</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

Add distilled H$_2$O to 750 ml, adjust to pH 8.8 with concentrated HCl. Add H$_2$O to 1000 ml. Store at 4°C.

### Resolving Gel Overlay

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>resolving gel buffer</td>
<td>10 ml</td>
</tr>
<tr>
<td>distilled H$_2$O</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

Store at 4°C.

### SDS-Running Buffer (10x)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tris base</td>
<td>30.3 g</td>
<td>0.025 M</td>
</tr>
</tbody>
</table>
glycine 144.0 g 0.192 M
SDS (use 10% stock sol.) 100 ml 0.1%

Store at RT. Dilute to working concentration (1x) as needed. Solution molarities given are for working dilution.

SDS Sample Buffer (2x)

tris base 1.51 g 0.125 M
glycerol 20.0 g 20%
SDS (use 10% stock sol.) 46 ml 4.6%

Add distilled H₂O to 75 ml volume. Adjust pH to 6.8. Add distilled H₂O to 100 ml. Add 10% 2-mercaptoethanol to small volumes just prior to use.

Stacking Gel Acrylamide (4.75%)

stacking gel buffer 2.5 ml
acrylamide stock 1.5 ml
distilled H₂O 6.0 ml
TEMED 10 ul
APS 30 ul

Mix the first three ingredients by swirling gently. Add TEMED and APS immediately prior to use. This volume is for one gel.
Stacking Gel Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>tris base</td>
<td>30.3 g</td>
<td>0.5 M</td>
</tr>
<tr>
<td>SDS (use 10% stock sol.)</td>
<td>20 ml</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

Add distilled H₂O to 350 ml, adjust pH to 6.8 with concentrated HCl. Add H₂O to 500 ml. Store at 4°C.

Stacking Gel Overlay

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>stacking gel buffer</td>
<td>10 ml</td>
</tr>
<tr>
<td>distilled H₂O</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

Store at 4°C.
Iso-electric Focusing in Tube Gels

1. Season new tubes before using by polymerizing acrylamide in them as described below (step 3).

2. Make sure tubes are thoroughly cleaned by first soaking overnight in 50% nitric acid, then soaking for 2 hr in KOH saturated ethanol. Rinse thoroughly and dry before using.

3. Fill tubes (1.5 x 6 x 150 mm) to 2 cm from top with IEF acrylamide solution. Allow to polymerize for 1 hr. Overlay with distilled water, then remove overlay and unpolymerized acrylamide.

4. At all times when overlaying tube gels or loading samples, ensure that no air bubbles are trapped in tube. Overlay with 20 ul IEF sample buffer, then with water and leave 1-2 hr.

5. Load tubes into upper tank. Remove overlay and replace with 20 ul IEF sample buffer, then overlay with cathode solution. Fill lower tank with anode solution and upper tank with cathode solution. Ensure that tops of glass tubes are immersed in cathode solution.

6. Connect electrophoresis tank to power supply. Pre-run gels at 200 v constant voltage for 15 min, then 300 v for 30 min, then 400 v for 30 min.

7. Disconnect tank from power supply. Remove cathode solution. Remove overlay from tubes. Load samples (10-
20 µl volume) in IEF sample buffer. Overlay with 10 µl sample overlay solution, then fill the remaining space in the tube with cathode solution. Refill upper tank with cathode solution. Reconnect tank and run at 300 v constant voltage for 16 hours (4800 volt hours).

8. Increase voltage to 800 v for 1 hr. Turn off power and remove tubes.

9. Extrude gels from glass tubes by forcing water into the cathodic (top) end of the tube using Tygon tubing attached to a syringe.

10. Equilibrate each tube gel in 1 ml equilibration buffer for 30 min to 2 hr prior to either using on the second dimension (see SDS-PAGE instructions) or freezing for storage.

11. The pH gradient established can be approximated by slicing a non-equilibrated tube gel without sample into 5 mm sections and placing each section into 1 ml distilled H₂O. Equilibrate gel sections with gentle shaking for 4 - 6 hr, then measure pH.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Amount</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEF Acrylamide Stock</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ultrapure acrylamide</td>
<td>28.4 g</td>
<td>28.4%</td>
</tr>
<tr>
<td>ultrapure bis-acrylamide</td>
<td>1.6 g</td>
<td>1.6%</td>
</tr>
</tbody>
</table>
Add distilled water to 100 ml. Filter through 0.2 um and store in foil covered bottle at 4°C.

**IEF Acrylamide Gel Solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>ultrapure urea</td>
<td>5.5 g</td>
<td>9.2 M</td>
</tr>
<tr>
<td>ampholines pH 5 - 7</td>
<td>0.4 ml</td>
<td>1.6%</td>
</tr>
<tr>
<td>ampholines pH 3.5 - 10</td>
<td>0.1 ml</td>
<td>0.4%</td>
</tr>
<tr>
<td>acrylamide stock</td>
<td>1.33 ml</td>
<td>4%</td>
</tr>
<tr>
<td>distilled H₂O</td>
<td>2 ml</td>
<td></td>
</tr>
<tr>
<td>Nonidet P40 (use 10% stock sol., w/v)</td>
<td>2 ml</td>
<td></td>
</tr>
<tr>
<td>APS</td>
<td>10 ul</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>7 ul</td>
<td></td>
</tr>
</tbody>
</table>

Prepare immediately prior to use. Ampholines come as 40% solutions, so use the volumes given. Combine first six ingredients, warm under hot running water to dissolve urea. Do not heat. Add APS and TEMED, swirl gently and use immediately. This volume is for 12 tube gels of 1.5 x 150 mm.

**Anode Solution**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>phosphoric acid</td>
<td>5 ml</td>
<td>0.01 M</td>
</tr>
<tr>
<td>distilled H₂O</td>
<td>7300 ml</td>
<td></td>
</tr>
</tbody>
</table>

Phosphoric acid comes as 85% solution.
APS

ammonium persulfate 1 g 10%
distilled H₂O 1 ml
Prepare fresh daily.

Cathode Solution

NaOH 0.8 g 0.02 M
distilled H₂O 1000 ml
Boil water 10 min, to decrease dissolved gases. Add
NaOH dissolved in 5 ml H₂O. Boil for an additional
5 min. Cool, then degas before using. Make up
fresh for each run.

IEF Equilibration Buffer

tris base 3 g 0.125 M
SDS (use 10% stock sol.) 40 ml 2%
glycerol 20 ml 10%
2-mercaptoethanol 75 ul/15 ml 0.5%
Add distilled H₂O to 150 ml, check pH and adjust to
6.8 then add water to 200 ml. Filter through 0.2
um. Freeze in 15 ml volumes. Add 75 ul 2
mercaptoethanol to 15 ml just prior to using.

IEF Sample Buffer

ultrapure urea 5.7 g 9.5 M
Nonidet P40 (use 10% stock sol., w/v) 2 ml 2% (w/v)

ampholines pH 5 - 7 0.4 ml 1.6%
ampholines pH 3.5 - 10 0.1 ml 0.4%
2-mercaptoethanol 0.5 ml 5%

Add distilled H$_2$O to make 10 ml vol. Store in 1 ml volumes at -70°C. Ampholines are available from Pharmacia.

IEF Sample Overlay Solution

ultrapure urea 2.7 g 9 M
ampholines pH 5 - 7 0.1 ml 0.8%
ampholines pH 3.5 - 10 25 ul 0.2%

Add distilled H$_2$O to make 5 ml volume. Store in 0.5 ml volumes at -70°C.


Suppliers

Amersham
2636 S. Clearbrook Dr.
Arlington Hts, Illinois 60005
800-323-9750

Bethyl Laboratories Inc.
PO Box 850
Montgomery, Texas 77356
800-338-9579

Cooper Biomedical
1230 Wilson
WestChester, Pennsylvania 19380
800-523-7620

ICN Biomedicals Inc.
3300 Hyland Ave.
Costa Mesa, California 92626
714-545-0113

Kirkegaard and Perry
2 Cessna Ct.
Gaithersburg, Maryland 20879
800-658-3167

Pharmacia Inc.
800 Centennial Ave.
Piscataway, New Jersey 08854
800-526-3593

Sigma Chemical Co.
PO Box 14508
St. Loius, Missouri 63178
800-325-3010

This list of suppliers is included only as an aid to locate products. Inclusion of a manufacturer does not necessarily imply recommendation or exclusive source.
ACKNOWLEDGEMENTS

I wish to acknowledge the many people who have assisted with various aspects of the research; the manager, Bill Larson, and the staff of the Poultry Experimental Research Station who maintained the birds and assisted with sample collection, technicians; Danielle LeBlanc, Mike Kaiser and Betty Young, and fellow graduate students; Shen Cheng, Marlene Ellis and Ed Steadham for their help with developing techniques, collecting samples and interpreting results.

I especially wish to thank my major professor, Dr. Susan J. Lamont for her support and encouragement throughout my doctoral studies. I also wish to thank the other members of my committee, Dr. Don Reynolds, Dr. Max Rothschild, Dr. Jerry Sell and Dr. William Welshons for their comments and suggestions regarding the thesis.

I am indebted to Dr. W.E. Briles and R.W. Briles for blood typing birds and for supplying alloantisera. I particularly wish to thank Dr. W.E. Briles for his enthusiasm, suggestions and many enlightening comments throughout the various stages of the research and also for his reviews of early editions of manuscripts.

I also wish to acknowledge Vicki Hall, technician at the flow cytometry facility, for her excellent assistance in designing the flow cytometric experiments, running the samples and interpreting the results.
Finally, I wish to acknowledge my family, my parents, Stan and Betty Fulton, for their encouragement and interest throughout my graduate career, and my children, Cameron and Jocelyn, for their patience with and acceptance of Mummy going to school. I am deeply indebted to my husband, Jeff Daly, who despite having assisted with the task of typing tables and proofreading my master's thesis, was willing to repeat the experience with a more monumentous doctoral dissertation. To him I am deeply grateful.