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Antibody to Streptococcal Group A Carbohydrate and on Other
Genetic Factors Which Influence the Immune Response**

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STUDIES ON THE INHERITANCE OF IDIOTYPIC MARKERS
OF MOUSE ANTIBODY TO STREPTOCOCCAL GROUP A
CARBOHYDRATE AND ON OTHER GENETIC FACTORS WHICH
INFLUENCE THE IMMUNE RESPONSE

A thesis submitted to the Faculty of The
Rockefeller University in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

by
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PREFACE

The generation of specific antibodies to an almost unlimited number of different antigens is certainly one of the most interesting unanswered questions in genetics. Even though many theories have been developed to explain the genetic mechanism underlying antibody diversity, there are insufficient data to unequivocally defend any one theory to the exclusion of all others.

One type of data that is now lacking, and may be of aid in this respect, would be a knowledge of the inheritance of the amino acid sequences in antigen binding regions (or variable regions) of antibody molecules. It has not yet been feasible to study this type of inheritance by direct amino acid sequencing of antibodies. It is possible, however, to use an indirect approach to this problem by studying the inheritance of antigenic markers (idiotypes) associated with the binding site. Experiments using this approach are described in this thesis.

Idiotypic specificities are detected by antisera directed against variable region determinants of antibody molecules. Such antisera are specific for determinants on antibodies to generally only one antigen, and generally to only a small fraction of the total number of different antibodies that can bind that antigen. The portion of an antibody variable region sequence that is recognized by a particular anti-idiotypic serum is called an idiotypic determinant. Thus, the inheritance of the ability to make a particular variable region sequence can be studied by following the inheritance of the idiotypic determinants of that sequence. The offspring from parents with antibody of known idiotypic specificities (idiotypes) are immunized with the same antigens as were the parents. The antibodies produced by the offspring are then tested for the parental idiotypes.

The anti-idiotypic antisera for the experiments described in this thesis were made in rabbits against relatively homogeneous mouse anti-Group A streptococcal carbohydrate antibodies. The antisera were rendered idiotypically specific by subsequent absorptions with normal mouse immunoglobulin. These adsorbed anti-idiotypic sera have been shown to bind to the antigen binding site of mouse anti-Group A antibodies.

Since at the start of these studies little was known about the response of mice to Group A streptococcal vaccine, it was first necessary to perform dose response studies involving several mouse strains. In the course of this work, strains were identified which were high and low responders to Group A carbohydrate. Certain strains were better than others in the production of anti-Group A antibodies with restricted heterogeneity. Breeding experiments were performed to examine the genetic control of the magnitude of the antibody response to Group A carbohydrate.

In order to obtain larger amounts of a single homogeneous antibody than could be obtained from a single mouse, a procedure was developed in which the response could be propagated to syngeneic mice by injecting them with immune spleen cells of the original mouse, followed by immunization with Group A vaccine. Antibody obtained in this manner proved to be very useful in the developments of systems to study the idiotype of mouse anti-Group A antibodies.

The final experiments of this thesis demonstrate the inheritance in backcross mice of the genetic predisposition to make antibodies of a particular idiootype.

These studies were greatly facilitated by the type of graduate program that Drs. Bronk and Brink established at The Rockefeller University. I am very grateful to them for allowing me to take part in this program. I am equally grateful to President Seitz and to Dean James A. Hirsch for their roles in continuing this unique graduate program. During my studies Associate Dean Connelly has been quite helpful with many of the procedural aspects of graduate research.

In my six years at this University, I have had occasion to make use of the advice and assistance of a very large number of individuals from many different laboratories.

Dr. John Nelson was always cheerfully willing to advise me on any problems relating to the health of the mouse colony used in these studies. The irradiation experiments were greatly expedited by the cooperation of Dr. Robert Franzl, who instructed me in the use of the University's cobalt source. Dr. Merrill Chase was of immeasurable help on many occasions

by providing me with advice on performing various techniques and helping me locate particular references. The idiotype experiments described in this thesis profited greatly from discussions with Dr. Henry Kunkel.

I was originally introduced to the concepts of modern immunogenetics by Dr. Curtis Williams, under whose direction I conducted several projects which introduced me to much of the immunochemistry utilized in this study. When I began my thesis project under Dr. Krause's supervision, Dr. Tatum and Dr. Williams very kindly allowed me to remain physically located in their laboratory. I would like to thank all of the members of Dr. Edward Tatum's laboratory for the advice and assistance they have given me, and for sharing their work space with me for the past several years. Of the members of Dr. Williams' laboratory, I would like to especially thank George Kuzmycz, who has taught me many immunological techniques and who was always willing to help me locate necessary equipment.

Even though these studies were performed to a large extent in Dr. Tatum's laboratory, it was the members of Drs. McCarty's, Krause's, and Lancefield's laboratories who provided me with the advice and constructive criticism which made it possible for me to do idiotypic studies with the streptococcal antibody system. Dr. John Zabriskie taught me the necessary procedures for handling lymphocytes; Dr. Emil Gotschlich provided advice concerning the preparation and utilization of radioactive antigens in immune assays; and Dr. Lancefield generously supplied me with cultures of Groups A, B, and C streptococci and with whatever advice I needed to make use of them. Dr. McCarty provided advice concerning the isolation of Group A carbohydrate and the use of various haptens appropriate to the Group A system.

The past and present members of Dr. Krause's laboratory have been very helpful with their suggestions and criticism during this thesis project. In particular, I would like to thank Dr. Viktor Bokisch, Dr. Thomas Kindt, Dr. David Klapper, Dr. Dietmar Braun, and Dr. Klaus Eichmann. The technical staff of the laboratory was also quite helpful in providing advice and assistance when needed. In particular, I would like to thank Mr. Henry Lackland, Ms. Shelly Seid, Mr. David Bernstein, and Mr. Jay Greenblatt.

My greatest debt of gratitude, however, goes to my advisor, Dr. Richard M. Krause, who allowed me the freedom to pursue experiments with inbred mice in a laboratory which was heavily committed to genetics studies in a well-defined rabbit population. Dr. Krause has been a continual source of advice and encouragement throughout these studies; they would have been very difficult, if not impossible, without him.

My wife, Eve, deserves a great deal of thanks for her patience and encouragement during this work. I must also acknowledge her assistance in proofreading, typing, and editing various drafts of my thesis.

Ms. Ryland Stacey must be acknowledged for her cheerful typing of several rough drafts of this thesis, as well as several abstracts and a paper.

Finally, I must acknowledge Mr. Henry Bedard for his expert typing of the final copy of this thesis.

ABSTRACT

The antibody response to Group A streptococcal carbohydrate was measured in 10 inbred mouse strains after immunization with whole heat-killed streptococcal vaccine. Using A/J and SWR/J mice, dose response studies were performed to determine optimal immunization conditions of dose, route of injection, and age of mice. In the course of these studies, inbred strains were identified which produced high and low levels of anti-Group A antibody after immunization with Group A vaccine. Subsequent genetic experiments demonstrated that the differences in antibody titer observed among some of these strains were under the genetic control of genes at several loci.

When A/J and SWR/J mice are optimally immunized, they often produce high titers of anti-Group A antibody (antibody concentrations ranging from 5 to 15 mg/ml in SWR/J mice and from 5 to 60 mg/ml in A/J mice) with restricted heterogeneity. The restriction of heterogeneity of the anti-Group A antibody in these sera has been documented by micro-zone electrophoresis of isolated antibodies and by disc gel electrophoresis of reduced and alkylated anti-Group A antibody.

Mice with this type of monodisperse anti-Group A antibody have been used as donors for spleen cell transfers (10^7 spleen cells per recipient) to irradiated (500 R) recipient syngeneic mice. The immunized recipient mice produced anti-Group A antibody identical to that of the donor mouse. Such cell transfer lines have been propagated for as many as three passages.

Antibodies isolated from the original donor, or from one of the recipients of such spleen cell transfer lines, have been used to prepare anti-idiotypic sera in rabbits. Idiotype was detected by passive hemagglutination inhibition and by the radio-immune tube binding assay. One of the idiotypes studies was found to be present in SWR/J anti-Group A antibody and generally absent from anti-Group A antibody produced by A/J mice. It has been possible to demonstrate the inheritance of this idiotypic marker in hybrid A/J x SWR/J and in A/J x (A/J x SWR/J) backcross mice. The pattern of inheritance that was observed is consistent with a small number of loci, possibly only one, controlling the inheritance of idiotypic determinants.

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INTRODUCTION

In the introduction various types of genetic control of the immune response have been reviewed. Included is a review of the inheritance of quantitative aspects of the immune response, since a few of the experiments described in this thesis are concerned with the genetics of high versus low antibody response to the streptococcal Group A carbohydrate. The review of the quantitative aspects of the immune response is followed by a description of the genes which control the synthesis of specific domains of the light and heavy chains of immunoglobulins. Included in this section is a description of immunoglobulin allotypes, since the genes responsible for the allotypes are thought to be synonymous with those coding for antibody constant region structure. Finally, the inheritance of idiotypic markers are described because this topic represents a major portion of this thesis and because the genes responsible for the inheritance of idiotypy either affect the expression of particular antibody variable regions or are the structural genes which code for the variable region amino acid sequences.

Early Studies on Quantitative Aspects of the Immune Response

Perhaps the first experiment suggesting genetic control of antibody production was performed by Haduck and McMaster (1934), who showed that two inbred lines of mice differed in the amount of antibody they produced to B. enteridis. Gorer and Schütza (1938), using 4 inbred mouse strains, also observed definite strain differences in antibody production. They studied anti-H and anti-O antibodies to S. typhimurium and found that among the strains tested, the amount of anti-H but not the amount of anti-O was correlated to resistance to infection with S. typhimurium.

Subsequently many other comparative studies have been done with mice. Fink and Quinn (1953) compared the response of 6 strains of mice to alum-precipitated ovalbumin. They found that the ordering of the strains from highest to lowest antibody producers was different if an intramuscular rather than intraperitoneal route of immunization was used. This suggested that different genes controlled the antibody responses via the different routes of immunization. Using foreign red blood cells,

Davidsohn and Stern (1954) and Dineen (1964) immunized 10 and 9 different strains of mice respectively. As expected, some strains made much more anti-red cell antibody than others.

Ipsen (1959) did a very thorough investigation of the anti-tetanus toxoid response of 4 inbred lines of mice. Using various injection schedules, he studied the responses over a 100 fold range of antigen concentration. In all cases the highest responding strain was Balb/cd followed in order by A/J, DBA/2 and A/Heston.

In all of these studies described above, and in studies using synthetic polypeptides as antigens to be described later, the differences in response among the various inbred strains indicated the existence of genetic differences in the ability to make specific antibodies.

It is interesting to note that in all of the above studies there was generally considerable variation in the antibody response within each of the inbred lines tested. It is likely that this variation is due to non-genetic factors, because highly inbred lines of mice should be homozygous at virtually all of their loci. Gasser (1970) tested this supposition by mating high and low responder mice, who, as the result of inbreeding, should have had identical genotypes. He observed that the average antibody titer of the offspring produced from high x high matings was identical to that from low x low matings.

This non-genetic variation serves to complicate any studies of the inheritance of high versus low antibody responses. The genetic differences seen in the comparative studies above could be established only because the variation between inbred lines was greater than the variation within inbred lines (see for example Dineen, 1964; Fink and Quinn, 1953).

Further evidence that quantitative aspects of the immune response are under genetic control is provided by numerous attempts to selectively breed outbred animals to establish high and low immune responder lines. Chase, in 1941, showed that random-bred guinea pigs could be bred to develop strong or weak skin sensitivity to 2,4-dinitrochlorobenzene. Scheibel (1943), also using random guinea pigs, showed that they could be bred for the production of high and low anti-diphtheria toxin responses.

Biozzi et al. (1968) have succeeded in selectively breeding, from random-bred mice, a line of mice which produce high titers of anti-sheep red cell antibodies and another line that produces low titer anti-sheep red cell antibodies. More recently, Eichmann et al. (1971) have demonstrated that outbred rabbits can be bred for high and low antibody responses to Group C streptococcal vaccine.

Table I shows the results of one of our own studies (Briles and Williams, 1968) using random bred mice immunized with bovine γ -globulin, in which it was possible to demonstrate a correlation between the antibody titers of young mice and those of their parents. The mice were bred and weaned prior to immunization of the parents so that circulating antibody of the mother could not affect the response in the offspring.

It is apparently possible to breed animals for the ability to develop immunological tolerance. A line of mice bred by Sobey et al. (1966) to be unresponsive to bovine serum albumin was subsequently shown by Hardy and Rowley (1968) to make anti-BSA when a low antigen dose was used. This indicated that Sobey had really been selecting for tolerance rather than unresponsiveness.

Another experiment demonstrating the possibility of breeding animals for the ability to be made tolerant was performed by Thorbecke and Benacerraf (1967). They used bovine serum albumin to produce tolerance in adult rabbits.

In other experiments, it has been possible to show that different strains of mice have different predispositions to develop tolerance to heterologous γ -globulins (Staples and Talal, 1969). Similar findings, depicted in Table II, were obtained by Briles and Williams (1968) using human serum albumin as an antigen. In this study, smaller tolerizing doses of human serum albumin were required to produce tolerance in BRVR and BSVS mice than in NCS mice.

The findings of these experiments on the inheritance of the ability to induce tolerance suggest that many of the "low titer" strains described above, might really be simply strains that are easily made tolerant. However, this certainly does not hold for all of the studies on

TABLE I

Correlation between Anti-Bovine γ -Globulin Titer of
Outbred NCS Parental Mice and the Fraction of
Offspring Making Anti-BGG*

| Mother's titer | Father's titer | | Totals |
|----------------|----------------|-----------------|-----------------|
| | < .1 | \geq .1 | |
| < .1 | $\frac{0}{19}$ | | $\frac{0}{19}$ |
| \geq .1 | $\frac{6}{43}$ | $\frac{10}{27}$ | $\frac{16}{70}$ |
| totals | $\frac{6}{62}$ | $\frac{10}{27}$ | $\frac{16}{89}$ |

* Ten litters, all born on the same day, and their parents were obtained from the Rockefeller Animal House. When the young mice were 33 days old they and their parents each were injected i.p. with 0.85 mg of bovine γ -globulin (BGG) in 0.1 ml Freund's complete adjuvant. The mice were all bled at 32 days post injection. Antibody titers were determined by performing double diffusion in agar between various concentrations of bovine γ -globulin and each immune serum. The titer is expressed as the concentration of BGG required to give a line of equivalence. The heterogeneity Chi-square for the fraction of positive mice in the three parental classes is 11.0, d.f. = 2, $P = 0.004$. In the comparison between the two paternal classes ($\frac{6}{62}$ vs $\frac{10}{27}$), $X^2 = 5.2$, d.f. = 1, $P = 0.02$. In the comparison between the two maternal classes ($\frac{0}{19}$ vs $\frac{16}{70}$), $X^2 = 9.4$, d.f. = 1, $P = 0.002$.

TABLE II

Ability of NCS, BRVR, and BSVS Mice to be
Made Tolerant to Human Serum Albumin

| Dose* | Fraction of Mice Making Anti-HSA Antibody | | |
|-------|--|------|------|
| | NCS | BRVR | BSVS |
| None | 5/5 | 5/6 | 3/4 |
| .1 mg | 4/4 | 2/5 | 3/4 |
| 1 mg | 3/5 | 0/4 | 0/3 |
| 10 mg | 3/5 | 0/5 | 0/7 |

* Toleragenic dose of HSA in PBS

Twenty-two days after tolerance induction the mice were each challenged with 1 mg/ml in 0.1 ml Freund's complete adjuvant. At 21 days post challenge, the mice were bled and the presence or absence of antibody was determined by double diffusion of the immune serum vs. 0.1, 0.3, and 0.9 mg/ml HSA. Tolerance was induced in the mice at 30 days of age by the injection of soluble HSA in PBS. Particulate material was removed by centrifugation of 48,000 g for 1 hour. The supernates were injected immediately after centrifugation.

the ability to make high and low titer antibody responses. For example, in Ipsen's experiments, decreasing the amount of antigen used for immunization only succeeded in decreasing the amount of antibody produced by the low as well as the high responding strains.

Number of Genes Controlling Antibody Titers

In the studies described above involving a large number of inbred lines such as those of Davidsohn and Stern (1954) and Dineen (1964), the average responses of the inbred lines could not be readily classified into discreet categories but formed a continuum from high to low response. Such findings are generally indicative of multigenic control, often called multigenic inheritance (Srb et al., 1965).

Two examples of multigenic control of differences in antibody response come from the work of Sobey et al. (1966) and Playfair (1968). Sobey et al. (1966) used breeding studies to show that the ability of rabbits and mice to make anti-BSA antibodies was dependent on genes at not less than 2 or 3 loci. One of the most clear-cut examples of multigenic control of an immune response comes from the studies of Playfair (1968). Sheep cells were injected into 7-day-old NZB and Balb/cJ mice. At 5 days post-injection, the spleen of each mouse was removed and the total number of anti-sheep red blood cell antibody producing cells in each spleen was determined by a plaque assay. The average plaque forming cell (PFC) count of the NZB mice was about 4 logs higher than that for the Balb/cJ mice. The average PFC count for the F₁ hybrid mice was intermediate between the NZB and Balb/cJ values, indicating additive inheritance rather than a simple dominance relationship. One hundred mice of each of the possible two backcrosses were tested. The backcross to the NZB parent yielded a distribution of PFC values intermediate between those of the F₁ and NZB mice. This result indicated that the difference in phenotype between the F₁ and F₁ x NZB animals was controlled by additive multigenic inheritance. The backcross to the Balb/cJ parent, on the other hand, yielded a distribution of values indistinguishable from those produced by the F₁ mice. Based on this result, Playfair (1968) concluded that an NZB gene from any one

of at least 3 loci yielded the F₁ phenotype when placed on the Balb/cJ genetic background.

Further evidence for multigenic control comes from additional experiments of Playfair (1968). The highest-titer F₁ x Balb/cJ mice were then backcrossed themselves to Balb/cJ mice, to produce a second-backcross generation. The PFC counts from these second-backcross mice were indistinguishable from those of the Balb/cJ mice. This finding indicated that more than one NZB gene is required on a Balb/cJ background to produce the F₁ genotype. This raises the total number of loci involved in the phenotypic difference between the Balb/cJ and the F₁ mice to at least 4 or 5. So, from the backcrosses to each parental strain evidence for multigenic control was observed.

Single Gene Effects in the Immune Response

As indicated by the experiments described above, the immune response is generally thought to be under the genetic control of many different loci (Benacerraf and McDevitt, 1972). To study individual genes controlling a multigenic response, it is necessary to do so under conditions where all of the loci affecting the response are homozygous except for the one locus whose genes are under investigation. In the case of the immune response, it is necessary to use parents who are homozygous for the same alleles at all of the loci controlling the immune response being studied except one. In this way, the phenotypic effects of genes at that one locus can be studied.

It is also important to use the optimum immunization conditions for the particular genes under investigation since, as has been shown by McDevitt and Sela (1965) and by Fink and Quinn (1953), the relative amount of antibody produced by animals of different genotypes is dependent on the procedure of immunization.

A number of genetic studies have been reported in which single gene effects on antibody responses have been studied. In many instances, numerous different test crosses between inbred strains were needed to identify a combination of strain, antigen, and dose that would allow a single gene effect to be observed. Another approach is also possible.

An antibody response in an outbred population which can always be classified into one or two very distinct categories, with the absence of intermediate responses, is indicative of possible single gene control. This type of approach was used by Kleczkowska and Kleczkowski (1939). They were able to show in rabbits that the ability to make a strong response to whole human serum was controlled by a dominant gene even though the magnitude of the "strong" response varied somewhat among the high titer animals. Their paper was probably the first to report a study of the inheritance of the ability to make a specific immune response, and should stand as a landmark in this field not only for its originality but for its careful design and logical genetic analysis.

Work of Amsbaugh et al. (1972) has demonstrated that the mouse IgM response to type III pneumococcal polysaccharide in mice is controlled in an all-or-none fashion by an X-linked gene. In this case, the magnitude of the antibody response in mice bearing the X-linked gene appears to be further regulated by autosomal genes.

Some of the best studies of a single gene controlling an antibody response have been performed using synthetic polypeptides as antigens (McDevitt and Benacerraf, 1969; Penschunk and Maurer, 1968). Most of the previous studies on the genetic control of antibody responses had been done with complex antigens such as bovine serum albumin or heterologous erythrocytes (Levine et al., 1963; McDevitt and Sela, 1965). It was felt by some (Levine et al., 1963; McDevitt and Sela, 1965) that the magnitude of an antibody response was more likely to be controlled by a single locus if the antigen contained a minimal number of different antigenic determinants. These workers, therefore, used synthetic polypeptide of polypeptide-hapten conjugates as antigens.

Immune response genes of guinea pigs The first study using this type of antigen was done by Kanter, Ojeda, and Benacerraf (1963). Random-bred Hartley strain guinea pigs were immunized with 2,4-dinitrophenylpoly-L-lysine (DNP-PLL) in complete Freund's adjuvant. Approximately 40% of the guinea pigs produced an immune response, as characterized by the development of delayed hypersensitivity to DNP-PLL and synthesis of 1-2 mg/ml of anti-DNP-PLL antibodies.

and affinity. Homogeneous characteristics would be expected if the immune response genes were antibody structural genes (McDevitt and Benacerraf, 1969). The structure of the variable region on the anti-DNP antibodies is, in theory, thought to be mediated by the antibody-producing B cells (Benacerraf and McDevitt, 1972).

Several other guinea pig immune response genes have been described which are linked to the major histocompatibility locus of the guinea pig. In the humoral response, these genes have carrier specificities, as in the case of the PLL gene. The genes also control the ability of the animals to make a cellular response to the substance employed as a carrier for the hapten (Benacerraf and McDevitt, 1972; Green and Benacerraf, 1971).

One of these immune response genes governs the ability of guinea pigs to respond to haptens bound to guinea pig albumin (GPA). This gene is found in strain 13 guinea pigs and is absent in strain 2 guinea pigs. Two cellular studies have been done involving the gene, to confirm the supposition by Benacerraf and McDevitt (1972) that the immune response gene effects are mediated by the T cells.

The first of these two studies, by Davie and Paul (1970), utilized lymph node cells from GPA responder guinea pigs and absorbents made by coupling DNP-GPA and several other DNP conjugates to agarose. They were able to show that the cells responsible for cellular immunity (T cells) could be absorbed only by DNP-GPA coupled to agarose and not by DNP conjugated to any other carriers. The B cells, responsible for antibody production, could however be absorbed by any of the DNP conjugates. This experiment thus confirms the theoretical supposition that the T cells show carrier specificity whereas the B cells do not.

The second of the two studies (Davie et al., 1971) is a demonstration that the strain 2 non-responder guinea pigs have the same number of DNP-GPA binding cells as the strain 13 responder guinea pigs. Since the antigen-binding cells are presumed to be the precursors of antibody-forming cells (Davie et al., 1971), this result implies that the GPA gene is not expressed in the antibody producing or B cell population, a finding in agreement with the theory of Benacerraf and McDevitt (1972).

Immune response genes of mice Studies on the immune response genes in mice, using branched-chain polypeptides as antigens, have yielded results strikingly similar to those obtained in guinea pigs (McDevitt and Sela, 1965; McDevitt and Benacerraf, 1969). A typical branched-chain polypeptide used in these experiments, which is abbreviated (T,G) A--L, is composed of a poly-L-lysine backbone with poly-D,L-alanine side chains. The side chains are terminated in a short copolymer of tyrosine and glutamic acid. Various other branched polypeptides are made by substituting different amino acids for the poly-D,L side chains or for the terminal polytyrosine-glutamic acid residues.

Using these polypeptides as antigens, it has been possible to demonstrate that in many, but not all cases, a single gene controls the ability to produce a humoral antibody response to a particular polypeptide (Moses et al., 1969; Benacerraf and McDevitt, 1972). The genes for most of these single gene effects are very closely linked to the major histocompatibility locus (H-2) of the mouse (McDevitt and Chinitz, 1969; Benacerraf and McDevitt, 1972), a finding reminiscent of the guinea pig immune response genes discussed above. In fact, some of the mouse immune response genes have been mapped within the H-2 locus itself (McDevitt et al., 1972). This group of H-2 linked genes are called the Ir-1 genes.

For one of these Ir-1 genes, Grumet (1972) has shown that its influence on antibody production involves the switch from IgM to IgG production. Both the high and low responder lines made comparable amounts of IgM antibody, but only the high responding line was able to switch to IgG production. Mitchell et al. (1972) has further shown that if responder mice are thymectomized, they produce only IgM antibody, similar to the non-responder mice. These two experiments taken together indicate that the Ir-1 genes probably act through the T cells as do the guinea pig immune response genes.

Evidence that the B cell is probably not affected by the Ir-1 genes comes from Dunham et al. (1972). The same number of antigen binding B cells were observed in responder and non-responder mice. All of these above observations suggest that the Ir-1 locus of the mouse may be analogous to the histocompatibility-linked immune response genes in the guinea pig (Benacerraf and McDevitt, 1972).

Other histocompatibility-linked immune response genes have been described that control immune responses in the guinea pig to low doses of complex antigens such as bovine serum albumin (Green and Benacerraf, 1971) and in the mouse to ovomucoid, bovine γ -globulin and ovalbumin (Vas and Levine, 1970; Vas et al., 1970; Vas et al., 1971). The occurrence of these single genes regulating the immune response to complex antigens fits in well with the suggestion of Benacerraf and McDevitt (1972) that the T cells are able to recognize "carrier" units that are considerably larger than individual haptens.

In the mouse, three immune response genes have been described which are not linked to the H-2 locus, although, interestingly enough, two of them appear to be linked to the H-3 or H-6 histocompatibility locus (Snell et al., 1967; Gasser, 1970; Benacerraf and McDevitt, 1972).

This frequent association of immune response genes with histocompatibility loci suggests that the immune response gene effects are simply manifestations of self-tolerance to histocompatibility antigens. This possibility is ruled out, however, by the fact that immune response genes are dominant in F₁ hybrids and that there does not appear to be any cross-reaction between H-2 antisera and the appropriate mouse anti-polypeptide antisera (McDevitt and Benacerraf, 1969).

Control of antibody specificity by immune response genes In two instances, one in the mouse (Moses et al., 1969) and one in the guinea pig (Blustein et al., 1972), histocompatibility-linked immune response genes appear to regulate humoral antibody specificity. In the study by Moses et al. (1969), DBA and SJL mice were injected with (Phe,G)-Pro--L. This polypeptide is similar to (T,G)-A--L described above except that phenylalanine and proline are substituted for tyrosine and alanine respectively. Both DBA and SJL mice were responders to this antigen. However, when the antisera were assayed with (T,G)-Pro--L for anti-Pro--L antibody, the DBA mice were nonresponders and the SJL mice were responders. Further genetic analysis showed this to be a single gene effect which was not linked to the H-2 locus. When the same antisera were analyzed with (Phe,G)-A--L to test for anti-(Phe,G) antibody, the DBA mice were responders and the SJL mice were nonresponders. This turned out to be a single gene effect, linked to the H-2 locus.

At first glance, these results suggest that these genes are controlling antibody specificity and may even be coding for primary antibody structure. However, such a conclusion is not compatible with the large amount of data on histocompatibility-linked immune response genes which imply that they function only to regulate the occurrence of a response (McDevitt and Benacerraf, 1969). In what follows, I have made an attempt to reinterpret these specificity experiments in terms of carrier effects.

It has been shown that mice that are unresponsive to a particular polypeptide antigen can be made responsive if the antigen is attached to a suitable immunogenic carrier such as methylated bovine serum albumin (McDevitt, 1968). Furthermore, Green and his colleagues (1966) showed that DNP-PLL nonresponder guinea pigs immunized with DNP-PLL coupled to an albumin carrier, produced not only antibodies to the DNP group, but also to the poly-L-lysine moiety. In the absence of the albumin carrier, the nonresponder guinea pigs are unable to make an immune response to the PLL carrier of DNP-PLL.

Thus, the hapten-carrier relationship does not appear to be a rigid one; that is, what serves as carrier in one situation can be the hapten in another, and vice-versa.

The apparent immune response gene control of antibody specificity could be explained if it were assumed that the T cells of SJL mice but not the DBA mice are able to recognize the (Phe,G) portion of the polypeptide as a carrier. Thus, the SJL mice would be able to make antibody to the Pro--L portion of the polypeptide whereas the DBA mice could not.

The specificity for the (Phe,G) portion of the carrier could be explained in a similar manner, if the T cells of the DBA mice but not the SJL mice could recognize the Pro--L portion of the polypeptide as a carrier. This would allow the DBA mice to make anti-(Phe,G) whereas the SJL mice could not.

The apparent inheritance of specificity in this case can be explained as simply the inheritance of the ability of T cells to recognize carriers, as is the case with other immune response loci. The example of immune response gene controlled antibody specificity in the guinea pig (Blustein

et al., 1972) could also be explained in terms of carrier effects. If this explanation is verified experimentally for the mouse and guinea pig immune response genes, then a major obstacle to the theory that the histocompatibility-linked immune response genes control carrier, but not antibody, specificity (Benacerraf and McDevitt, 1972) would be removed.

Are the immune response gene effects mediated only by the T cells?

The contention that guinea pig histocompatibility-linked immune response genes and the mouse Ir-1 immune response genes express their effect only in the thymus-derived cells, but not in the antibody producing cells (Benacerraf and McDevitt, 1972), is seriously questioned by experiments with guinea pigs (Ellman et al., 1970b) and with mice (Bechtol et al., 1972). In these experiments, chimeric responder/non-responder animals were immunized with the appropriate polypeptide antigens. Since the immune response genes were thought to be expressed only in T cells, it was expected that in chimeric animals containing some responder T cells both responder and non-responder B cells would produce antibody. However, in every case, animals producing antibody did so only with B cells of responder origin.

Molecular basis of immune response gene effects The exact nature of the supposed T cell "carrier" receptors, or how they are affected by the immune response genes, is not known. The Ir-1 genes of the mouse map within the portion of the chromosome controlling H-2 specificities, but are not identical with any of the known histocompatibility regions of this portion of the chromosome (McDevitt, 1972).

Two possible explanations have been suggested for the nature of the gene products of the Ir-1 and guinea pig immune response genes (Benacerraf and McDevitt, 1972). One proposed explanation is that the Ir-1 genes code for T cell antigen receptors. This explanation appears somewhat unlikely in view of the very small region of the chromosome occupied by the Ir-1 genes. The other explanation suggested by Benacerraf and McDevitt (1972) is that the Ir-1 locus controls the production of an as yet undetected cell surface substance in a manner similar to the genetic control of histocompatibility antigens. This proposed "Ir-1 substance" would affect the specificity of the T cell carrier receptor through a local modification of the cell surface.

Genetic Control of Antibody Specificity

In addition to the experiments described above, several other antigens have been used to demonstrate apparent genetic control over antibody specificity. Arquilla and Finn (1963) demonstrated that strains 2 and 13 guinea pigs make anti-insulin antibodies of slightly different specificities. They subsequently demonstrated (Arquilla and Finn, 1965) that hybrid strain 2 x strain 13 guinea pigs made anti-insulin antibodies with specificities which were not made by animals of the parental strains. The significance of these observations is not completely understood (Benacerraf and McDevitt, 1972). Using Salmonella lipopolysaccharides as antigens, Di Pauli (1972) demonstrated that the specificity of the antibodies produced was under genetic control. His experiments also show clearly that the control was at the recognition level and not the result of genes coding for antibody structure.

It seems likely that the results of Arquilla and Finn (1963, 1965) as well as those of Di Pauli (1972) were due to the control of antibody specificity by immune response type genes, as was the case in the mouse and guinea pig experiments described above. There is no persuasive evidence that any of the experiments involving the study of the genetic control of antibody specificity have been following the inheritance of structural genes coding for variable region sequences.

Structure of Immunoglobulins

The first insight into the molecular nature of antibodies was obtained when Tiselius (1937) demonstrated that anti-ovalbumin antibodies from a hyperimmunized rabbit could be classified by their electrophoretic mobility as γ -globulins. It is very likely that the predominant antibody component observed by Tiselius was rabbit 7S IgG.

The structure of human and rabbit IgG immunoglobulins has been studied in considerable detail (Cohen and Milstein, 1967; Edelman, 1971). The fundamental structures of the other human immunoglobulins (IgA, IgM, IgD, and IgE) are quite similar to that of human and rabbit IgG, although important differences do exist (Cohen and Milstein, 1967; Kochwa et al.,

1971). In fact, the basic structure of mammalian immunoglobulins in general, as well as those of lower vertebrates, are structurally similar to human and rabbit IgG (Gray, 1969).

The structure of 7S immunoglobulin, IgG (figure 1), was originally proposed by Porter (1962). His model of antibody structure was based on an analysis of the fragments obtained by enzymatic cleavage of immunoglobulins (Porter, 1959), the demonstration that immunoglobulins have a multichain structure (Edelman, 1959) and results of others (see review by Cohen and Porter, 1964).

With the complete sequence of an entire IgG molecule (Edelman et al., 1969), it was possible to unequivocally confirm the model proposed by Porter (1962), and to obtain a much more detailed knowledge of the structure of immunoglobulins (Edelman, 1971).

Figure 1 is a diagrammatic representation of the structure of IgG. This stylized drawing, based on the models of Porter (1962), Cohen and Milstein (1967), and Edelman et al. (1969), has been constructed to emphasize the features of immunoglobulin molecules that are of importance to us here. The basic monomer, as shown, consists of two light and two heavy chains, each composed of about 215 and 450 amino acid residues, respectively. The N-terminal 107-122 amino acids of the light and heavy chains are variable in their amino acid sequence from one immunoglobulin molecule to another. The remaining amino acids in the light and heavy chains are constant in all light and heavy chains of the same class and allotype. The light chain variable and constant regions were first described by Hilschmann and Craig (1965) on the basis of sequence studies with light chains (Bence-Jones proteins secreted in the urine of patients with multiple myeloma¹). The size of the heavy chain variable region was

¹ Following the demonstration (Deutsch et al., 1955; Korngold and Lapuri, 1956b) that Bence-Jones proteins were immunochemically related to immunoglobulins, it was shown by Edelman and Gally (1962) that Bence-Jones proteins were identical with light chains of the myeloma proteins of patients with multiple myeloma. Myeloma proteins themselves had been previously shown to be representative of immunoglobulins by Kunkel et al. (1951; see also Kunkel, 1965).

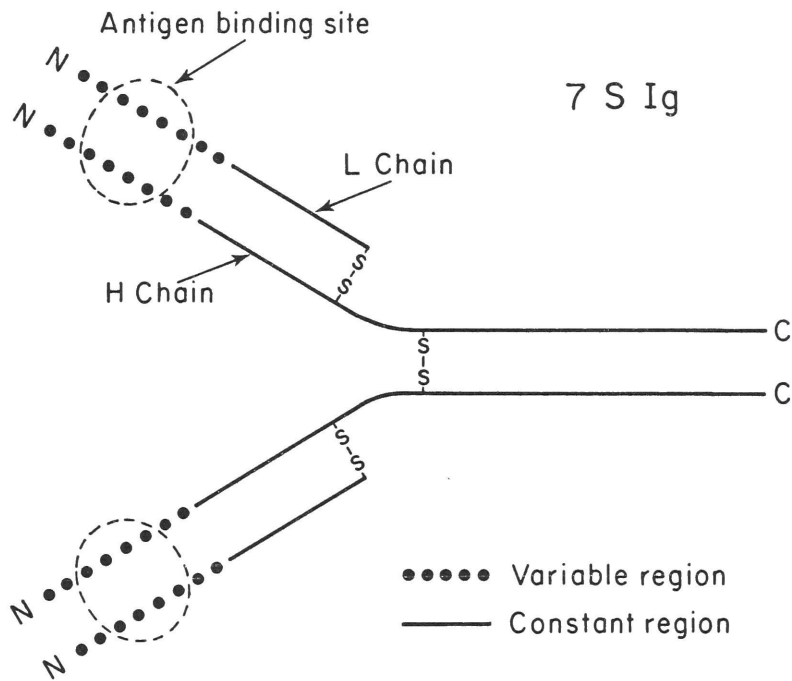


Figure 1. A diagrammatic representation of a 7S immunoglobulin adapted from Cohen and Milstein (1967). The immunoglobulin is made up of heavy (H) and light (L) chains held together by disulfide bonds (-S-S-) between half cystines.

revealed by comparison of amino acid sequences of heavy chains from two different myeloma proteins (Edelman et al., 1969).

In immunoglobulins, there are two basic types of immunochemically different light chains, κ and λ (see Hood et al., 1967). Antibodies in each of the classes (IgG, IgA, IgM, IgE, and IgD) of human immunoglobulins have either two κ or two λ light chains (Mannik and Kunkel, 1963; Fahey, 1963).

Each of the five human antibody classes, IgG, IgM, IgA, IgD, and IgE, are characterized by their own type of heavy chain, which can be distinguished from each other immunochemically (Franklin and Kunkel, 1957; Heremans and Heremans, 1961; Rowe and Fahey, 1965; Bennich and Johanssen, 1971).

The IgG immunoglobulins have been further shown to be composed of subclasses, IgG₁, IgG₂, IgG₃, IgG₄, differing from each other in the immunochemical properties of their heavy chains (Grey and Kunkel, 1964; Natvig and Kunkel, 1968).

Two subclasses of IgA have also been demonstrated by Kunkel and Pendergast (1966).

IgD (7S) and IgE (8S) molecules are composed of two light and two heavy chains with an overall structure similar to that of IgG (Rowe and Fahey, 1965; Bennich and Johanssen, 1971). The IgA immunoglobulins originally described by Williams and Grabar (1955a,b) were shown to occur in the serum as a four-chain 7S monomer similar to IgG, as well as various polymers of the four chain unit (Heremans et al., 1959).

IgM antibodies with a molecular weight of around 900,000 were originally described in anti-pneumococcal sera by Heidelberger and Pedersen (1937) and by Kabat (1939). These antibodies are generally composed of five 4-chain subunits each with a molecular weight of about 185,000 (Miller and Metzger, 1965a; Feinstein et al., 1971). Each subunit is composed of two heavy chains (molecular weight 70,000) and two κ or λ light chains in a structure analogous to IgG (Miller and Metzger, 1965b).

The general level of complexity of the human immunoglobulins is also seen in other vertebrate species, except for some of the very primitive vertebrates whose sera appear to contain only IgM-like antibody (Grey, 1969).

Mouse immunoglobulins The mouse has both λ and κ light chains, but at least 90% of the mouse immunoglobulins appear to be of the κ type. This is in contrast to the human situation, where approximately one third of the immunoglobulins have λ light chains (Hood et al., 1967). The mouse heavy chain classes IgG, IgG_{2a}, IgG_{2b}, IgA and IgM were originally described by Fahey et al. (1964a,b). The mouse IgA and IgM appear to be analogous to human IgA and IgM in that they are composed of similar type polymers of a basic four chain unit. The IgG, IgG_{2a}, and IgG_{2b} immunoglobulins, on the other hand, all have a molecular weight of around 150,000 and a basic structure analogous to human IgG (Potter and Lieberman, 1967).

Immunoglobulin variable regions By the use of affinity-labeling techniques, the antigen binding site was located in the variable region of the light and heavy chains (Goetzl and Metzger, 1970; Franek, 1971; Ray and Cebra, 1971). Porter (1959) demonstrated that enzymatic cleavage of antibody molecules with papain yielded two monovalent antigen binding fragments per monomer. The monovalent fragments (now called Fab) were shown by Fleischman et al. (1963) to contain the light chains and a portion of the heavy chains (now called Fd). It was subsequently demonstrated that in the Fab fragment both the heavy and light chains contributed to the antibody binding site (Edelman et al., 1963; Rohult et al., 1966).

From these studies it was learned that the antigen binding sites of antibodies were formed by an interaction between the variable regions of light and heavy chains. It is now generally assumed that the antigen binding specificity of individual antibodies is the result of the particular amino acid sequences in the variable regions of their light and heavy chains (Cohen and Milstein, 1967).

From comparisons of a large number of light chain amino acid sequences, it has become apparent that the sequence variability in the light chain variable region is not randomly distributed, but is largely localized in

three hypervariable regions (Wu and Kabat, 1970). The same situation appears to hold true for the heavy chain, in which most of the sequence variation occurs in three hypervariable regions within the heavy chain variable region (Capra, 1971; Kehoe and Capra, 1971).

The authors of the above three papers contend that the hypervariable regions, because of their high variability, very likely play a major role in the actual formation of antibody binding sites. These authors pointed out that the known intrachain disulfide bonds bring the hypervariable regions within each polypeptide chain into close proximity, so that the three hypervariable regions of each chain could cooperate in the formation of an antigen binding site. This view, that the hypervariable regions make up the antigen binding site, is supported by affinity labeling studies, in which the affinity labels have been observed to bind to known hypervariable regions in the light and heavy chains (Goetzl and Metzger, 1970; Franek, 1971; Ray and Cebra, 1971).

Constant Region Genes, Immunoglobulin Allotypes

Immunoglobulin allotypes are the immunochemical specificities found on antibodies of a particular class (or classes) in some individuals and not found on the immunoglobulins of the same class (or classes) in other individuals of the same species (from Oudin, 1966a,b). The genes controlling immunoglobulin allotypes are thought to be the same genes that code for the amino acid sequences of antibody constant regions (Oudin, 1966a). Immunoglobulin allotypy was discovered independently by Grubb and Laurell (1956) and by Oudin (1956).

Oudin (1956) discovered immunoglobulin allotypy in rabbits. For the detection of rabbit allotypes he used antisera prepared by immunizing rabbits with immunoglobulins isolated from other rabbits. For a review of the current status of rabbit allotypy see Prahl and Todd (1971).

Grubb and Laurell (1956) used, as their allotypic reagents, the serum from certain patients with rheumatoid arthritis which contained rheumatoid factors (anti-IgG's). These sera identified various allotypic (Gm) determinants on the constant regions of human IgG immunoglobulins (see review by Natvig and Kunkel, 1968).

An additional group of human allotypic specificities, InV factors, were described by Ropartz et al. (1961). Alleles at the InV locus do not appear to be genetically linked with the factors at the Gm locus (Steinberg, 1968). The Gm factors were shown to be only on IgG (7S) immunoglobulins whereas the InV factors were found on IgG, IgM, and IgA immunoglobulins as well as on Bence-Jones proteins. This indicated that the Gm factors were located on the IgG heavy chains and that the InV factors were located on light chains, thus allowing the latter to be present on all classes of immunoglobulins (Harboe et al., 1962; Franklin et al., 1962). Further studies indicated that the InV factors were present on κ but not λ light chains.

By making use of myeloma proteins of various IgG subclasses, it was possible to assign each of the 8 Gm factors to one of three different IgG subclasses (Natvig and Kunkel, 1968). Linkage studies showed that the Gm factors of the different IgG subclasses were very closely linked (Natvig et al., 1967). Since the genes controlling the expression of Gm factors are thought to be synonymous with genes coding for heavy chain constant regions, the above finding indicates that the IgG₁, IgG₂, and IgG₃ constant region genes are very closely linked (Natvig and Kunkel, 1968).

An allotypic marker in IgA₂ immunoglobulin has also been shown to be closely linked to the Gm locus (Kunkel et al., 1969).

Allotropy in mice was first demonstrated by Kelus and Moor-Jankowski (1961). They used antisera produced by immunizing mice with anti-B. proteus antibodies raised in mice. The allotypic determinant was identified in the immunoglobulin of some strains and not in others. A second allotypic determinant in mouse immunoglobulin was described by Dubiski and Cinider (1963). Wunderlich and Herzenberg (1963) demonstrated in backcross studies that the inheritance of the allotypic determinant they had identified was controlled by a single gene. This was also observed by Dray et al. (1963), who demonstrated that two different allotypic determinants found in different inbred strains were inherited as alleles.

Subsequent studies in many laboratories have greatly expanded our understanding of mouse immunoglobulin allotypes (see reviews by Potter and Lieberman, 1967; Herzenberg et al., 1968).

Of the five major mouse immunoglobulin classes IgG_1 , IgG_{2a} , IgG_{2b} , IgA and IgM (Fahey et al., 1964a,b), heavy chain allotypes have been shown to be associated with three of them: IgA , IgG_{2a} , and IgG_{2b} .

Eight allelic types of IgG_{2a} are found in inbred mice (Herzenberg et al., 1965). Two additional allelic types of mouse IgG_{2a} have been identified in wild mouse populations (Lieberman and Potter, 1966a). In inbred strains, the 8 allelic types of IgG_{2a} share 11 different specificities. A similar situation is seen with IgG_{2b} immunoglobulins, where 6 allelic types share 7 specificities. In the case of IgA , 5 allelic types have been identified that share 4 different specificities (Herzenberg et al., 1968).

The genes controlling the allotypic determinants located on the IgG_{2a} and IgA heavy chains were shown to be very closely linked (Herzenberg, 1964). Herzenberg examined 149 backcross progeny and observed no recombinants between the genes coding for the IgG_{2a} and IgA allotypes. This finding was confirmed by Lieberman and Potter (1966b), who examined 1054 backcross mice and found no recombinants. There were also no recombinants observed in linkage studies of genes controlling the allotypic determinants on the mouse IgG_{2a} and IgM immunoglobulins (Lieberman et al., 1965; Herzenberg et al., 1967). By comparing the electrophoretic mobility of the Fc fragment of IgG_1 immunoglobulins from different inbred strains, Herzenberg and his co-workers (1967) identified two structural variants which were inherited as though controlled by a single gene. They were able to demonstrate complete linkage of this IgG_1 marker to the IgG_{2a} allotype of the same strains. It was thus apparent that the genes controlling the constant region structure of the heavy chains of mouse IgG_{2a} , IgG_{2b} , IgG_1 and IgA immunoglobulins were all very closely linked together as was also the case with the human loci controlling allelic types of IgG and IgA .

Blomberg et al. (1972) and Lieberman et al. (1971) have demonstrated linkage of certain immune response genes to the heavy chain allotype locus of the mouse.

Variable Region Genetic Markers

Attention has centered on variable region genetic markers since Dreyer and Bennett (1965) suggested that separate genes code for the constant and variable regions of antibody polypeptide chains. Once it was learned that constant region allotypes were inherited as if controlled by single genes, it was of interest to determine if variable region markers would map in the same chromosome region.

Two variable region allotypic markers have been described, one on light chains of the mouse (Edelman and Gottlieb, 1970) and one on the rabbit heavy chain (see review by Prahl and Todd, 1971). It was shown that the rabbit variable region allotypic marker was associated with multiple amino acid substitutions in the variable region of the rabbit heavy chain (Koshland, 1967; Wilkinson, 1969).

This allotype, termed group a, is present on the heavy chain variable region of rabbit IgG, IgM, and IgA immunoglobulins (Todd, 1963; Feinstein, 1963) and IgE (Kindt and Todd, 1969).

The occurrence of this same rabbit allotype on four different classes of rabbit heavy chains has been interpreted to indicate that different genes code for the variable and constant region of the rabbit heavy chain (Prahl and Todd, 1971; Gally and Edelman, 1970).

Although the group a allotype is closely linked to the other rabbit heavy chain allotypic markers (Tosi *et al.*, 1970), there is evidence for a low frequency of crossovers between it and the other markers (Mage *et al.*, 1971; Kindt and Mandy, 1972). The occurrence of these crossovers is further evidence that separate genes code for variable and constant regions of immunoglobulin molecules.

In the mouse, it has been possible to use peptide maps to identify an allotypic marker in the variable region of mouse light chains (Edelman and Gottlieb, 1970). There have not yet been any reports of linkage studies involving this mouse variable region marker.

In the case of both the mouse and the rabbit, the variable region allotypic markers do not appear to be associated with the hypervariable

region, since they are found in preimmune γ -globulin as well as specific antibody (Edelman and Gottlieb, 1970; Kindt and Mandy, 1972). In fact, the peptide isolated by Edelman and Gottlieb (1970) does not fall into one of the light chain hypervariable regions described by Wu and Kabat (1970).

Idiotypic Determinants of Antibody Molecules

Although the variable region allotypic markers described above have given information about the inheritance of the structure of the more conservative portions of the variable region, these markers cannot be employed for studies on the genetic control of structural variations associated with the antigen binding site, or hypervariable region. Studies on the genetic control of this region require a genetic marker in the hypervariable region itself. One way to obtain such a marker is to consider the hypervariable region as an antigen in its own right. An antiserum directed against the hypervariable region of a single antibody molecule could be used to determine if antibodies from related animals had a similar binding site structure.

Antisera capable of recognizing this type of determinant have been prepared against myeloma proteins (Slater et al., 1955; Korngold and Lapari, 1956a). These antisera were prepared in rabbits against isolated myeloma proteins, and subsequently absorbed with human immunoglobulin. The antisera gave precipitin reactions only with the human myeloma proteins against which they were made, and not with other myeloma proteins or with normal human immunoglobulin.

Such antisera are now called idiotypic antisera, and the determinants which they recognize are called idiotypic determinants (Oudin, 1966b). The idiotypic determinants detected in this manner have been localized in the Fab fragments of myeloma proteins (Grey et al., 1965; Wang et al., 1970).

Using mouse myeloma proteins as antigens, it has also been possible to produce idiotypic antisera to mouse myeloma proteins (Potter and Lieberman, 1967). Like the idiotypic antisera prepared against human myeloma proteins, these antisera react only with the myeloma protein against which they were made and not with other mouse myeloma proteins or with normal mouse immunoglobulin. It has also been possible to produce idiotypic

antisera against isolated Bence-Jones proteins that do not react with other Bence-Jones proteins (Stein et al., 1963).

The idiotypic antisera to these homogeneous immunoglobulins and Bence-Jones proteins probably detect antigenic determinants in the hypervariable region of these molecules, since they do not react with other homogeneous proteins of the same type or with normal immunoglobulin.

Further evidence that idiotypic markers were in the hypervariable, or antigen binding, region of immunoglobulin molecules came from the study of Williams et al. (1968). These workers demonstrated that partially absorbed idiotypic antisera to 19S human cold agglutinins occasionally cross-reacted with other cold agglutinins with similar specificity, but did not react with any of 50 Waldenstrom macroglobulins tested.

Similar findings have been subsequently made using mouse myeloma proteins with specificity for certain ligands. Balb/cJ myeloma proteins known to bind the phosphoryl choline determinant of pneumococcal C-carbohydrate (Cohn et al., 1969; Potter and Lieberman, 1970), 2,4-dinitrophenyl compounds (Sirisinha and Eisen, 1971; Brient et al., 1971), and α -1,3-dextran (Carson and Weigert, 1973) have all been used to prepare anti-idiotypic antisera. These antisera have been shown to cross-react with other myeloma proteins with specificity for the same antigens as the myeloma protein used to elicit the idiotypic antisera. The antisera, however, do not react with unrelated myeloma proteins or with normal immunoglobulins.

It has been possible to demonstrate that the binding of many of these anti-myeloma idiotypic antisera to their myeloma protein can be inhibited with the appropriate haptens (Sirisinha and Eisen, 1971; Brient et al., 1971; Carson and Weigert, 1973). These studies have all provided additional evidence that the idiotypic determinants are associated with the antigen binding site of the myeloma proteins. In an extension of this work, Sirisinha and Eisen (1971) demonstrated that a myeloma protein with an affinity labeling reagent in the antigen binding site did not elicit antibodies to the idiotypic determinants normally associated with the myeloma protein.

It has also been possible to produce antisera that detect specificities present on antibodies (Kunkel et al., 1963, 1966; Oudin and Michel, 1963; Gell and Kelus, 1964; Kelus and Gell, 1968).

Kunkel et al. (1963) demonstrated the presence of idiotypic specificities in isolated human antibodies to hog gastric A substance, dextran, and levan. Oudin and Michel (1963) prepared idiotypic antisera against rabbit anti-Salmonella typhi antibodies. Gell and Kelus (1964) used rabbit anti-proteus antibody to make idiotypic antisera. In all of these studies, each idiotypic antiserum reacted only with the antibody used for immunization and not with antibodies to the same antigen in other sera, or with normal serum from the same species. This finding indicates that antibodies to a particular antigen have many different idiotypes.

It seems likely that the idiotypic determinants on antibody molecules are associated with the antigen binding site, as are those on myeloma proteins. Using idiotypic antisera directed against anti-para-azobenzoate antibodies, Brient and Nisonoff (1970) were able to inhibit the reaction of the idiotypic antiserum with anti-para-azobenzoate antibodies with the appropriate azobenzoate haptens. Kunkel et al. (1963) demonstrated that idiotypic determinants of antibodies were located on the Fab fragments. Fab fragments are known to contain the antigen binding sites of antibody molecules.

The idiotypic specificities present in the mouse myeloma proteins described above have also been shown to be present in Balb/cJ mouse antibodies produced against the antigens known to bind certain myeloma proteins (Cohn et al., 1969; Sirisinha and Eisen, 1971; Carson and Weigert, 1973).

The idiotypy experiments that have been described in this introduction have all used antisera specific for determinants in the variable region of the immunizing antibody. But these reagents have not all been prepared in the same manner. Idiotypic antisera may be prepared in the same species which was the source of the antibody. On the other hand, as mentioned earlier, Kunkel and collaborators commonly use rabbits to produce idiotypic antisera to isolated human immunoglobulins (see Slater et al., 1955;

Kunkel, 1965). The antisera produced are absorbed with normal human γ -globulin or human serum to render them specific for idiotypic determinants.

In the idiotypy experiments described in this thesis, essentially this same procedure has been used, except that the rabbit idiotypic antisera are made against isolated mouse antibodies. This is diagrammatically illustrated in figure 2. The hypervariable regions of the light and heavy chains of the immunizing immunoglobulin have been schematically illustrated by X's in the light chain and zig-zags in the heavy chain. The non-hypervariable region portions of the light and heavy chain variable regions are indicated by dashed lines, and the constant regions of the light and heavy chains are represented by solid lines.

The rabbit antiserum produced as a result of this immunization contains antibodies to antigenic determinants on both the constant and variable regions of the injected mouse immunoglobulin. Some of the rabbit antibodies directed against the variable region bind antigenic determinants in the hypervariable region. These hypervariable region determinants are called idiotypic determinants, and are generally somewhat unique to the immunizing immunoglobulin. To make the rabbit antiserum specific for these idiotypic determinants, it is absorbed with normal immunoglobulin from the same species as the immunizing immunoglobulin. When preparing rabbit idiotypic antisera to mouse antibodies, the absorption is generally done with normal serum from the same inbred strain as the immunizing immunoglobulin. This absorption technique renders the idiotypic antiserum specific for antigenic (idiotypic) determinants of the immunizing immunoglobulin which are very rare in normal immunoglobulin. The idiotypic determinants, as indicated previously, are thought to be associated with the hypervariable region or antigenic binding site of the immunizing immunoglobulin molecule.

As noted above, an alternative approach for making idiotypic antisera has been to immunize an allotypically matched animal of the same species. This approach was first utilized by Oudin and Michel (1963) and

Preparation of Idiotypic Antiserum

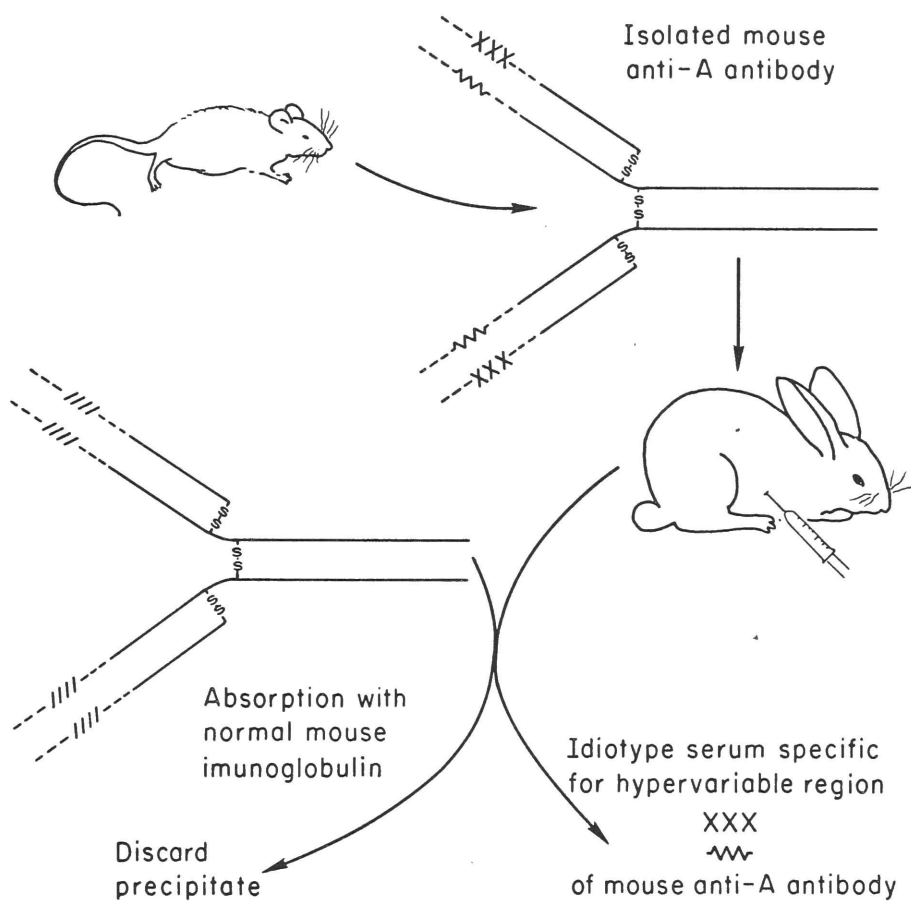


Figure 2. Procedure used to prepare anti-idiotypic sera to mouse anti-Group A antibody.

Gell and Kelus (1964). Since the immunized and donor animals are allotypically matched, antibodies are produced only to the unique or idiotypic determinants on the immunizing antibody. As Kunkel has pointed out (personal communication), in this case there is in vivo "self absorption", and such antisera do not require subsequent absorption. Sirisinha and Eisen (1971) have used a similar approach in mice. They have immunized Balb/cJ mice with Balb/cJ myeloma proteins.

Others have used Balb/cJ myeloma proteins to immunize other inbred strains of mice. The antisera obtained are then absorbed to remove anti-allotype antibody (Potter and Lieberman, 1970; Blomberg et al., 1972). This approach is intermediate between immunizing allotypically matched animals and immunizing across species barriers. In general, if the immunizations are made between two species, the antisera are referred to as heterologous idiotypic antisera. If the immunizations are made within the same species, the antisera are referred to as homologous idiotypic antisera. For the purpose of this thesis, the heterologous idiotypy employed here will be termed idiotypy. A review of the literature of the inheritance of idiotypy will be presented in the discussion.

Idiotypic Determinants of Anti-Streptococcal Group Carbohydrate Antibodies

Much of the work to be described in this thesis deals with the idiotypy of mouse antibodies to streptococcal Group A carbohydrate. This work with mouse idiotypy stems directly from a series of findings in Dr. Krause's laboratory.

It was demonstrated that rabbits immunized with pepsinized streptococcal vaccines often produced monodisperse antibody to the streptococcal group-carbohydrate (Osterland et al., 1966; Krause, 1970b). It was subsequently shown by Braun and Krause (1968) that antisera could be raised that detected idiotypic determinants of monodisperse streptococcal antibodies. In a subsequent development, it was shown that the ability to make anti-streptococcal antibodies with a particular idiotypic specificity was inherited (Eichmann and Kindt, 1971). Unpublished observations of Krause and Eichmann (personal communication) demonstrated that outbred mice could be induced to make monodisperse antibody to streptococcal

Group C carbohydrate. These results suggested the possibility of studying the inheritance of idiotypic determinants in anti-streptococcal antibodies produced by established inbred mouse strains.

From several points of view, the inbred mouse has several advantages over the rabbit for studies on the inheritance of idiotype. Two of these advantages are of primary importance: 1) the use of highly inbred animals permits a search for idiotypic determinants in a large number of animals of essentially identical genotypes, and 2) much higher resolution genetic studies can be performed with mice than with rabbits because larger numbers of mice can be used. The primary disadvantage of mice is the small amount of immune serum that can be obtained from a single animal.

Anti-streptococcal group carbohydrate antibodies are particularly useful for idiotypic studies in the rabbit for two reasons:

1) anti-streptococcal antibodies are readily obtained as the result of immunization, and they react with well-defined natural antigens (Krause, 1963, 1970b), and 2) it is possible to obtain anti-streptococcal carbohydrate antibodies with remarkable molecular uniformity (Krause, 1970a).

In these studies antibodies to the cell wall carbohydrate antigen of Group A streptococci were produced in mice by immunizing them with streptococcal vaccine made according to the method described by McCarty and Lancefield (1955).

The Group A carbohydrate, against which these antibodies are directed, is composed of 60% rhamnose and 30% N-acetyl-D-glucosamine (Krause and McCarty, 1961; Krause, 1963). Figure 3 is a schematic representation of the structure for Group A carbohydrate as proposed by Krause (1970a). McCarty (1958) demonstrated that terminal β -N-acetyl-glucosaminide residues were the immunodominant determinants of Group A carbohydrate. Part of the evidence for this is the fact that N-acetyl-D-glucosamine and β -phenyl-N-acetyl-D-glucosaminide readily inhibit the precipitation reaction between Group A carbohydrate and its antibody. β -phenyl-N-acetyl-D-glucosaminide was several times more effective as an inhibitor than was N-acetyl-D-glucosamine.

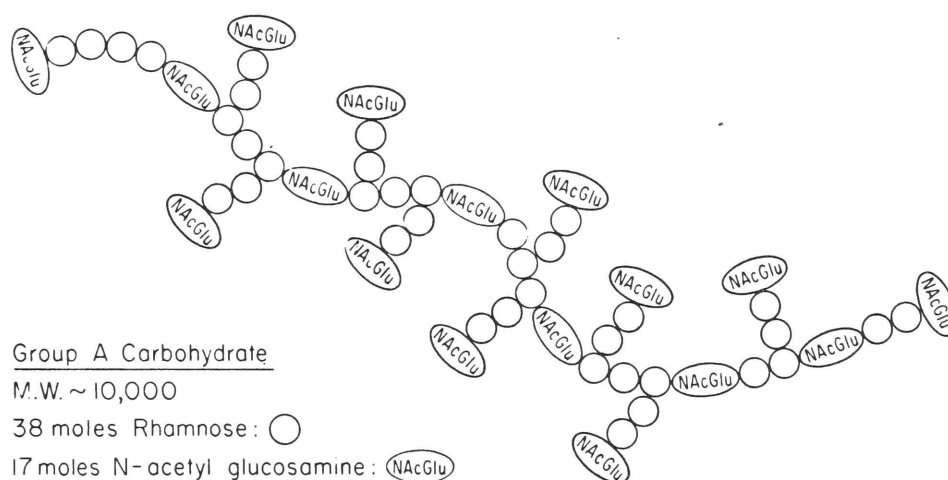


Figure 3. Schematic diagram of the chemical structure of the Group A carbohydrate antigen extracted from streptococcal cell wall (Krause, 1970b).

The Group A carbohydrate is a component of the cell wall of Group A streptococci (McCarty, 1952), as is seen in figure 4 (Krause, 1970a). Live streptococci have a hyaluronic acid capsule surrounding the cell wall. The cell wall itself is composed of mucopeptide overlaid with Group A carbohydrate and the external protein antigens (McCarty, 1971).

A diagram of the streptococcal vaccine used in these experiments is also seen in figure 4. The outer protein antigens are removed by treatment with pepsin. After the pepsin treatment, the Group A carbohydrate is the outermost surface element of the cell wall, and as such, is highly immunogenic (Krause, 1970b).

In the section on Results, I will describe the means by which this anti-streptococcal Group A system was adapted to studies with inbred mice, and eventually utilized to study the inheritance of an idiotypic specificity of anti-A antibody. After these studies were under way, other investigators also began to use inbred strains of mice to study the inheritance of idiotype. As was mentioned previously, their work will be discussed in the Discussion section of this thesis.

Living Group A Strep.

Dead Vaccine

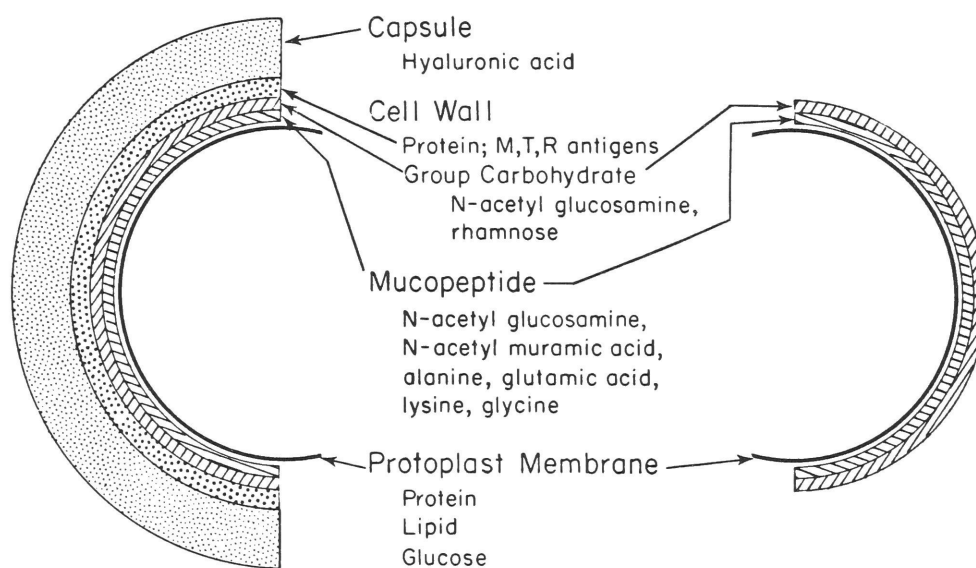


Figure 4. Schematic diagram of a living Group A streptococcus and the dead vaccine prepared from it (Krause, 1970b).

MATERIALS AND METHODS

Streptococcal Group A vaccine Strain J17A4 Group A streptococci were used to prepare the vaccine. This strain was originally isolated from a patient with sore throat and rheumatic fever by Coburn and Pauli (1932) and designated RPH-1. The strain was subsequently classified as a Group A streptococcus and designated J17A4 by Lancefield (1933), who has generously supplied me with samples of it from time to time. The vaccine was prepared according to the procedure of McCarty and Lancefield (1955; see also Krause 1970b). During the preparation of the vaccine, streptococci are heat-killed and the hyaluronic acid capsule is washed away. Pepsin is then used to remove the surface protein antigens M and T (Lancefield et al., 1946; Lancefield, 1962). What remains is composed of heat-killed streptococci with a superficial cell wall surface of carbohydrate antigen which encloses the underlying mucopeptide matrix and residual intracellular material (Krause and McCarty, 1961).

In these studies, rhamnose has been used as a measure of vaccine concentration since it comprises 60% of the Group A carbohydrate (Krause and McCarty, 1961) and is not found elsewhere in the streptococcus (Krause and McCarty, 1961).

Isolation of Group A carbohydrate Group A carbohydrate was extracted from lyophilized Group A streptococci with formamice at 180°C (Fuller, 1938) and purified as described by Krause and McCarty (1961).

Injection of Group A vaccine into mice Streptococcal vaccine was diluted into phosphate buffered saline, pH 7.2, and injected in 0.1 ml doses. Injections were either given intraperitoneally (i.p.) or intravenously (i.v.). For the i.v. injections, a 27 gauge needle was used and the mice were restrained in a glass tube (see Chase, 1967) with an inside diameter of 3.1 cm which was drawn so that one end had only a 1.0 cm opening. A #7 rubber stopper, which fits the 3.1 cm opening, was notched to allow the mouse's tail to hang outside for manipulation. The tube was mounted on a ring stand. It was found that if the tube was oriented so that the mouse's head was down rather than up, the mouse tended to jump less, thus greatly facilitating the i.v. injection via

the tail veins. By placing a small pan under the lower end (small hole) of the tube, it was possible to rinse out the tube with 70% ethanol between mice to keep it clean.

Mice All inbred mice used in these experiments, except for BRVR and BSVS mice, were obtained from Jackson Laboratory in Bar Harbor, Maine. For most experimental purposes, female "retired breeders" generally over 30 weeks old, were used unless otherwise indicated. These older mice had the advantage of a lower cost and greater body size.

The BSVS and BRVR mice originally developed by Webster (1937) were obtained from two small colonies jointly maintained by Dr. Curtis Williams and myself. These colonies have now been discontinued at The Rockefeller University. BSVS and BRVR mice can presently be obtained from the NIH.

All of the inbred mice used had been brother-sister mated for over 90 generations, except for the RF/J and SJL/J mice. These had been inbred for over 50 generations (Green, 1968).

Outbred specific-pathogen-free NCS mice (see Lynch, 1969) were obtained from The Rockefeller University Animal House. Hybrid and back-cross mice were bred from parents obtained either from Jackson Laboratory or from our own BRVR and BSVS strains. Mice were generally caged 3-5 per cage and numbered by a system of dye marks. During experiments in which the mice were subjected to more than one set of experimental conditions, an effort was always made to put a mouse subjected to each set of conditions into each cage so that "cage effects" could be ruled out in all experiments involving more than one cage of mice.

Mature male mice were generally caged individually because they tended to fight each other. SWR/J mice over 6 months old were also housed individually because of congenital polyuria (Green, 1968) which occurs at this age. (If several were kept together, their bedding would become saturated with urine within 24 hours.) All mice were given Purina Lab Chow and water ad lib.

Collection of mouse antisera Antisera were collected from mice by orbital bleeding (Riley, 1960). Etherized mice were bled with 5-1/2 inch Pasteur pipettes. The blood was immediately expelled from the pipettes

into numbered 10x75 mm culture tubes and was allowed to clot at room temperature for 2-6 hours. It was found that by using disposable glass tubes, the clots always retracted away from the smooth glass surface. Generally, the volume of blood obtained from each mouse by this procedure is 0.6-1.0 ml. If more than 1.0 ml of blood was required, the mouse was rebled after injection i.v. with 1.0 ml of saline or Ringer's injection solution. This procedure can often be repeated a third time but the mouse usually dies. After bleeding is completed, the mice are each given 1.0 ml of sterile saline i.p. and returned to their cages. The mice can be rebled if necessary after 4-7 days, without a high risk of killing them.

The serum obtained after clot retraction was centrifuged at room temperature in disposable 6x50 mm glass tubes (Kimble Products Art. No. 45060). The clarified serum was then transferred to another 6x50 mm tube and stoppered with a size 000 cork. The serum was then immediately frozen and stored frozen at all times. Mouse serum deteriorates rapidly at 4°C, and is never stored unfrozen. If it is anticipated that a sample will be used frequently, aliquots are frozen separately so that the entire sample will not have to be thawed with each use.

Collection of ascites fluid The yield of antibody from SWR/J mice was increased by the inoculation with the ascites tumor, sarcoma 180/TG (Sartorelli *et al.*, 1966). The tumor line was obtained from Dr. Charles W. Todd in C57Bl/6 mice and adapted to SWR/J mice. The mice were inoculated with the tumor 10-15 days after the last injection of vaccine. Two weeks later, up to 35 ml of ascites fluid was drained by paracentesis with a 16 or 18 gauge needle. Fluid may be drained every 3-5 days until the death of the mouse, 4-5 weeks after inoculation with the tumor.

Several prolonged attempts were made to adapt the tumor to A/J mice, but it never consistently produced more than a few ml of fluid in A/J mice immunized with Group A vaccine.

Microzone electrophoresis Microzone electrophoresis of sera, serum proteins, and ascites fluid employed a Beckman model R101 microzone cell. Samples were run on cellulose acetate membranes in 0.043 M sodium barbital

adjusted to pH 8.6 with HCl. Samples were delivered to the membrane with an applicator calibrated to deliver 2 μ l and electrophoresis was performed at a constant voltage of 5mA (250-350 volts) for 25 minutes.

The membranes were stained for 10 minutes in 0.2% Ponceau S. stain dissolved in 3% trichloroacetic acid and 3% sulfosalicylic acid. The membranes were destained in 5% glacial acetic acid. If a densitometric tracing of the electrophoretic pattern was desired, the membrane was cleared by sequential 1 minute incubations in absolute alcohol and a 7:3 mixture of absolute alcohol and glacial acetic acid. The membranes were then dried on a glass plate at 100°C for 10-15 minutes, and scanned by a Beckman analytrol recording densitometer.

The Ponceau S. stain detects as little as 0.25 mg/ml of protein migrating as a single component. When greater sensitivity was desired, the membranes were stained with 0.25% coomasie brilliant blue in water after fixation for 1 minute in 20% sulfosalicylic acid (Fazekas de St. Groth et al., 1963). These membranes were destained in water. The coomasie brilliant blue stain has approximately 10 times the sensitivity of the Ponceau S. stain.

Protein determinations The concentration of isolated antibody in solution was determined by measuring the optical density at 280 m μ and converting to mg/ml with the extinction coefficient of $E_{1\text{cm}}^{1\%} = 14$. Determinations of protein concentration on whole serum were done by the biuret method (Gornall et al., 1949).

In vivo cloning of antibody-forming cells The basic procedure used to clone antibody-forming cells is similar to that of Askonas et al. (1970). Hyperimmunized mice which made particularly monodisperse anti-A antibody served as spleen cell donors. Such mice were bled out, and their spleen cells (usually $10\text{--}30 \times 10^7$) were teased through a stainless steel mesh into Ringer's Injection solution (Abbott Labs). Nucleated spleen cells were counted in a 2% acetic acid solution. 10^7 or more nucleated spleen cells were injected intraperitoneally into each of 6-30 recipient mice which had been irradiated 48 hours earlier. SWR/J mice were given 540 roentgens of γ -irradiation from a cobalt-60 source. A/J

mice were given 450 roentgens. In general, better recipient responses were observed if more than 10^7 nucleated spleen cells were injected, although this decreased the number of possible recipient mice from a single donor. Twenty-four hours after the cell transfer, the recipient mice were immunized with Group A vaccine containing 6-30 μ g of rhamnose. The higher dose was more effective in producing high antibody responses in the recipients. Vaccine has been given both intravenously and intraperitoneally to the recipient mice. Recent experiments have indicated that intraperitoneal immunization may be somewhat superior. The recipients are given four to five injections of vaccine spread over two weeks, and bled 10 days after the last injection. The antisera from the recipients are subjected to microzone electrophoresis and the recipients with the best responses are used for the next passage.

Rabbit anti-mouse sera Rabbit antisera were all made by injecting the appropriate antigens intraperitoneally in 1.5 ml of Freund's complete adjuvant. All rabbits were bled 30 days later. Anti-whole mouse sera were made by immunizing rabbits with 0.5 ml normal SWR/J serum in Freund's complete adjuvant. Anti-mouse γ -globulin was made by two methods: 1) approximately 2 mg of SWR/J anti-human serum albumin antibodies were precipitated with human serum albumin at equivalence and injected into rabbits, and 2) two milligrams of Pentex mouse γ -globulins were injected into rabbits.

Preparation of anti-idiotypic sera Rabbits were immunized intraperitoneally with 0.5-1.0 mg of electrophoretically monodisperse mouse anti-A antibody in 1.5-3.0 ml of Freund's complete adjuvant. The rabbits were bled thirty days later. Rabbit antisera giving a strong precipitation with normal mouse sera were subjected to further analysis as possible idiotypic antisera. To render these antisera idiotypically specific, they were absorbed overnight at 4°C within normal mouse serum or normal mouse ascites fluid. Three idiotypic antisera are discussed in this thesis. One was made to an isolated A/J anti-Group A antibody. This antiserum was absorbed with the largest volume of normal A/J sera that still permitted a precipitating cross-idiotypic reaction between the idiotypic antiserum

and pooled A/J anti-Group A serum. The absorbed idiotypic serum no longer precipitated with normal mouse γ -globulin. A second idiotypic antiserum was prepared against an SWR/J anti-Group A antibody isolated from the ascites fluid of an SWR/J cell line 3 mouse. This antiserum was absorbed with the ratio of normal SWR/J ascites fluid giving the largest precipitate. The third idiotypic antiserum was prepared against an SWR/J anti-Group A antibody isolated from the ascites fluid of an SWR/J cell line 1 mouse. The amount of normal ascites fluid used to absorb this anti-idiotypic serum was the largest amount still permitting a precipitation reaction with the proband antibody. This was twice as much as was required to prevent precipitation reactions with other SWR/J normal or anti-Group A sera.

Phosphate buffered saline Unless otherwise indicated, the phosphate buffered saline (PBS) used in this work is 0.056 M Na_2HPO_4 , 0.01 M KH_2PO_4 , and 0.078 M NaCl.

Determination of precipitating anti-Group A antibody by quantitative precipitin analysis Quantitative precipitins were done according to the method of McCarty and Lancefield (1955), except that the protein in the precipitates was determined by the Lowry method (Lowry *et al.*, 1951), adapted for use on a Technicon auto-analyzer (Hood *et al.*, 1969).

Isolation of mouse anti-Group A antibody by absorption and elution from Group A vaccine or Group A cell walls Approximately 10 μl of packed Group A cell walls were required to absorb 50-100 μg of mouse Group A antibody. Absorptions were performed at 4°C for 4-12 hours. The mixture was then centrifuged at 7,000 rpm for 10 minutes and the supernatant was removed. The vaccine was then washed three times with PBS. The antibody was eluted from the vaccine with 5-10% N-acetyl-D-glucosamine in PBS for 12 hours at 4°C. A single elution step is performed to obtain a high concentration of isolated antibody. For more complete recovery the vaccine or cell walls are eluted several times until the vaccine or cell walls are no longer clumped by the adsorbed antibody. The absorption and elution steps are generally carried out in 6x50 tubes siliconized with Siliclad. The supernatants obtained are pooled. The eluted antibody is also stored in siliconized 6x50 tubes. To increase the stability of the isolated antibody, elutions are often carried

in the presence of 0.1% BSA. When vaccine is used as an absorbent it is first heat-treated at 90°C for 10 minutes to inactivate any pepsin left over from preparation of the vaccine.

Immuno-electrophoresis and immunodiffusion Immuno-electrophoresis was performed on microscope slides in 1.5% washed Nobel agar as described by Williams (1971). The buffer used for immunodiffusion is the same as was described for microzone electrophoresis. Immunodiffusion (Ouchterlony, 1958) was performed on microscope slides using special punches made for this purpose by Gelman Instrument Co., Ann Arbor, Mich.

Autoradiography Autoradiography of immuno-electrophoretic and immunodiffusion patterns was based on a procedure described by Thorbecke et al (1971). To localize anti-Group A antibodies bound in precipitin lines of immuno-electrophoretic or immunodiffusion patterns, the slides are first washed in several changes of phosphate buffered saline (PBS) over 24 hours. Then the wells and/or troughs of the slide are filled with approximately 1 µc of ¹²⁵I-labeled Group A streptococcal carbohydrate. The slides are incubated overnight at 4°C, followed by 24 hours of washing in several changes of PBS. The PBS wash was followed by 24 hours of washing versus in changes of distilled water. The slides were dried with their agar surface in contact with a piece of wet filter paper to prevent cracking of the gels. The dried slides were then taken into a dark room and the agar side of each slide was placed in contact with the emulsion on 1x3" glass Kodak Autoradiographic plates, type No Screen. The slides and plates were wrapped in black paper. After exposure times of from 24 hours to 3 weeks, the plates are developed for 7 minutes in Kodak D-11 developer diluted 1:1 with water. The developed plates were then air-dried. After autoradiography, the dried immunodiffusion and immuno-electrophoresis slides were stained for 5-10 minutes with 0.1% Amido Black in 10% acetic acid - 70% methanol. The slides were destained in 10% acetic acid - 70% methanol for about 1 hour.

Disc electrophoresis of reduced and alkylated antibody preparations Antibody molecules were reduced and alkylated and electrophoresed in alkaline urea disc gels according to the procedure of Reisfeld and Small (1966).

SDS gels for the determination of molecular weights Molecular weight estimates were performed by SDS electrophoresis (Shapiro *et al.*, 1967). Samples were run in 6% acrylamide, 0.24% N,N'-methylenebisacrylamide gels in 0.1 M tris-HCl buffer, pH 8, in the presence of 0.1% sodium dodecyl sulfate.

¹²⁵I-labeled Group A carbohydrate The labeled carbohydrate was prepared by Dr. Thomas Kindt and myself according to an unpublished procedure of Dr. Emil Gotschlich. Fifty mg of Group A carbohydrate was dissolved in 10 ml H₂O. The pH was adjusted to 11 with 0.1 N NaOH and 10 mg of CNBr were added. The addition of the CNBr was performed during constant stirring and the pH was maintained at 11 for 5 minutes by the addition of 0.1 N NaOH. Fifty mg of tyramine HCl were added in 1.5 ml of 0.5 M NaHCO₃. The pH was adjusted to 8.5 and the mixture was allowed to stir overnight at room temperature. The tyramine-carbohydrate was then dialyzed against H₂O. The tyramine-carbohydrate was stored frozen in water. The addition of ¹²⁵I was as follows: 100 µl of 0.5 M Na₂HPO₄, pH 7.7 buffer was added to 20 µl of a solution containing 10 mg/ml of the tyramine-carbohydrate prepared as above. To this was added 0.5-1.0 mc of carrier-free ¹²⁵I and 10 µl of a solution containing 20 mg/ml of choramine-T in the pH 7.7 buffer. After 10 minutes, 50 µl of 100 µg/ml sodium hydrosulfite and 10 µl of 200 µg/ml KI were added.

The reaction mixture was then loaded onto a Sephadex G-50 fine column, 0.8 cm x 26 cm bed. The column was equilibrated with PBS and pre-treated by washing a few ml of 10% bovine serum albumin through it in the PBS buffer. Two peaks of radioactivity were obtained from the column. The first contained the ¹²⁵I-labeled carbohydrate and the second contained the unbound label.

Iodination of anti-Group A antibody The iodination of proteins with Na¹²⁵I was performed according to the procedure of McFarlane (1958), in the presence of iodine monochloride in a pH 8.5 glycine-NaOH buffer.

Radioimmunoassay for measurement of antibodies to Group A carbohydrate A modified Farr procedure (Farr, 1958), which employed a very large antigen excess as suggested by Osler (1971), was used to measure antibody to Group

A carbohydrate. Radiolabeled Group A carbohydrate was prepared by de-acetylation (Kristiansen *et al.*, 1969) followed by reacetylation with ^3H -acetic anhydride (Kuhn and Kirschenlohr, 1956). The acetylated material was passed over a Bio-Gel P-60 column in hemagglutination buffer, pH 7.2, to remove radioactive low-molecular-weight degradation products. A peak component corresponding to a molecular weight of about 10,000 was recovered. 98% of the radioactivity in this material was precipitable with an excess of rabbit anti-Group A antibody. The specific activity was about 17,000 cpm per microgram of rhamnose in the carbohydrate. The stock solution of labeled carbohydrate contained 4 million cpm/ml or about 270 μg CHO/ml.

The concentration of radioactive carbohydrate employed in the test was adjusted so that 1.0 ml of PBS contained 200,000 cpm. The solution containing the radioactive carbohydrate also contained bovine γ -globulin at a final concentration of 2.5 mg/ml as a carrier.

The test was carried out in a series of siliconized 6 x 50 mm glass tubes in which 5 μl of solution containing from 0.5 to 50 μg /ml of Group A antibody were added to 0.1 ml of the mixture of labeled carbohydrate plus bovine γ -globulin. After incubation at 4°C for 30 minutes, 0.15 ml of saturated ammonium sulfate at 4°C was added. After one hour at 4°C , the precipitates were recovered by centrifugation at $1500 \times g$ for 10 minutes. The precipitates were washed three times in 0.5 ml of 60% saturated ammonium sulfate. After the final wash, the precipitates were removed from the cold and dissolved at room temperature in 0.4 ml Soluene (Packard) and counted for 10 minutes in 5% Liquiflor (New England Nuclear)-95% toluene in a refrigerated scintillation spectrophotometer.

All samples were tested in duplicate. The number of counts per minute precipitated by the carrier alone was subtracted from the number of counts per minute obtained from each sample. A standard curve was constructed as described in the results, and was used to estimate the antibody concentration of the serum sample from the number of counts it precipitates in the assay.

Tube binding assay The tube binding assay was performed according to the procedure described by Askenase and Leonard (1970), as adapted for

idiotypic studies by Carson and Weigert (1972). The γ -globulin fraction of the rabbit idiotypic antiserum was isolated by passage over a DEAE-cellulose column equilibrated with 0.025 M sodium phosphate buffer, pH 7. The column fractions containing the rabbit γ -globulin were identified by microzone electrophoresis of each fraction. The microzone electrophoresis membranes were stained with coomasie brilliant blue.

The isolated rabbit γ -globulin was vacuum concentrated at room temperature in a collodion bag (Schleicher & Schuell No. 100), and restored to its original serum volume with PBS. The isolated idiotypic γ -globulin was stored frozen in aliquots. For use in the tube binding assay, the isolated idiotypic γ -globulin (antibody) was diluted 1/500 with phosphate-buffered saline containing 0.005 molar potassium phosphate, pH 7.2. One ml of this idiotypic antibody dilution was added to each of several 12x75 mm plastic disposable culture tubes (Falcon Plastics, tube 2058). The tubes were then incubated at room temperature for 4 hours to allow some of the antibody in the idiotypic serum to adhere to the side of the plastic tube. The tubes were then drained and rinsed once with the 0.005 M phosphate buffered saline. The tubes were then filled with several ml of 1% bovine serum albumin and incubated at room temperature. The tubes were emptied and filled with 1 ml of a solution of 1% BSA, 0.1% fresh normal mouse serum, 30,000 cpm of ^{125}I -labeled anti-Group A antibody, and various inhibitors all in the 0.005 M phosphate buffered saline. The tubes were incubated for 18 hours at 37°C, to allow time for the labeled anti-A antibody to be bound by the idiotypic antiserum coated on the inside of the plastic tubes. After this incubation, the tubes were emptied and rinsed three times with the 0.005 M phosphate-buffered saline. The labeled anti-Group A antibody which remained bound to the antiserum-coated tube was determined by counting the tubes in a gamma counter. In practice, this test was used as an inhibition assay, as described in the results.

Passive hemagglutination inhibition Freshly washed rabbit red blood cells were coated with isolated anti-A antibody by the chromic chloride method of Gold and Fudenberg (1967). It was found that isolated anti-Group A antibody preparations should be centrifuged (20,000 rpm for 30 minutes)

immediately prior to the coating procedure to prevent clumping of the cells during the coating process. The concentration of anti-Group A antibodies used was generally between 0.1 and 1.0 mg/ml. The solution containing these isolated antibodies also contained 1 mg/ml bovine serum albumin, which helped to stabilize the isolated antibody and prevent spontaneous clumping of the coated red cells.

Whenever whole antisera or whole ascites fluids were used to inhibit a hemagglutination reaction, they were first absorbed with an equal volume of rabbit cells coated with normal mouse serum or normal mouse ascites fluid, according to the procedure of Gold and Fudenberg (1967).

All titrations were performed in microtiter plates.

Identification of hemoglobin type by electrophoresis Mouse restricted and diffuse hemoglobulin types (Russel and Bernstein, 1966) could be distinguished by microzone electrophoresis of isolated mouse hemoglobin. Red cells from 0.075 ml of heparinized blood were washed in saline and lysed in 0.5 ml distilled water. To the 0.5 ml lysate was added 1.0 ml of the pH 8.6 veronal buffer used for microzone electrophoresis. Microzone electrophoresis was performed on this solution of hemoglobin for 20 minutes at a constant current of 5 milliamps, as described above, and stained with Ponceau S. In this manner it was possible to distinguish three genotypes: ss, single (or restricted hemoglobin); dd, diffuse hemoglobin; and sd, intermediate.

RESULTS

Specificity and quantitation of the radioimmunoassay for the anti-Group A antibodies For screening mouse antisera for anti-Group A antibody, a quantitative assay was needed that utilized very small amounts of serum and required as little time per sample as possible. The assay system chosen was a modification of the Farr antigen binding assay (Farr, 1958). In the traditional antigen binding assay (Farr, 1971) an approximately two-fold antigen excess is used. A difficulty with this procedure is that different antibody titers can be obtained for two antisera with the same antibody concentrations if they have antibodies with different avidities.

To minimize this problem, Osler (1971) has used very large antigen excesses to prevent formation of large antigen-antibody complexes in which several antibody molecules would be bound to each complexed antigen molecule. Under conditions of high antigen excess, the number of radioactive antigen molecules bound is directly proportional to the number of antibody binding sites in the test antiserum. The amount of antibody in the test sample of hyperimmune serum can then be estimated directly, by comparing its antigen binding capacity to that of a standard antiserum.

The antigen binding system developed here for use with anti-Group A carbohydrate antibodies is patterned after that of Osler (1971). Since Osler was studying the antibody response to bovine serum albumin he used ^{125}I bovine serum albumin as his antigen. For our purposes we prepared tritium labeled Group A carbohydrate as the antigen.

The optimal amount of labeled carbohydrate to be used in the assay was determined by the experiment shown in figure 5. Increasing concentrations of radioactive carbohydrate were added to a series of tubes containing a constant amount of a standard mouse anti-Group A serum. The amount of antiserum added to each tube contained 5 μg of anti-Group A antibody, as determined by a quantitative precipitin assay.

From figure 5, it can be seen that the amount of labeled carbohydrate bound to the antibody began to fall off when less than 20,000 counts per tube, or 0.5 μg of carbohydrate, were used. For this reason, 20,000 counts

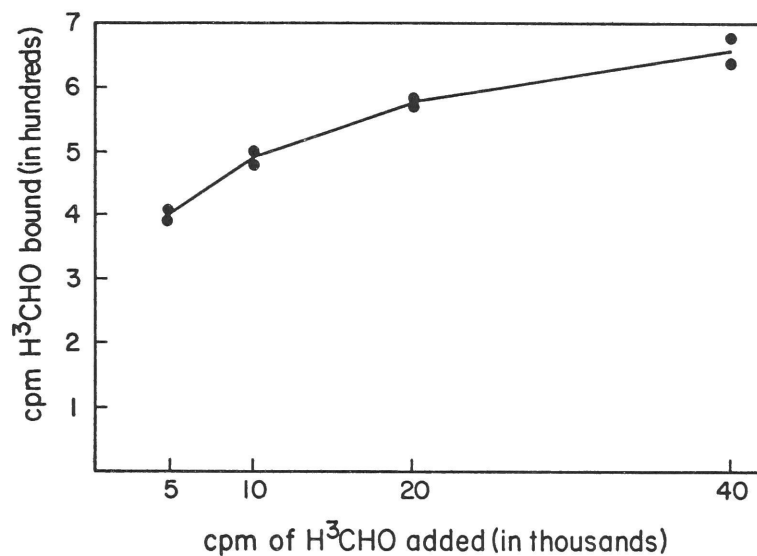


Figure 5. Relationship between the amount of 3H -labeled Group A carbohydrate added, and the amount bound by 5 μg of pooled anti-Group A antibody from F_1 (A/J \times SWR/J) mice. The antibody-antigen complexes in this experiment were precipitated with 50% saturated cold ammonium sulfate.

per tube were used in all subsequent experiments. It should be noted that this is over 30 times as much label as was bound by the 5 μ g of anti-Group A antibody.

The initial experiments were performed by precipitating the antigen-antibody complex with 50% saturated cold $(\text{NH}_4)_2\text{SO}_4$, since this concentration was used by Siskind *et al.* (1967) in a binding assay with mouse antisera. However, it was found that at $(\text{NH}_4)_2\text{SO}_4$ concentrations greater than 50% saturated, significantly higher specific binding was observed. All subsequent experiments were done at 60% saturated $(\text{NH}_4)_2\text{SO}_4$.

Several lines of evidence indicated that the antigen binding assay was specific for Group A antibody. The labeled carbohydrate, for example, was bound only to Group A antisera, and was not bound by normal mouse sera. Furthermore, as shown in figure 6, the binding of the labeled Group A carbohydrate to mouse anti-Group A antibody was inhibited by unlabeled Group A carbohydrate. There was minimal inhibition with unlabeled Group C carbohydrate. The concentration of the unlabeled Group A carbohydrate required for 50% inhibition was about 0.5 μ g/ml, or approximately 1/2.4 of the concentration of labeled carbohydrate in the test system. As shown in figure 7, it was also possible to inhibit the binding of the labeled carbohydrate by N-acetyl-D-glucosamine, the immunodominant determinant of the Group A carbohydrate (McCarty, 1958). Inhibition was not seen with corresponding amounts of N-acetyl-D-galactosamine, the immunodominant determinant of the Group C carbohydrate (Krause and McCarty, 1962).

Standard sera of known antibody content were needed in order to estimate, by the antigen binding assay, the concentration of antibody in unknown mouse sera. Three pools of mouse anti-Group A antibody were prepared from A/J, SWR/J, and SWR/JxA/J F₁ mice. The concentrations of precipitating antibody in each of these pools were determined by quantitative precipitation with unlabeled Group A carbohydrate. The antibody concentration for the three pools were respectively 2.75 mg/ml, 1.65 mg/ml, and 1.70 mg/ml.

Before using these three antiserum pools as standards, it was necessary to determine whether or not they contained large amounts of anti-IgGs which might distort the results of the assay. It has been shown by Bokisch

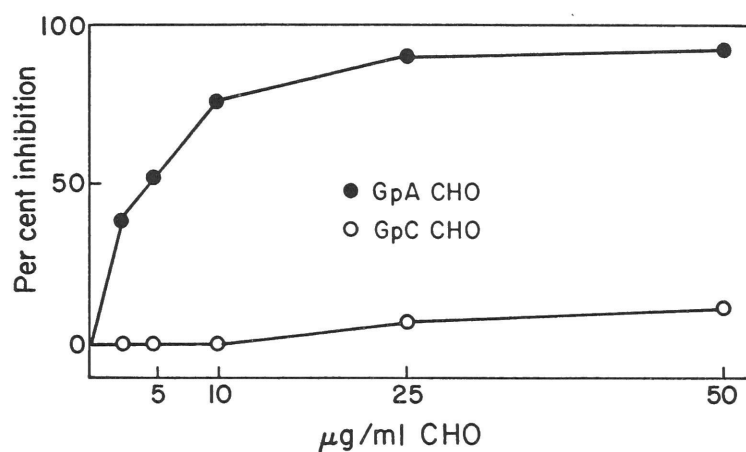


Figure 6. Inhibition by unlabeled Groups A and C carbohydrate of the binding of ^3H -labeled Group A carbohydrate to mouse anti-Group A antibody. In addition to the various inhibitors, each tube contained 20,000 cpm of labeled Group A carbohydrate and 3.4 μg of pooled A/J anti-Group A antibody.

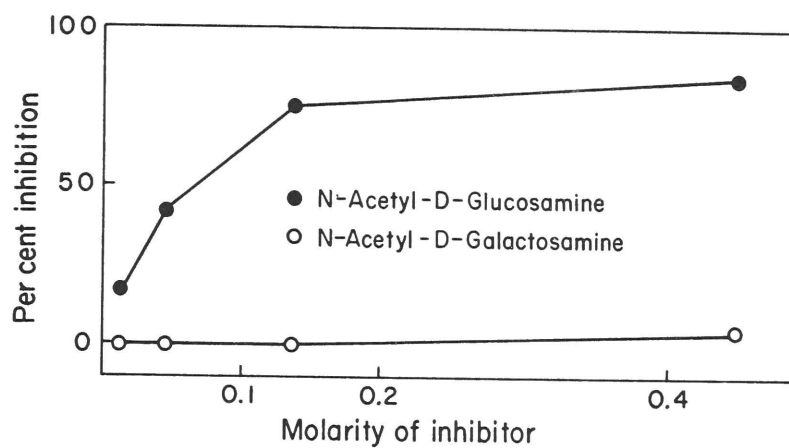


Figure 7. Inhibition by N-acetyl-D-glucosamine and N-acetyl-D-galactosamine of the binding of ^3H -labeled Group A carbohydrate to mouse anti-Group A antibody. In addition to the various inhibitors, each tube contained 20,000 cpm of labeled Group A carbohydrate and 3.4 μg of pooled A/J anti-Group A antibody.

et al. (1972) that rabbits immunized with streptococcal vaccines of the type used here developed high serum concentrations of 7S and 19S anti-IgG antibodies. They further observed that the anti-IgGs would co-precipitate with the anti-streptococcal antibodies in the quantitative precipitin test. This can frequently lead to an over-estimation of the amount of anti-streptococcal antibody in an antiserum.

A co-precipitation test was used to determine that the concentration of anti-IgGs in the mouse antisera was insufficient to lead to false estimates of the antibodies to Group A carbohydrate in mouse antisera. Immune complexes of human serum albumin and mouse anti-human serum albumin were incubated with the anti-streptococcal sera for 16 hours at 0°C. If the anti-streptococcal sera had contained any anti-IgG, it would have been expected to co-precipitate with the human serum albumin-anti-human serum albumin complexes. The protein content of the complexes, however, did not increase in the presence of the anti-streptococcal sera. From the results obtained, it was clear that less than 10% of the protein, in immune precipitates of the streptococcal antisera with Group A carbohydrate, could have been due to anti-IgGs.

By testing the standard antisera at various dilutions, it was possible to establish a linear relationship between antibody concentration and the amount of radioactive carbohydrate bound in counts per minute (figure 8). This experiment was performed by adding 5 μ l of antiserum dilutions containing between 0.05 and 3.0 mg/ml precipitating antibody, to a series of tubes to which had been added 1.2 μ g of H^3 -Group A carbohydrate.

In figure 8 the data are plotted in counts per minute versus antibody concentration of these 5 μ l aliquots. The values for the SWR/J and F₁ anti-A pools fall on the same straight line. The values for the A/J anti-A pools are consistently higher. This difference may be due to other factors which influence the determination of the Group A antibody by the quantitative precipitin test in the case of the A/J pool. It is possible, for example, that this serum contains nonprecipitating antibody which would not be detected by the quantitative precipitin test, but would be detected by the antigen binding assay. Nonprecipitating antibody to Group A carbohydrate is commonly seen in rabbit antisera (Krause, personal communication).

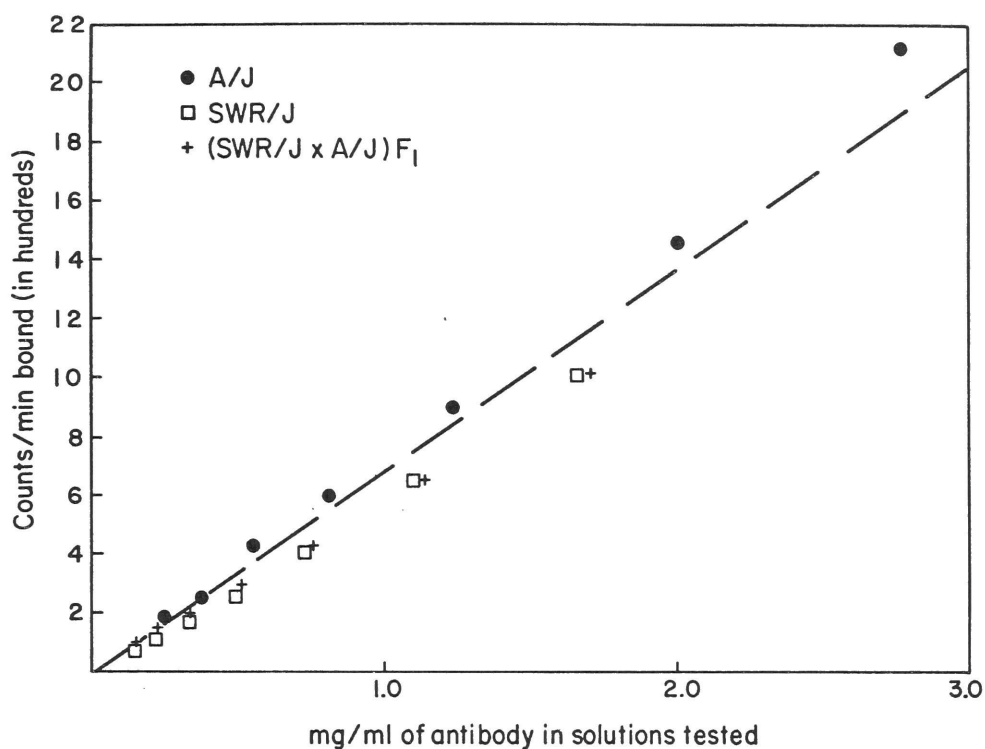


Figure 8. Counts per minute of ^3H -labeled Group A carbohydrate bound by antibody in 5 μl of various dilutions of pooled anti-Group A sera from A/J, SWR/J, and F_1 (A/J x SWR/J). The anti-Group A antibody concentrations of the 3 antisera pools, and hence the dilutions, were determined by quantitative precipitation with Group A carbohydrate. Each tube contained a total of 20,000 cpm of labeled Group A carbohydrate.

The linear relationship between the amount of antibody added and the amount of antigen bound was observed over a range of 0.2 to 3.0 mg of antibody per ml. A serum with 29 mg of precipitating antibody was used to determine the higher range of linearity. As shown in figure 9, dilutions of this serum containing 13 mg/ml or less antibody bound amounts of radioactive antigen which fell on a linear plot. Because of the absence of linearity above 13 mg/ml, all sera with antibody levels in the upper ranges were diluted four-fold for testing with the antigen binding assay.

Since the relationship between antibody concentration and antigen binding is linear, only a single dilution of the standard was run with each set of samples. The amount of antibody in the unknown samples was calculated as follows:

$$\text{ANTIBODY}_{\text{unknown}} = \frac{\text{CPM}_{\text{unknown}}}{\text{CPM}_{\text{standard}}} \times \text{ANTIBODY}_{\text{standard}}$$

Dose response studies In rabbits, it has been possible to use heat-killed streptococcal vaccines to elicit the production of anti-Group carbohydrate antibodies (Lancefield, 1928) with greatly restricted heterogeneity. Antibody concentrations ranged from 10-60 mg of antibody per ml (Krause, 1970). In this thesis, various experiments are described which make use of mouse anti-streptococcal Group A antibodies with restricted electrophoretic mobility. Thus a prerequisite of these studies was the development of an adequate means of immunization, and the identification of inbred strains that produce such antibody. To obtain this information, dose response studies have been conducted throughout these investigations, with various inbred strains, in an effort to optimize the immunization procedures. The most satisfactory results obtained to date have been with intravenous (i.v.) immunizations in 30-week-old female A/J mice (figures 10-14).

Figure 10 shows the antibody titers obtained by giving mice 4 intravenous injections with various dosages of vaccine. The injections were given once every two weeks and the mice were bled 10 days after the last

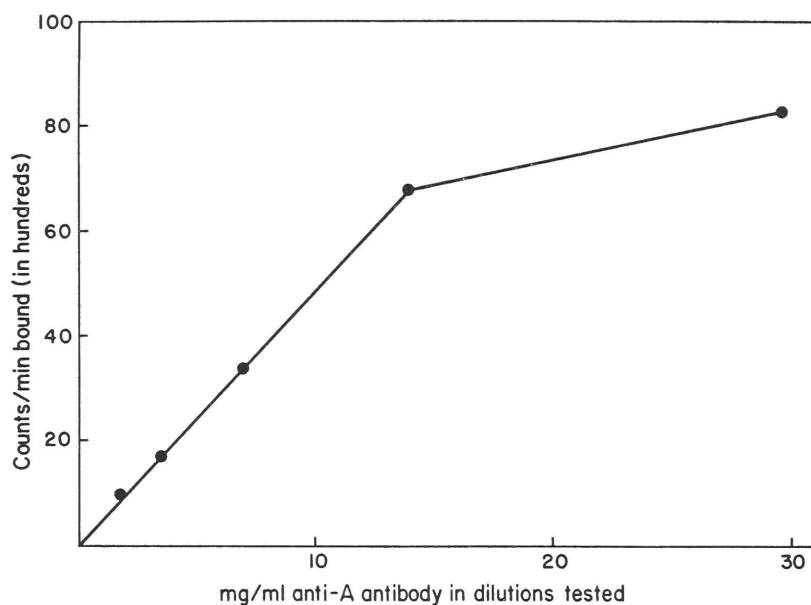


Figure 9. Counts per minute of ^3H -labeled Group A carbohydrate bound by antibody in 5 μl of various dilutions of an anti-Group A serum from a single A/J mouse. The anti-Group A antibody titer of this serum was found to be 29.5 mg/ml, by comparing the number of counts bound by this serum with the number of counts bound by a standard mouse anti-Group A serum tested in the same assay. Each tube contained a total of 20,000 cpm of labeled Group A carbohydrate.

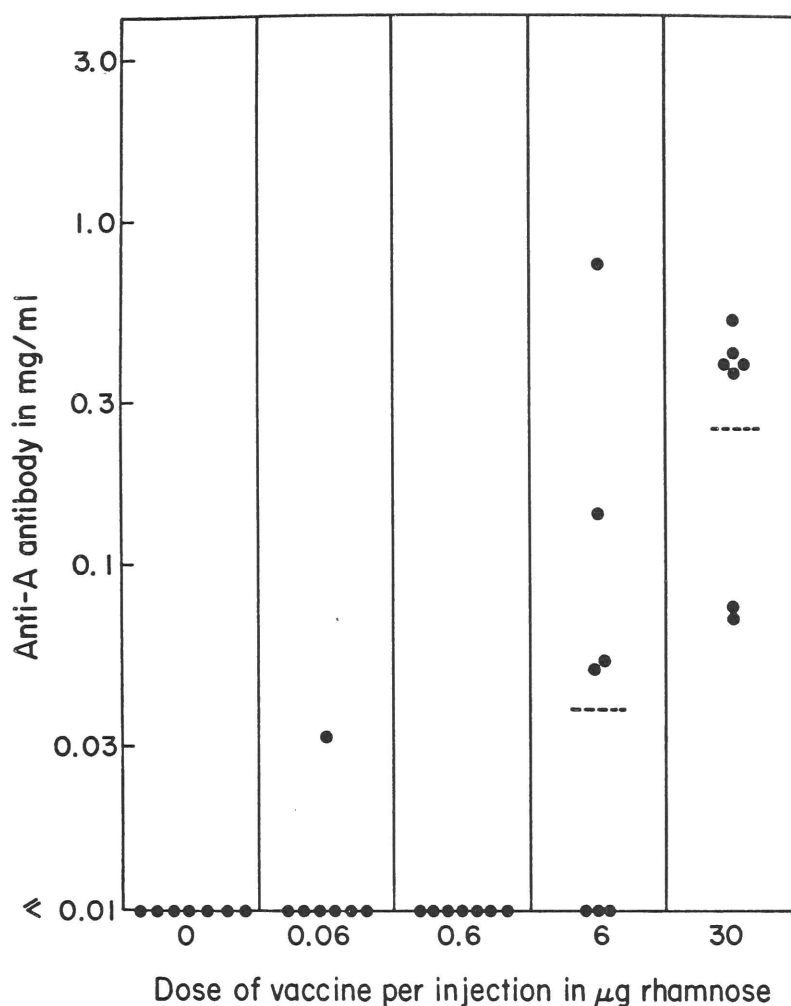


Figure 10. Anti-streptococcal Group A carbohydrate antibody responses of female retired breeder strain A mice as determined by the antigen binding assay. All mice were approximately 30 weeks old at the start of immunization. Four intravenous injections of various doses of streptococcal vaccine were given once every two weeks, and the mice were bled 10 days after the last injection. The antibody concentrations of these sera are depicted above. The horizontal dashed lines are the geometric mean responses for each group. The mice were then allowed to rest for 1 month and reinjected with a single intravenous injection containing the same dosage as before. Ten days after this booster injection, the mice were bled and the amount of anti-Group A antibody was determined. The antibody titers for the booster injection are depicted in figure 11.

injection. The dose of vaccine given per injection is expressed as the amount of rhamnose contained in the vaccine, since rhamnose comprises 60% of the Group A carbohydrate (Krause and McCarty, 1961). It was observed that only those mice injected with doses of vaccine containing 13 μ g of rhamnose or greater made significant quantities of antibody. In fact, a higher proportion of mice who received vaccine doses containing 30 μ g of rhamnose made antibody than those whose vaccine doses contained only 6 μ g of rhamnose.

The mice were allowed to rest for one month and then reinjected with one i.v. injection containing the same dosage as before. Ten days after this single booster injection the mice were bled. The antibody concentration for each mouse is shown in figure 11. After the booster immunization, the antibody levels increased in all of the mice that had previously made antibody. Many of the mice that had received low dosages of vaccine in the first immunization and had not produced detectable antibody, began to make antibody after the booster injection. The highest titers were observed in the mice who had received the largest doses of vaccine. The seven control mice that were not immunized during the experiments never made any detectable antibody.

Microzone electrophoresis patterns of the sera from the mice injected with the highest dosage of vaccine were indicative of the production of antibodies with restricted heterogeneity. This type of microzone pattern was not seen in the sera of mice receiving vaccine dosages containing less than 6 μ g of rhamnose. The topic of restricted antibody heterogeneity in these and other A/J antisera will be taken up in more detail later.

Braun et al. (1972) reported that intraperitoneal (i.p.) immunizations of streptococcal Group A vaccine yielded high titers of antibody in Balb/cJ mice. In pilot experiments using SWR/J and A/J mice, we observed that with vaccine dosages containing 6 μ g or less rhamnose per injection, the antibody titers resulting from i.p. injections were generally equal to or slightly greater than those obtained when equal doses were given i.v. Because of the comparative speed and reproducibility with which i.p. injections can be performed, it was of interest to find out whether or not the

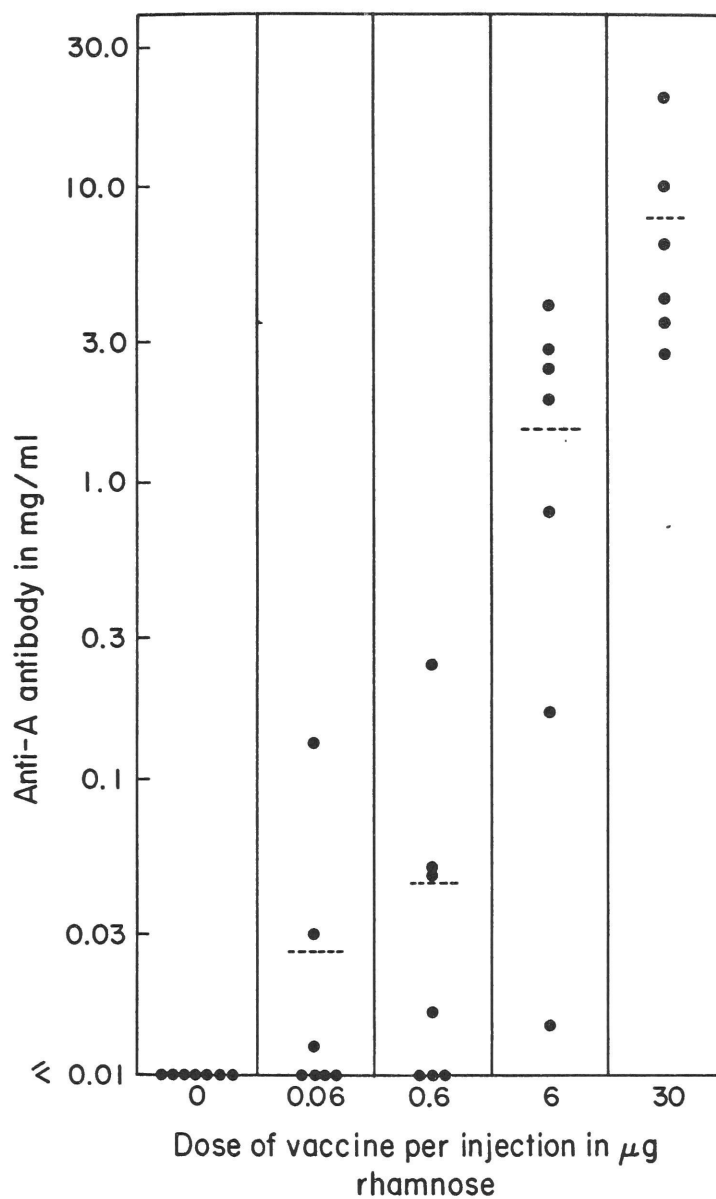


Figure 11. Antibody titers 10 days after booster injection (see figure 10).

high-titer, restricted-heterogeneity antibody responses readily obtained with high i.v. doses of vaccine could also be obtained if the vaccine were given i.p.

An experiment to test this possibility was set up, using A/J mice. The immunization schedule followed is given in Table III. The dosages of vaccine per injection were 3, 30, and 300 μ g of rhamnose. This experiment differed from the previous one in that animals receiving the higher doses were gradually raised to those doses over the first several injections. As a control, some of the mice given the 30 μ g dosage were injected i.v. The resulting antibody titers of these mice are shown in figure 12. In examining the data it is clear that the mice given i.v. immunizations responded much better than those injected i.p. The 300 μ g dose i.p. appeared to be too high, since three of the five mice died after the second booster injection. The two surviving mice had titers of 0.8 and 6.5 mg/ml antibody, well within the range observed for the other i.p. injected mice.

The mice injected i.v. responded even better than those in figure 11 which had been injected with the same lot of vaccine at the same maximum dosage. Three factors may have influenced this enhancement of the response: 1) the rest period was extended by 17 days, 2) the initial injection was reduced to 3 μ g to try to decrease any possibility of partial tolerance induction, 3) two booster injections were given instead of one. Which, if any, of these factors may have enhanced the response has not yet been determined.

Microzone electrophoresis of the antisera of 5 mice given i.v. injections with doses of vaccine containing 30 μ g of rhamnose are shown in figure 13. To be noted are the prominent γ -globulin bands which were later shown to be composed of anti-Group A antibody.

While these experiments were in progress, Klaus Eichmann (1972) reported that he had been able to achieve anti-Group A streptococcal antibody responses of up to 40 mg/ml in strain A mice by using a slightly different immunization protocol. An attempt was made to duplicate his findings as closely as possible to see how his method would compare with our own.

TABLE III

Immunization Schedules for A/J Mice in Figure 12

| | | Dose of vaccine in μ g rhamnose | | | | | |
|-----------------------------|------|-------------------------------------|----|-----|-----|-----|--------------|
| | | i.p. | 3 | 3 | 3 | 3 | 3 |
| Route of Immunization | i.p. | 3 | 30 | 30 | 30 | 30 | Mice Bled |
| | i.v. | 3 | 30 | 30 | 30 | 30 | |
| | i.p. | 3 | 30 | 300 | 300 | 300 | |
| Days after 1st injection | | 0 | 7 | 14 | 61 | 64 | 75 |

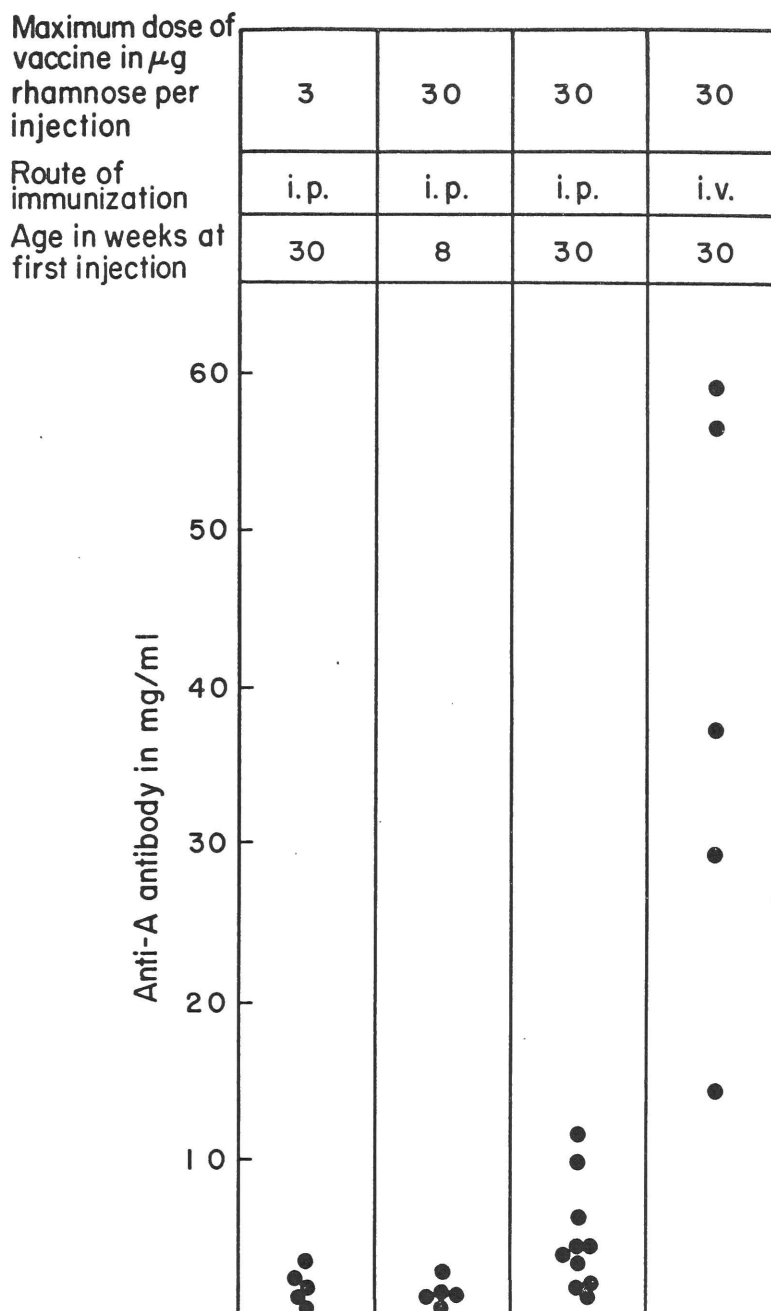


Figure 12. Anti-streptococcal Group-A carbohydrate antibody responses of female strain A mice as determined by the antigen binding assay. The mice were given various dosages of Group A vaccine according to the appropriate immunization schedule as listed in Table III. The mice were bled 11 days after the last immunization. In this experiment five mice were given i.p. injections of an amount of vaccine containing 300 μg of rhamnose. Three of these mice died after the booster injection and the two others (not shown) had antibody titers of 0.8 and 6.5 mg/ml.

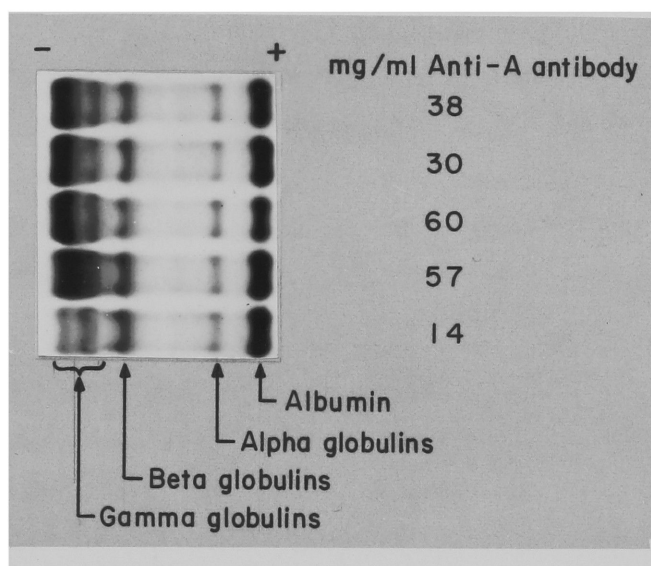


Figure 13. Microzone electrophoresis patterns of whole serum from five female A/J mice that had been immunized with Group A streptococcal vaccine. All five mice were given i.v. injections of vaccine containing 3 μ g of rhamnose in the first injection and an amount of vaccine containing 30 μ g of rhamnose in the remaining 4 injections according to the schedule described in Table III. The mice were bled 11 days after the last injection. The antibody titers of these sera, as determined by the antibody binding assay, are depicted in the right-hand column of figure 12 as well as listed above.

Seven- to eight-week-old A/J mice and 30-week-old retired breeder A/J and SWR/J mice were used in this experiment. All of the mice used in our previous experiments were also "retired breeders", about 30 weeks old. Some of the mice in each age group were injected i.p. to see if the difference in response between i.v. and i.p. injections would be observed with this protocol also. The mice were all injected 5 times over a period of two weeks. They were then allowed to rest for one month, reinjected 5 times over two more weeks and bled 11 days after the last injection. The dose of vaccine in each injection was 13.5 μ g of rhamnose. Thus this protocol differs from the one I developed in that it calls for twice as many injections and administers slightly less than half as much vaccine per injection.

The results obtained were somewhat unexpected (figure 14). The only mice that made really high levels of antibody were the 30-week-old A/J mice that had been injected i.v. All three of them made over 20 mg/ml of antibody. The six 8-week-old A/J mice injected i.v., interestingly enough, had the same low levels as were produced by both the 8- and 30-week-old mice that had been injected i.p.

This apparent requirement for the use of older mice to obtain high levels of anti-A antibody production is consistent with the fact that the high-responding mice in the previous experiments (figures 11 and 12) were all at least 30 weeks old at the time of their first injection.

Thus, taking all three of these dose response experiments together, it appears that for an optimal response in A/J mice, the dose of vaccine per injection must be large enough to contain at least 13 μ g of rhamnose. The injections should be given intravenously, and aged, 30-week-old mice should be used. While the apparent need to use aged mice certainly warrants further study, these older mice appear to be the best bet if one is trying to obtain high titers of anti-Group A antibody in strain A mice.

Some dose response data using comparable dosages have also been obtained with "retired breeder" SWR/J mice (figures 14 and 15). In one experiment (figure 15), the doses of vaccine and the immunization schedule were exactly the same as for the A/J mice in figure 12 (see Table III).

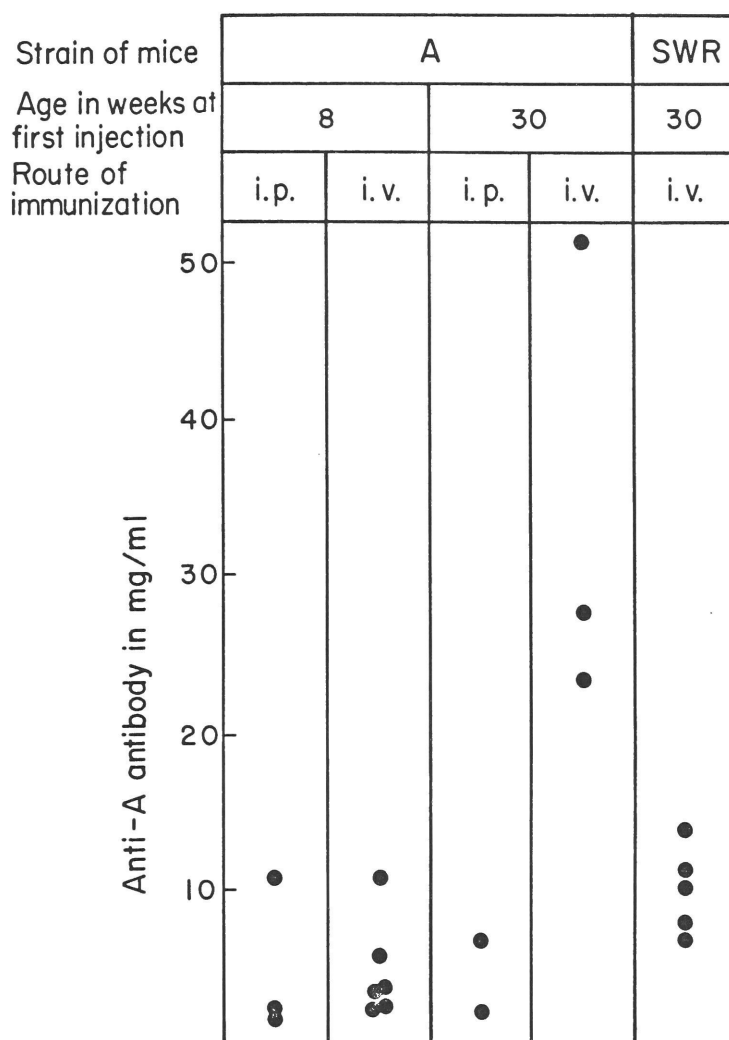


Figure 14. Anti-streptococcal Group A carbohydrate antibody responses of female SWR/J and A/J mice, as determined by the antigen binding assay. All mice were injected 5 times over a period of 2 weeks, rested for 1 month and given 5 more injections over a period of 2 weeks. The mice were bled 11 days after the last injection. At each injection the mice were injected with a quantity of vaccine containing 13.5 μ g of rhamnose.

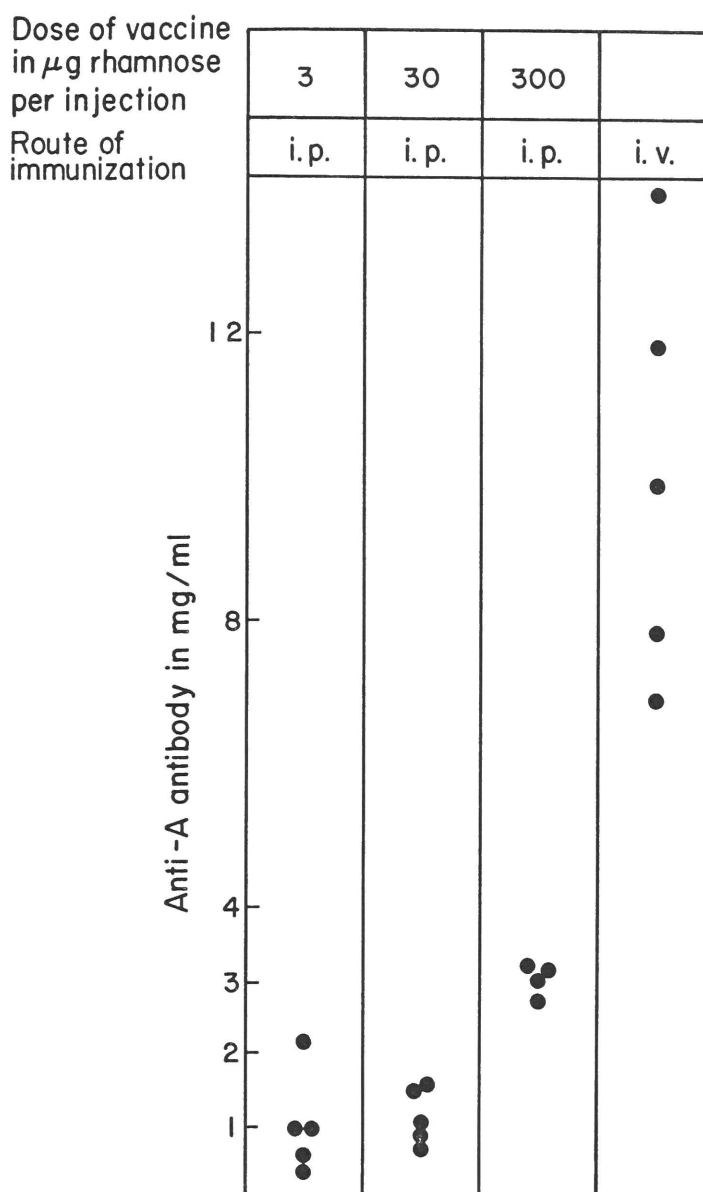


Figure 15. Anti-streptococcal Group A carbohydrate antibody responses of female SWR/J retired breeder (30-week-old) mice, as determined by the antigen binding assay. The mice in the three left-hand columns were given i.p. immunizations of Group A vaccine according to the appropriate dosage and immunization schedule listed in Table III. The data in the right-hand column are re-plots of the responses of the SWR/J mice in figure 14.

All of the SWR/J mice in this experiment were injected i.p. In contrast to the A/J mice, only one of the five SWR/J mice died after receiving the highest dose of vaccine. In general, the higher the dosage given, the higher the antibody response that was observed. However, none of the mice had antibody titers above 4 mg/ml.

In a separate experiment (see figure 14), 5 SWR/J mice were given i.v. injections of vaccine. These SWR/J mice gave a much higher response (7-14 mg/ml antibody) than those immunized i.p. The responses of these 5 i.v. immunized mice have also been plotted in figure 15 for comparison.

Two of these i.v. immunized SWR/J mice showed restricted-heterogeneity antibody components by microzone electrophoresis. These fragmentary results with SWR/J mice suggest that these mice also have higher antibody responses after i.v. than after i.p. immunization.

Figure 14 also demonstrates that under these immunization conditions, A/J mice generally produce more antibody than do SWR/J mice. This difference has also been observed in other experiments.

Evidence for production of mouse anti-A antibodies with restricted heterogeneity Many of the experiments described in this thesis were performed before it had been determined that the highest antibody responses were obtained with 30-week-old A/J mice given intravenous dosages of vaccine containing at least 13 μ g of rhamnose per injection. However, even before these immunization conditions had been established, mouse anti-streptococcal Group A antibodies with restricted electrophoretic heterogeneity had been observed (Briles, 1971).

In one of the earlier dose response studies, 100 eight-week-old A/J mice were given two series of injections with one month's rest in-between. The number of injections given to different mice varied from 5 to 13, and the doses of vaccine per injection were adjusted to contain 2, 6, or 12 μ g of rhamnose. In this experiment there was little correlation between the immunization schedule used and the fraction of mice making antibody of restricted heterogeneity. Figure 16 contains microzone electrophoresis patterns of the antisera from 3 of the 100 mice. The top pattern in the figure had the most prominent immunoglobulin component of all the 100

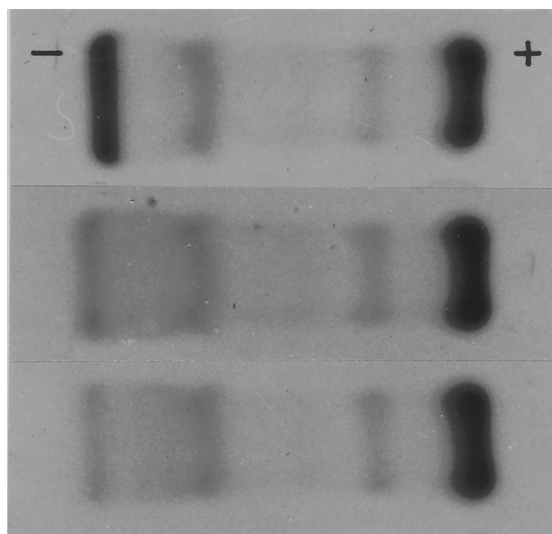


Figure 16. Microzone electrophoresis of sera from three A/J mice immunized i.v. with Group A vaccine as described in the text. The slow migrating band to the left in each serum could be shown to be antibody by absorption with Group A vaccine.

antisera. Five other sera were observed which gave an electrophoretic pattern comparable to the third serum. None of the 82 other sera showed evidence of antibody components restricted to a single electrophoretic band.

Absorption experiments utilizing whole vaccine or isolated streptococcal cell walls were employed to demonstrate that the monodisperse immunoglobulin components in A/J and SWR/J antisera were antibody to Group A carbohydrate. A typical experiment for a serum of a single A/J mouse is depicted in figure 17.

Absorption of the serum with streptococcal Group A cell walls removed the slow migrating γ -globulin component, whereas absorption with Group C cell walls (not shown in figure 17) did not. Further evidence that the slow-migrating material was anti-Group A antibody was obtained by eluting the material from the cell walls with 10% N-acetyl-D-glucosamine, the immunodominant determinant of Group A streptococcal carbohydrate (McCarty, 1958). The antibody eluted from the cell walls was identified as immunoglobulin by immunoelectrophoresis (figure 18). The whole mouse anti-streptococcal serum, the isolated antibody, and the cell-wall-absorbed antiserum were placed in the wells in the upper frame of figure 18. After electrophoresis, the troughs were cut and filled with anti-whole mouse serum and anti-mouse immunoglobulin. The isolated antibody gave one predominant arc with anti-mouse immunoglobulin. Furthermore, the isolated antibody gave only an immunoglobulin reaction with anti-whole mouse serum.

Further evidence that the eluted immunoglobulin was antibody to Group A carbohydrate was obtained by an additional experiment with this immunoelectrophoretic pattern. The immunoelectrophoretic slide was washed in buffered saline for 24 hours to remove any unprecipitated antibodies. The troughs were then filled with ^{125}I -labeled Group A carbohydrate in PBS. The slide was incubated for 24 hours at 4°C to allow the anti-Group A antibodies bound in the immunoelectrophoresis precipitin lines to bind the labeled carbohydrate. After this, the slides were then washed again in buffered saline for 24 hours, followed by a 24-hour wash in distilled water. The slides were then dried and placed in contact with photographic plates for autoradiography.

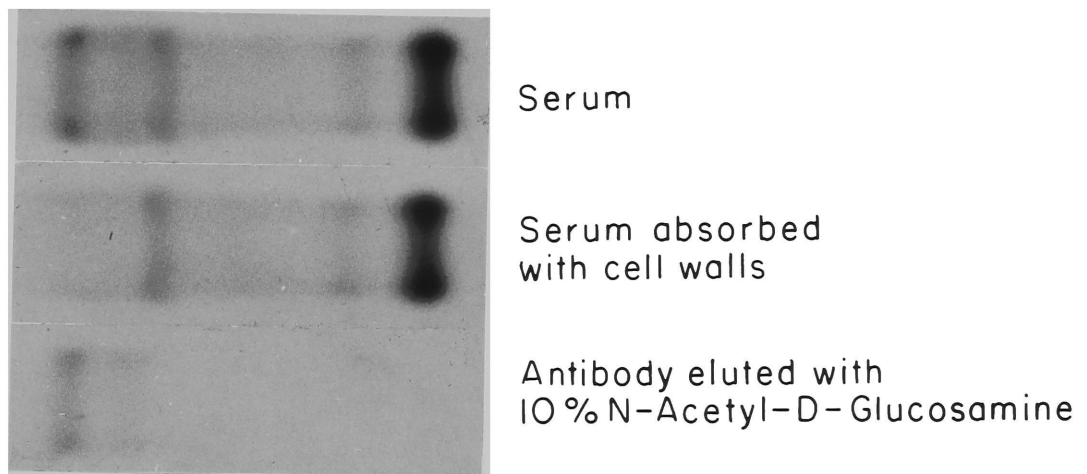


Figure 17. Microzone electrophoresis patterns of absorbed and unabsorbed anti-Group A serum from an A/J mouse immunized with Group A streptococcal vaccine. Serum was absorbed with Group A cell walls and the antibody was eluted from the cell walls with 10% (0.46 M) N-acetyl-D-glucosamine.

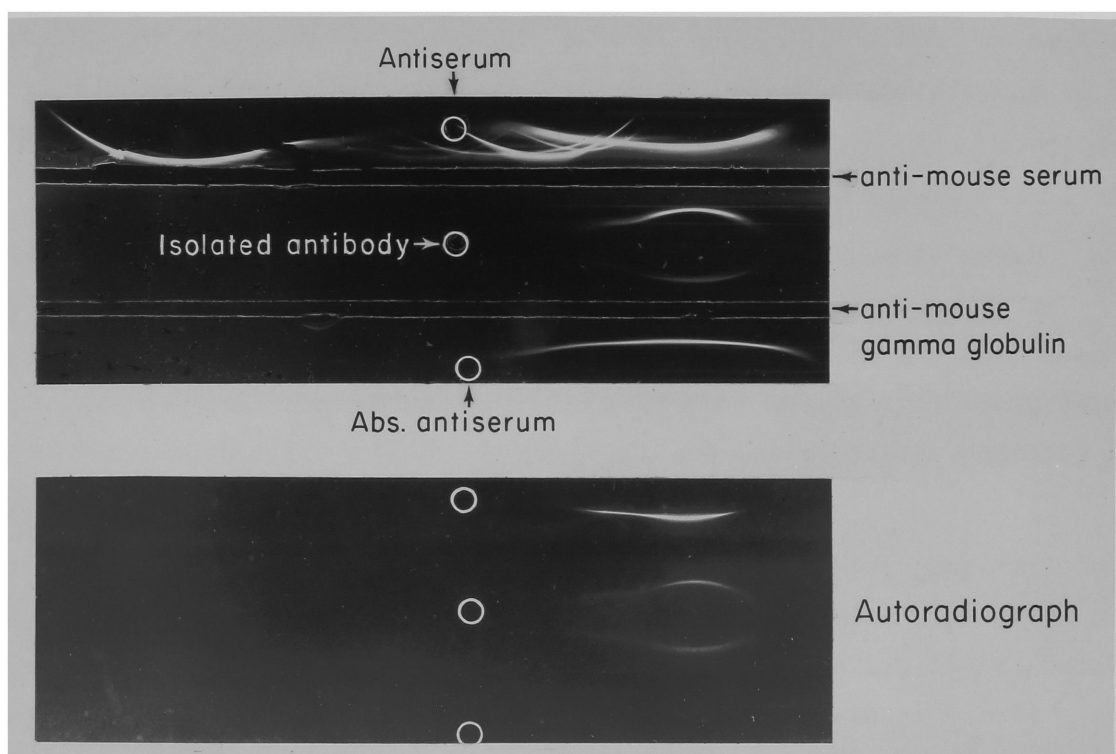


Figure 18. Immunoelectrophoresis of the same mouse anti-Group A serum, isolated antibody, and absorbed anti-Group A serum, as shown in figure 17. The cathode is to the right. The antisera in the troughs were rabbit anti-whole mouse serum and rabbit anti-mouse γ -globulin. After immunoelectrophoresis the slide was washed in PBS and the troughs were filled with about 1 μc of ^{125}I -labeled Group A carbohydrate in PBS. After a 24 hour incubation at 4°C , the slide was washed, dried, and autoradiographed.

The lower frame of figure 18 is the resulting autoradiograph, which shows that the immunoglobulin precipitin arcs formed by the whole anti-streptococcal serum and the eluted antibody have bound the labeled carbohydrate. The specificity of the binding is demonstrated by the fact that immunoglobulin arcs of the absorbed anti-streptococcal serum, as well as the arcs produced by other serum proteins, did not bind the labeled antigens.

The degree of restricted heterogeneity of antibody isolated from vaccine was further characterized by disc gel electrophoresis after reduction and alkylation. Alkaline urea disc gel electrophoresis of reduced and alkylated antibody (Reisfeld and Small, 1966) has been used previously to study the light and heavy chains of rabbit anti-bacterial carbohydrate antibodies exhibiting restricted electrophoretic mobility (Krause, 1970).

When reduced and alkylated rabbit γ -globulin antibody preparations were analyzed by this technique, there is generally a good separation of the slower migrating heavy chains from the faster migrating light chains (figure 19-c). The light chains from relatively homogeneous rabbit antibodies were resolved into one or more distinct bands, most commonly a major band with one or more minor bands alongside (Krause, 1970). When this procedure was applied to mouse antibodies, somewhat different patterns were obtained than had been seen with reduced and alkylated rabbit antibodies.

In figure 19-b it can be seen that the light and heavy chains of reduced and alkylated pooled mouse γ -globulins do not separate into distinctly different domains of the gel after alkaline urea disc gel electrophoresis. By SDS electrophoresis it was possible to show that this was not the result of incomplete separation of the mouse light and heavy chains by the reduction and alkylation step. This was demonstrated by running reduced and alkylated mouse γ -globulins, reduced and alkylated mouse anti-Group A antibody, and reduced and alkylated rabbit γ -globulins in 0.1% SDS gels. Both the rabbit and mouse gels gave identical light and heavy chain bands. Furthermore, by running lysozyme and human serum albumin

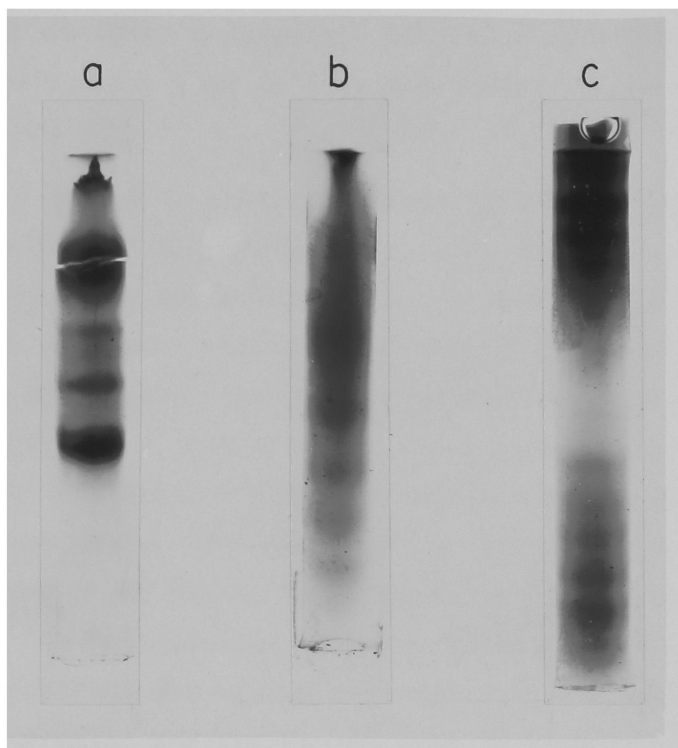


Figure 19. Alkaline urea disc electrophoresis of reduced and alkylated immunoglobulins: isolated mouse anti-Group A antibody (a); pooled mouse immunoglobulin (b); pooled rabbit immunoglobulin (c). In the rabbit pool the lower banding pattern is light chains and the upper pattern is heavy chains.

as molecular weight markers, it was shown that the light and heavy chain bands corresponded to around 20,000 and 50,000 daltons respectively, a result consistent with the well-characterized molecular weights of light and heavy chains (Cohen and Porter, 1964).

Many of the reduced and alkylated mouse anti-Group A antibodies with restricted electrophoretic mobility exhibited a much simpler banding pattern in alkaline urea gels than did reduced and alkylated pooled mouse γ -globulin. Many of these reduced and alkylated anti-Group A antibodies exhibited one predominant band in what was thought to be the light chain region of the gels.

The alkaline urea disc electrophoresis pattern of one such reduced and alkylated isolated SWR/J antibody is shown in figures 19 and 20. In this pattern there are a few major bands rather than the large number of overlapping minor ones seen with reduced and alkylated mouse pooled γ -globulin. The electrophoretic pattern of the sample exhibits a single major fast migrating band, and a single major slow migrating band. The pattern also shows two lighter staining intermediate bands and a general background of light stain. To determine the light and/or heavy chain identity of the two major bands, two disc gels of the same sample were run in parallel. One was stained (figures 19 and 20) and the other was cut into 0.5 cm sections and eluted overnight with 1% SDS in 10 M urea. When the eluted material was run on SDS gels in parallel with some of the original reduced and alkylated antibody (figure 20), it became evident that the fast migrating band in the alkaline urea gel was primarily composed of light chains as had been expected. The slow migrating band appeared to be composed primarily of heavy chains. The experiment also indicated that there was a considerable overlap between the banding patterns of contaminating mouse light and heavy chains in the sample.

This overlap in the disc gel banding patterns of light and heavy chains was also observed when alkaline urea disc gels of reduced and alkylated pooled mouse γ -globulin were sliced, eluted, and the eluted materials run on SDS gels.

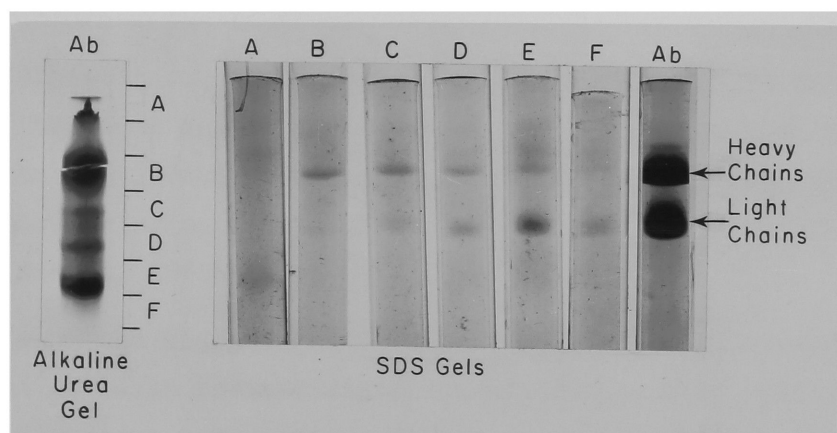


Figure 20. Electrophoresis of reduced and alkylated anti-Group A antibody (Ab) isolated from an immunized SWR/J mouse, on an alkaline urea and 0.1% SDS gels. An unstained alkaline urea gel, run in parallel with the stained alkaline urea gel shown, was sliced into 0.5 cm slices. The slices were eluted with 1% SDS in 10 M urea. The eluted protein was run on 0.1% SDS gels A-F in parallel with the original material, as shown.

Thus, as had been seen with rabbit anti-streptococcal carbohydrate antibody (Krause, 1970), the SWR/J antibody studied above appeared to have predominantly a single light chain. The heavy chains also appeared to migrate as a major band consistent with the presence of a single heavy chain in the sample.

However, because mouse light and heavy chains do not migrate in distinct domains in alkaline urea gels during electrophoresis, the technique has not been used for a general screening of isolated mouse anti-A antibodies.

The interpretation of the electrophoretic data is that most of the antibody in the mouse antisera is the product of predominant clones of antibody-producing lymphocytes. This also appears to be the case in the much more thoroughly documented studies with rabbit anti-streptococcal antibodies (Krause, 1970b; Hood et al., 1970).

In spite of the high degree of inbreeding in the mice used, anti-Group A antibodies of at least several different electrophoretic mobilities were observed in different individuals of the A/J and SWR/J inbred lines. This is illustrated in figures 21 and 22. Figure 21 shows the microzone electrophoresis patterns of seven different A/J anti-Group A sera. The electrophoretic mobilities of the predominant slow migrating anti-Group A antibodies (at the right in each pattern) almost form a continuum from fast to slow electrophoretic mobilities.

This same point is illustrated in figure 22, using isolated anti-Group A antibody from three different SWR/J mice. This test was repeated several times and the electrophoretic mobility of antibody preparation A was consistently intermediate to that of preparations B and C. Similar findings have been obtained with other SWR/J and A/J anti-Group A sera. It therefore appears certain that mice within inbred strains are capable of making different anti-A antibodies, but the number with different idiotypic specificities has yet to be determined.

Genetic control of the immune response to Group A carbohydrate Early in our studies on the anti-Group A response, we were not aware that immunization with doses of vaccine containing at least 13 $\mu\text{g/ml}$ rhamnose had to

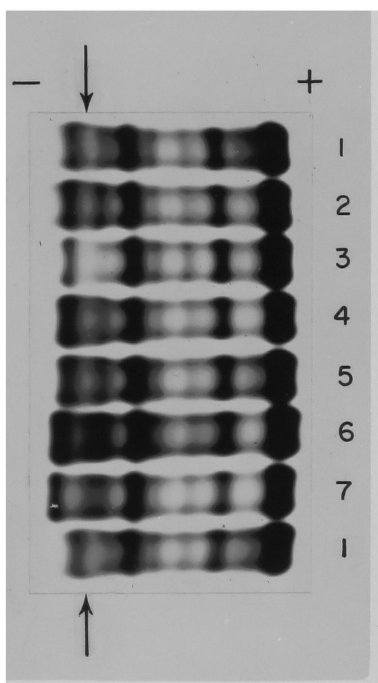


Figure 21. A stained microzone electrophoresis membrane of anti-Group A sera from 7 different A/J mice immunized with Group A vaccine. Serum #1 has been positioned at both the top and bottom of the membrane. By subsequent absorption, it was possible to demonstrate that the densely staining bands at the left are anti-Group A antibody.

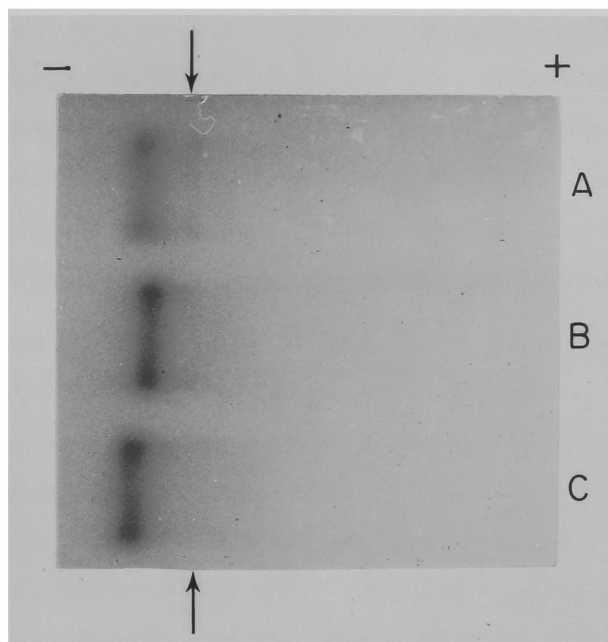


Figure 22. Microzone electrophoresis of 3 different anti-Group A antibodies isolated from the immune sera of 3 SWR/J mice. Preparation A came from an immunized normal SWR/J mouse. Preparation B came from mouse #4 of the first passage of cell line 3. Preparation C came from mouse #1 of the second passage of cell line 1.

be given to get an optimal response. Our preliminary dose response studies had been done with Balb/cJ, BSVS, and BRVR mice, all of which turned out to be very poor responders to Group A carbohydrate. None of these mice showed an increase in their antibody response with vaccine dosages of greater than 6 $\mu\text{g}/\text{ml}$.

On the basis of those early dose response studies, other inbred strains were immunized in a search for inbred mice producing higher titers of anti-Group A antibody. Mice from 9 inbred strains were immunized with Group A vaccine according to the schedule in Table IV. Although each mouse was given 15 injections over a period of 127 days, the vaccine dose in none of the injections contained more than 6 μg of rhamnose. From the dose response studies described earlier in the thesis, it is now apparent that the mice in this experiment were given a sub-optimal level of immunization.

All mice were five weeks of age at the first injection and were immunized with the same lot of vaccine on the same days. The mice were all bled 10 days after the last injection and their antibody titers were determined by the antigen binding assay. The geometric mean responses of these mice are given in Table V. Individual data for mice from 7 of the inbred strains is depicted in figure 23. The mean serum antibody concentrations of the various strains varied from a high of 1.9 mg/ml for the SJL/J mice to a low of 0.037 mg/ml for the Balb/cJ mice. Many of the differences in mean response were statistically significant by the Student's T test. For example, the difference between the mean of the SJL/J mice and that of the C3H/J mice is statistically significant at the 0.1% level. The mean of the C3H/J mice is in turn statistically different from that of the DBA mice at the 2% level. The mean response of the A/J mice is statistically significant from that of both the Balb/cJ and the SJL/J mice at the 5% level. Other experiments have confirmed this latter comparison, showing that with an immunization similar to that described here, SJL/J mice have a higher mean titer than the A/J mice which in turn have a higher titer than Balb/cJ mice. The data also demonstrate that within at least some of the strains, such as C57Bl/6J and Balb/cJ, there is considerable variation in the antibody responses of individual mice. The quantitative differences in antibody response of these strains are indicative of genetic differences in their ability to respond to the immunization procedure used.

TABLE IV

Injection Schedule for Mice in Figure 24 and Table V

| | | | | | | | | | | | | | | | | |
|-------|-----|-----|-----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|------|
| Day | 0 | 3 | 6 | 12 | 16 | 41 | 44 | 51 | 54 | 58 | 104 | 108 | 116 | 123 | 127 | 137 |
| Dose* | 1.5 | 1.5 | 1.5 | 3 | 3 | 6 | 6 | 6 | 6 | 6 | 4.6 | 6 | 6 | 6 | 6 | bled |

*vaccine dose in μg of rhamnose per injection

TABLE V

Anti-Group A Response of Inbred Mice

| Inbred Strain | No. of Mice | Mean Titer (geometric) $\mu\text{g/ml}$ |
|---------------|-------------|---|
| SJL/J | 6 | 1860 |
| C3H/J | 6 | 515 |
| C57B1/6J | 6 | 469 |
| A/J | 6 | 428 |
| SWR/J | 3 | 410 |
| C57Br/cdJ | 5 | 290 |
| DBA/J | 5 | 270 |
| BRVR | 4 | 142 |
| Balb/cJ | 6 | 37 |

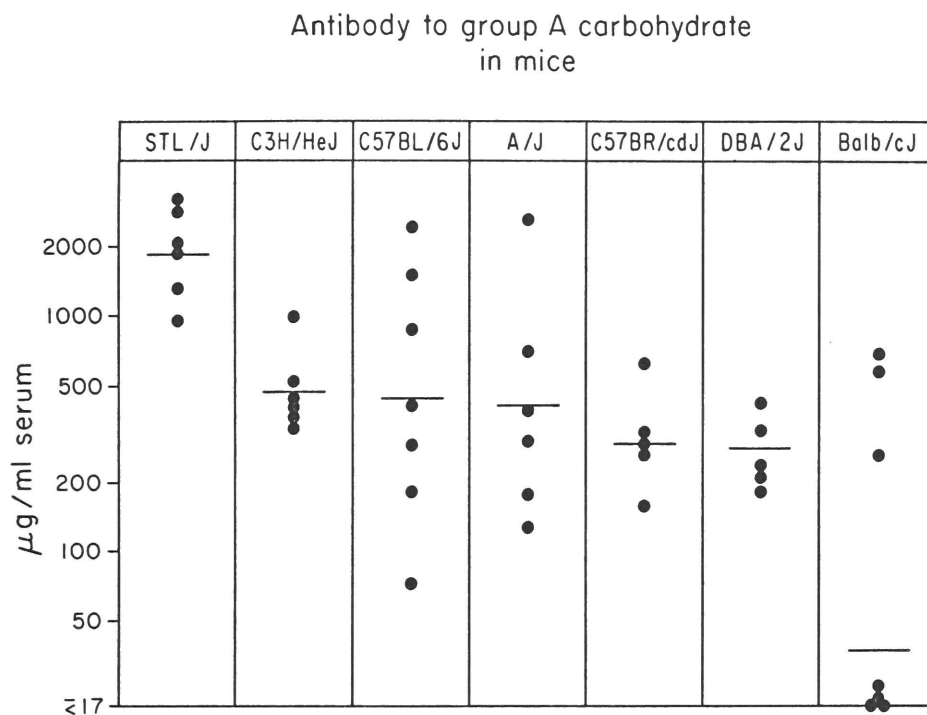


Figure 23. The anti-Group A antibody response of female mice of 7 inbred strains. The mice were immunized with Group A vaccine according to the schedule given in Table IV. All mice were 5 weeks old at the start of immunization. Mice were bled 10 days after the last injection and their antibody titer was determined by the antigen binding assay. Three SWR/J mice, with average titers of 410 $\mu\text{g/ml}$, and 4 BRVR mice, with average titers of 142 $\mu\text{g/ml}$, were included in the experiments, but their responses do not appear in the figure. The horizontal lines indicate geometric means.

Genetic experiments were designed to determine if the anti-Group A antibody response in inbred mice was under simple genetic control.

In the first experiment, the immune response was studied in BSVS and Balb/cJ mice. Both of these strains produced low anti-Group A antibody responses, but the responses of BSVS mice were significantly lower than those of the Balb/cJ mice.

For this study mice of both inbred strains, F_1 hybrids, and both types of backcross mice (F_1 x Balb/cJ and F_1 x BSVS) were all raised at the same time under as nearly identical conditions as possible. When the mice were 4-5 weeks old, they were all immunized as a group according to the schedule given in Table VI. The mice were all bled 10 days after the last injection and their antibody titers were determined by the antigen binding assay.

The antibody responses of these mice are shown in figures 24 and 25. The geometric mean responses that have been calculated include the data from both male and female mice. The mean response for the Balb/cJ mice was about 7.5 times that of the BSVS mice, although the ranges in response of the two strains overlapped.

The mean response for the F_1 mice falls in between the responses of the Balb/cJ and the BSVS mice. In general the response of the F_1 mice appears to correspond a little more closely to the response of the BSVS mice than to the response to Balb/cJ mice. Thus, the higher responsiveness of the Balb/cJ mice appears to be co-dominant with respect to the BSVS genotype.

The nature of the gene action regulating the antibody response in the F_1 mice relative to the Balb/cJ and BSVS mice was further studied in the two groups of backcross mice. The backcross F_1 x BSVS will be discussed first.

The average titer of the F_1 x BSVS backcross mice was midway between the average titer of the F_1 mice and the BSVS mice. This made it likely that only a small number of Balb/cJ genes were required for the difference in responses of the F_1 mice and the BSVS mice. Otherwise few, if any, of

TABLE VI

Immunization Schedule for Balb/cJ-BSVS
Genetics Experiment

| Day | 0 | 3 | 6 | 9 | 15 | 50 | 82 | 123 | 133 |
|-------|-----|-----|---|---|----|----|----|-----|------|
| Dose* | 1.5 | 1.5 | 3 | 3 | 6 | 6 | 6 | 6 | bled |

*vaccine dose in μg rhamnose per injection

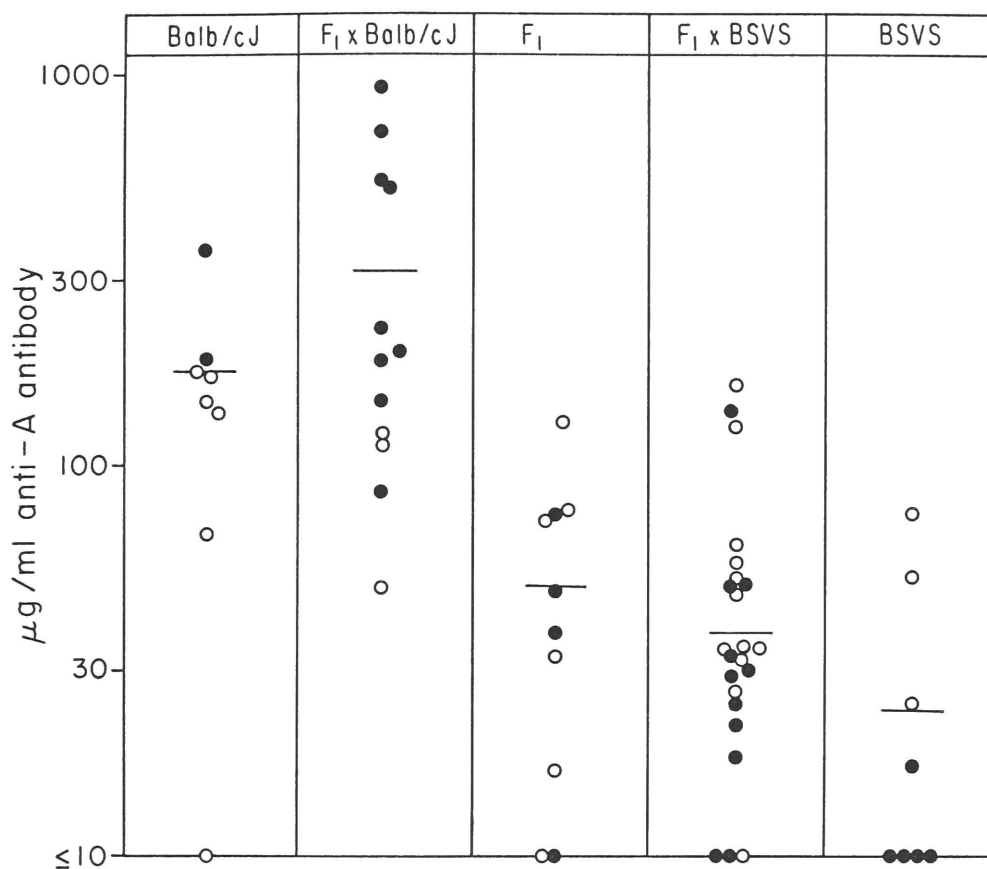


Figure 24. Anti-Group A antibody responses of Balb/cJ, BSVS, F₁, F₁ x Balb/cJ, and F₁ x BSVS mice after immunization with Group A vaccine according to the schedule in Table VI. All mice were 4-5 weeks old at the first injection and were bled 10 days after the last injection. Antibody titers were determined by the antigen binding assay. The horizontal lines indicate geometric means. Solid circles indicate males, open circles indicate females. Mice 1, 2, 3, 4 and 5 were used in subsequent breeding studies.

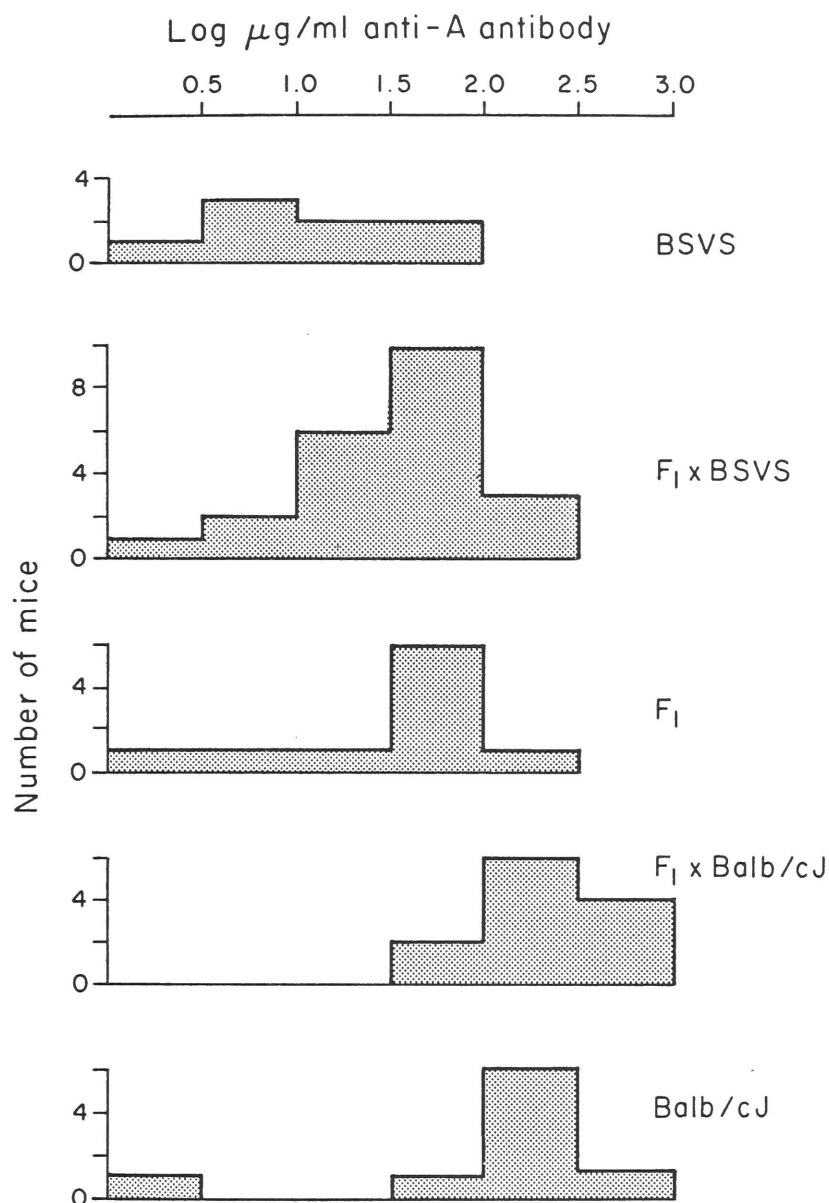


Figure 25. Histogram of the anti-Group A antibody responses for the mice depicted in figure 24. The first vertical bar in each plot represents all of those mice with antibody responses of equal to or less than 3.2 $\mu\text{g/ml}$. The next bar contains all of those with a response of 3.3-10.0 $\mu\text{g/ml}$ inclusive, and so on for the following bars.

the backcross mice would have inherited enough of the relevant Balb/cJ genes to alter their response from a typical BSVS response.

However, because of the small number of BSVS mice tested, the difference in the antibody responses of the BSVS and F_1 x Balb/cJ mice was only of border-line significance. To clarify this problem, the three F_1 x BSVS mice with the highest antibody titer (mice 3, 4, and 5 in figure 24) were themselves backcrossed to BSVS mice to produce a second-backcross generation. In all, 25 second-backcross mice were raised. At the same time, 21 BSVS mice were raised under similar conditions. The antibody responses of these mice are shown in figure 26. In at least three of the second-backcross litters, X, Y, and Z, there were a greater proportion of "high" responding mice than were observed in the BSVS litters. These three litters were either fathered or mothered by backcross parents 3 or 4. The fourth litter, W, was mothered by backcross parent 5.

The observation of an enhanced antibody response in the second-backcross mice, as compared to BSVS mice, indicates that the high titers of at least mice 3 and 4 were very likely the result of immune response genes they inherited from their Balb/cJ grandparent. The higher average titers of the second-backcross mice as compared to BSVS mice indicates that this gene (or genes) was subsequently inherited by many of the second-backcross mice.

Figure 27 shows the plot obtained if the data of all the F_1 x BSVS mice are compared as a group with the data from the three BSVS litters immunized at the same time. From this representation of the data, it can be readily seen that the BSVS mice form a unimodal distribution centering around 0-3 $\mu\text{g/ml}$ antibody. The responses of the second-backcross mice form a clearly bimodal distribution. This data indicate that only a small number of Balb/cJ genes are required to enhance the immune response of the second-backcross mice. While the data are consistent with a single gene mechanism, further experiments would be required in order to completely rule out the requirement of more than one Balb/cJ gene.

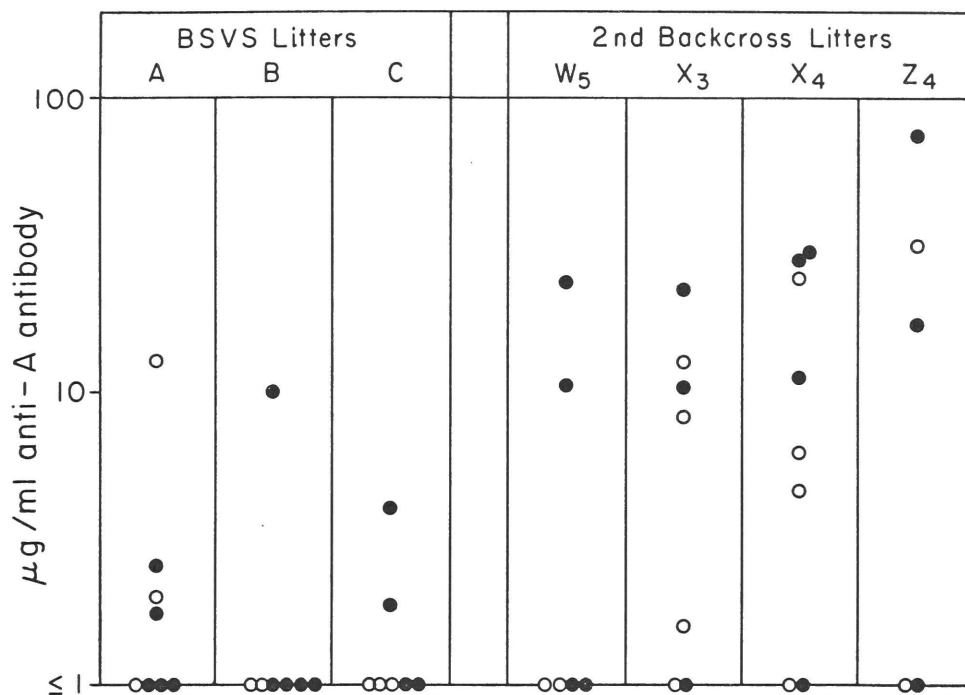


Figure 26. Anti-Group A antibody titers of second-backcross mice and BSVS mice. The second-backcross mice were obtained by mating first-backcross mice, BSVS x (Balb/cJ x BSVS) mice 3, 4 and 5 (see figure 24), with BSVS mice of the appropriate sex. The litters obtained are designated W, X, Y and Z. The subscripts 3, 4, 5 on W, X, Y and Z indicate which of the first-backcross mice were parents of the litter. The antibody titers were determined by the antigen binding assay. Solid circles indicate males; open circles indicate females.

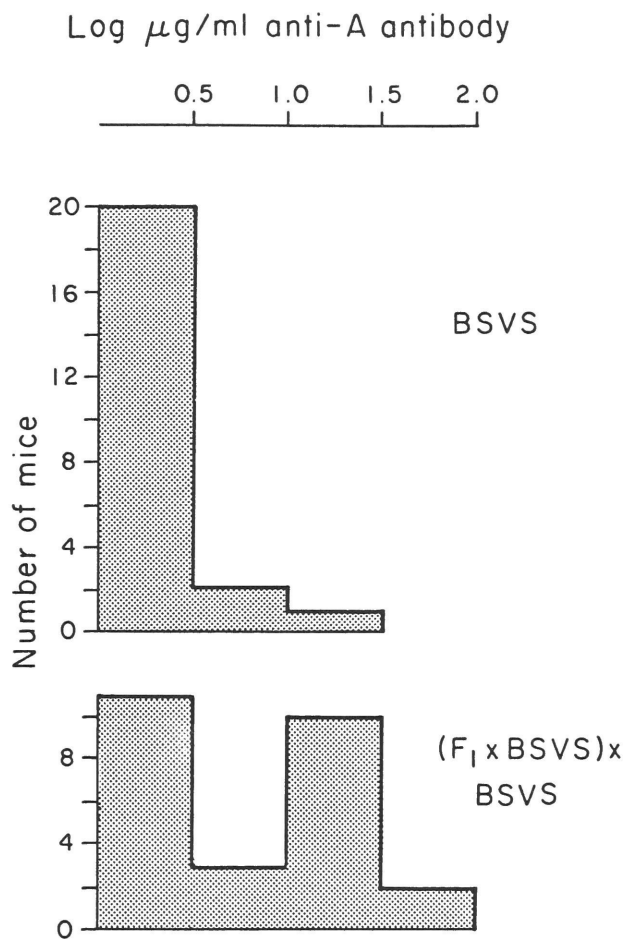


Figure 27. Histogram of anti-Group A antibody responses for individual BSVS and second-backcross [(BSVS x Balb/cJ) x BSVS] x BSVS mice. The first vertical bar in each plot represents all of those mice with antibody responses of equal to or less than $3.2 \mu\text{g/ml}$. The next bar contains all of those with a response of $3.3\text{--}10.0 \mu\text{g/ml}$ inclusive, and so on for the following bars. This figure is a cumulative plot of the points in litters A, B and C, and litters X, Y and Z in figure 26.

By tracing the parentage of mice 3 and 4 from the F_1 x BSVS backcross, it was possible to establish that this gene(s) was not on the X chromosome. The pedigree of mice 3 and 4 (figure 28) reveals that their X chromosomes had to be of BSVS origin, and therefore could not carry any Balb/cJ genes. The gene also could not be on the Y chromosome because litter X was mothered by female mouse 3. It therefore appears as though this gene (or genes) is autosomal.

One explanation for the observation that the F_1 mice have a lower antibody response than the Balb/cJ mice would be that the gene studied in the F_1 x BSVS backcross is only co-dominant and does not fully express itself in the heterozygote. If this were the case, however, then the presence of its BSVS allele would show up in the F_1 x Balb/cJ backcross mice. Backcross mice carrying the BSVS allele would be expected to have an antibody titer similar to the F_1 mice rather than like the Balb/cJ mice. From figures 24 and 25, it is apparent that there is no evidence for the segregation of a single co-dominant BSVS gene. In fact, the shortage of F_1 x Balb/cJ mice with low antibody titers indicates that multiple BSVS genes are required to produce the low titer antibody responses seen in the F_1 mice. Some F_1 x Balb/cJ mice had even higher responses than any of the 8 Balb/cJ mice. The high titer of these 4 mice could very likely have been simply the result of statistical variation. To check this out, several of the high and low responding F_1 x Balb/cJ mice were backcrossed to Balb/cJ mice.

Litters were obtained only from matings involving mice 1 and 2 (see figure 25). Eleven second-backcross mice were fathered by mouse 1, and 10 were fathered by mouse 2. Twelve Balb/cJ mice were raised at the same time and immunized along with the second-backcross mice as controls. The geometric anti-Group A antibody titers of the three groups of mice were: 61 $\mu\text{g/ml}$ for the mice fathered by the high responding mouse 1; 94 $\mu\text{g/ml}$ for the mice fathered by the medium responding mouse 2; and 81 $\mu\text{g/ml}$ for the Balb/cJ mice. In other words, there seemed to be no positive correlation between the antibody titers of the offspring and those of the parents. Both second-backcross litters simply behaved as if they were Balb/cJ mice.

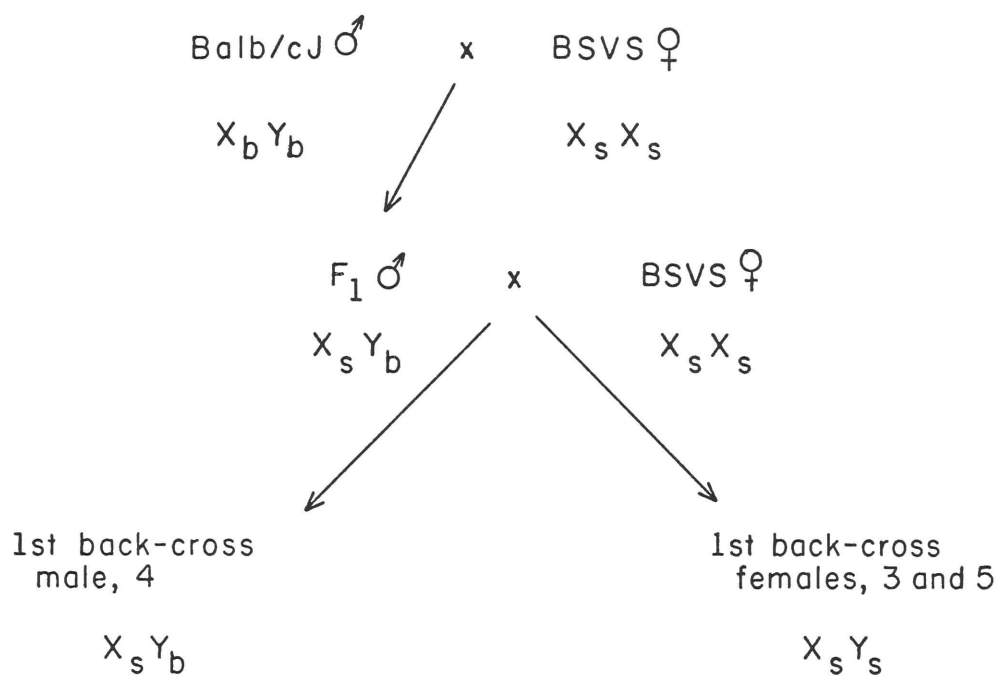


Figure 28. Pedigrees of first-backcross mice 3, 4 and 5. Also indicated are the X and Y chromosomes of each mouse of the pedigree. Subscripts on the X and Y chromosomes indicate whether they are of Balb/cJ or BSVS origin.

b = Balb/cJ; s = BSVS

It was possible that some pedigree error was made and the original F_1 x Balb/cJ mice were really Balb/cJ mice. To rule this out, an anti-BSVS red cell serum was made in Balb/cJ mice. With this antiserum it was possible to demonstrate the presence of BSVS red cell antigens in some of the F_1 x Balb/cJ mice, but not in any of the Balb/cJ mice. Thus, the original pedigree was confirmed.

On the basis of these genetic experiments with BSVS and Balb/cJ mice, the following working hypothesis concerning the genes regulating the antibody response in these mice has been formulated. It is suggested that Balb/cJ mice have a gene, R, which favors the production of anti-Group A antibody. The BSVS mice have the recessive allele, r, which does not favor the production of anti-Group A antibody. In addition it is hypothesized that the BSVS mouse has a group of unlinked genes which act together to diminish the effect of R, and that if any of this group of genes were absent, no effect on R would be observed. The action of these genes would explain why the F_1 mice have an average response so much lower than is observed in Balb/cJ mice. The requirement that all of these unlinked BSVS genes be present in order for them to exert their effect on R, would help explain why some of the 12 individual F_1 x Balb/cJ mice did not have the lower antibody responses characteristic of the F_1 mice. This hypothesis for multiple BSVS genes requires confirmation by additional backcross experiments.

The lack of high antibody production in the BSVS mice was further studied in a breeding experiment involving A/J mice. BSVS, A/J, A/J x BSVS, and both types of backcross mice were raised at the same time under as nearly identical conditions as possible. The mice were immunized intravenously at 4-5 weeks of age according to the immunization schedule in Table VII.

The injection schedule consisted of two series of injections separated by 128 days. Twelve days after each series of injections, the mice were bled and their anti-Group A antibody levels were determined by the antigen binding assay. The antibody concentrations of the sera from the first bleeding are summarized in figure 29. Although there is complete overlap of the BSVS and A/J anti-Group A antibody responses, it is clear that the A/J and F_1 x A/J mice in general had higher antibody titers than did the BSVS, F_1 , and F_1 x BSVS

TABLE VII

Immunization Schedule for A/J-BSVS
Genetics Experiment

| Day | 0 | 8 | 15 | 22 | 36 | 48 | 164 | 176 | 188 |
|-------|---|---|----|----|----|------|-----|-----|------|
| Dose* | 2 | 2 | 3 | 6 | 6 | bled | 6 | 6 | bled |

*vaccine dose in μ g rhamnose per injection

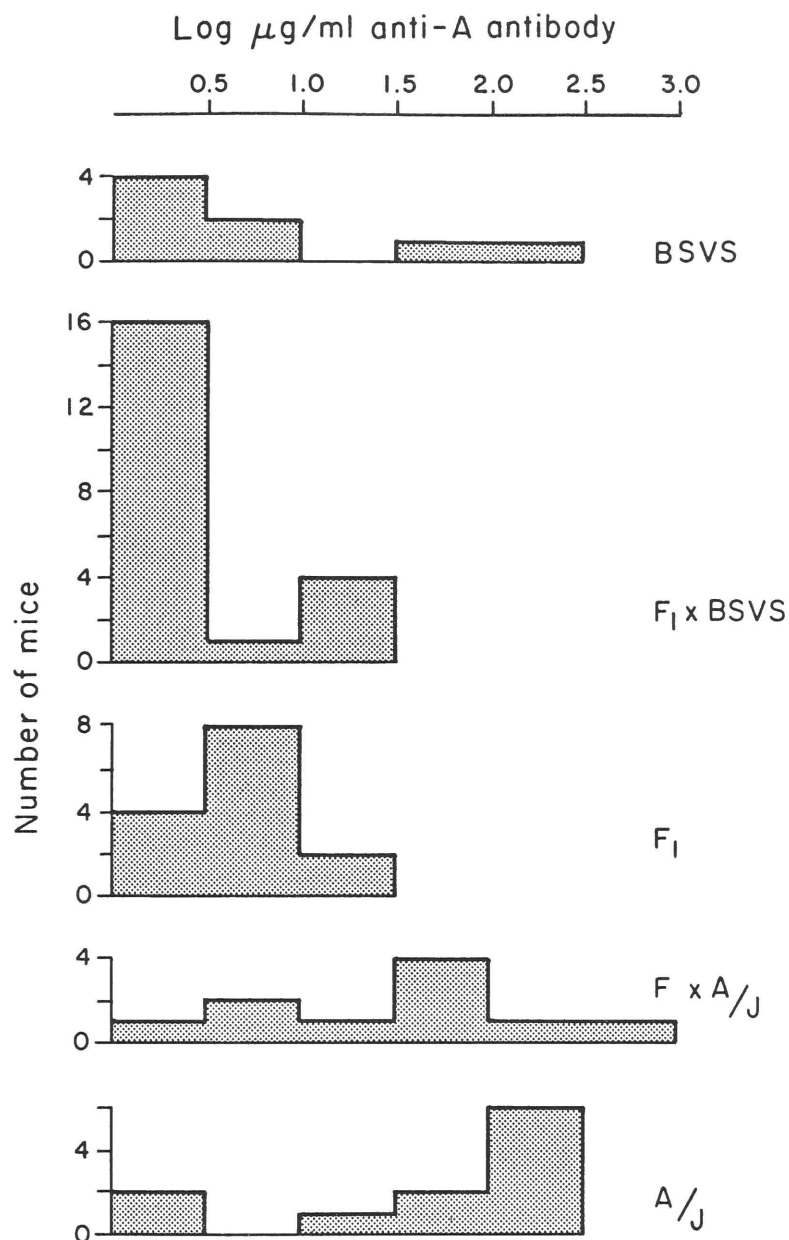


Figure 29. Histogram of anti-Group A antibody responses for individual BSVS, $(\text{BSVS} \times A/J) \times \text{BSVS}$, $(\text{BSVS} \times A/J) \times A/J$, and A/J mice after one series of immunizations (bled day 48). See Table VII for immunization schedule. The first vertical bar in each plot represents all of those mice with an antibody response of equal to or less than $3.2 \mu\text{g/ml}$. The next bar contains all of those mice with responses from 3.3 – $10.0 \mu\text{g/ml}$ inclusive, and so on for the following bars.

mice. It appears as though the low response imparted by the BSVS genotype is dominant in the F_1 animals. Not enough F_1 x A/J mice were tested to determine whether or not they showed a segregation of high and low responses. In general however, the response of the F_1 x A/J mice appears to be more like the A/J response than the F_1 or the BSVS responses.

After the second series of injections, the mean response of all the groups of mice increased except for the BSVS mice. Their responses stayed at the same low level (see Table VIII)! Therefore, it appeared that perhaps the reason the BSVS mice generally give a low response to the Group A vaccine is because they fail to make a typical secondary anti-Group A response. In examining figure 30 a result quite different from that of the first bleeding is seen. The distribution of antibody titers in the hyperimmune F_1 sera is readily distinguishable from that of the BSVS mice, and is essentially indistinguishable from the distribution of titers in the hyperimmune sera of the A/J mice and the F_1 x A/J mice. Thus, in the boosted response the genetic contribution of the A/J mice appears to be dominant over that of the BSVS mice. The responses of the F_1 x BSVS mice are intermediate to those of the F_1 and BSVS mice, as might be expected. However, the lack of low titer (0-10 $\mu\text{g/ml}$) antibody responses in any of these 12 backcross mice indicates that homozygosity of BSVS genes at a number of loci might be required in order to prevent the high hyperimmune response.

While these data do not permit a detailed analysis of the number of genes involved, it does indicate that different genes control different phases of the mouse anti-Group A response. For example, in the F_1 mice, the BSVS genes are of primary importance in determining the amount of antibody produced after the priming immunization series. In these same mice, the level of antibody produced after the two booster injections appeared to be regulated by genes from the A/J mice.

Adoptive transfer of spleen cells from mice immunized with Group-A sterptococci, to syngeneic recipients: Evidence for propagation of clones of antibody To obtain more of a particular monodisperse anti-Group A antibody than could be obtained from the serum of a single mouse, we have utilized the technique of adoptive transfer (Chase, 1951; see also Cochrane and

TABLE VIII

Comparison of Anti-Group A Antibody Response
After 1 and 2 Series of Immunizations*

| Mice | Geometric mean antibody titers in $\mu\text{g/ml}$ | |
|-----------------------|---|------------|
| | bleeding 1 | bleeding 2 |
| BSVS | 6 | 4 |
| F ₁ x BSVS | 2 | 67 |
| F ₁ | 4 | 130 |
| F ₁ x A/J | 45 | 324 |
| A/J | 52 | 525 |

* Bleedings 1 and 2 were made 12 days after the 1st and 2nd series of immunizations respectively.

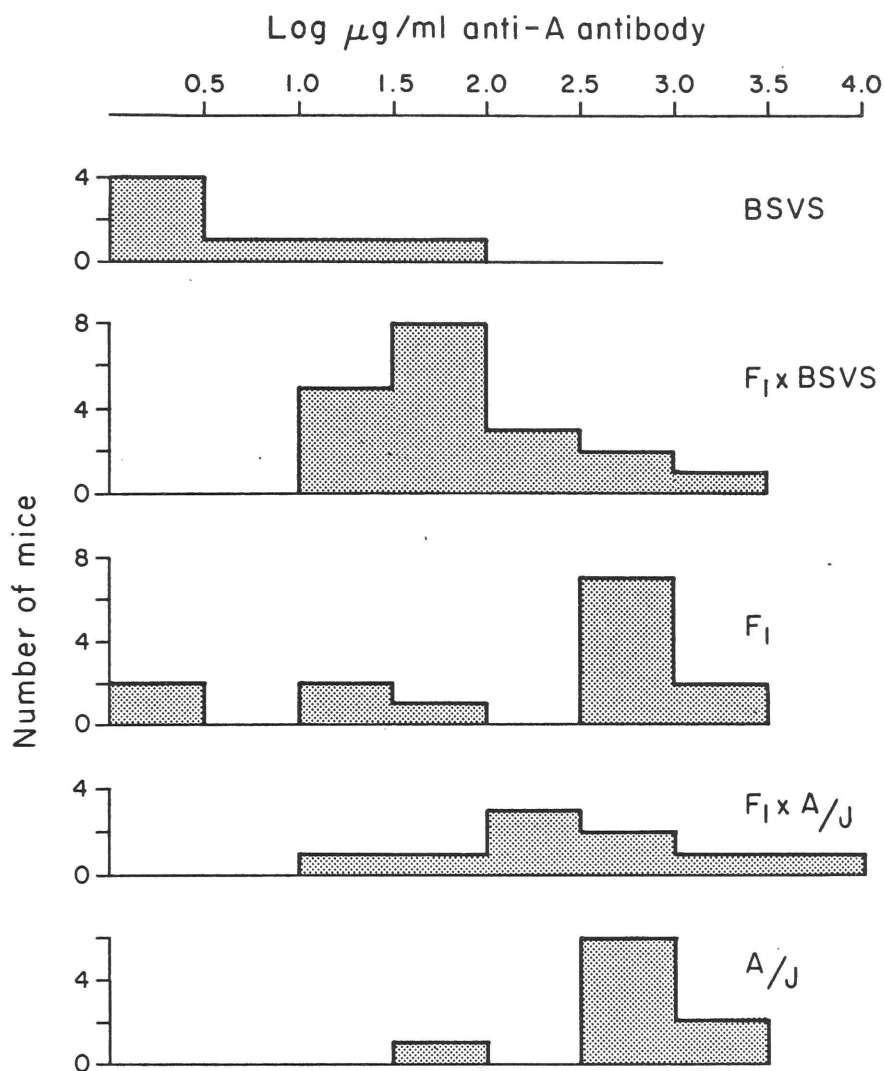


Figure 30. Histogram of anti-Group A antibody responses for individual BSVS, (BSVS \times A/J) \times BSVS, BSVS \times A/J, (BSVS \times A/J) \times A/J, and A/J mice after two series of immunizations (bled on day 188). See Table VII for immunization schedule. The bars are plotted as in figure 29.

Dixon, 1962). The technique employs the spleen cells from a mouse making a particularly monodisperse anti-Group A antibody. These cells were injected intraperitoneally into 6-30 recipient syngeneic mice that had been irradiated with about 500 roentgens 48 hours prior to transfer. The recipient mice were then immunized, and their antisera were screened by microzone electrophoresis to detect those which made antibody similar to that of the donor mouse (Briles, 1971). From the recipient mice, certain individuals were selected as donors for the next passage. This transfer procedure is similar to that employed by Askonas et al. (1970) to propagate clones of anti-DNP antibody.

Figure 31 depicts the microzone electrophoresis patterns of the anti-A sera of a donor mouse and some of its recipient mice (Briles and Krause, 1972). In this transfer, 10^7 nucleated donor spleen cells were given to each of 18 irradiated recipient mice. After immunization, the sera of 14 of the recipient mice showed the same slow antibody component as the donor mouse. Microzone electrophoretic patterns of the sera from 4 of these 14 recipient mice are shown in figure 31. Figure 31 also depicts a microzone electrophoretic pattern of a control mouse which was not reconstituted with immune spleen cells.

In all, 26 control mice were run in conjunction with this experiment. Twenty of the control mice received no spleen cells and six of the mice were reconstituted with 10^7 nucleated nonimmune spleen cells. All of the control mice were then immunized in the same manner as the recipients of the immune spleen cells. There was no indication of the occurrence of the slow antibody component of the donor in any of the 26 control mice.

The irradiation step is not absolutely essential for adoptive transfer. This is seen in figure 32, where the sera from the first passage of two A/J clones have been microzone electrophoresed. In cell transfer line 8, half of the donor spleen cells were given to each of two recipients. In cell transfer line 9, the donor spleen cells were divided among 6 recipients. All recipient antisera have a monodisperse antibody component. The monodisperse antibody component of cell line 9 has a more rapid mobility than that of cell line 8. This difference in the mobility of the antibody produced by different clones was also seen for the two different SWR/J cell

TRANSFER OF IMMUNE SPLEEN CELLS
IN SWR/J MICE

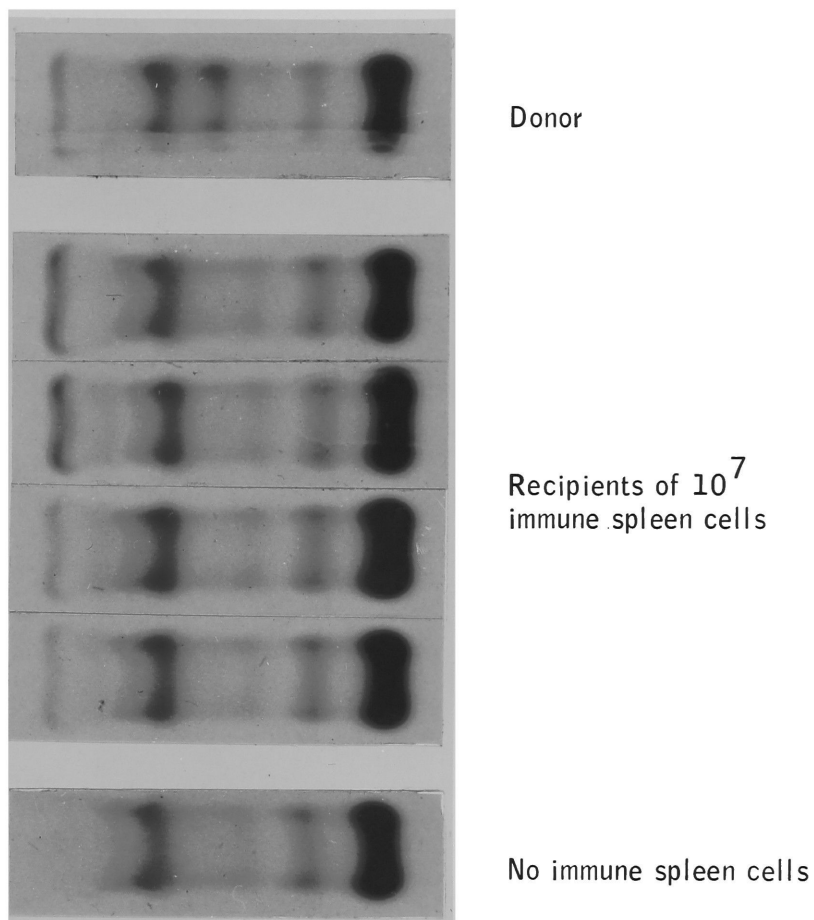


Figure 31. Microzone electrophoresis patterns of antisera of donor and recipient mice from a spleen cell transfer experiment. Absorption experiments revealed that all of the protein in the thin bands at the left of the pattern is antibody to Group A carbohydrate. Also shown is the microzone electrophoresis pattern of a control mouse who was not reconstituted with immune spleen cells. The extra beta band in the donor serum is hemoglobin.

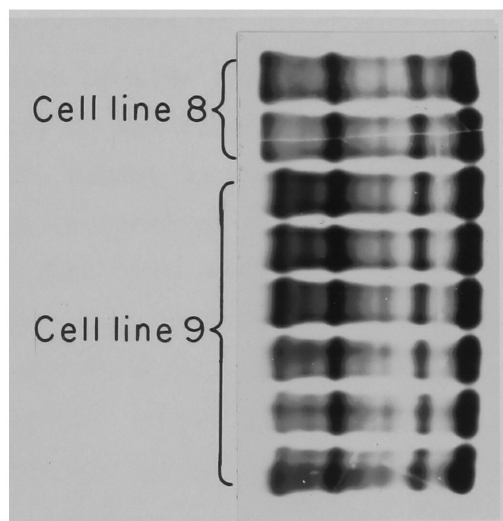


Figure 32. Microzone electrophoresis patterns of anti-Group A sera from two different A/J spleen cell transfer lines. Absorption experiments revealed that the dense staining protein bands to the left in each sera are antibody to Group A carbohydrate. The recipients for these two transfers were nonirradiated A/J mice.

transfer lines depicted in figure 22. To date, successful passage has been accomplished for five SWR/J and four A/J cell lines. The antibodies produced by these 9 cell lines are different from each other, as judged by microzone electrophoresis, and, in some cases to be described later, by idiotype.

The fact that different cell lines make different anti-Group A antibodies indicates that the cell transfer system is transferring the ability to make a particular antibody, and not a general anti-Group A immun response.

The interpretation of this type of experiment is that a clone of cells which has proliferated in the original donor is propagated in the recipients. These clones, however, cannot be passed continuously. In fact, high titers of antibody cannot be obtained after more than two or three passages. This is demonstrated with data from a single A/J clone presented in Table IX. In this transfer, the original donor has an antibody concentration of 61 mg/ml of serum. In the first passage, the average response was 15.2 mg anti-Group A antibody/ml. By the second passage, the average antibody concentration was only 4.9 mg/ml and in the third passage it declined to less than 1 mg/ml.

This limited life span of transferred clones has also been observed with anti-DNP clones, by Williamson and Askonas (1972).

Idiotypy of mouse anti-Group A antibodies Prior to the findings reported here, the passive hemagglutination technique was used to detect inbred mouse strain-specific idiotype of antibodies to Group A carbohydrate, and to monitor spleen cell transfers in syngeneic mice. Much of this work has been reported elsewhere (Briles and Krause, 1972) and has been summarized in Appendix I along with some more recent findings with the same system.

The Group A antibodies of A/J and SWR/J mice were used to examine the genetic control of idiotype. Isolated SWR/J and A/J anti-Group A antibodies which exhibited restricted electrophoretic mobility were used for the preparation of anti-idiotypic sera in rabbits. The isolated antibodies were emulsified in Freund's complete adjuvant and injected into rabbits. The antisera were made idiotypically specific for variable region

TABLE IX

Decline of Antibody Titer
with Passage of Cell Clone

| Passage | Number of mice | Average Ab titer mg/ml |
|----------|----------------|---------------------------|
| Original | Donor | 61 |
| 1 | 5 | 15.2 (8.5-24)* |
| 2 | 6 | 4.9 (1.2-10.1) |
| 3 | 12 | 0.7 (.07-1.3) |

*Numbers in parentheses are the observed range in antibody titers. The mouse with the highest anti-Group A titer in each passage was used as the donor for the next passage.

determinants by absorption with syngeneic normal mouse serum or normal ascites fluid. In these experiments, an isolated antibody used to prepare a particular anti-idiotypic serum is referred to as a proband antibody. The mice from which the proband antibodies are isolated are called probands.

Three idiotypic antisera will be described in this thesis. Two were made against SWR/J anti-Group A antibodies and one was made against A/J anti-Group A antibodies. The findings with the A/J anti-idiotypic serum will be described first.

Depicted in figure 33 is the microzone electrophoresis pattern of the isolated A/J anti-Group A antibody used for the preparation of this anti-idiotypic serum. The antibody was isolated by adsorption to Group A vaccine and subsequent elution with 10% N-acetyl-D-glucosamine in saline.

As shown in the top frame of figure 34, the immunoelectrophoresis pattern developed with anti-whole mouse serum and anti-mouse immunoglobulins indicated that the isolated proband antibody was indeed mouse immunoglobulin. The isolated proband immunoglobulin was shown to be anti-Group A antibody by an autoradiographic experiment involving ^{125}I -labeled Group A carbohydrate.

After the immunoelectrophoresis pattern shown in figure 34 had been developed, about 1 μc of ^{125}I -Group A carbohydrate was allowed to diffuse into the agar gel. The gel was then washed and autoradiographed. From the autoradiograph, shown in the lower portion of figure 34, it can be seen that the labeled Group A carbohydrate bound to the precipitin bands containing the isolated antibody. The carbohydrate did not, however, bind to the immunoglobulin arcs of the normal SWR/J serum that had been placed in the upper and lower immunoelectrophoresis wells.

The proband antibody could be precipitated with the idiotypic serum made against it. In immunoelectrophoresis, the isolated antibody gave similar precipitin arcs with the idiotypic antiserum and with anti-mouse immunoglobulin (figure 35). This anti-A/J idiotypic serum did not give a precipitin reaction with proband serum that had been absorbed with Group A vaccine. The antiserum also did not give a precipitation reaction with immunoglobulin in normal A/J mouse serum (figure 36).

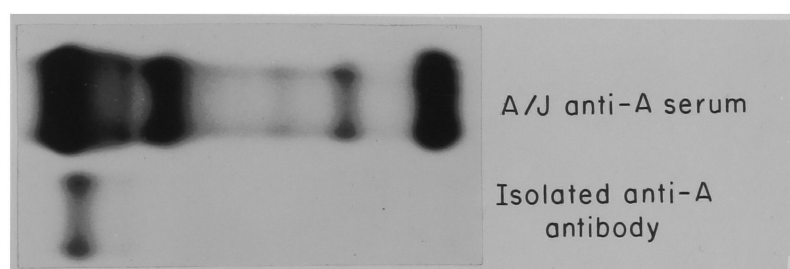


Figure 33. Microzone electrophoresis pattern of an A/J anti-Group A serum and the anti-Group A antibody isolated from it. This isolated A/J anti-Group A antibody was used to prepare the anti-A/J idiotypic serum.

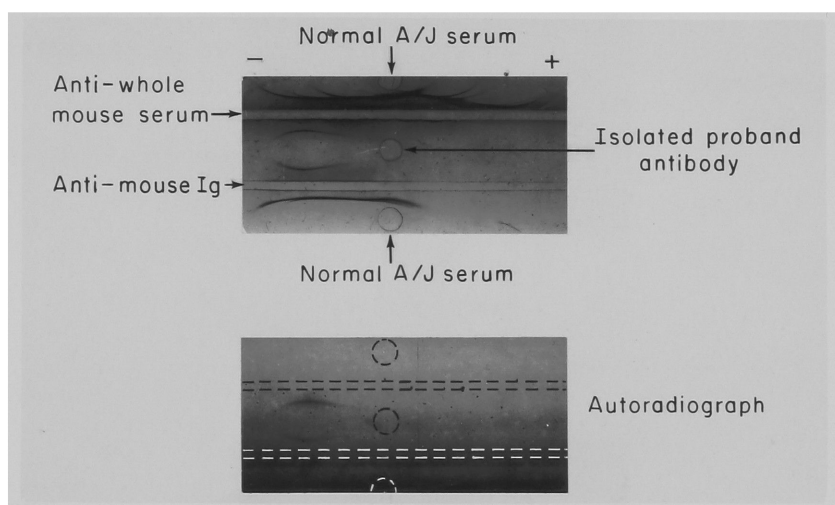


Figure 34. Immunoelectrophoresis of the same (A/J) isolated proband anti-Group A antibody shown in figure 33. The isolated antibody was placed in the center well. The top and bottom wells contained normal A/J serum. The upper antiserum trough contained rabbit anti-whole mouse serum, and the lower antiserum trough contained rabbit anti-mouse γ -globulin. After immunoelectrophoresis, the troughs were filled with about 1 μ c of 125 I-labeled Group A carbohydrate in PBS. After a 24-hour incubation at 4°C, the slide was washed, dried, and autoradiographed.

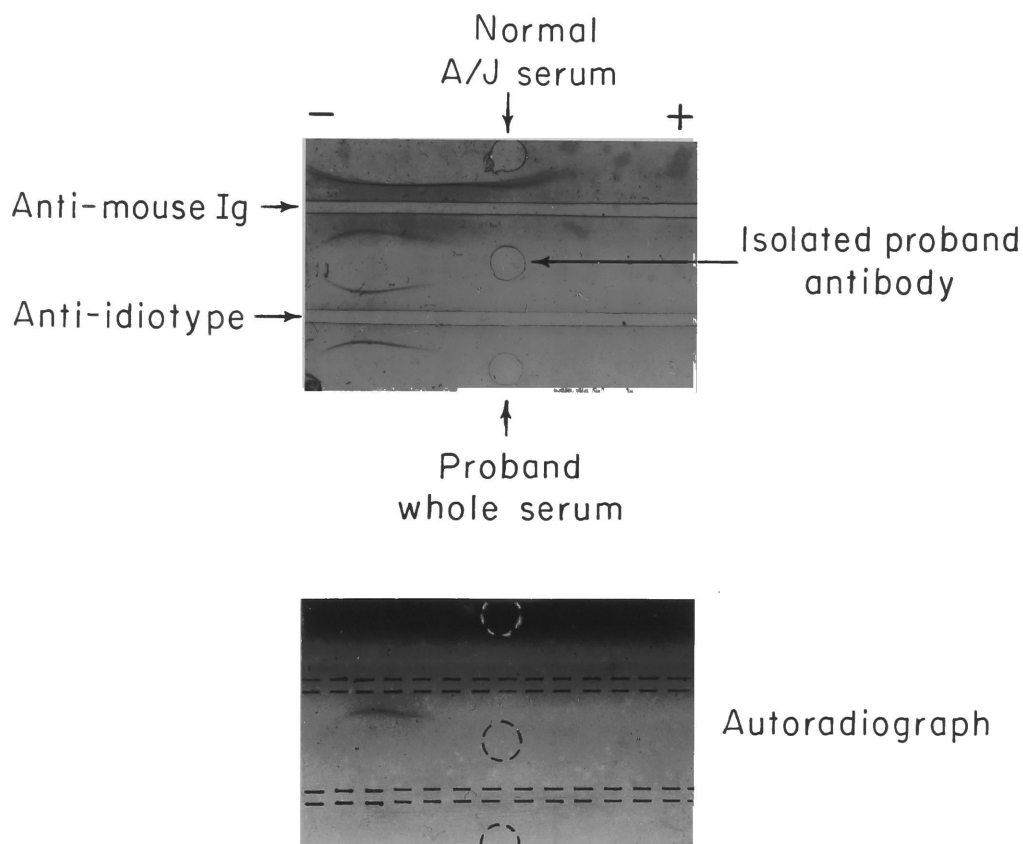


Figure 35. Immunoelectrophoresis of the same (A/J) proband anti-Group A serum and isolated proband anti-Group A antibody shown in figure 33. The proband anti-Group A serum was placed in the lower well. The isolated proband antibody was placed in the center well. The top well contained normal A/J serum. The upper antiserum trough contained rabbit anti-mouse γ -globulin, and the lower antiserum trough contained the anti-A/J idiotypic serum. After a 24-hour incubation at 4°C, the slide was washed, dried, and autoradiographed.

Specificity of Idiotypic Reaction with Respect to Normal Mouse Serum

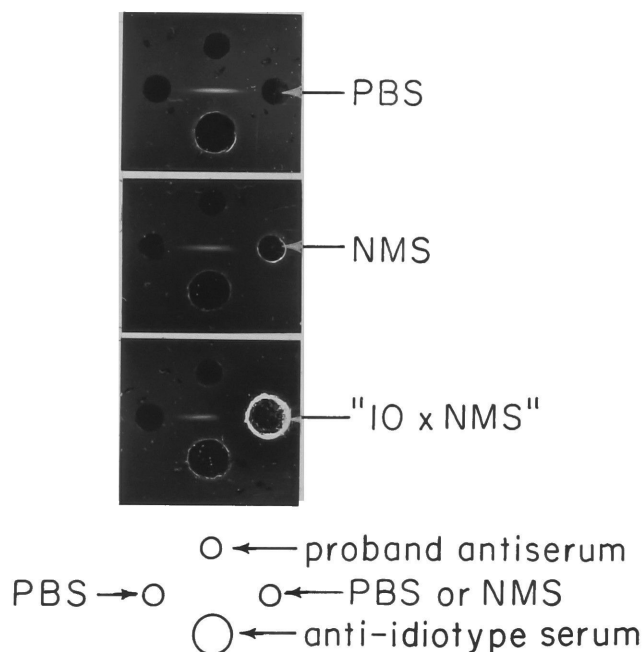


Figure 36. Double diffusion reactions between the anti-A/J idiotypic serum, the (A/J) proband anti-Group A serum, and normal A/J mouse serum. The proband anti-Group A serum contained 20 mg/ml anti-Group A antibody, and was diluted 1/20 for this assay. The upper pattern contains only the anti-idiotypic serum and the proband anti-Group A serum. In the center pattern, the left-hand well has been filled with normal A/J serum (containing about 2 mg/ml immunoglobulin). In the lower pattern the large left-hand well has been filled with concentrated normal A/J serum. By placing the concentrated normal serum in a larger well, it was possible to apply 10 times more normal serum to the well in the lower pattern than had been applied to the well in the center pattern.

However, high concentrations of normal A/J serum inhibited the precipitation reaction of the idiotypic antiserum with the proband serum. This is shown by the shortened precipitin line in the lower frame of figure 36. Inhibition of precipitation with normal serum, or normal immunoglobulin, is also characteristic of precipitating idiotypic studies in other systems (Grey et al., 1965; Wilson et al., 1971; Eichmann and Kindt, 1971).

Evidence that the idiotypic antiserum reacted with the antigen binding site was obtained by several techniques. The first evidence was obtained from autoradiography of the immunoelectrophoresis pattern in figure 35. This pattern had been equilibrated with ^{125}I -Group A carbohydrate and subsequently autoradiographed in the same manner as the immunoelectrophoresis pattern in figure 34. From the autoradiograph (see lower frame of figure 35), it was observed that the labeled carbohydrate did not bind the anti-Group A antibody in the precipitin arcs produced by the idiotypic serum, whereas it did bind the anti-Group A antibody precipitated by anti-mouse Ig serum. This indicated that the idiotypic antisera had blocked the antigen binding site of mouse anti-Group A antibodies.

In figure 37 are shown the double diffusion reactions between isolated proband anti-Group A antibody, the anti-idiotypic serum, and two anti-mouse immunoglobulin sera. It was observed that the precipitin reaction of the isolated antibody with the idiotypic antiserum gave a line of identity with the major precipitin line produced by each of the anti-mouse immunoglobulin sera. No spurs were observed. This indicated that the idiotypic antiserum was reacting with the same component of the isolated antibody preparation that formed the major precipitin line with each of the two anti-mouse immunoglobulin sera.

By the same type of autoradiography experiment described above, it was possible to demonstrate that the major precipitin line formed by the anti-immunoglobulin sera contained anti-Group A antibody as had been expected (figure 36). The portion of the precipitin line where the anti-Group A antibody was precipitated by the idiotypic serum did not bind the labeled carbohydrate as had been observed in the autoradiograph of the immunoelectrophoresis pattern (figure 35).

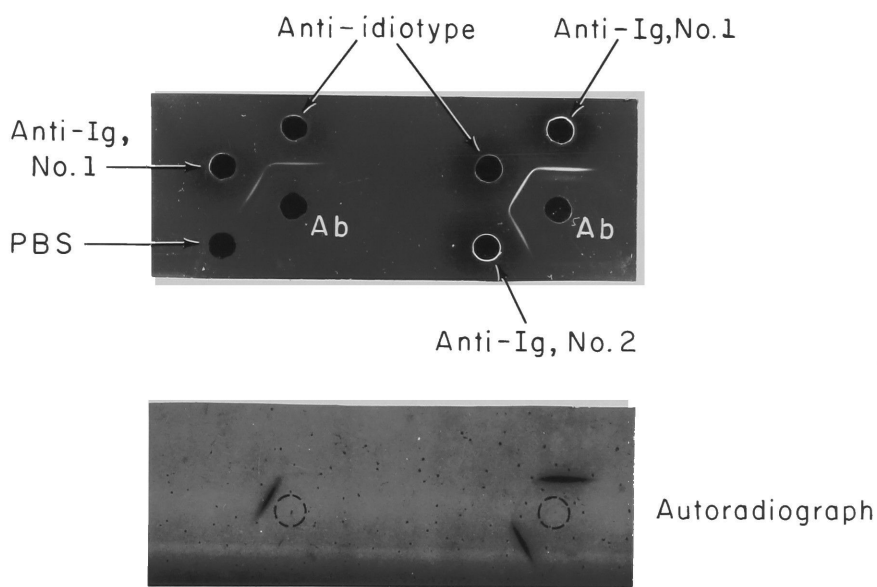


Figure 37. Double diffusion reactions between isolated (A/J) proband anti-Group A antibody, anti-A/J idiotypic serum, and two anti-mouse immunoglobulin sera. In the pattern at the left, the concentration of isolated proband antibody (Ab) was 0.7 mg/ml. In the pattern at the right, the concentration of isolated proband antibody was 2.7 mg/ml. The concentrations of the other reagents were adjusted to give sharp lines of equivalence. After the double diffusion patterns had developed, the wells were filled with about 1 μ c of 125 I-labeled Group A carbohydrates. After 24 hours of incubation at 4°C, the slide was washed, dried, and autoradiographed.

These studies suggest that the idiotypic antisera binds to the variable region of the anti-Group A antibodies and possibly within the antigen binding site itself. More definitive evidence that the idiotypic specificity includes the binding site came from inhibition studies using N-acetyl-D-glucosamine, the immunodominant monosaccharide of Group A carbohydrate (McCarty, 1958).

Figure 38 illustrates the N-acetyl-D-glucosamine almost completely inhibited the double diffusion reaction between proband serum and its idiotypic antiserum. N-acetyl-D-galactosamine, the immunodominant determinant of Group C carbohydrate (Krause and McCarty, 1962) gave very little inhibition.

As had been anticipated, the anti-idiotypic serum gave a cross-idiotypic precipitation reaction with anti-Group A antibody from other A/J mice. This cross-reaction is shown in figure 39 (lower left). What occurred, which was unexpected, were cross-idiotypic reactions between this anti-A/J idiotypic serum and anti-Group A antibody of SWR/J mice (figure 39).

In the upper right hand panel of the same figure, it can be seen that the precipitating reactions of the antisera with A/J, SWR/J, and SWR/J x A/J anti-Group A sera could be inhibited with N-acetyl-D-glucosamine. This inhibition indicated that these cross-idiotypic reactions were the result of anti-binding site antibodies. The major portion of these cross-idiotypic reactions appeared to be due to a single subgroup of antibodies in idiotypic antiserum. This is evidenced by the fact that adsorption of the idiotypic antiserum with SWR/J anti-Group A antibodies greatly reduced its cross-idiotypic reactions with pooled A/J and SWR/J x A/J anti-Group A antibodies (as seen in figure 39, lower right). It is possible that further absorption would have reduced or eliminated these cross-reactions.

Quantitative data on the cross-idiotypic reaction was obtained with a solid phase tube-binding assay (Askenase and Leonard, 1970; Carson and Weigert, 1973). The assay was carried out by coating the inside of polystyrene tubes with the immunoglobulin fraction of the idiotypic antisera. ¹²⁵I-labeled Group A antibody was then added to the antiserum-coated tubes

Inhibition of Idiotypic Reaction with N-Acetyl-Hexosamines

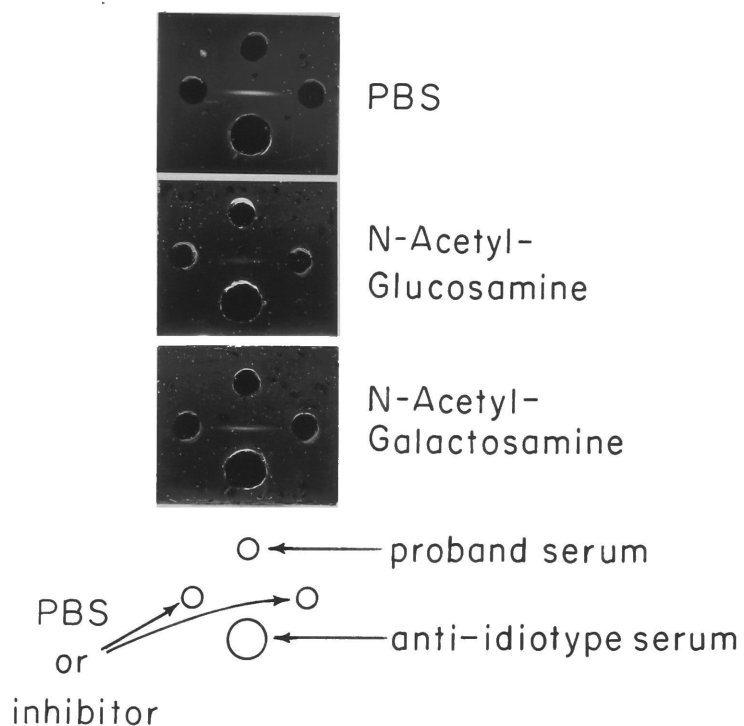
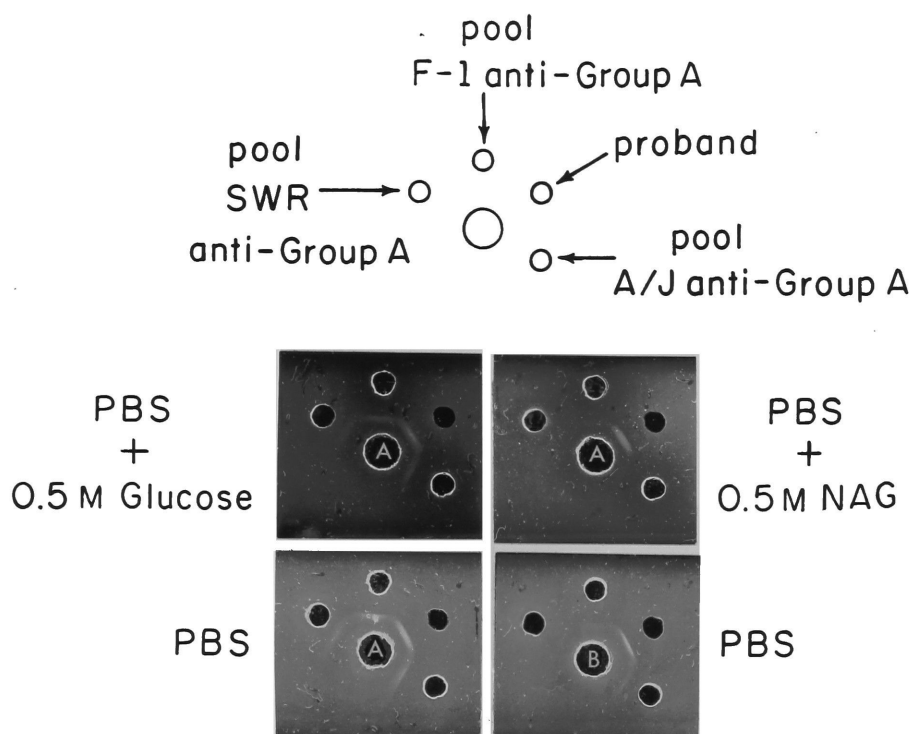


Figure 38. The effect of inhibitors, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine, on the double diffusion reaction between the (A/J) proband anti-Group A serum and the anti-A/J idiotypic serum. The lateral wells were filled with phosphate buffered saline (PBS), 0.5 M N-acetyl-D-glucosamine in PBS, or 0.5 M N-acetyl-D-galactosamine in PBS, as indicated. These patterns were photographed after 36 hours incubation at 4°C.



A = anti-idiotype absorbed with
normal A/J serum

B = anti-idiotype absorbed with
normal A/J serum + SWR anti-Group A serum

Figure 39. Cross-idiotypic reactions between the anti-A/J idiotypic serum and anti-Group A antibodies from A/J and SWR/J mice. The antiserum (A) in the center well of three of the patterns is the same preparation of idiotypic antiserum used in the earlier figures. The antiserum (B) was prepared by absorbing the rabbit antiserum with some SWR/J anti-Group A serum at the same time it was being absorbed with normal A/J serum. Each anti-Group A serum tested was diluted to contain 0.7 mg/ml anti-Group A antibody. The two bottom patterns were run in the normal manner at 4°C for 36 hours. The upper two patterns were made with agar containing 0.5 M inhibitors (about 9%) in PBS. Because of the slowed diffusion caused by the presence of the high concentration of solute, the two upper patterns were allowed to develop for 90 hours or until the glucose inhibited pattern looked very similar to the pattern obtained in PBS for 36 hours.

in the presence of various inhibitors. After 18 hours of incubation, the tubes were emptied and rinsed, and the amount of ^{125}I -labeled antigen bound to the antiserum-coated tubes was determined.

^{125}I -labeled pooled A/J anti-Group A antibodies were used as the reference antigen, rather than the proband material. Use of the pool was possible because at least some of the A/J anti-Group A antibody in the pool cross-reacted with the idiotypic antiserum, as was shown in the double diffusion assay above. By using the pooled A/J anti-Group A antibody as the reference antigen it was possible to preserve the limited supply of proband antibody, and enabled the cross-idiotypic reaction to be studied, without possible interference from idiotypic determinants which might be present on the proband antibody but rare or nonexistent in the pooled A/J anti-Group A antibody.

The binding of labeled A/J pooled anti-Group A antibody to the antiserum-coated tubes could be inhibited up to 60% with 200 mM N-acetyl-D-glucosamine. This confirmed the earlier observations that the specificity of the idiotypic antiserum was directed at least in part to the anti-Group A binding site of the mouse anti-Group A antibodies.

As was expected, the binding of the label could also be inhibited with pooled A/J anti-Group A sera (figure 40). This inhibition was due to the anti-Group A antibody in the anti-Group A sera, since absorption with Group A vaccine, but not with Group B vaccine, removed its inhibitory ability. Further evidence that the anti-Group A antibodies bear the idiotypic determinant comes from the observation that isolated A/J anti-Group A antibody is as effective as inhibitor of the tube binding assay as the whole immune serum.

Also shown in figure 40 is the fact that normal pre-immune pooled A/J and SWR/J sera were not good inhibitors of this idiotypic reaction.

From the inhibition curves in figure 40, it is apparent that at least 100 times as much normal A/J immunoglobulin as A/J anti-Group A antibody is required for 50-60% inhibition of the binding. This inhibition of the assay by high concentrations of whole A/J and SWR/J normal serum is probably due, in part at least, to nonspecific effects, or to some type of

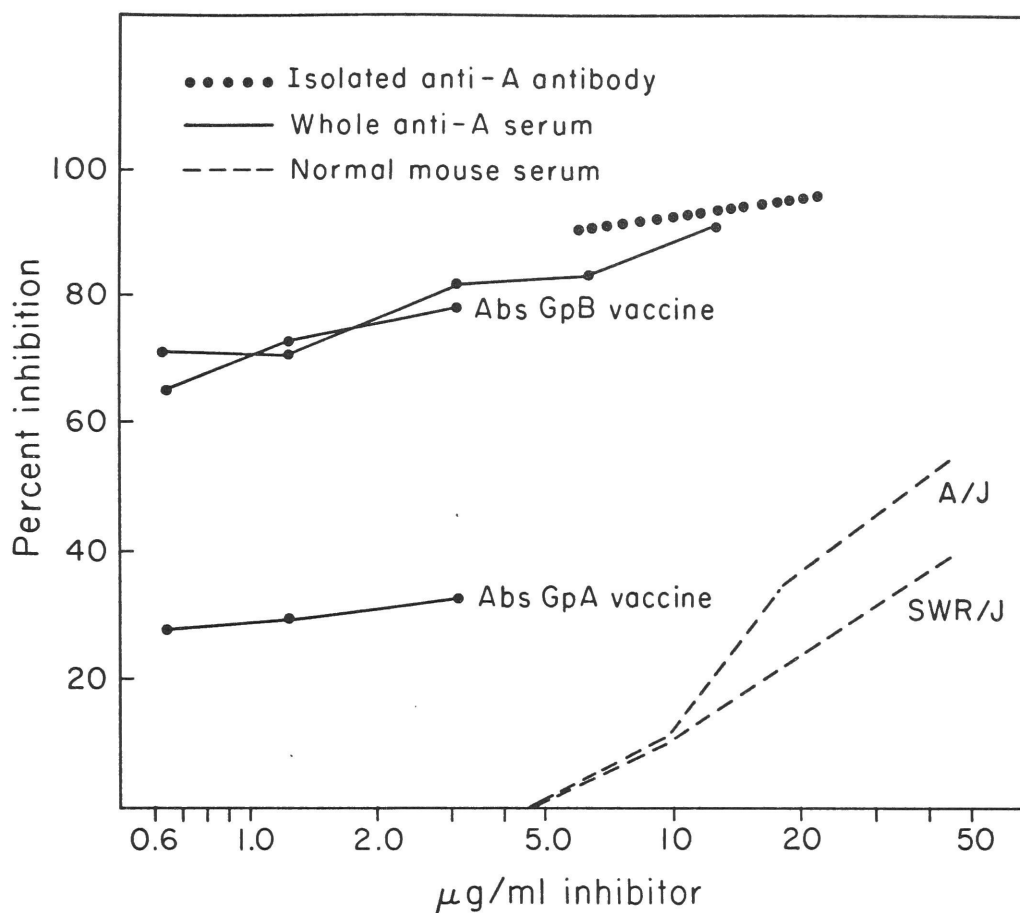


Figure 40. Inhibition of binding of ^{125}I -labeled pooled A/J anti-Group A antibody to tubes coated with the anti-A/J idiotypic serum. The anti-Group A serum was obtained from a pool of A/J anti-Group A sera. The inhibition curves for this serum and the anti-Group A antibody isolated from it are plotted in terms of $\mu\text{g/ml}$ of anti-Group A antibody. The anti-Group A serum pool was absorbed with Group A and Group B vaccine. The inhibition curves for the absorbed anti-Group A sera are plotted in terms of amount of anti-Group A antibody in the serum prior to absorption. The inhibition curves for the A/J and SWR/J normal sera pools are plotted in terms of $\mu\text{g/ml}$ mouse immunoglobulins.

weak cross-reactions, rather than to a low concentration of molecules of identical idiotypy.

The inhibition observed in figure 40 with high concentrations of normal mouse serum is consistent with results of Carson and Weigert (1973) involving idiotypy of mouse antibodies and myeloma proteins with anti-dextran activity. They observed that high concentrations of normal mouse serum, similar to those used here, also caused inhibition of specific idiotypic reactions in the tube binding assay. They also demonstrated that unrelated purified mouse myeloma proteins inhibited the assay when tested at concentrations greater than 10 $\mu\text{g/ml}$. This latter finding indicates that the inhibition with various immunoglobulin preparations in high concentration is probably due to some type of weak cross-reaction rather than to a low concentration of molecules with identical idiotypy. Because of this rather non-specific inhibition observed at high immunoglobulin concentrations, the assay is not useful for detecting very low levels of a particular idioype.

As shown in figure 41, pools of SWR/J and SWR/J x A/J Group A antisera were good inhibitors of the A/J idiotypic reaction. The SWR/J anti-Group A pool, however, was a less efficient inhibitor of the idiotypic reaction than the A/J or SWR/J x A/J anti-Group A pool, a result consistent with the double diffusion data in figure 39.

Information on the expression of the idioype in some sera from individual mice is seen in figure 42. Three of the four A/J antisera were more efficient inhibitors of the idiotypic reaction than any of the SWR/J sera. In one of the SWR/J antisera, the idioype was probably present only as a minor component.

As mentioned previously, the strong cross-idiotypic reaction between the anti-Group A idiotypic antiserum and the SWR/J anti-Group A antibodies had been unexpected. To investigate this further, an additional experiment was conducted to determine whether or not this cross-idiotypic reaction involved determinants associated with the antigen binding site of the SWR/J anti-Group A antibodies. For this experiment, ^{125}I -labeled SWR/J pooled anti-Group A antibodies were used as the reference antigen in the

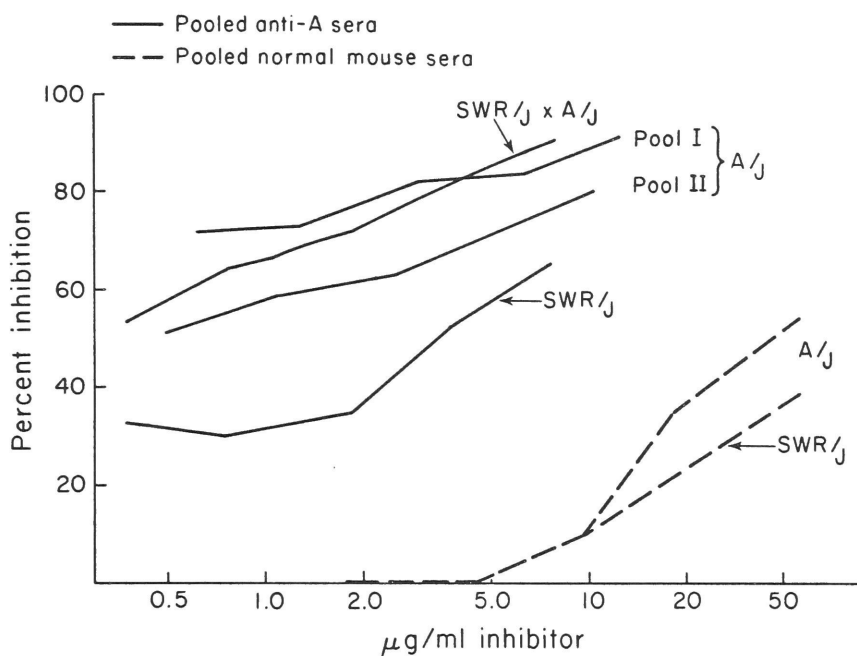


Figure 41. Inhibition of binding of ^{125}I -labeled pooled A/J anti-Group A antibody to tubes coated with anti-A/J idiotypic serum. The inhibition curves for the pooled anti-Group A sera are plotted in terms of $\mu\text{g/ml}$ anti-Group A antibody. The inhibition curves for normal A/J and SWR/J sera are the same ones as seen in figure 40, and are plotted in terms of $\mu\text{g/ml}$ mouse immunoglobulins.

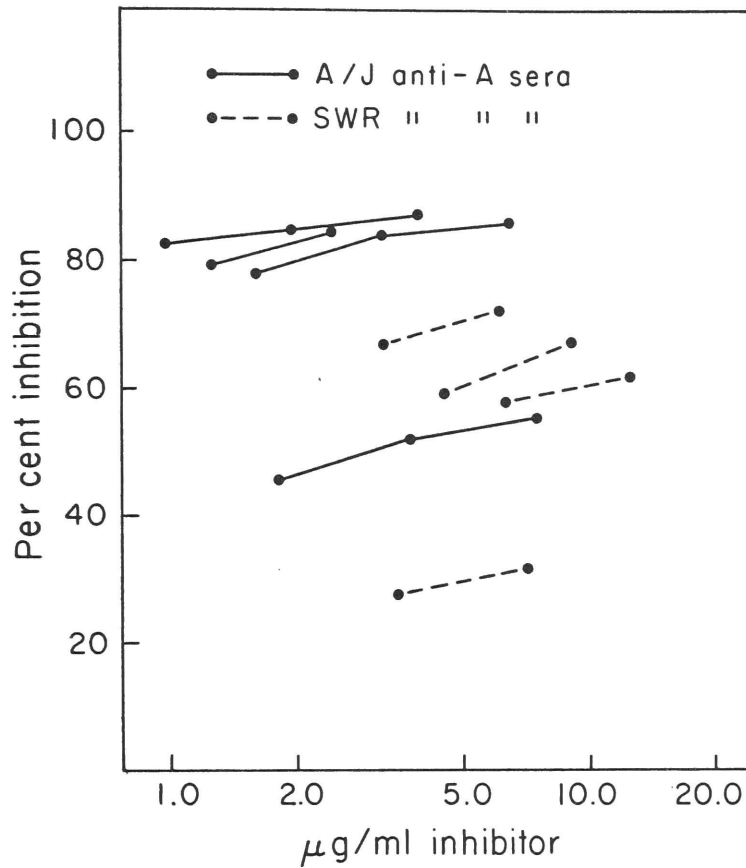


Figure 42. Inhibition of the binding of ^{125}I -labeled pooled A/J anti-Group A antibody to tubes coated with anti-A/J idiotypic serum. The anti-Group A sera were obtained from individual A/J and SWR/J mice. These inhibition curves are all plotted in terms of $\mu\text{g/ml}$ anti-Group A antibody.

tube binding assay, rather than the ^{125}I -labeled A/J anti-Group A antibodies described previously.

Using the tube binding assay, it could be demonstrated that the binding of the ^{125}I -labeled SWR/J anti-Group A antibodies, by the anti-A/J idiotypic serum, could be inhibited with N-acetyl-D-glucosamine (figure 43).

The observation that N-acetyl-D-glucosamine could specifically inhibit the cross-idiotypic reaction indicated that the idiotypic antiserum was recognizing an idiotypic determinant associated with the antigen binding site of the SWR/J anti-Group A antibodies. This conclusion was further supported by the fact that β -phenyl-N-acetyl-D-glucosaminide was an even better inhibitor of the binding than was N-acetyl-D-glucosamine. In the Group A system, β -phenyl-N-acetyl-D-glucosaminide is a better hapten than N-acetyl-glucosamine (McCarty, 1958), as was mentioned earlier. Glucose, galactose, and rhamnose (the other monosaccharide in Group A carbohydrate) had little inhibitory activity.

To determine whether or not anti-A antibodies from other strains of mice shared the same cross-idiotypic determinants as the SWR/J and A/J anti-Group A antibodies, the following tube binding inhibition experiment was conducted. In this experiment, the ^{125}I -labeled SWR/J anti-Group A antibodies were used as the reference antigen and the anti-A/J idiotypic serum was used to coat the tubes. Fourteen anti-Group A sera from individual mice of 4 other inbred lines and one outbred NCS mouse were used to inhibit the cross-idiotypic reaction between the ^{125}I -labeled SWR/J anti-Group A antibody and the anti-A/J idiotypic antiserum.

Table X gives the percent inhibition of binding, at 2 $\mu\text{g}/\text{ml}$ of anti-Group A antibody, for each of these 14 anti-Group A sera, as well as the inhibition values for 4 SWR/J and 4 A/J anti-Group A sera. From these data, it is apparent that outbred NCS mice and mice of each of the six inbred strains (A/J, SWR/J, RF/J, Balb/cJ, SJL/J, C57B1/6J) were equally capable of making the cross-idiotypic determinant detected by this assay. Also included in Table X are the results of a direct precipitation assay, which indicate that, by this technique as well, the anti-Group A sera of many of these mice react with the anti-A/J idiotypic antiserum.

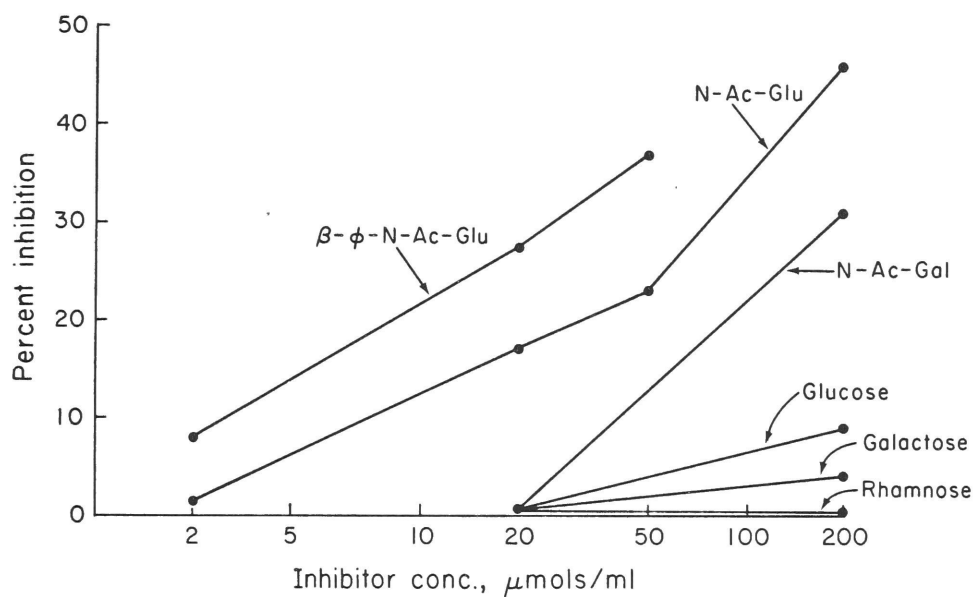


Figure 43. Inhibition of binding of ^{125}I -labeled pooled SWR/J anti-Group A antibody to tubes coated with anti-A/J idiotypic serum. The concentrations of the inhibitors are plotted in terms of $\mu\text{moles/ml}$ (millimolar).

TABLE X
Cross Idiotypic Reactions of Anti-Group A Sera
from Different Mouse Strains

| Strain | Individual Mouse | Percent ¹ Inhibition at 2 μ g/ml Ab | Positive ² Precipitin Reaction |
|----------------------|---------------------|--|---|
| A/J | 1 | 55 | N.D. |
| | 2 | 47 | N.D. |
| | 3 | 43 | N.D. |
| | 4 | 29 | N.D. |
| SWR/J | 1 | 59 | N.D. |
| | 2 | 38 | N.D. |
| | 3 | 35 | N.D. |
| | 4 | 33 | N.D. |
| RF/J | 1 | 53 | + |
| | 2 | 42 | 0 |
| Balb/cJ | 1 | 56 | 0 |
| | 2 | 46 | + |
| | 3 | 42 | 0 |
| | 4 | 38 | + |
| | 5 | N.D. | + |
| SJL/J | 1 | 57 | + |
| | 2 | 55 | + |
| | 3 | 51 | + |
| | 4 | 43 | 0 |
| C57B1/6J | 1 | 50 | + |
| | 2 | 57 | + |
| NCS | 1 | 51 | + |
| SWR NMS ³ | | 0 | 0 |
| A/J NMS ³ | | 1 | 0 |

¹ Inhibition of binding of ¹²⁵I-pooled SWR/J anti-Group A antibody to tubes coated with idiotypic antisera directed against isolated A/J anti-Group A antibody. Percent inhibition is that obtained with 2 μ g/ml anti-Group A antibody (Ab). For the normal mouse sera the percent inhibition is that obtained at 2 μ g/ml mouse immunoglobulin.

² Results of double diffusion in agar. All were tested at 1.5 mg/ml anti-Group A antibody versus the idiotypic serum. The normal sera were tested undiluted.

³ NMS = normal (preimmune) mouse serum. The normal mouse sera tested all came from pools of over 10 mice.

Although this A/J idiootype appeared to be widespread among mouse strains, it has not been detected in two rather heterogeneous rabbit anti-Group A sera. Further investigations will be required to determine whether or not the idiotypic determinants detected by this antiserum are species-specific.

In the study described above, the idiotypic antiserum was absorbed in such a manner as to allow the detection of the SWR/J-A/J cross-idiotypic specificity. It is possible that more thorough absorption of this antiserum could eliminate much of its cross-idiotypic reaction with SWR/J anti-Group A antibody, and yield an idiotypic antiserum reactive against A/J but not SWR/J anti-Group A antibody. However, preliminary attempts to do so, utilizing passive hemagglutination inhibition as the assay, have not proved encouraging.

However, an idiotypic antiserum detecting inbred strain specific determinants found in SWR/J anti-Group A antibody (Briles and Krause, 1972; see also Appendix I) has been useful for genetic studies.

The proband mouse used in these genetic studies was a spleen cell recipient in SWR/J cell transfer line 1. The mouse was given as ascites tumor to increase the yield of anti-Group A antibody.

Figure 44 shows the microzone electrophoresis pattern of the whole ascites fluid obtained from this mouse. The figure also depicts the ascites fluid after absorption with Group A vaccine, as well as the proband antibody isolated by eluting the vaccine with 5% N-acetyl-D-glucosamine. The alkaline urea disc gel electrophoresis pattern of this antibody, after reduction and alkylation, is shown in figure 19.

By immunoelectrophoresis, the unabsorbed rabbit antiserum against this antibody reacted with a γ -globulin component of the proband ascites fluid and with the isolated proband antibody (figure 45, upper pattern). By double diffusion analysis the unabsorbed antiserum was shown to react with the normal SWR/J ascites fluid (figure 46, upper pattern).

The rabbit antiserum was rendered idiotypically specific by absorption with the largest amount of normal SWR/J ascites fluid still permitting a precipitin reaction between the proband anti-Group A antibody and the

Ascites Fluid from Immunized SWR Mouse

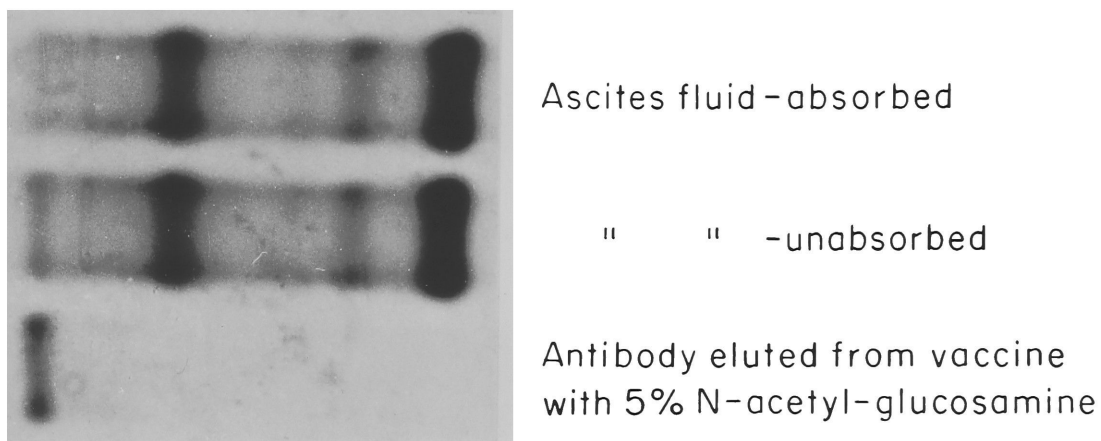


Figure 44. Microzone electrophoresis patterns of absorbed and unabsorbed ascites fluid from a recipient mouse of the second passage of SWR/J cell line 1. After spleen cell transfer, this mouse was immunized with Group A vaccine and subsequently inoculated with an ascites tumor (sarcoma TG/180). The ascites fluid was absorbed with Group A vaccine, and the absorbed anti-Group A antibody was eluted from the vaccine with 5% (0.23 M) N-acetyl-D-glucosamine. This isolated antibody was used to prepare an idiotypic serum against SWR/J cell line 1 antibody.

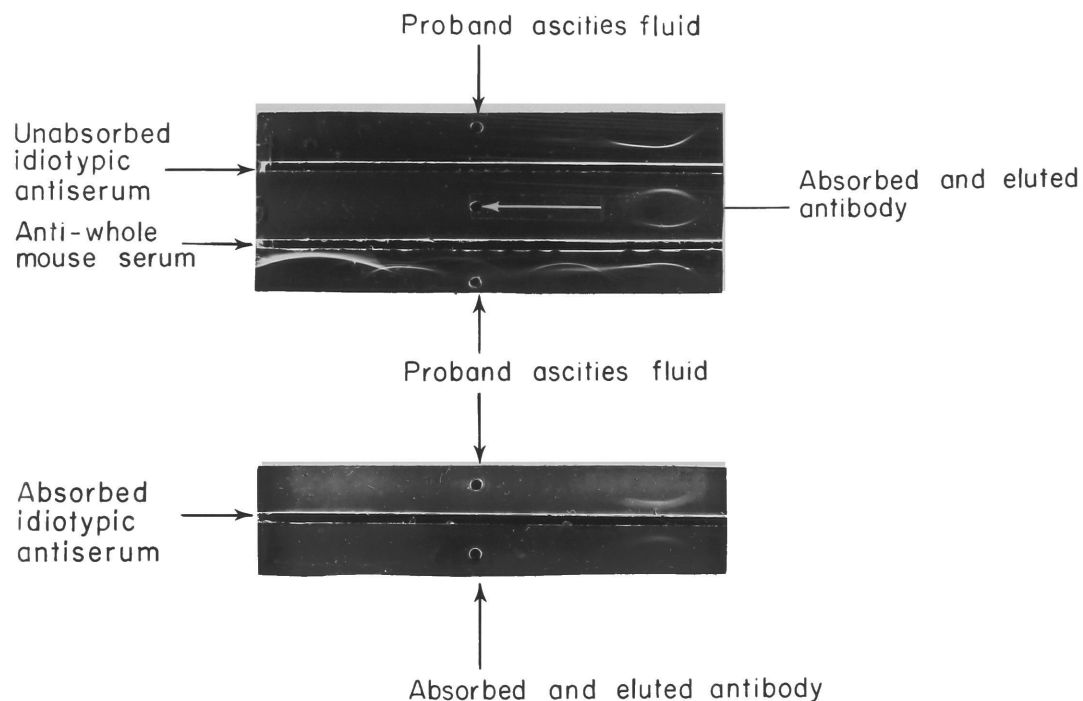


Figure 45. Immunoelectrophoresis of whole (SWR/J cell line 1) proband ascites fluid and isolated proband anti-Group A antibody, developed with anti-cell line 1 idiotypic serum and anti-whole mouse serum. In the upper pattern, the idiotypic antiserum is unabsorbed. In the lower pattern the idiotypic antiserum has been absorbed with normal mouse ascites fluid to render it idiotypically specific. The upper and lower patterns were photographed after 24 and 72 hours respectively at 4°C.

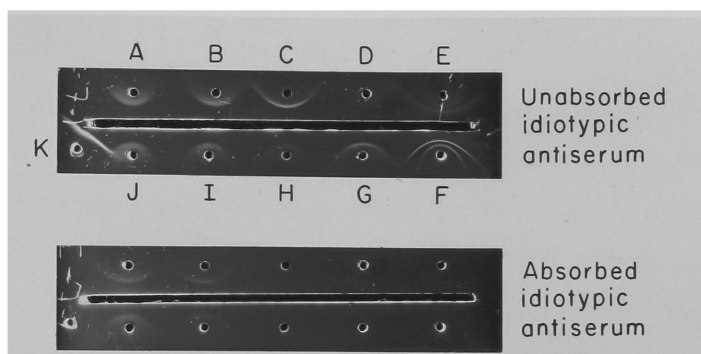


Figure 46. Double diffusion reaction between the anti-SWR/J cell line 1 idiotypic serum, isolated anti-Group A antibody from various sources, and normal mouse serum. In the upper pattern, the idiotypic antiserum is unabsorbed. In the lower pattern, the idiotypic antiserum has been absorbed with normal mouse ascites fluid to render it idiotypically specific. Well E contained normal SWR/J ascites fluid (containing about 0.7 mg/ml mouse immunoglobulin). Well F contained concentrated SWR/J ascites fluid (containing about 3.0 mg/ml mouse immunoglobulin). Well D contained a preparation of anti-Group A antibody that was shown to have deteriorated during storage. Thus, the result with well D should be discounted. All of the other wells were filled with solutions containing 0.3 mg/ml isolated anti-Group A antibody. The sources of the isolated anti-Group A antibody are as follows: A, mouse 5 from second passage SWR/J cell line 1; B and I, proband mouse from second passage SWR/J cell line 1; C and G, pooled A/J anti-Group A serum; H, SWR/J cell line 3; J, mouse 5 from first passage SWR/J cell line 1; K, SWR/J cell line 4. The upper and lower patterns were photographed after 24 and 72 hours respectively at 4°C.

idiotypic antiserum. The precipitation reaction between the idiotypic antiserum and the proband antibody was shown by immunoelectrophoresis (figure 45, lower pattern) and by double diffusion (figure 46, lower pattern).

In figure 46 (lower pattern), it can be seen that the idiotypic serum also gave precipitin lines when tested against isolated antibodies from two other mice from the same cell transfer line. It did not give precipitin lines, however, with anti-Group A antibodies isolated from two other SWR/J cell transfer lines or with normal SWR/J ascites fluid.

Thus, the precipitin reaction of the absorbed idiotypic serum appeared to be specific for the proband anti-Group A antibody.

In the studies which follow, the tube binding assay was used in genetic studies to detect this SWR/J idio-type in SWR/J, A/J, F₁, and backcross mice. All the mice were immunized with Group A vaccine and the antisera tested for the idiotypic cross-reactions with the absorbed SWR/J anti-idiotype serum. Before the results of the genetic studies are described, the conditions of the tube binding assay will be discussed.

As stated above, this idiotypic antiserum was one prepared against the SWR/J cell line 1 antibody (see figure 44). In the test, however, the reference (¹²⁵I-labeled)Group A antibody is from 10 pooled SWR/J anti-Group A antisera. This provided an adequate supply of Group A antibody to perform the tests which follow. Furthermore, use of the pool of SWR/J antibody as the reference antigen enhances the use of the assay to detect idiotypic cross-reactions. This permits detection of that portion of the proband idiotypic specificity which is commonly expressed in many SWR/J anti-Group A antibodies. Inhibition of the assay would indicate the presence of molecules in the test antiserum which were idiotypically similar but not necessarily idiotypically identical to the proband antibody. The technique of detecting idiotypic similarities by using non-proband antibodies as the reference antigen was originally described by Kunkel et al. (1973).¹

¹ Kunkel et al. (1973) used the passive hemagglutination test to detect idiotypic similarities by coating their red cells with homogeneous immunoglobulins which cross-reacted with the idiotypic antiserum, but which were not idiotypically identical with the proband immunoglobulin.

Under the assay conditions used, approximately 10% of the ^{125}I -labeled pooled SWR/J anti-Group A antibody was bound to the idiotypic antiserum in the tubes. If proband antibody had been employed, a higher percentage of binding would have been expected. The low percentage of binding with the ^{125}I -labeled pooled SWR/J anti-Group A antibody is due, in part, to the fact that not all SWR/J anti-Group A antibody have the idiotypic determinant(s) detected by this anti-idiotypic serum. This could be seen in the double diffusion experiment in figure 46 and will be further substantiated later.

To investigate the specificity of idiotypic antiserum in the tube binding assay, anti-Group A antibody from three SWR/J cell transfer lines and from SWR/J, A/J, and SWR/J x A/J pools were used.

The binding of the ^{125}I -labeled Group A antibody to the antiserum-coated tubes could be inhibited with either pooled SWR/J anti-Group A serum or isolated pooled SWR/J anti-Group A antibody, as is shown in figure 47. As in the previous study, normal SWR/J immunoglobulin gave significant inhibition only at high concentrations. Absorption of the SWR/J anti-Group A serum with Group A vaccine reduced the inhibitory capacity to 1/30 of what it had been.

In figure 48, it can be seen that binding of the labeled SWR/J anti-Group A antibody to the antiserum-coated tubes can be readily inhibited with β -phenyl-N-acetyl-D-glucosaminide and N-acetyl-D-glucosamine, but not with N-acetyl-D-galactosamine, glucose, or rhamnose. From the data in these two figures (47 and 48), it can be concluded that the idiotypic determinant detected by this assay is associated with the antigen binding site of the labeled Group A antibody. Inhibition of the assay by other anti-Group A antibodies would therefore indicate that their idiotypic determinants cross-react with the idiotypic specificities detected by the assay.

Figure 49 shows a comparison between the inhibitory power of the pooled SWR/J and A/J anti-Group A serum. Also shown are the inhibition curves for the anti-Group A antibody isolated from each of these serum pools. Unlike the hemagglutination inhibition experiments with this antiserum, the A/J

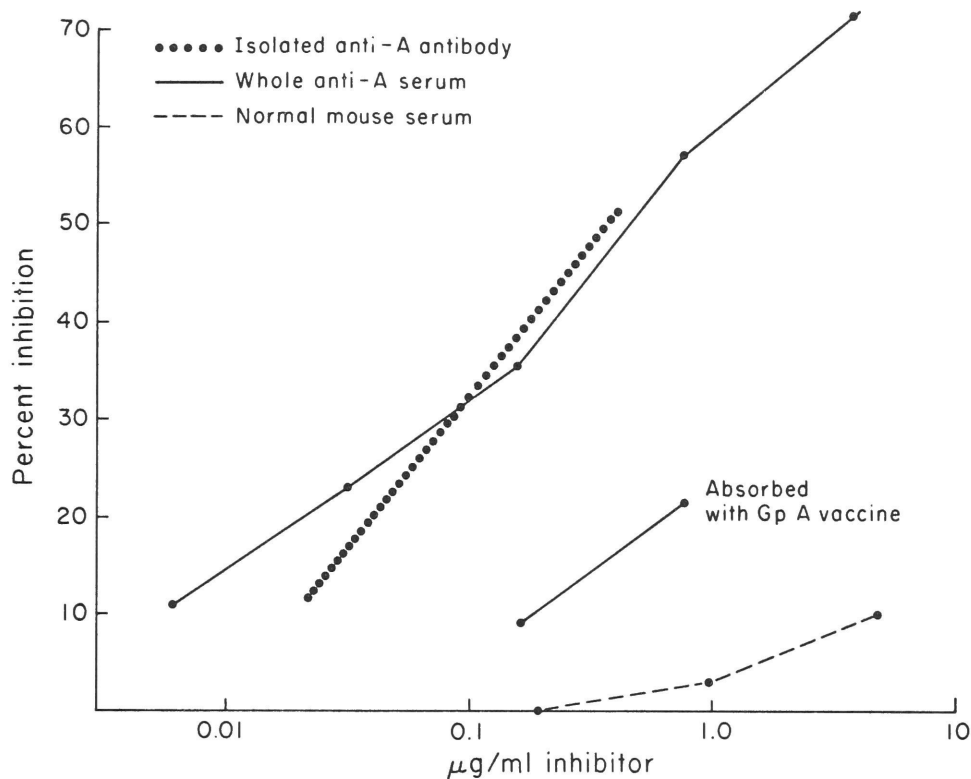


Figure 47. Inhibition of binding of ^{125}I -labeled SWR/J anti-Group A antibody to tubes coated with anti-SWR/J cell line 1 idiotypic serum. Anti-Group A serum was obtained from a pool of SWR/J anti-Group A sera. Inhibition curves for this serum and the anti-Group A antibody isolated from it are plotted in terms of $\mu\text{g/ml}$ anti-Group A antibody. The anti-Group A serum was absorbed with Group A vaccine. The inhibition curve for the absorbed anti-Group A serum is plotted in terms of the amount of anti-Group A antibody in the serum prior to absorption. The inhibition curve for the SWR/J normal serum pool is plotted in terms of $\mu\text{g/ml}$ mouse immunoglobulin.

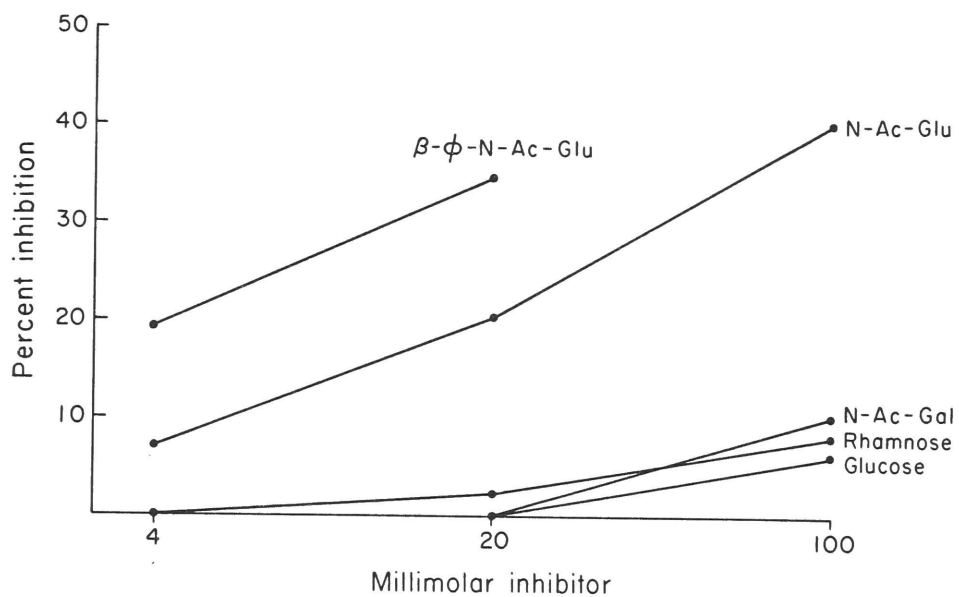


Figure 48. Inhibition of binding of ^{125}I -labeled pooled SWR/J anti-Group A antibody to tubes coated with anti-cell line 1 idiotypic serum.

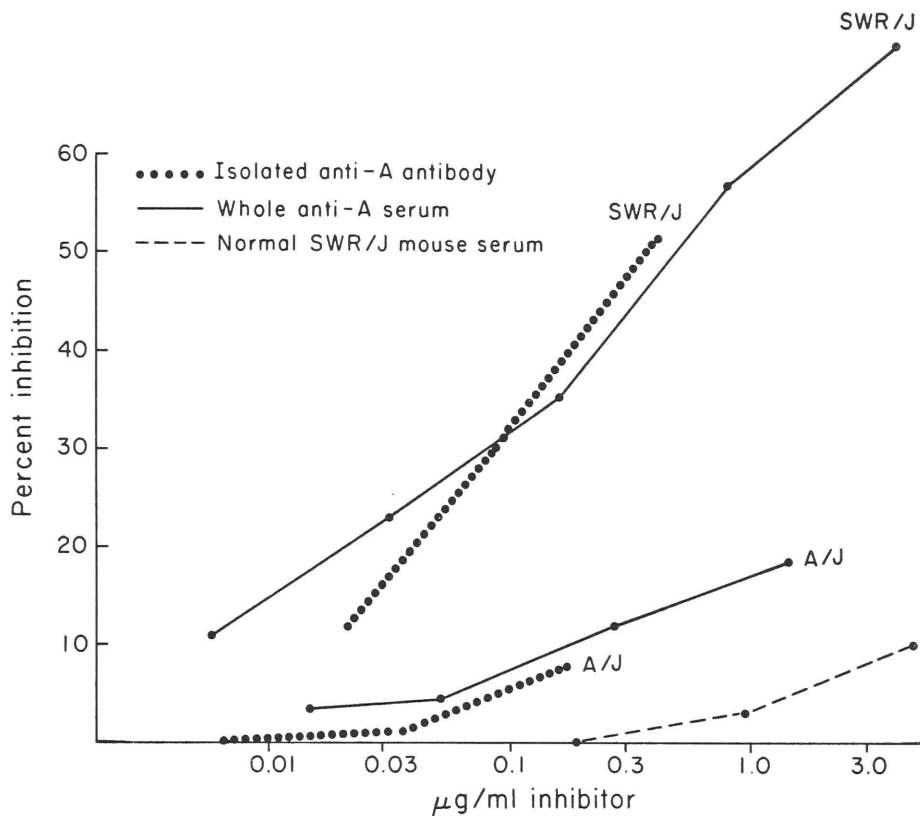


Figure 49. Inhibition of binding of ^{125}I -labeled SWR/J anti-Group A antibody to tubes coated with anti-SWR/J cell line 1 idiotypic serum. The SWR/J and A/J anti-Group A sera were obtained from serum pools. The isolated SWR/J and A/J anti-Group A antibodies were isolated from these same serum pools. The inhibition curves for the isolated anti-Group A antibodies and the anti-Group A sera are plotted in terms of $\mu\text{g/ml}$ anti-Group A antibody. The inhibition curve for the SWR/J normal serum pool is plotted in terms of $\mu\text{g/ml}$ mouse immunoglobulin.

anti-Group A antibody did appear to weakly inhibit the idiotypic reaction. In an effort to remove the idiotypic cross-reaction between the idiotypic antiserum and the A/J anti-Group A pool, the idiotypic serum was absorbed with some of the A/J anti-Group A pool at the same time that the routine absorption with normal SWR/J serum was performed.

This doubly absorbed idiotypic antiserum appeared to still be specific for the anti-Group A binding site, since the reaction of the anti-idiotypic serum with the ^{125}I -labeled SWR/J anti-Group A antibody could be 51% inhibited with 100 millimolar N-acetyl-D-glucosamine. The additional absorption however appeared to have increased the anti-idiotypic serum's specificity for SWR/J anti-Group A antibody with respect to A/J anti-Group A antibody (figure 50). The inhibition by the pooled A/J anti-Group A antibody appeared to level off at less than 10%. Subsequent studies with individual A/J sera also showed the same leveling off below 10%. This was interpreted to mean that the A/J anti-Group A antibody could cross-react with only a small fraction of the anti-binding site antibodies in the idiotypic serum.

In contrast to the A/J anti-Group A antibody, SWR/J and SWR/J x A/J anti-Group A antibody gave strong inhibition reactions. At concentrations of 1 $\mu\text{g}/\text{ml}$, both were able to inhibit the tube binding assay by more than 50%. Thus, it appeared as though inhibition of more than 10% might be an indication of the presence of an idio type found in the anti-Group A antibody of mice bearing SWR/J genes.

Thus, it appeared that this idiotypic system would be useful for studying the inheritance of the SWR/J idio type in crosses with A/J mice.

For this experiment, a total of 87 SWR/J x A/J, SWR/J (SWR/J x A/J), and A/J (SWR/J x A/J) mice were raised at the same time. When these mice were all three to four months old, they were given the immunization series shown in Table XI. The A/J and SWR/J mice were obtained from Jackson Laboratory and were immunized in a similar manner.

The antisera obtained from these mice were used to inhibit the tube binding assay. The inhibition curves for these were plotted versus the anti-Group A antibody concentration in the serum dilutions tested.

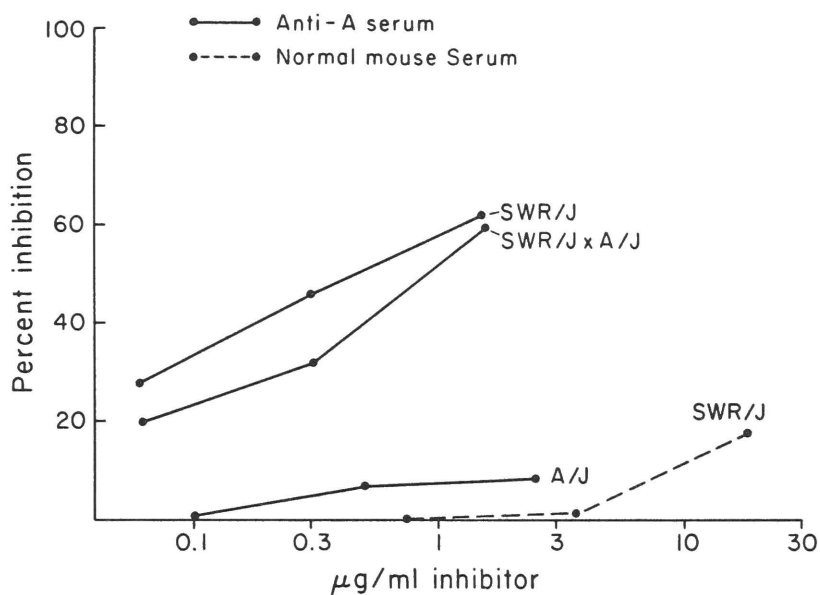


Figure 50. Inhibition of binding of ^{125}I -labeled SWR/J anti-Group A antibody to tubes coated with anti-SWR/J cell line 1 idiotypic antiserum. In addition to the usual absorption with normal SWR/J ascites fluid, the idiotypic antiserum was also absorbed with some pooled A/J anti-Group A antibody. The SWR/J, A/J, and SWR/J x A/J anti-Group A sera were obtained from serum pools. The inhibition curves for the anti-Group A sera are plotted in terms of $\mu\text{g/ml}$ anti-Group A antibody. The inhibition curve for the SWR/J normal serum pool is plotted in terms of $\mu\text{g/ml}$ mouse immunoglobulin.

TABLE XI

Injection Schedule for Mice in Inheritance
of Idiotype Experiment (see Figure 52)

| | | | | | | | |
|-------|---|----|----|-----|-----|-----|------|
| Day | 0 | 33 | 59 | 104 | 106 | 108 | 119 |
| Dose* | 3 | 30 | 30 | 30 | 30 | 30 | Bled |

*vaccine dose in μg rhamnose per injection

The percent inhibition obtained with 1 $\mu\text{g/ml}$ anti-Group A antibody of each of these 87 sera is plotted in figure 51 as a black dot (figure 51). It should be noted that at a concentration of 1 $\mu\text{g/ml}$, normal mouse immunoglobulin gives little or no inhibition. Since the inhibition curves for 17 out of the 18 A/J anti-Group A sera leveled off below 10% inhibition, an arbitrary line was drawn at 10% inhibition. Any inhibition greater than 10% was taken as an indication of the presence of the SWR/J idiotypic.

The observed inhibition (at 1 $\mu\text{g/ml}$ anti-Group A antibody) for the 10 individual SWR/J anti-Group A sera ranged from 10-70%. Proband (cell line 1) anti-Group A antibody itself gave only 70% inhibition when tested at a concentration of 1 $\mu\text{g/ml}$ (Table XII). Therefore, in figure 51, sera giving an inhibition of around 70% are inhibiting with an efficiency equal to that of the proband antibody. Some idea of the range of the amount of idiotypic expression depicted in figure 51 can be obtained by examining figures 47 and 50. From figures 47 and 50, it is apparent that several hundred times more pooled SWR/J anti-Group A antibody was required to inhibit the idiotypic reaction 70% rather than 10%.

The data in figure 51 clearly show an inherited predisposition to make the SWR/J idiotypic. Nine out of 10 of the SWR/J anti-Group A sera gave inhibitions of greater than 10%, whereas only 1 in 18, or 5.5%, of the A/J anti-Group A sera gave an inhibition of greater than 10%. The F_1 anti-Group A sera appear to be good inhibitors of the idiotypic reaction, as had been expected from the data in figure 50. The $F_1 \times$ SWR/J anti-Group A sera were also good inhibitors, as one would expect.

In the backcross to the A/J mice, 34% of the sera gave inhibition values of greater than 10%. This suggests segregation of the SWR/J idiotypic genes in the backcross mice. A statistical evaluation was performed using a 2x2 heterogeneity Chi square on the points above and below the 10% line in the A/J and $F_1 \times$ A/J groups. The results indicated that the distribution was significantly different from random at the 2% level.

From the observation that only 78% of the F_1 mice gave a positive inhibition and 5.5% of the A/J mice gave a positive inhibition, the number of backcross mice expected to give a positive inhibition could be calculated.

TABLE XII

Inhibition of Binding of ^{125}I -Labeled Pooled SWR/J
Anti-Group A Antibody to Anti-Cell Line 1 Idiotypic Serum

| Source of anti- Group A sera | % Inhibition | |
|---------------------------------|----------------------------|----------------------------|
| | at 0.1 $\mu\text{g/ml}$ Ab | at 1.0 $\mu\text{g/ml}$ Ab |
| Cell line 1 | 59 | 70 |
| Cell line 3 | 0 | 1.5 |
| Cell line 7 | 1 | N.D. |
| Pool SWR/J anti-Group A | 35 | 59 |
| | at 0.1 $\mu\text{g/ml}$ Ig | at 1.0 $\mu\text{g/ml}$ Ig |
| Pool SWR/J normal serum | 0 | 1.0 |

For a one gene system this came out to 16.8 positive backcross mice. This is not significantly different from the value of 14 positive backcross mice that were observed. However, the observed value is also not significantly different from the expected value of 9.85 positive mice that would have been expected if the inheritance of two genes were required for expression of the idiomorph.

It therefore appears that the difference in idiotypic expression between the SWR/J and A/J mice is controlled by genes at a small number of major loci, possibly a single major locus.

The inhibition value for the one positive A/J anti-Group A sera was rechecked and found not to be in error. Thus from the present data, we must conclude that A/J mice may be capable of making an idiotypic specificity that cross-reacts with the cell line 1 idiomorph, although they make this specificity only rarely.

The pedigree of the F_1 x A/J mice was confirmed, utilizing the difference in the beta chain of hemoglobin known to exist between A/J and SWR/J mice (Green, 1968). SWR/J mice are homozygous for the s allele (hemoglobin bands sharply in electrophoresis), and A/J mice are homozygous for the d allele (hemoglobin exhibits diffuse banding pattern in electrophoresis). By the procedure described in the Methods section, it was possible to distinguish the sd heterozygotes from the dd homozygotes.

Using this procedure, hemoglobins from 15 of the F_1 x A/J backcross mice were tested. Eight were shown to be sd heterozygotes, and the inheritance of the hemoglobin difference segregated independently of the ability to make the SWR/J idiomorph.

The fact that all of the SWR/J anti-Group A antibodies did not inhibit the idiotypic reaction with equal efficiency had been expected, since it had already been demonstrated (see figure 46) that not all SWR/J anti-Group A antibodies carried the idiotypic specificity. This was also demonstrated by using the tube binding assay to test anti-Group A sera from cell transfer lines 1, 3, and 7 for their inhibitory capacity. Table XII shows the results of these tests.

Only the cell transfer line 1 antibody and the pooled SWR/J anti-Group A antibody were inhibitory at 0.1 and 1.0 $\mu\text{g/ml}$. It therefore appeared that at least some of the SWR/J anti-Group A antibodies did not carry the idiotypic determinant.

Since 10 times more of the pooled SWR/J anti-Group A was required than proband antibody for 59% inhibition (see Table XII), it appeared as though only about 1/10 of the anti-Group A antibodies in the SWR/J pool carried the SWR/J idiootype.

DISCUSSION

From the results of the studies described in this thesis, and from the results of studies from other laboratories (Braun et al., 1972; Eichmann, 1972), it has become apparent that the mouse anti-streptococcal group carbohydrate antibodies are useful for examining questions concerning homogeneous antibody, idiotypy, and antibody-producing cell clones.

The usefulness of the mouse anti-streptococcal antibodies stems from the fact that several strains of inbred mice, including A/J and SWR/J, readily produce high serum levels of antibodies with restricted heterogeneity against streptococcal Group A carbohydrate (Briles, 1971; Briles and Krause, 1972). This finding was confirmed and extended by work in other laboratories (Braun et al., 1972; Eichmann, 1972). Eichmann (1972) also demonstrated that a homogeneous antibody response could be obtained to streptococcal Group C carbohydrate. The route of immunization, the dose of vaccine, and secondary immunizations are important variables which influence the anti-Group A carbohydrate response.

From this work, and that of Braun et al. (1972) and Eichmann (1972), it is apparent that different strains of mice differ in their ability to make humoral anti-Group A antibody after immunization with streptococcal Group A vaccine. The genetic control of the anti-Group A response to sub-optimal doses of Group A vaccine has been examined here in two genetic studies. In each study, BSVS mice were the low-responding strain, and A/J and Balb/cJ mice served as high and intermediate-responding strains, respectively.

From Table VIII, it is apparent that, after the first series of immunizations, the A/J mice produced much more anti-Group A antibody than did the BSVS mice. What is most remarkable is that after the booster injection, the BSVS mice had no higher anti-Group A titers than they had after the first series of immunizations. The A/J mice, on the other hand, increased their average titer ten-fold. Thus, the BSVS mice not only responded poorly to the initial stimulation, but they also failed to develop a secondary-type response after the booster immunization.

These findings indicated that the lower antibody response of the BSVS mice may be due in part to an inability to make a secondary response to Group A carbohydrate. A comparison of the antibody responses of hybrid A/J x BSVS mice with those of the two parental strains, for both the priming and booster injections (figures 29 and 30), suggests that the anti-Group A responses to the priming and booster immunizations were under separate genetic control. It would be of interest in this connection to determine whether the anti-Group A antibody produced by the BSVS mice is primarily 7s or 19s immunoglobulin. Grumet (1972) has demonstrated that one of the Ir-1 genes in the mouse controls the ability to switch from IgM (primary response) to IgG (secondary response) antibody production.

From the genetics experiment involving Balb/cJ and BSVS mice, it was observed that hybrid Balb/cJ x BSVS mice gave an average antibody titer intermediate to that obtained with Balb/cJ and BSVS mice. From backcross studies, it was concluded that the difference in response between the F_1 and BSVS mice was probably the result of a gene at a single major locus. The difference in response between the F_1 and Balb/cJ mice, on the other hand, appeared to require BSVS genes at several loci.

From these genetics studies, it is apparent that the ability to make anti-Group A antibody is under genetic control. Genes at several loci are probably involved, but the number appears to be small enough to make further studies possible.

As pointed out above, the primary reason for studying the antibody responses to streptococcal group carbohydrate in mice was the hope that they would produce antibodies with restricted heterogeneity, as had been observed in rabbits (Osterland et al., 1966; Krause, 1970b). From the earliest studies on anti-Group A antibody produced in inbred mice, it was apparent that many of these antibodies exhibited marked restriction of electrophoretic mobility (Briles, 1971; Braun et al., 1972; Eichmann, 1972). These findings confirmed an unpublished finding of Eichmann and Krause that outbred NCS mice often produce rather homogeneous antibody when immunized with Group C streptococcal vaccine.

Eichmann (1972) has been able to demonstrate that some mouse anti-Group A antibodies give an isoelectric focusing pattern characteristic of a single molecular species. These data, coupled with our own, indicate that many of the anti-Group A antibodies exhibit quite restricted heterogeneity.

In 1970, Askonas et al. demonstrated that cell clones producing anti-DNP antibody could be propagated in mice by serial passage of spleen cells from donor to recipient. Briles (1971) reported that cell clones producing anti-Group A antibody could be propagated in a similar manner. This was subsequently confirmed by additional studies (Eichmann, 1972; Briles and Krause, 1972). These clones, however, cannot be passed indefinitely. Of the 10 clones from A/J and SWR/J mice that have been passed, none persisted for more than two or three passages. There is a decline in the antibody concentration from one mouse passage to the next. Such a limited clone life span has also been observed with anti-DNP clones (Williamson and Askonas, 1972).

In spite of the short life span of these clones, it should be possible to obtain a considerable amount of anti-Group A antibody from a single clone. In the A/J anti-Group A clone described in Table IX, the average antibody concentration in the second transfer recipients was about 5 mg/ml. If all of the first transfer recipients, rather than just one of them, had been used as donors for the second passage, there could have been 100 second-passage recipients. From 100 recipients, each with a titer of 5 mg/ml, it would have been possible to obtain as much as 1 gram of antibody for structural studies. Further refinements in the cellular transfer technique should make it possible to obtain even more antibody from the second transfer mice. Although the cellular transfer technique has not been fully exploited to obtain large amounts of antibody, it has yielded sufficient antibody to develop the idiotypic studies reported here.

Before discussing the results of idiotypy experiments with mouse anti-Group A antibodies, I would like to discuss the nature of the idiotypic reactions in general. In the Results section, I often referred to idiotypic determinants as though they were single discreet entities. Idiotypic determinants are actually not single defined units, but whole portions of the

hypervariable region. Idiotypic antisera are also generally complex, and contain many different antibodies that recognize various overlapping portions of the same hypervariable region. In fact, different idiotypic antisera to the same antibody can recognize different portions of the hypervariable region. Of the idiotypic specificities in the variable region, there are undoubtedly some that are quite unique to the proband molecule (and other molecules of identical variable region structure), whereas others are more or less common to other antibodies with specificity for the same antigens.

In the studies described in this thesis, idiotypic antisera to mouse anti-Group A antibodies have been prepared in rabbits. These antisera require absorption with normal mouse serum (containing normal mouse γ -globulin) to remove antibody directed against all but the idiotypic determinants of the immunizing mouse anti-Group A antibody. Various degrees of absorption and various assay procedures are used to study different subsets of the idiotypic specificities detected by the antiserum. For example, extensive absorption of idiotypic antisera generally diminishes the number of cross-reactions with other antibodies with specificity for the same antigen. Idiotypic antisera absorbed to a lesser degree cross-react more widely with other antibodies of the same specificity.

The type of assay used also has an effect on the idiotypic specificities studied. For example, in the passive hemagglutination inhibition system, if the proband antibody is used to coat the erythrocytes, the inhibitor must be essentially identical to the proband to give complete hemagglutination inhibition (Kunkel, 1970). However, in an inhibition of precipitation assay, complete identity of the inhibitor is not required for inhibition (see Eichmann and Kindt, 1971), because the precipitation lattice will be disrupted if only a few of the major specificities of the proband are inhibited (Wilson *et al.*, 1971).

As was indicated in the Results section, in order to study idiotypic specificities common to the proband as well as other antibodies to the same antigen, it is preferable to perform the inhibition assay with a cross-reacting immunoglobulin as the reference antigen (Kunkel *et al.*, 1973). In this way, any other immunoglobulins having the idiotypic

specificity common to the proband and the reference antigen will readily inhibit the idiotypic assay.

Idiotypic studies are further complicated by the fact that some types of idiotypic assays, such as inhibition of precipitation, are quite susceptible to inhibition with high concentrations of normal immunoglobulin (Grey et al., 1965; Eichmann and Kindt, 1971; Wilson et al., 1971). From the data presented in this thesis, and by Carson and Weigert (1973), it is apparent that the tube binding idiotypic assay is also susceptible to inhibition by high concentrations of normal gamma globulin.

An experiment was performed which indicated that inhibition of the tube binding assay with high concentrations of normal immunoglobulin shows some specificity for immunoglobulin from closely related animals (see Appendix II, figure 53). Immunoglobulin from syngeneic mice gave stronger inhibition than immunoglobulin from unrelated mice. Rabbit and bovine immunoglobulins gave even less inhibition. This finding is in close agreement with a similar finding by Eichmann and Kindt (1971), who demonstrated that preimmune rabbit serum from the proband and related rabbits gave stronger inhibition of precipitating idiotypic reactions than did preimmune sera from unrelated rabbits.

The first series of idiotypic experiments described in this thesis employed an idiotypic antiserum prepared against A/J anti-Group A antibodies with greatly restricted electrophoretic mobility (see figure 33). At least some of the idiotypic determinants detected by this absorbed anti-idiotypic antiserum were associated with the antigen binding site of the proband antibody, since N-acetyl-D-glucosamine could partially inhibit the precipitation reaction between the idiotypic antiserum and the proband antibody (see figure 38). The anti-idiotypic serum was absorbed with sufficient normal A/J mouse serum so that it no longer precipitated with normal mouse γ -globulin. The absorbed antiserum still gave a good precipitation reaction with pooled A/J anti-Group A antibody, and a weaker reaction with pooled SWR/J anti-Group A antibody (figure 39).

The cross-idiotypic reaction with SWR/J anti-A antibody had been unexpected, since shared idiotypy is generally found only in closely

related inbred strains (Blomberg et al., 1972; Sher and Cohn, 1972; Pawlak et al., 1973; Eichmann, 1973). A number of experiments were performed to examine the nature of the A/J and SWR/J cross-idiotypic determinants (idiotypic determinants on non-proband anti-Group A antibodies) detected by this anti-idiotypic serum.

In these experiments, the tube binding assay of Askenase and Leonard (1970) was utilized as an idiotypic assay, as described by Carson and Weigert (1973). Because interest centered on cross-idiotypic specificity of the antiserum, the proband anti-Group A antibody was not used as the reference antigen. Instead, separate pools of A/J and SWR/J anti-Group A antibodies were iodine-(^{125}I -)labeled for use as reference antigens. It was observed that the binding of the ^{125}I -labeled pooled A/J anti-Group A antibody to the anti-A/J idiotypic antiserum could be specifically inhibited with N-acetyl-D-glucosamine. This indicated that the idiotypic antiserum was directed at least in part to the antigen binding site of the pooled anti-Group A antibodies. A/J and SWR/J anti-Group A sera could also inhibit the binding of the labeled A/J anti-Group A antibody to the antiserum-coated tubes, although the SWR/J anti-Group A sera were generally much less effective inhibitors (figures 40, 41, 42).

Since idiotypic cross-reactions are not commonly seen between mouse antisera with the same specificity from unrelated individuals, it was of interest to determine whether or not the cross-idiotypic reaction between the idiotypic antiserum and the SWR/J anti-Group A antibodies was directed against the antigen binding site, or was simply due to constant region cross-reactions which occurred because of inadequate absorption.

This cross-idiotypic reaction was investigated directly by using ^{125}I -labeled pooled SWR/J anti-Group A antibodies as the reference antigen in the tube binding assay. From inhibition experiments using N-acetyl-D-glucosamine and related haptens, it could be demonstrated that the idiotypic antiserum was in fact directed, at least in part, against the antigen binding site of the ^{125}I -labeled pooled SWR/J anti-Group A antibodies (see figure 43).

This same cross-idiotypic reaction (between ^{125}I -labeled pooled SWR/J anti-Group A antibody and the anti-A/J idiotypic serum) could be inhibited by mouse anti-Group A antibody from several sources. From Table X (page 117), it is apparent that all of the 6 strains tested were equally capable of producing anti-Group A antibody capable of inhibiting the idiotypic reaction. This result stands in contrast with that obtained when pooled A/J anti-Group A antibody is used as the reference antigen (see figure 42).¹ This indicates that the anti-A/J idiotypic serum also detects additional specificities in the A/J anti-Group A antibody that are rare or nonexistent in the SWR/J anti-Group A antibody.

What is of interest is that by not extensively absorbing the anti-A/J idiotypic serum, cross-idiotypic determinants associated with the antigen binding site were detected for anti-Group A antibodies produced by many different (and not closely related -- see Green, 1968; Lynch, 1969) strains of mice.

The full significance of this finding is not clear. One possible explanation is that this cross-idiotypic determinant is so closely associated with the ability of anti-Group A antibodies to bind terminal β -N-acetyl-D-glucosaminide residues in bacterial antigens, that there has been strong selection pressure on all mice to be able to produce this determinant.

The second series of idiotypic experiments described in this thesis were done with an idiotypic antiserum directed against an isolated SWR/J anti-Group A antibody (from a recipient mouse of SWR/J cell line 1). In contrast to the anti-A/J idiotypic serum described above, this idiotypic antiserum was quite specific for the proband anti-Group A antibody and did not give a precipitation reaction with monodisperse anti-Group A antibodies from two other SWR/J cell transfer lines.

Using the tube binding assay, it was possible to demonstrate the presence of cross-reacting idiotypic determinants in the anti-Group A antibody of other SWR/J mice but generally not in A/J mice. It was also possible to demonstrate the inheritance of this idiotypic specificity in immunized

¹ The results in figure 42 and in Table X were obtained with the same 8 SWR/J and A/J anti-Group A sera.

mice from crosses and backcrosses between SWR/J and A/J mice (figure 52). These more extensive studies were consistent with previous experiments using passive hemagglutination inhibition as an idiotypic assay (see Appendix I).

In the tube binding assay with which this genetics study was done, ^{125}I -labeled pooled SWR/J anti-Group A antibody was used as the reference antigen. It was possible to demonstrate that the idiotypic determinant detected by this assay was associated with the antigen binding site of some, but not all, SWR/J anti-Group A antibodies.

In this assay, an inhibition of greater than 10% was interpreted as an indication of the presence of the SWR/J idiotypic specificity. Using this criterion, it is apparent that immunized SWR/J mice have a much greater predisposition to produce this idio type than do immunized A/J mice. The hybrid A/J x SWR/J mice clearly demonstrated inheritance of the ability to produce the SWR/J idiotypic determinant. In the backcross, F_1 x A/J mice, 34% of the mice showed inhibition titers of greater than 10%, as compared to 5.5% of the A/J mice. This difference was statistically significant at the 2% level. The numbers of backcross mice that showed the SWR/J idio type was consistent with a single gene mode of inheritance. Studies are currently underway to examine possible genetic linkage between the ability to make the SWR/J idio type and the mouse heavy chain allotypic locus.

The variation in expression of the idio type in different mice of the same genotype is interpreted to reflect the fact that not all SWR/J anti-Group A antibodies carry the idiotypic specificity detected by the assay (see Table XII, page 132), and that different SWR/J and F_1 anti-Group A sera possess different ratios of antibody with and without the idiotypic determinant. Both from the studies presented in this thesis and in a recent report by Eichmann (1973), it is clear that an inbred strain of mouse has the capability of making at least several different antibodies to the Group A antigen. The determination of the number of different anti-Group A antibodies that can be produced by a single inbred strain will have to await further study.

Since this work was undertaken, several other studies on the inheritance of idiotypic markers in inbred mice have been reported. These reports are summarized in Table XXI on the following page. Blomberg et al. (1972) studied the idiotypy of mouse antibodies to α -1,3-dextran. Their anti-idiotypic serum was prepared against a Balb/c myeloma protein known to bind α -1,3-dextran. They used this idiotypic serum to look for the presence of the idiotypic determinant in Balb/c mice immunized with α -1,3-dextran. The idiotypic determinant of the myeloma protein was found in Balb/c anti-dextran antibodies but not in C57Bl/6 anti-dextran antibodies. The idiotpe was expressed in the hybrid obtained by crossing the two strains. The idiotpe was also expressed in two of Bailey's (1971) seven recombinant inbred (RI) strains derived by inbreeding hybrid Balb/cJ x C57Bl/6J mice. Both of these strains carried the Balb/cJ heavy chain allotypes, suggesting that there might be close linkage between the heavy chain allotypic markers and the locus controlling the idiotypic specificity of the Balb/cJ idiotpe.

A similar study has been done by Sher and Cohn (1972), using a Balb/cJ myeloma protein known to bind the phosphoryl choline determinant of pneumococcal C polysaccharide. The idiotypic specificity detected by this assay was shown to be present in Balb/cJ mice immunized with pneumococcal C polysaccharide and absent in A/J mice immunized with the same antigen. By performing a genetic breeding experiment of the type described in this thesis, they were able to demonstrate that the ability to produce the Balb/cJ idiotpe is linked to the heavy chain allotype locus of the Balb/cJ mice.

In the study by Pawlak et al. (1973), antibody to p-azophenyl-arsenate was used to prepare an idiotypic serum. The idiotpe detected by this antiserum was shown to be present in AL/N mice but not in Balb/cJ mice. The idiotpe was also present in AL/N x Balb/cJ mice. By making use of a single congenic strain, Pawlak et al. were able to demonstrate probable linkage of the ability to produce the AL/N idiotpe to the heavy chain allotype locus.

TABLE XXI

Genetic Studies of Idiotype in Inbred Mice

| <u>Antigen</u> | <u>Mouse Strains</u> | | | |
|------------------------------------|----------------------|---------|----------------|-----------------|
| $\alpha,1,3$ dextran ¹ | Balb/cJ | C57B/6 | F ₁ | RI ⁵ |
| | + | - | + | +, - |
| phosphoryl-choline ² | Balb/cJ | A/J | F ₁ | Backcross |
| | + | - | + | +, - |
| Group A CHO ³ | A/J | Balb/cJ | F ₁ | |
| | + | - | + | |
| Group C CHO ³ | + | - | + | |
| p-azo-phenyl-arsonate ⁴ | AL/N | Balb/cJ | F ₁ | Congenic |
| | + | - | + | + |

¹ Blombert et al. (1972)² Sher and Cohn (1972)³ Eichmann (1973)⁴ Pawlak et al. (1973)⁵ RI = Recombinant Inbred Strains (Bailey, 1971; also see text)

Eichmann (1973), in a study similar to that reported here, has identified idiotypic markers in A/J mice that are present on anti-streptococcal Group A and streptococcal Group C antibodies. These markers are present in A/J mice and absent from Balb/cJ mice. These idiotypic markers were also shown to be present on some of the anti-Group A or anti-Group C antibodies produced by hybrid A/J x Balb/cJ mice. Eichmann (1973) demonstrated that the antibodies in the hybrid mice that carried the A/J idio-type also possessed the A/J constant region allotype, thus showing a phenotypic linkage between the idiotypic and allotypic markers of the A/J mouse.

As was indicated in the Introduction, the allotypic markers of the mouse are located in the constant region of the heavy chains. The allotypic markers in the various heavy chain subclasses are so closely linked that studies in several laboratories have been unable to detect cross-overs between them, even though over 1,000 backcross mice have been studied (see Hertzzenberg and McDevitt, 1968).

Present data indicate that major genes controlling idiotypic expression are linked to this same chromosome region. Just as the genes regulating the expression of allotypy are implicated in controlling the structure of constant regions, it seems likely that the idiotypy genes play a role in determining the structure of the hypervariable region (see Sher and Cohn, 1972; Carson and Weigert, 1973).

From the studies of Sher and Cohn (1972) and Carson and Weigert (1973), there is some evidence that cross-overs occur between the genes controlling idiotypy and those controlling heavy chain allotypes. If this turns out to be the case, it would be additional evidence in favor of the theory that separate genes code for constant and variable region portions of the heavy chain (Dryer and Bennett, 1965).

Cohn (1970) postulated that hypervariable region structure is the result of somatic mutation of a relatively small number of germ line genes. In the studies of Sher and Cohn (1972), and those of Carson and Weigert (1973), this type of theory has been used to explain their findings with idiotypic studies of myeloma proteins and antibodies directed against phosphoryl choline and α -1,3-dextran.

From studies in several laboratories, it has been observed that only occasionally is an inbred mouse strain found whose antibody never cross-reacts with the idiotypic specificity (present in another strain) that is being studied (Kuettner *et al.*, 1972; Blomberg *et al.*, 1972; Sher and Cohn, 1972; Eichmann, 1973; Pawlak, 1973). Even in some of the genetics studies summarized in Table XXI, the designation of strains as idiotypically negative must be qualified, because a small proportion of antibody was reactive with the idiotypic serum. For example, in the idiotypy genetics studies of Blomberg *et al.* (1972) and of Sher and Cohn (1972), where idiotypy was studied at the level of the antibody-producing cells, it was found that in the "negative" strains, as many as 20% of the antibody-producing lymphocytes were producing antibody with the idiotype of the "positive" strain. In the positive strains, 90-100% of the antibody-producing lymphocytes were producing antibody with the idiotype being studied.

This type of result could be explained by the type of somatic generation of hypervariable region structure suggested by Cohn (1970) if one assumed that in the case of the "negative" strain, a larger number of random somatic events were required to produce the particular idiotype from its germ line genes than were required to produce the same idiotypic specificity from the germ line genes of the positive strain. The net effect would be that a higher frequency of the lymphocytes in the positive strain would be able to make the idiotype than in the negative strain. Such an explanation could also explain the fact that one of the eighteen A/J anti-Group A sera tested in our experiment inhibited the idiotypic assay, compared with 90% of the SWR/J anti-Group A sera.

Since it is clear from several studies that both light and heavy chains generally contribute to the antigen binding site (Edelman *et al.*, 1963; Rohult *et al.*, 1966) and to the idiotypic determinant (see Grey *et al.*, 1965; Carson and Weigert, 1973), it had been expected that the inheritance of gene products at two different loci might be required for the inheritance of idiotypy. However, the data of Blomberg *et al.* (1972), Sher and Cohn (1972), and Pawlak *et al.* (1972) all indicate that a single major locus, linked to the heavy chain allotypic locus, controls idiotypic expression.

The data presented in this thesis are totally consistent with idiotypic expression being controlled by a single major locus. Two possible explanations for the control of idiotype in the mouse at only one locus (Carson and Weigert, 1973; Pawlak *et al.*, 1973) are: 1) the genes controlling the light and heavy chain variable regions are very closely linked, and 2) only the genes controlling the heavy chain hypervariable region need to be inherited because all of the inbred mice tested have the capability of making suitable light chain hypervariable regions. Sher and Cohn (1972) have presented data that an additional locus, not linked to the allotypic locus, may play a minor role in the inheritance of idiotype. They have suggested that this gene may be a light chain variable region structural gene. However, their data are not quite what one would expect if this were the case, because the second locus is not required for idiotypic expression and can, in fact, apparently cause some (but not full) idiotypic expression in the absence of the allotype-linked gene.

Many additional experiments will be required to resolve some of these problems and to get a better understanding of the genetic control of the genes regulating the expression of antibody hypervariable region structure. While much has yet to be learned about the nature of the genes controlling idiotypic expression, these genes are likely candidates for the genes that determine hypervariable region sequences. Further studies on idiotype may aid in unravelling the genetic mechanism of the generation of antibody diversity.

APPENDIX I

Use of Passive Hemagglutination to Detect
an SWR/J Idiotypic Present on Some SWR/J Anti-Group A Antibodies
and to Monitor Spleen Cell Transfer in Syngeneic Mice*

Summary

The antibody response to the Group A carbohydrate antigen was measured in SWR/J and A/J mice after intravenous (i.v.) immunization with whole heat-killed streptococcal vaccines. With the immunization procedure employed here, most SWR/J mice produced less than 1 mg of antibody per ml. The occasional mouse, however, produced anti-Group A antibody with restricted heterogeneity; such mice were used as donors for spleen cell transfers (10^7 spleen cells per recipient) to irradiated (500 R) recipient SWR/J mice. Antisera of recipient mice had antibody components identical to that of the donor as detected by microzone electrophoresis. Isolated antibody from an SWR/J recipient of spleen cell transfer line 1 was used to prepare anti-idiotypic sera in rabbits. Idiotype was detected by a hemagglutination inhibition assay. Antibody with the same idiotypic was detected in the original donor mouse of the cell transfer experiments and in the majority of the mice of the first and second passage. This idiotypic specificity was not apparent in the antisera of SWR/J mice which received immune cells from a different donor.

By use of the hemagglutination inhibition test, at least 5 out of 14 immunized SWR/J mice had a minor antibody component with the same idiotypic as that of the proband. This idiotypic was not detected in the Group A antibodies of 16 A/J mice immunized with the same antigen. This SWR/J idiotypic marker may be useful for genetic studies, because an antibody component with this idiotypic occurred in the hybrid SWR/J x A/J. This idiotypic specificity was also observed in the antibody produced by BSVS mice.

* Much of the data in Appendix I has already been published (Briles and Krause, 1972).

Materials and Methods

All of the procedures used in this Appendix have been described in the body of the thesis.

Results

Specificity of idiotypic assay Studies to detect idiotypic determinants of SWR/J anti-Group A antibodies employed a passive hemagglutination inhibition (HI) test. This test was selected because of its high sensitivity and specificity (Kunkel, 1970). These idiotypic studies employed the same anti-SWR/J idiotypic serum used in the body of the thesis. This antiserum, it should be remembered, was made against anti-Group A antibody isolated from SWR/J cell transfer line 1. Isolated proband streptococcal antibody was used to coat rabbit erythrocytes. Hemagglutination of these coated erythrocytes was readily inhibited by the addition of the isolated proband antibody. Inhibition of hemagglutination by antisera, ascites fluids, and isolated antibodies from other immunized SWR/J mice was an indication that these other mice produced antibodies with the same idiotypic determinant as the antibodies of the proband.

The specificity of the HI test for the detection of an idiotypic determinant is shown in Table XIII. Proband ascites fluid with an antibody concentration of 0.13 mg/ml had an HI titer of 2048, whereas the fluid after absorption with Group A vaccine had a titer of <8. Isolated antibody with a concentration of 0.05 mg/ml had an HI titer of 512. No HI titer was seen with a pool of non-immune SWR/J ascites fluid containing 4.7 mg of Ig per ml, or a pool of ascites fluid containing 3 mg/ml from Ig from SWR/J mice immunized with human serum albumin.

The detection of an idiotypic determinant has been a useful way to monitor the successful passage of a predominant plasma cell clone from an immunized SWR/J donor mouse to irradiated SWR/J recipient mice. Such a study is illustrated in Table XIV. The anti-idiotypic serum was prepared against the antibody recovered from proband mouse #1. The proband was a mouse from the second spleen cell passage of cell line #1. Ascites fluid from 10 mice of cell line #1 inhibited the hemagglutination reaction, whereas ascites fluid from none of the cell line #2 mice gave inhibition.

TABLE XIII

Agglutination Inhibition of RBC Coated with
 Proband Anti-CHO (SWR)⁴

| Inhibitor | HI titer reciprocal ¹ | Anti-CHO ³ mg/ml | Ig mg/ml |
|-----------------------------|-------------------------------------|--------------------------------|-------------|
| Proband SWR, anti-CHO | | | |
| Ascites fluid | 2048 | 0.13 | |
| Absorbed fluid ² | <8 | <0.003 | |
| Isolated anti-CHO | 512 | 0.05 | |
| Pool SWR, non-immune | | | |
| 1. Ascites fluid | <8 | | 1.0 |
| 2. Ascites fluid | <8 | | 4.7 |
| Pool SWR, anti-HSA | | | |
| Ascites fluid | <4 | | 3.0 |

¹ The HI titer is the highest dilution of the inhibitor giving complete inhibition of the passive hemagglutination reaction. The inhibitors were tested by using 2-fold dilutions.

² Ascites fluid absorbed with Group A vaccine.

³ Anti-CHO, antibody to Group A carbohydrate.

⁴ Coated cells agglutinated with anti-SWR/J cell line 1 idiotypic serum.

TABLE XIV

Use of HI Test to Detect Transfer of Spleen Cell Line

| Spleen Cell Line #1 | | Spleen Cell Line #2 | |
|---------------------------------|----------------------------------|---------------------|----------------------------------|
| Source of anti-CHO ³ | HI titer reciprocal ¹ | Source of anti-CHO | HI titer reciprocal ¹ |
| Donor | 512 | Donor | <1 |
| Passage 1 | | Passage 1 | |
| Mouse 1 | 32 | Mouse 1 | <6 |
| Mouse 2 | 320 | Mouse 2 | <2 |
| Mouse 3 | 256 | Mouse 3 | <2 |
| Mouse 4 | 128 | | |
| Passage 2 | | Passage 2 | |
| Mouse 1 ² | 2048 | Mouse 1 | <2 |
| Mouse 2 | 1024 | Mouse 2 | <2 |
| Mouse 3 | 320 | Mouse 3 | <4 |
| Mouse 4 | 320 | Mouse 4 | <4 |
| Mouse 5 | 960 | | |

¹ The HI titer employed here was obtained by multiplying the initial dilution of the inhibitor by the highest subsequent 2-fold dilution which gave inhibition.

² The anti-idiotypic antisera were prepared against anti-Group A antibody of this mouse.

³ Anti-CHO; antibody to Group A carbohydrate.

All mice had detectable anti-Group A antibodies in their ascites fluid as determined by the capillary precipitin test. For the line #1 cell transfers, the mouse chosen as the proband was the one with the largest amount of anti-Group A antibody in the ascites fluid when examined by microzone electrophoresis. As can be seen from the electrophoretic patterns in figure 52, there is less antibody in the mouse #3 preparation than in the proband, and this is reflected in the difference in the HI titer. The absence of detectable HI titers with ascites fluids from mice of spleen cell line #2 indicates that these antibodies lack the idiotypic determinant of the antibodies in cell line #1. Immune ascites fluid and isolated antibody from mice of a third cell transfer line also failed to inhibit the hemagglutination reaction.

Microzone electrophoresis has shown that the predominant antibody component produced by the 8 mice of cell line #2 is slightly more negatively charged than the antibodies produced by 10 cell line #1 mice. Although this difference in mobility was only 0.5 mm, it was consistently reproducible as shown by two or more electrophoretic patterns for each antiserum. This is demonstrated in figure 52, which shows the microzone electrophoretic patterns of antibodies isolated from representative mice of spleen cell line #1 and spleen cell line #2.

The specificity of the passive hemagglutination assay, with this antiserum, was further investigated by making use of isolated antibody preparations from mice carrying cell lines 1 and 3 (Table XV). In this experiment, 1.8 $\mu\text{g/ml}$ of proband antibody was required for complete inhibition of hemagglutination. A preparation of antibody from another cell line 1 mouse required 2.4 $\mu\text{g/ml}$ antibody for complete inhibition. In contrast, 580 $\mu\text{g/ml}$ of isolated antibody from a mouse of cell line 3 did not give complete hemagglutination inhibition. The antibody produced by cell lines 3 and 1 have different electrophoretic mobilities, as is illustrated in figure 23, where isolated antibody from a cell line 1 mouse and a cell line 3 mouse are compared.

In order to demonstrate that the idiotype detected was not just an artifact of the cell line 1 system, a second anti-idiotypic serum was prepared against an isolated antibody from mouse #4 of SWR/J cell line 3.

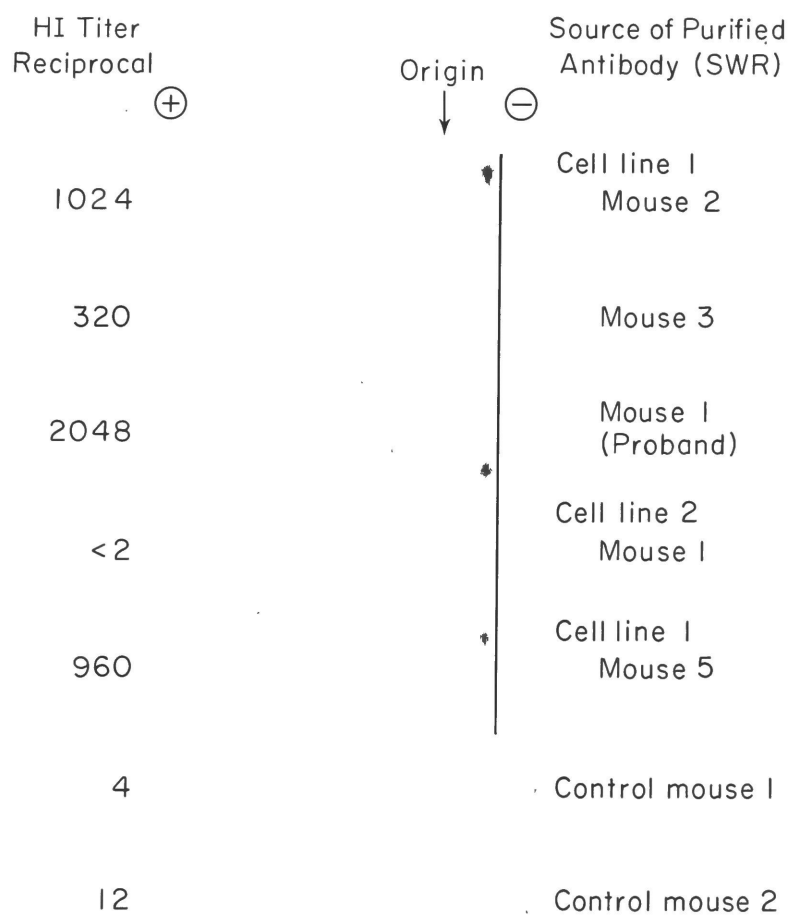


Figure 52. Microzone electrophoresis of isolated anti-Group A antibody from 4 mice of spleen cell line 1; 1 mouse from spleen cell line 2; and 2 control mice.

TABLE XV

Specificity of the Anti-Cell Line 1 Idiotypic
Antiserum for the Cell Line 1 Idiotypic

| Source of isolated anti- Group A antibody ¹ | μg/ml isolated antibody required for inhibition of passive hemagglu- tination ² |
|--|--|
| Cell line 1 passage 2 | |
| Mouse 1 | 1.8 |
| Mouse 2 | 2.4 |
| Cell line 3 passage 1 | |
| Mouse 4 | >580 |

¹ Anti-Group A antibody isolated from immune ascites fluids by absorption to and elution from vaccine.

² Concentration of antibody in eluate determined by optical density at 280 mμ prior to titration.

This idiotypic serum was shown by the HI assay to be specific for immune sera from cell line 3 mice (Table XVI). Antisera from cell line 1 and SWR/J cell line 7 mice did not inhibit the passive hemagglutination reaction even though all of these sera were shown to contain anti-Group A antibody by capillary precipitin tests. The agglutination also could not be inhibited with pooled SWR/J anti-Group A sera. This antiserum, therefore, appeared to be specific for the anti-Group A antibody produced by the cell transfer line 3 mice.

The anti-Group A antibody from these three cell lines differ in their electrophoretic mobilities. For example, in figure 22 the electrophoretic mobility of isolated antibodies from cell lines 1 and 3 are compared.

Inbred strain specificity of idiotypic The HI test was used to determine if some of the anti-Group A streptococcal carbohydrate antibodies in the serum of immunized SWR/J mice would have the idiotypic specificity of the cell transfer line #1 antibodies. For this study, SWR/J, A/J, and SWR/J x A/J mice were immunized intravenously according to the schedule in Table VII. Fourteen immunized SWR/J mice were tested (Table XVII). The serum antibody levels of these 14 SWR/J mice varied from less than 3 $\mu\text{g/ml}$ to 453 $\mu\text{g/ml}$. Five mice had an HI serum titer of 72, and four had a titer of 24. Although these titers are considerably lower than the proband titer, they were reproducible with different batches of sensitized cells. Absorption of selected SWR/J antisera with Group A vaccine removed this inhibitory activity. Furthermore, antibody eluted from the vaccine with 5% N-acetyl-D-glucosamine inhibited the hemagglutination reaction. Thus, it appeared that at least some of the 14 sera from the 14 immunized mice had antibody components with the same idiotypic as the proband.

There was a great deal of variation, however, in the efficiency with which the SWR/J sera inhibited the hemagglutination reactions (Table XVIII). A few of them required no more anti-Group A antibody for complete inhibition than was required of the proband antibody. Others required up to several hundred, and in one case, 1,700 times as much anti-Group A antibody as was required of the proband.

TABLE XVI

Specificity of the Anti-Cell Line 3 Idiotypic
Antiserum for the Cell Line 3 Idiotypic in HI Test

| Source of anti-CHO ¹ | HI titer reciprocal ² |
|-----------------------------------|----------------------------------|
| CELL LINE 3 | |
| Passage 1 | |
| Mouse 4 (proband) | <u>>12,000</u> |
| Passage 2 | |
| Mouse 1 | <u>>12,000</u> |
| Mouse 10 | <u>>12,000</u> |
| Passage 3 | |
| Mouse 1 | 96 |
| Mouse 5 | 1,500 |
| CELL LINE 1 | |
| Passage 2 | |
| Mouse 1 | <12 |
| CELL LINE 7 | |
| 9 antisera tested | <12 |
| Pooled SWR/J anti-Group A sera | <12 |

¹ All anti-Group A sera contained anti-Group A antibody by the capillary precipitin test.

² The HI titer was obtained by multiplying the initial dilution of the inhibitor by the highest subsequent 2-fold dilution which gave inhibition. Titers are rounded off to two significant figures.

TABLE XVII

Use of HI Test to Detect the Mouse Strain Specificity
of Idiotypic of Antibody to Streptococcal Carbohydrate²

Experiment A

| Source of anti-CHO | No. of mice | HI titer reciprocal ¹ | Conc. of anti-CHO in each serum ³ |
|--------------------|----------------|----------------------------------|--|
| | | | µg/ml |
| Proband (SWR) | 1 ² | 3888 | 129 |
| SWR | 5 | 72 | <3, 3, 14, 30, 42 |
| | 4 | 24 | <3, 8, 25, 73 |
| | 3 | <8 | 40, 64, 453 |
| | 2 | <8 | <3, <3 |
| A/J | 16 | 8 | 25 to 3060 (av. = 1206) |
| SWR x A/J | 2 | 72 | 690, 3600 |
| | 1 | 24 | 730 |
| | 2 | 8 | <3, <3 |

¹ The HI titer was obtained by multiplying the initial dilution of the inhibitor by the highest subsequent 3-fold dilution which gave inhibition.

² Anti-idiotypic antisera prepared against proband (cell line 1) anti-Group A antibody. Cells were coated with proband (cell line 1) anti-Group A antibody.

³ Anti-CHO antibody to Group C carbohydrate.

TABLE XVIII

Relative Inhibitory Capacity of Mouse Anti-Group A sera in HI Assay^a

| Source of anti-CHO | | µg/ml anti-Group A CHO required for inhibition | Relative amount of anti-Group A CHO required for inhibition |
|--------------------|----|--|---|
| Proband | | 0.033 | 1.0 |
| SWR/J | 1 | < .04 ^b | < 1.2 |
| | 2 | < .125 ^b | < 3.8 |
| | 3 | .041 | 1.2 |
| | 4 | .198 | 5.8 |
| | 5 | .33 | 10 |
| | 6 | .42 | 13 |
| | 7 | .58 | 18 |
| | 8 | 1.0 | 30 |
| | 9 | 3.3 | 100 |
| | 10 | 5.0 | 150 |
| | 11 | 8.0 | 240 |
| | 12 | 57.0 | 1700 |
| | 13 | c | |
| | 14 | c | |
| A/J | 1 | >3 | >91 |
| | 2 | >50 | >1500 |
| | 3 | >66 | >2000 |
| | 4 | >67 | >2000 |
| | 5 | >85 | >2600 |
| | 6 | >90 | >2700 |
| | 7 | >91 | >2800 |
| | 8 | >100 | >3000 |
| | 9 | >120 | >3900 |
| | 10 | >130 | >4500 |
| | 11 | >150 | >4500 |
| | 12 | >200 | >6100 |
| | 13 | >230 | >7100 |
| | 14 | >290 | >8800 |
| | 15 | >350 | >10600 |
| | 16 | >380 | >11500 |
| SWR x A/J | 1 | < .4 ^b | < 8.2 |
| | 2 | < .4 ^b | < 8.2 |
| | 3 | 9.6 | 290 |
| | 4 | 30 | 900 |
| | 5 | 50 | 1500 |

^a The data in this table was calculated from the data in Table XVII.^b Less than 3 µg/ml anti-Group A antibody.^c Less than 3 µg/ml anti-Group A antibody and no HI.

None of the sera from the 16 immunized A/J mice inhibited the hemagglutination reaction in spite of the fact that, for 14 of these sera, it was possible to test higher concentrations of anti-Group A antibody than were required for complete inhibition by even the least efficient SWR/J serum.

It therefore appeared that the A/J mice were very likely incapable of making anti-Group A antibodies having the cell line 1 idiotypic as detected by this assay.

Anti-Group A sera from five hybrid SWR/J x A/J mice were tested for the presence of the idiotypic; two had received HI titers as high as 72. All five of them had reciprocal HI titers of greater than 8. This indicated that the SWR/J gene(s) regulating this idiotype specificity(s) is still expressed in the presence of the A/J genotype.

This general finding, that the cell line 1 idiotypic was strain-specific and inheritable, was confirmed by a second experiment with another group of mice immunized in a manner similar to those above. These results are shown in Table XIX. Of the four SWR/J anti-Group A sera tested, two had reciprocal inhibition titers of 384 or greater. Five F₁ mice were tested, and two of them had similarly high inhibition titers. A pool of A/J anti-Group A sera was tested and showed no hemagglutination inhibition, in spite of the fact that it had an anti-Group A antibody concentration of more than 10 times that of the highest titer SWR/J sera tested. A group of 10 F₁ x A/J mice were also assayed for the presence of the cell line 1 idiotypic. Five of them showed HI titers of 12 or 24.

These data indicate that some of the backcross mice may have inherited the ability to make the cell line 1 idiotypic. However, the very low HI titers in these mice make it necessary to do further experiments to confirm this finding.

Presence of the cell line 1 idiotypic in BSVS mice Sera of immunized BSVS and BSVS x A/J mice were assayed by the HI technique to see whether or not any of them contained antibody bearing the SWR/J cell line 1 idiotypic. The results are given in Table XX. Eight BSVS sera were tested.

TABLE XIX

Use of HI Test to Detect the Mouse Strain Specificity
of Idiotypic of Antibody to Streptococcal Carbohydrate²

| Experiment B | | | |
|-----------------------|----------------|-------------------------------------|---|
| Source of anti-CHO | No. of sera | HI titer reciprocal ¹ | Conc. anti-CHO in serum µg/ml ³ |
| Proband (SWR/J) | 1 ² | ≥384 | 129 |
| SWR/J | 2 | ≥384 | 78, 226 |
| | 1 | 48 | 183 |
| | 1 | 24 | 97 |
| F ₁ | 2 | ≥384 | 847, 1061 |
| | 1 | 24 | 278 |
| | 1 | 12 | 899 |
| | 1 | < 12 | 2656 |
| F ₁ x A/J | 2 | 24 | 102, 142 |
| | 3 | 12 | 320, 164, 110 |
| | 5 | < 12 | 330, 73, 88, 58, 40 |
| A/J (pool) | | < 12 | 2750 |

¹ The HI titer was obtained by multiplying the initial dilution of the inhibitor by the highest subsequent 2-fold dilution which gave inhibition; the highest dilution of any sera tested was 1/384.

² Anti-idiotypic antisera prepared against proband (cell line 1) anti-Group A antibody. Cells were coated with proband (cell line 1) anti-Group A antibody.

³ Anti-CHO antibody to Group C carbohydrate.

TABLE XX

Use of HI Test to Detect the Mouse Strain Specificity
of Idiotype of Antibody to Streptococcal Carbohydrate²

| Experiment C | | | |
|--------------------------|-------------|----------------------------------|---|
| Source of anti-CHO | No. of sera | HI titer reciprocal ¹ | Conc. anti-CHO in serum $\mu\text{g/ml}$ ³ |
| Proband (SWR/J) | 1 | 3900 | 129 |
| BSVS | 1 | 216 | < 3 |
| | 2 | 24 | < 3, 24 |
| | 5 | 8 | < 3, < 3, < 3, 9, 24 |
| BSVS x A/J | 2 | 216 | 532, 614 |
| | 1 | 24 | 1490 |
| | 6 | 8 | < 3, < 3, 21, 330, 590, 1500 |
| | 2 | < 8 | 13, 715 |
| A/J | 16 | < 8 | 25 to 3060 (av. = 1206) |
| Pooled normal BSVS serum | 1 | < 8 | ~2000 $\mu\text{g/ml}$ Ig |

¹ The HI titer employed here was obtained by multiplying the initial dilution of the inhibitor by the highest subsequent 3-fold dilution which gave inhibition.

² Anti-idiotypic antisera prepared against proband (cell line 1) anti-Group A antibody. Cells were coated with proband (cell line 1) anti-Group A antibody.

³ Anti-CHO antibody to Group C carbohydrate.

All of them had very low antibody titers. One of the sera gave an HI titer of 216 and two other sera gave HI titers of 24. No inhibition titer was observed in any of the 16 A/J sera tested, as was explained previously.

It therefore appeared as though the SWR/J cell line 1 idiotypic was capable of being expressed by BSVS mice and that it could also be expressed in BSVS x A/J mice. It was therefore important to demonstrate that the hemagglutination inhibition in these BSVS and F₁ sera was in fact due to anti-Group A antibodies. Using one of the two sera with a reciprocal HI titer of 216, it was observed that the inhibiting material in this serum could be isolated by absorption to and elution from Group A vaccine.

Conclusions

These results have demonstrated that both the BSVS and SWR/J mice, but not the A/J mice, are capable of producing the SWR/J cell line 1 idiotypic marker detected by this assay. These results have also demonstrated that BSVS and SWR/J mice can genetically transmit the ability to make the cell line 1 idiotypic to hybrid animals in crosses with A/J mice.

These idiotypic markers, as detected by the HI assay, have also proved to be useful for following the in vivo propagation of cell clones producing anti-Group A antibody. As had been expected from the previous electrophoretic data (see figure 22), these idiotypic experiments demonstrated that SWR/J mice were capable of making more than one anti-Group A antibody (Tables XV and XVI). By the passive hemagglutination inhibition assay the antibody produced by cell lines 1, 3, and 7 had individual (non-cross-reacting) idiotypes. Further evidence that SWR/J mice were capable of making different anti-Group A antibodies came from the observation that the idiotypes of cell line 1 and 3 antibodies were generally quite rare in other SWR/J mice immunized with Group A vaccine (Tables XV and XVIII).

APPENDIX II

Inhibition of the Tube Binding Idiotypic Assay
with High Concentrations of Non-immune Gamma Globulin

This experiment was performed using the same anti-SWR/J cell line 1 idiotypic antiserum, the same ^{125}I -labeled pooled SWR/J anti-Group A antibody, and the same assay conditions employed in the experiment depicted in figures 48 and 50. Normal γ -globulin from various sources were used to inhibit the idiotypic assay. The concentrations required were several hundred times greater than the concentrations of pooled SWR/J anti-Group A antibody required to give the same inhibition. The inhibitors tested were: normal SWR/J serum, a globulin fraction of normal SWR/J serum obtained by precipitation with 42% cold saturated ammonium sulfate, commercial (Pentex) mouse γ -globulin, rabbit γ -globulin (Pentex), and bovine γ -globulin (Pentex). The Pentex mouse γ -globulin undoubtedly did not come from SWR/J mice. The γ -globulin concentrations in the SWR/J normal serum and the SWR/J globulin fraction were calculated from the total proteins of these preparations and the densitometric tracings of their microzone electrophoresis patterns. From figure 53, it is apparent that the SWR/J γ -globulin inhibits the tube binding idiotypic assay much more efficiently than did the other γ -globulin preparations. The fact that the non-syngeneic mouse γ -globulin was a poorer inhibitor than the syngeneic γ -globulin, and that the γ -globulin from the other species hardly inhibited at all, indicates that the inhibition with non-immune syngeneic gamma globulin is not simply a non-specific effect. The specificity could be explained in two ways. One possibility is that normal SWR/J γ -globulin contains a small amount of the idio type detected by the anti-idiotypic serum. Another possibility is that the normal syngeneic serum contains idiotypic specificities similar, but not identical to, the idiotypic specificity detected by the assay. In either case, much higher concentrations of the normal serum would be required to get the same inhibition that is obtained with the proband anti-Group A antibody. It is, of course, likely that some combination of these two possibilities is acting on the case.

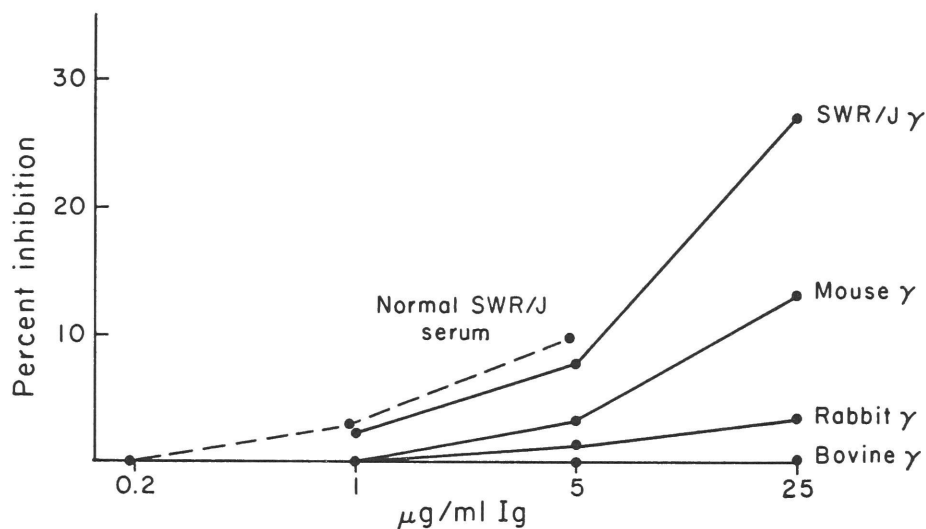


Figure 53. Inhibition of an idiotypic assay with non-immune γ -globulins.

All curves are plotted in terms of $\mu\text{g/ml}$ γ -globulin in the preparations tested. The dashed curve is normal SWR/J serum. The curve labeled SWR/J γ is a globulin fraction from normal SWR/J serum. The other three curves are commercial preparations of γ -globulin (Pentex). Anti-cell line 1 idiotypic serum has been coated to the tubes. The reference antigen is ^{125}I -labeled pooled SWR/J anti-Group A antibody.

Thus, rather than inhibition with normal serum being a problem, it might turn out to be of great value in genetic studies. For example, if the normal serum of two inbred strains showed distinctly different efficiencies of inhibition of an idiotypic assay, this might prove to be a satisfactory means of studying the inheritance of hypervariable region markers, without having to contend with the variable expression of idiotypes in immune sera. Such an approach has already been demonstrated by Eichmann and Kindt (1971). They observed that the pre-immune sera of their proband and proband-related rabbits were more efficient inhibitors of a precipitating idiotypic reaction than were pre-immune sera from unrelated rabbits.

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